IMMUNITY TO CYTOMEGALOVIRUS INFECTIONS: CHALLENGES AND THERAPEUTIC OPPORTUNITIES

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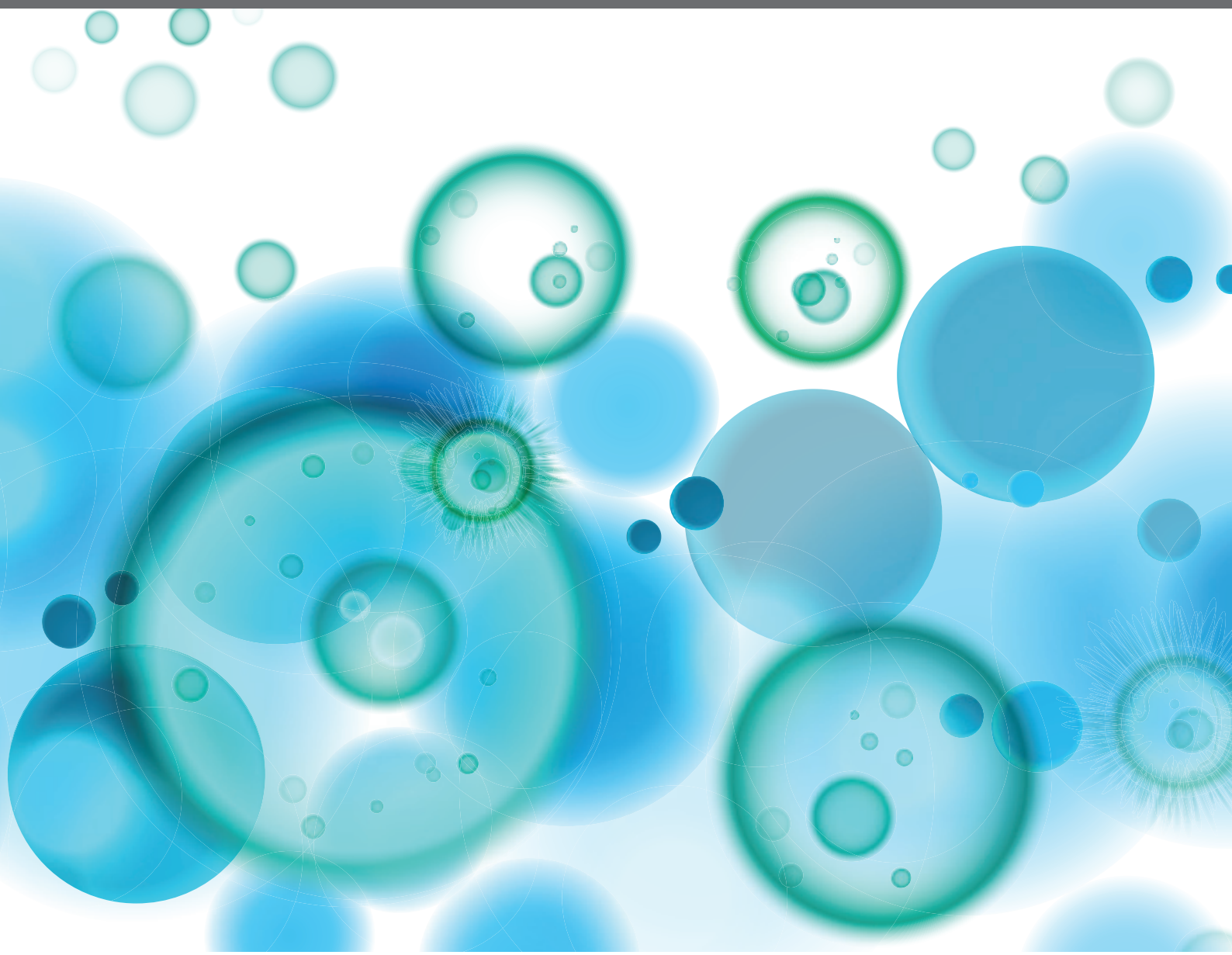

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IMMUNITY TO CYTOMEGALOVIRUS INFECTIONS: CHALLENGES AND THERAPEUTIC OPPORTUNITIES

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EDITORIAL
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Editorial: Immunity to Cytomegalovirus Infections: Challenges and Therapeutic Opportunities

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Keywords: cytomegalovirus (CMV), inflammatory diseases, immunodeficiencies, immunosenescence, T lymphocytes

Editorial on the Research Topic

Immunity to Cytomegalovirus Infections: Challenges and Therapeutic Opportunities

Human cytomegalovirus (CMV) is a double-stranded DNA virus belonging to the *Herpesviridea* family. Its transmission, through different fluids such as saliva, sexual contact, blood, and breast milk, makes its prevalence high, and the probability of infection increases with age. The implications of acute infection are of relevance for immunocompromised individuals (such as neonates and transplantation patients), and such CMV infections can lead to long lasting complications and even mortality. CMV is also of importance for the healthy population due to its chronicity and latency throughout the life of the infected individual. The development of an inflationary immune response over the years, which is not fully clarified, is a wide field of research where we can deepen our knowledge on the CMV-host interactions.

This Research Topic gives us a comprehensive overview of the current knowledge of CMV infection in various situations, as well as its possible solution or attenuation. This monograph includes thirteen articles: nine original articles and four review articles. The authors invited scientific collaborators to this collection based on their unique and specific findings on CMV under physiological and pathological conditions including: (a) mechanisms regulating CMV immune evasion, (b) the role of CMV in the alterations suffered by the immune system in patients with inflammatory chronic diseases, (c) CMV infection in the context of primary and secondary immunodeficiencies, (d) influence of CMV infection on the immunosenescence process associated to aging and (e) therapeutic opportunities in the management of CMV disease.

In the article by Reus et al., sex differences in the response of T lymphocytes to different CMV proteins are explored. The response of both CD4+ and CD8+ T lymphocytes was greater and more proinflammatory in men than in women. These findings may help understand sex differences in CMV-associated pathologies. Jackson et al. analyzed the effect of latent CMV infection on the secretome of CD14+ monocytes, finding an increase in the production of the chemokines CCL8 and CXCL10. The CD14+ latency-associated secretome also suppresses the anti-viral activity of stimulated CD4+ T cells. Moreover, co-culture of activated autologous CD4+ T cells with

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latently infected monocytes resulted in reactivation of CMV at levels comparable to those observed using M-CSF and Il-1b cytokines. This mechanism could be a strategy of CMV to reactivate and achieve local dissemination in peripheral tissues. Another article that studies the CMV latency period is the work by Griessl et al. showing that the IE, E and L genes are transcribed during latency, but in a stochastic manner, not following the IE-E-L cascade pattern. In addition, the transcripts that code for memory inflation-driving antigenic peptides rarely coincide with those that code for immune evasion proteins. This stochastic expression could explain why immune evasion is not operative in latently infected cells, however, it does not interfere with memory inflation. Another possible evasion mechanism studied in this monograph is the one analyzed by Zhang et al. where they observe that the expression of certain microRNAs that interact with IFN receptor 1 (IFNAR1) could achieve immune evasion, both in lytic and latent infection. van den Berg et al. investigated the impact of CMV infection on influenza-specific CD8+ T cells and the response to influenza infection in the elderly. CMV infection does not appear to diminish the influenza-specific response in acute CMV infection.

In the section on CMV infection associated with other pathologies, we find several very interesting works such as that of García-Torre et al. that relates the level of proinflammatory cytokines with the functional status of patients with chronic heart failure and with CMV infection. They found higher levels of all cytokines in patients with heart failure compared to healthy controls, as well as a direct correlation between levels of proinflammatory cytokines and worse functional status in patients. This cytokine production was much higher in CMV-infected patients, and anti-CMV antibodies correlated with levels of proinflammatory cytokines. The review presented by Alonso-Álvarez et al. tells us about the relationship between CMV infection and hematological tumors. The evolutionary symbiotic relationship between herpesviruses and humans disappears in immunosuppressed patients, especially in hematological patients. New procedures in transplantation have been introduced as well as new treatments to manipulate the composition of the graft and its functionality. In addition, new drugs have also been introduced to treat CMV infection. Another point discussed in this interesting review is the effects of CMV in terms of mortality or progression in patients with hematological tumors treated with immunochemotherapy or new molecules or in patients who have received SCT. El Baba and Herbein review the immunological profile in cancer patients infected by CMV. The changes promoted by CMV infection are related to immunosenescence and phenotypes that lead to immunosuppressive tumor environments and oncomodulation. CMV-induced evasion mechanisms play a major role in developing new approaches in tailored therapeutics against CMV, especially since immunotherapy has revolutionized therapeutic strategies against cancer.

Luo et al. discuss CMV infection and CMV-specific immune reconstitution in the context of haploidentical stem cell transplantation (SCT). The cure in haploidentical stem cell transplantation (haploSCT) is seriously hampered by CMV infection and delayed immune reconstitution compared to HLA-Matched stem cell transplantation. The authors provided an update on CMV infection and CMV-specific immune recovery in this fast-evolving field. Another very interesting original work is the one carried out by Gergely et al. where therapeutic vaccination against CMV in hematopoietic cell transplantation (HCT) recipients is reviewed. Therapeutic vaccination aims at restimulation and expansion of specific transferred CD8+ T lymphocytes in the recipient of HCT. Their preclinical research data provide an argument for using pre-emptive therapeutic vaccination to improve antiviral protection by adoptive cell transfer in HCT recipients with diagnosed CMV reactivation. In the review written by García-Ríos et al. the possibilities of using CMV-specific T-cell adoptive transfer as a treatment against CMV infection in solid organ transplantation (SOT) recipients are described. This option may be a therapeutic alternative to reconstitute the specific T cell response against CMV and control CMV viremia in SOT patients.

Pardieck et al. declare in their article that a vaccine that induces immunity against CMV in an immunocompromised or immunoimmature population is highly needed. Current insight encourages that a protective immune response to CMV might benefit from the induction of virus-specific T cells. The combination of antibodies and CD8+ T cell-eliciting vaccines provides a collaborative improvement of humoral and cellular immunity allowing to improve protection against CMV. In the article by Šustić et al. the role of memory CD8+ T cells, generated by a CMV vaccine vector expressing NKG2D ligand, with an effector-like phenotype and distinct functional features is studied. CMV is an attractive vaccine vector due to its large genome with few non-essential genes that can be easily manipulated. Their results shed new insights into the phenotypical and functional distinctness of memory CD8+ T cells induced with CMV vectors expressing cellular ligands for the NKG2D receptor.

We hope that all articles in this Frontiers in Immunology Research Topic will serve to broaden the knowledge of researchers and clinicians in the field of CMV infection. In addition, we hope that these works will serve as a stimulus to expand research on the mechanisms behind infection by this virus that will result in an improvement in the lives of patients.

AUTHOR CONTRIBUTIONS

MM-G wrote the editorial. RA-A and RA reviewed and corrected the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Human Cytomegalovirus miR-US33as-5p Targets IFNAR1 to Achieve Immune Evasion During Both Lytic and Latent Infection

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As the first line of antiviral defense, type I interferon (IFN) binds IFN receptor 1 (IFNAR1) and IFNAR2 to activate the Jak-STAT signal transduction pathway, producing IFN-stimulated genes (ISGs) to control viral infection. The mechanisms by which human cytomegalovirus (HCMV) counteracts the IFN pathway are only partially defined. We show that miR-US33as-5p encoded by HCMV is expressed in both lytic and latent infection. By analysis with RNA hybrid and screening with luciferase reporter assays, we identified IFNAR1 as a target of hcmv-miR-US33as-5p, which was further verified by examining the expression of two IFNAR1 mutants and the binding of IFNAR1 to miR-US33as-5p/miR-US33as-5p-M1/miR-US33as-5p-M2. We found that after the transfection of miR-US33as-5p mimics into different cell lines, the phosphorylation of downstream proteins and ISG expression were downregulated. Immunofluorescence showed that the miR-US33as-5p mimics also inhibited STAT1 translocation into the nucleus. Furthermore, we constructed HCMV with mutant miR-US33as-5p and determined that the mutation did not affect HCMV replication. We found that MRC-5/human foreskin fibroblast (HFF) cells infected with ΔmiRNA HCMV exhibited higher IFNAR1 and ISG expression and a reduced viral load in the presence of exogenous IFN than cells infected with WT HCMV did, confirming that the knockout of miR-US33as-5p impaired viral resistance to IFN. Finally, we tested the effect of ΔmiRNA HCMV on THP-1 and d-THP-1 cells, common *in vitro* models of latent infection and reactivation, respectively. Again, we found that cells infected with ΔmiRNA HCMV showed a reduced viral load in the presence of IFN than the control cells did, confirming that miR-US33as-5p also affects IFN resistance during both latency and reactivation. These results indicate a new microRNA (miRNA)-based immune evasion mechanism employed by HCMV to achieve lifelong infection.

Keywords: cytomegalovirus, viral miRNAs, US33as-5p, IFNAR1, immune evasion

INTRODUCTION

Human cytomegalovirus (HCMV), a prevalent human pathogen, is a member of the subfamily of β-herpesviruses, which are enveloped, double-stranded (ds) DNA viruses that maintains persistent latent infection for the duration of the host's lifetime (1). HCMV infection can be asymptomatic in healthy individuals but is the major cause of morbidity and mortality in immunocompromised patients and the leading cause of congenital birth abnormalities (2). Current antiviral drugs (such as ganciclovir) have been proven efficacious to control viral infection, but there are few approaches to thoroughly eliminate the virus (3). Therefore, further investigation to identify the mechanism linking viral persistence and immune evasion remains necessary.

MicroRNAs (miRNAs) are short non-coding RNAs (19–22 nucleotides in length) that post-transcriptionally regulate gene expression, causing the degradation and translational inhibition of target mRNAs by base-pairing with the 3′-untranslated region (3′-UTR) through the RNA-induced silencing complex (RISC) (4, 5). More than 230 viral miRNAs have been discovered, but the target genes of these viral miRNAs have not been extensively studied (6). HCMV has a large genome of 230–250 kb and is currently known to encode 26 miRNAs from 16 precursors. However, several miRNAs not included in the miRBase database (http://www.mirbase.org/) have been discovered. For example, we and other researchers found that miR-US33as-5p is encoded by HCMV and expressed in both lytic and latent infection (7).

In previous studies on herpesviruses, many viral miRNAs were found to be expressed to avoid host immune mechanisms, allowing persistent infection to be maintained, which is particularly common in β- and γ-herpesviruses (8). UL-112-1, miR-BART2-5p, and miR-K12-7, which are encoded by HCMV, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), respectively, were found to target MHC class I-related chain B (MICB), a stress-induced ligand that is targeted by natural killer (NK) cells through the ligand NKG2D (9, 10). miR-K12-9, encoded by KSHV, reduces NF-κB signaling by binding IRAK1 and MyD88, leading to reduced levels of the inflammatory cytokines IL-6 and IL-8 (11). miR-UL112-1, US5-1, and US5-2, encoded by HCMV, targets multiple cellular targets, reducing the secretion of the inflammatory cytokines TNF-α and IL-6 (12). miR-UL148D-1 targets RANTES, regulating activation, and normal T-cell expression and secretion (13, 14). Although some HCMV miRNAs and their targets have been reported, many more miRNAs, their target antiviral mechanisms, and their biological functions remain unclear.

In this study, we found that hcmv-miR-US33as-5p targets interferon (IFN) receptor 1 (IFNAR1) to evade host immunity by interfering with the canonical IFN signaling pathway. Type I IFNs play a key role in innate immune responses against viral infection through the production and secretion of IFNs by host cells upon the recognition of viral nucleic acids. Moreover, IFNs protect cells from further enhanced viral infection by inducing ISGs, many of which encode antiviral proteins. We showed that overexpression of hcmv-miR-US33as-5p obviously downregulated the expression of IFNAR1, further leading to inhibition of the Jak-STAT signal transduction pathway. Besides, mutation of miR-US33as-5p in HCMV obviously decreased viral resistance to IFN in both lytic and latent infection. These results establish a mechanism of hcmv-miR-US33as-5p-based immune evasion utilized by HCMV.

MATERIALS AND METHODS

Cells and Viruses

MRC-5 cells were cultured in minimum Eagle's medium (MEM, Gibco, Shanghai, China), HEK293 (293) cells and human foreskin fibroblast (HFF) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco), and THP-1 cells were cultured in RPMI 1640 medium (Gibco). All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, and all cells were cultured at 37°C under a 5% CO_2 atmosphere. Confluent cell monolayers were starved of serum for 4 h before drug treatment. Cells were treated with 10 μg/mL cycloheximide (CHX, Monmouth Junction, NJ, USA) for 0.5 h, maintained in serum-free DMEM, washed with phosphate-buffered saline (PBS) three times, and then stimulated with 1,500/500 IU/mL IFNα (Sigma-Aldrich, St. Louis, MO, USA) in medium supplemented with 10% FBS until harvesting.

HCMV (Toledo strain) was routinely inoculated and propagated in MRC-5 cells, and aliquots were stored at −80°C. MRC-5 and HFF cells were infected with HCMV at a multiplicity of infection (MOI) of 1, and THP-1 cells were infected with HCMV at an MOI of 10. For HCMV infection, the cells were infected with HCMV in a medium without FBS for 2 h, after which the medium was replaced with a medium containing 5% FBS.

Plasmid Construction

The 3′-UTRs of hcmv-miR-US33as-5p targets predicted by the RNA hybrid procedure were amplified from mRNA-derived complementary DNA (cDNA) with the corresponding primers listed in **Supplementary 1**. After purification and digestion, the fragments were cloned into the pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). In addition, vectors encoding IFNAR1 with one of two binding-site mutations were produced as described in **Supplementary 2** and named IFNAR1-M1 and IFNAR1-M2 (depicted in **Figure 2B**).

The sequence of hcmv-miR-US33as-5p was similarly amplified with miR-US33as-5p-specific primers, and the fragment was cloned into the GV251 vector (GeneChem, Shanghai, China), with the resulting vector named GV251-miR-US33as-5p. Two vectors encoding the mutants IFNAR1-M1 and IFNAR1-M2 were individually generated and named US33as-M1 and US33as-M2, respectively, as described in **Supplementary 3**.

To construct a RED-flagged STAT1 expression vector, STAT1 was amplified using semi-nested PCR (outer forward primer: 5′-TGCGTAGCTGCTCCTTTGGT-3′, outer reverse primer: 5′-GTCAGGATCCACTTCAGACACAGAAATCAA-3′; inner forward primer: 5′-GTCAGTCGACATGTCTCAGTGGT ACGAACT-3′, inner reverse primer: 5′-GTCAGGATCCACTT CAGACACAGAAATCAA-3′). Fragments were cloned into the

pDsRed1-N1 vector (Takara), and the resultant vector was named pDs-RED-STAT1. Insertion of the construct was confirmed by the company AuGCT.

Dual-Luciferase Reporter Assays

To confirm the target of hcmv-US33as-5p, 293 cells were cultured in 24-well plates. The cells were co-transfected with 150 ng of pmirGLO vector containing the 3′-UTR of a predicted target gene and 350 ng of GV251 blank vector or vector containing US33as-5p/US33as-5p-M1/US33as-5p-M2 using jetPRIME (Polyplus, Illkirch, France) in triplicate wells according to the manufacturer's instructions. The cell medium was replaced after 4 h of transfection. Luciferase activities were determined at 48 h post-transfection (hpi) according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega) using a luminometer. The transfection efficiency was normalized by the Renilla luciferase activity in the corresponding well.

Western Blot Analysis

Cell pellets were lysed in RIPA lysis buffer (CWBio, Beijing, China) with a cocktail of protein inhibitors and phosphatase inhibitors (CWBio). Extracts were separated by 10% sodium dodecyl sulfate-polymerase gel electrophoresis (SDS-PAGE), after which proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich) and visualized with antibodies specific for IFNAR1 (A1715), IFNAR2 (A1769), STAT1 (A0027), STAT2 (A14995), and β-actin (A2319) (all from ABclonal, Wuhan, Hubei, China), p-STAT1 (7649T), p-STAT2 (4441T), p-JAK1 (74129S), JAK1 (3344T), p-Tyk2 (68790S), and Tyk2 (14193S) (all from CST, Boston, MA, USA); and HRP-conjugated goat anti-rabbit IgG (ABclonal) via an electrochemiluminescence (ECL) detection system, followed by exposure using a ChemiDoc^{TM} XRS+ imaging system (Bio-Rad, Hercules, CA, USA).

RT-qPCR

Total RNA was isolated from cells using TRIzol reagent (Sigma-Aldrich), chloroform, and isopropanol according to the manufacturer's protocols. RNA purity and quantity were detected by a UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan). cDNA was reverse transcribed from the extracted RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan).

The expression levels of IFNAR1, IFNAR2, and 6 ISGs (primers listed in **Supplementary 4**) were screened and normalized to GAPDH mRNA expression (forward primer: 5′-TCGCTCTCTGCTCCTCCTGTTC-3′, reverse primer: 5′-CGCCCAATACGACCAAATCC-3′). The expression levels of other genes relative to the control were calculated as fold changes.

Real-Time PCR

The total viral copy number in cell supernatants of infected cells was determined with a genomic DNA extraction kit (BioTeke, Beijing, China) according to the manufacturer's instructions. Changes in HCMV DNA load were monitored by absolute quantitative real-time PCR with SYBR Green (TOYOBO). HCMV DNA levels were detected using primers specific for the HCMV IE1 gene (forward primer: 5′-ATGTACGGGGGCATCTCTCT-3′, reverse primer: 5′-GGCTTGGTTATCAGAGGCCG-3′).

mRNA Stability

MRC-5 cells transfected with mimics were treated with 10 μg/10^6 cells/mL actinomycin D (ActD) at 48 hpi. Samples were taken at 0, 2, 4, 6, and 8 h post-treatment and processed for qPCR analysis. MRC-5 cells treated with ActD at the specific concentration for 8 h did not show signs of overt ActD toxicity.

Examination of miRNA

miRNA expression was determined with a modified protocol from our previous report (15). Briefly, cDNA synthesis such that a poly(A) tail followed each miRNA was performed by reverse transcription (RT) using a tagged poly(T) primer (5′-CAGGTCCAGTTTTTTTTTTTTTTTVN-3′) and the One-Step miRNA cDNA Synthesis Kit (Hai-gene Bio Inc., Harbin, China). The generated cDNA was then used for miRNA examination (primers listed in **Supplementary 5**). The relative expression level of each miRNA was normalized to the expression of U6, and the cycle threshold (CT) ranged from 15 to 17 cycles.

Immunofluorescence

Two hundred and ninety three cells were transfected with GV251 blank vector or vector containing US33as-5p, followed by selection with G418 (0.5 μg/mL) for 3 days. The cells were then transfected with the pDs-RED-STAT1 vector for 24 h and then stimulated with or without IFNα (1,500 IU/mL) for 12 h. STAT1 nuclear localization was detected by indirect immunofluorescence using an Immunol Fluorescence Staining Kit (Beyotime, Beijing, China) according to the manufacturer's instructions. Fluorescence was visualized with an Olympus FluoView 1000 confocal microscope.

hcmv-33as-5p Knockout With the CRISPR-Cas9 System

hcmv-33as-5p was knocked out with the lentiCRISPR v2 plasmid as described previously (16, 17). Briefly, sgRNAs targeting hcmv-US33as-5p sequences capped by a 5′-N_{20}GG PAM sequence was designed using an online tool (https://zlab.bio/guide-design-resources). The sgRNAs were then synthesized and annealed and ligated to the linearized vector. Lentivirus was produced at a high titer in 293 cells by co-transfection with 2.6 μg of CRISPRv2 vector, 0.25 μg of pVSVg plasmid (Addgene 8454), and 2.5 μg psPAX2 plasmid (Addgene 12260). MRC-5 cells were infected with the Cas9/sgRNA lentivirus (MOI = 0.5), and stable transfectants were selected using puromycin (2.5 μg/mL). The MRC-5 cells were then infected with HCMV (Toledo strain, MOI = 1), and the supernatant, which contained the mutant virus, was collected at 72 hpi. The mutant virus was seeded in 96-well plates containing MRC-5 cells for plaque assays and isolated as described previously (18).

TABLE 1 | Putative hcmv-miR-US33as-5p targets identified by RNA hybrid.

Putative target mRNA	Accession number	Position of binding site	Mfe
Homo sapiens TNF receptor associated factor 5 (TRAF5)	NM_145759	3′UTR	−28.2 kcal/mol
Homo sapiens interferon alpha and beta receptor subunit 1 (IFNAR1)	NM_000629	3′UTR	−30.9 kcal/mol
Homo sapiens signal transducer and activator of transcription 5A (STAT5A)	NM_003152	3′UTR	−35.7 kcal/mol
Homo sapiens adhesion G protein-coupled receptor G1 (ADGRG1)	NM_201524	5′UTR	−33.0 kcal/mol
Homo sapiens serine active site containing 1 (SERAC1)	NM_032861	3′UTR	−29.3 kcal/mol
Homo sapiens kinesin family member 1B (KIF1B)	NM_015074	3′UTR	−35.7 kcal/mol
Homo sapiens upstream binding protein 1 (UBP1)	NM_014517	3′UTR	−32.1 kcal/mol
Homo sapiens BCL2 like 14 (BCL2L14)	NM_030766	3′UTR	−29.2 kcal/mol
Homo sapiens regulator of G protein signaling 20 (RGS20)	NM_170587	3′UTR	−30.5 kcal/mol
Homo sapiens cytochrome p450 oxidoreductase (POR)	NM_000941	3′UTR	−30.9 kcal/mol
Homo sapiens Rho GTPase activating protein 45 (ARHGAP45)	NM_012292	3′UTR	−29.7 kcal/mol
Homo sapiens interferon regulatory factor 8 (IRF8)	NM_002163	3′UTR	−27.9 kcal/mol
Homo sapiens heat shock protein family B (small) member 8 (HSPB8)	NM_014365	3′UTR	−29.1 kcal/mol

Statistical Analyses

All analyses were performed using SPSS software. All data are shown as the means ± standard deviation (SD). Significant differences ($p < 0.05$) were determined by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test or Kruskal-Wallis test followed by the Mann-Whitney U test. All experiments in this study were performed at least twice.

RESULTS

IFNAR1 Is a Putative Target of hcmv-miR-US33as-5p

To investigate the influence of hcmv-miR-US33as-5p on viral infection, 13 putative target mRNAs and their hcmv-miR-US33as-5p-binding sites were identified using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). Detailed information on the targets is provided in **Table 1**, and all putative targets were selected from the human genome database. To further validate these miRNA targets, we constructed 13 pmirGLO-reporter plasmids with primers analyzed by BLAST (**Supplementary 1**). To determine potential targets, the ability of hcmv-miR-US33as-5p to bind the 3′-UTR of each putative mRNA was verified by dual-luciferase reporter assay. From the 13 predictive targets, two targets were confirmed, and the relative luciferase activity in cells co-transfected with pmirGLO-STAT5A-UTR or pmirGLO-IFNAR1-UTR and hcmv-miR-US33as-5p mimics was obviously downregulated by 14.2% ($p = 0.072$) and 27.3% ($p < 0.05$), respectively, compared with that in cells co-transfected with negative control miRNA (**Figure 1A**). Since STAT5 is a transcription factor involved in IFN signaling, we concentrated on the IFNAR1-encoded protein, which activates Jak-STAT signaling and functions as an antiviral factor.

We next investigated the expression of hcmv-miR-US33as-5p during HCMV replication. MRC-5 cells were infected with HCMV (Toledo strain), and total RNA and protein were collected at different time points following infection. Based on melt curve analysis, the relative expression levels were calculated as fold changes relative to the level at 72 hpi. The expression of hcmv-miR-US33as-5p was increased with HCMV replication (**Figure 1B**). In addition, 293 cells were transfected with hcmv-miR-US33as-5p mimics/negative control (NC)-RNA (100 nM) and incubated with 1,500 U/mL IFNα The expression of INFAR1 was determined by western blot analysis. The results showed that the expression of IFNAR1 was reduced at different times postinfection (**Figure 1C**). These results indicate that hcmv-miR-US33as-5p may target IFNAR1 by binding the 3′-UTR of IFNAR1 mRNA.

Mutation of IFNAR1-Binding Sites Abolished the Targeting of IFNAR1 by hcmv-miR-US33as-5p, Which Was Restored by Back Mutation of the hcmv-miR-US33as-5p Seed Region

The putative IFNAR1-binding sites in hcmv-miR-US33as-5p identified by RNAhybrid are listed in **Figure 2A**. To confirm the exact binding site in hcmv-miR-US33as-5p for IFNAR1, we designed two mutations in the IFNAR1-binding site. To generate the first mutant, the nucleotides GCA were mutated to CGC, and to generate the second, the nucleotides AUC were mutated to CCG; these sequences were cloned into the pmirGLO vector, and the resultant plasmids were named IFNAR1-M1 and IFNAR1-M2, respectively. The complementary nucleotide sequences of the hcmv-miR-US33as-5p seed region corresponding to the mutated sites in IFNAR1 were mutated into GCG (US33as-M1) or CGG (US33as-M2) and then cloned into the GV251 vector. The mutagenesis and back mutagenesis protocols are explicitly described in **Figure 2B**.

The pmirGLO-IFNAR1-Mutation-UTR vectors (for the expression of WT IFNAR1, IFNAR1-M1, or IFNAR1-M2) and GV251-US33as-Mutation-UTR vectors (for the expression of blank vector, US33as, US33as-M1, and US33as-M2) were co-transfected into 293 cells cultured in 24-well plates. Dual-luciferase reporter assays were used to determine the fluorescence intensity. The grouping and results are listed in **Figure 2C**. Compared with 293 cells co-transfected with IFNAR1+blank

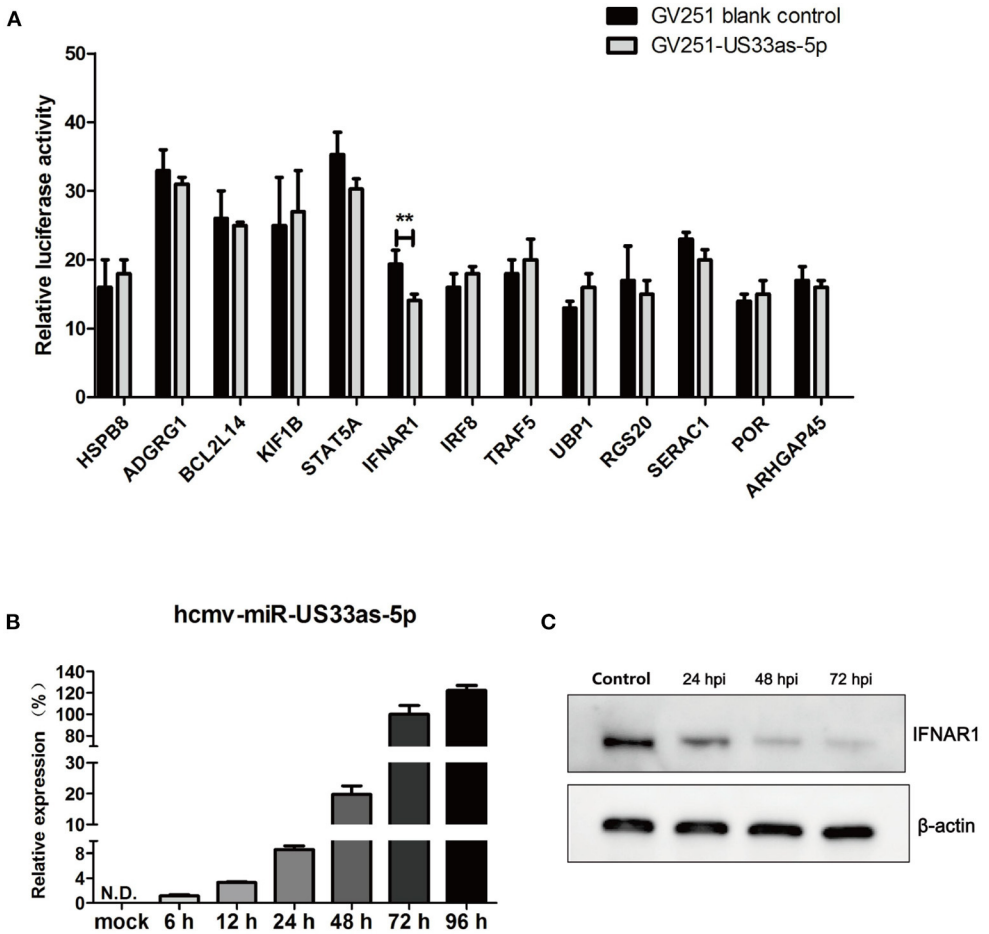

FIGURE 1 | hcmv-miR-US33as-5p inhibited the expression of IFNAR1 by targeting the 3′-UTR of IFNAR1. **(A)** Reporter vectors containing the 3′-UTRs of 13 predicted target genes were co-transfected with GV251 blank vector or vector containing US33as-5p into 293 cells. The dual-luciferase reporter assay was used to determine the relative Renilla luciferase activity normalized to firefly luciferase activity in cells in the corresponding wells at 48 h post-transfection (hpi) when cell lysates were harvested. **(B)** The expression of hcmv-US33as-5p during HCMV infection at different time points was determined by qPCR. U6 was used as a loading control. **(C)** IFNAR1 protein expression at different time points after the transfection of US33as-5p mimics was examined by western blot analysis. The sample of the control group comes from cells transfected with NC-RNA for 72 h. β-Actin was used as a loading control. The assays were performed in triplicate wells, and data were collected from two different experiments and are represented as the means ± SDs; $^{**}p < 0.05$.

vector, 293 cells co-transfected with IFNAR1+US33as showed significantly decreased relative luciferase activity, which was then found to be significantly increased in IFNAR1-M1/IFNAR1-M2+US33as-co-transfected 293 cells. These results indicate that base pairing between the seed region of US33as-5p and the 3′-UTR of IFNAR1 (regardless of the nucleotides at the M1 or M2 site) plays a key role in miRNA function. This is consistent with the findings that mutation of IFNAR1-binding sites disturbed hcmv-miR-US33as-5p binding to IFNAR1 and that back mutation of the hcmv-miR-US33as-5p seed region restored this binding ability, which was validated by western blot analysis (**Figure 2D**). Expression of the IFNAR1 protein was clearly reduced in GV251-US33as-UTR-transfected 293 cells compared to negative control- or GV251-transfected 293 cells, but this reduction was abolished by GV251-US33as-Mutation-UTR transfection.

Interestingly, although base pairing between both IFNAR1-M1+US33as-M1 and IFNAR1-M2+US33as-M2 was correct, the relative luciferase activity upon the transfection of IFNAR1-M1+US33as-M1 did not differ from that of the control group, while relative luciferase activity upon the transfection of IFNAR1-M2+US33as-M2 showed a significant difference compared with that of the control group, indicating that the base sequence at the M1 site plays a more important role in miRNA function than that at the M2 site.

hcmv-miR-US33as-5p Blocks the Jak-STAT Signaling Transduction Pathway by Targeting IFNAR1

Jak-STAT signaling is a necessary cytokine receptor signaling pathway that is important in many processes, including growth

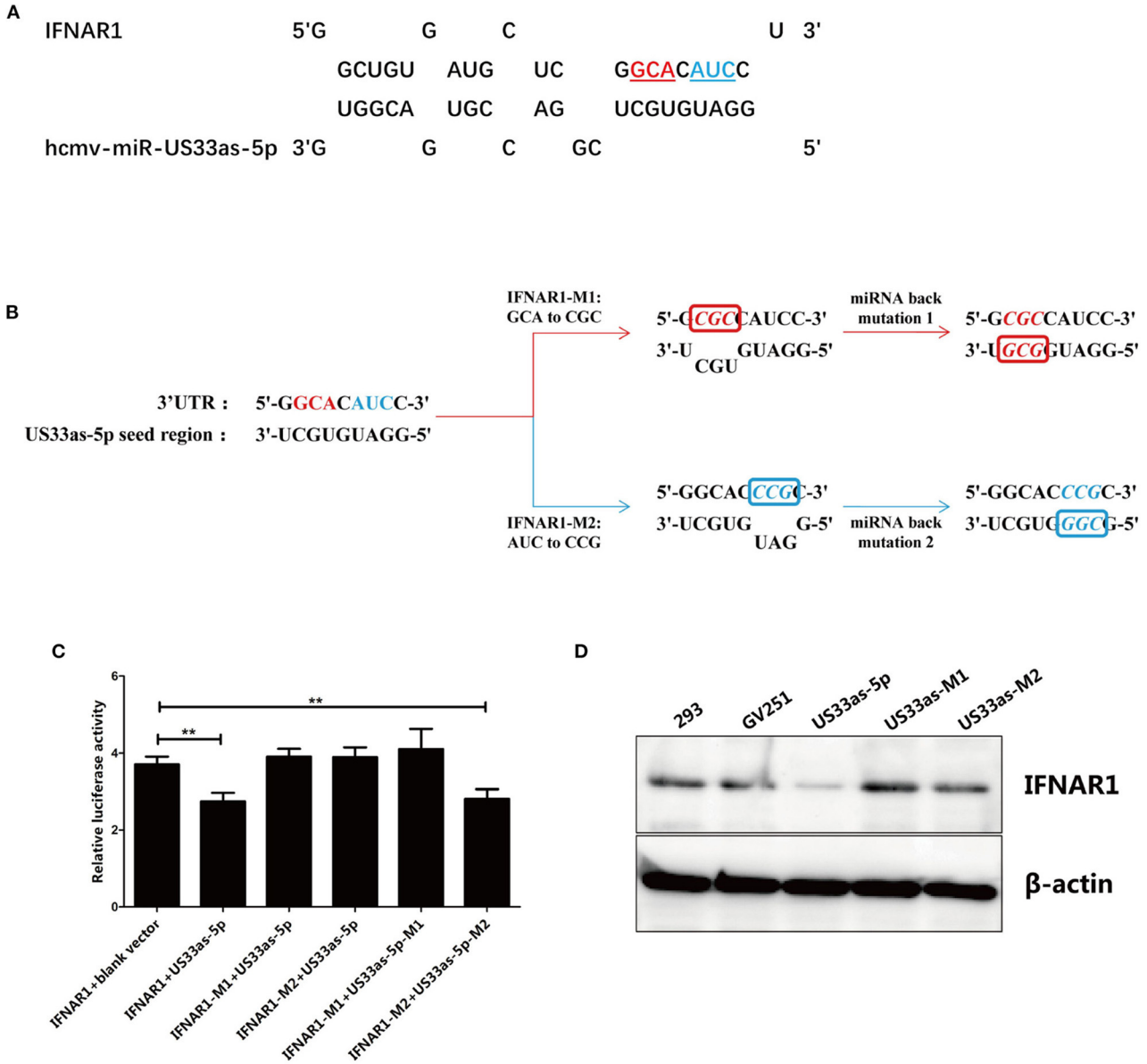

FIGURE 2 | Main binding site in hcmv-miR-US33as-5p for IFNAR1 mRNA. **(A)** Schematic of the targeting of IFNAR1 by hcmv-miR-US33as-5p. Mutated nucleotides are shown in color and underlined. **(B)** Mutation of the IFNAR1-binding site and back mutation of the hcmv-miR-US33as-5p seed region. For IFNAR1 mutagenesis 1, GCA was mutated to CGC, and the miRNA was complementarily mutated to GCG for miR-US33as-5p back mutagenesis 1. For IFNAR1 mutagenesis 2, AUC was mutated to CCG, and the miRNA was complementarily mutated to CGG for miR-US33as-5p back mutagenesis 2. The pmirGLO-IFNAR1-Mutation-UTR vector and GV251-US33as-Mutation-UTR vector were co-transfected into 293 cells cultured in 24-well plates. **(C)** Dual-luciferase reporter assays to determine relative firefly luciferase activity in 293 cells co-transfected with pmirGLO-IFNAR1-Mutation-UTR vector and GV251-US33as-Mutation-UTR vector were performed, and the calculated data are shown in **Figure 1A**. **(D)** Western blot showing the expression of IFNAR1 in 293 cells co-transfected with pmirGLO-IFNAR1(-Mutation)-UTR vector and GV251-US33as(-Mutation)-UTR vector harvested at the same time, with β-actin used as a loading control. The assays were performed in triplicate wells, and data were collected from two different experiments and are represented as the means ± SDs; $^{**}p < 0.05$.

regulation, survival, differentiation, and especially pathogen clearance. Blockade of the binding of type I IFNs to their cell surface receptor (IFNAR1) reduces a series of signaling events in the target cells, therefore undoubtedly inhibiting the conserved Jak-STAT pathway. Notably, HCMV has been demonstrated to encode several factors that counteract the IFN signaling pathway, including HCMV tegument protein pp65 (19–22) and pp71 (23); therefore, a subsequent series of experiments were performed by the transfection of miRNA mimics without HCMV infection, with IFNAR2 also examined as a control target.

Both MRC-5 and HFF cells were cultured in 24-well plates and transiently transfected with hcmv-US33as-5p mimics or

NC-RNA at a concentration of 100 nM for 24 h, followed by 12 h co-culture of serum-free mediums. The cells were then treated with 10 μg/mL CHX to inhibit the synthesis of endogenous IFN, followed by stimulation with 1,500 U/mL IFNα for 1 h until harvest. The mRNA levels were examined by qPCR, and the results showed that both MRC-5 and HFF cells had significantly lower INFAR1 mRNA expression than NC-transfected cells. In contrast, the expression of IFNAR2, which does not have a binding site for miRNA, was not affected by hcmv-miR-US33as-5p mimics (**Figure 3A**). To investigate this further, we treated the transfected MRC-5 cells with ActD, a transcription inhibitor. The cells transfected with hcmv-miR-US33as-5p mimics showed a rapid decline in IFNAR1 expression but no difference in IFNAR2 expression (**Figure 3B**). Taken together, these results suggest that the hcmv-miR-US33as-5p mimics obviously reduced IFNAR1 expression.

Prolonged IFN stimulation for 6 h was carried out to further investigate changes in the downstream pathway. We next evaluated differences in the expression of IFNAR1 and its downstream proteins, which are key proteins in the Jak-STAT signaling pathway, by western blot analysis. The cells transfected with hcmv-miR-US33as-5p showed lower levels of IFNAR1, phosphorylated STAT1, STAT2, Jak1 and Tyk2, suggesting decreased activation of the Jak-STAT signaling pathway, while IFNAR2 showed no difference among groups (**Figure 3C**). In addition, the ISGs Mx1, RSAD2, DDX58, BST2, IFIT2, and ISG20 were selected to analyze changes in ISG mRNA levels by qRT-PCR. The mRNA expression of these ISGs in cells transfected with hcmv-miR-US33as-5p mimics was lower than that in cells transfected with NC-RNA (**Figure 3D**). Therefore, these results showed that hcmv-miR-US33as-5p inhibits the Jak-STAT signaling pathway.

Moreover, immunofluorescence was performed to determine whether hcmv-miR-US33as-5p could inhibit the nuclear translocation of STAT1. As **Figures 4A,B** shows, without IFNα treatment, STAT1 was retained in the cytoplasm in both GV251 blank- and GV251-US33as-5p-transfected cells. After stimulation with IFNα, STAT1 translocated into the nucleus in the GV251 blank-transfected control group but was still retained in the cytoplasm of GV251-US33as-5p-transfected cells. All of the above results reveal that hcmv-miR-US33as-5p can downregulate IFNAR1, STAT1 and ISGs and inhibit STAT1 nuclear translocation by targeting IFNAR1.

HCMV in Which hcmv-miR-US33as-5p Was Knocked Out Was Produced and Verified

To further investigate the effect of hcmv-miR-US33as-5p on viral infection, HCMV with hcmv-miR-US33as-5p knockout was established by the CRISPR-cas9 system. A lentivirus CRISPR-cas9 system targeting HCMV miR-US33as-5p was designed by constructing Cas9- and sgRNA-co-expressing lentiviruses. After recombined lentiviral infection and selection with puromycin, the CRISPR-cas9 system was constructed in MRC-5 cells. These cells were then infected with WT HCMV, and mutant viruses in the supernatant were harvested at 72 hpi. After further plaque assays and the isolation of viral mutants three times, the pure ΔmiRNA HCMV strain was obtained, amplified, and stocked.

To determine whether the targeting region was mutated, DNA was extracted from the three mutant strains of HCMV and Sanger sequenced (**Figure 5A**, forward primer: 5′-AGCGGTCGTGCTTGTCTTTA-3′; reverse primer: 5′-ACGTGGTCCGTCGAAATTGA-3′). We observed that the CRISPR-cas9 system-induced indel mutations in the regions surrounding hcmv-miR-US33as-5p. To further evaluate whether genome editing affected viral proliferation and other miRNAs, MRC-5 cells were separately infected with mutated and WT HCMV, and the viral load was determined at different times (**Figure 5B**). The results indicate that genome editing did not impair viral replication. In addition, analysis of several viral miRNAs at 72 hpi showed no differences in the expression of these other viral miRNAs (**Figure 5C**). HCMV mutant strain #1 (named ΔmiRNA HCMV) was selected and investigated in the next series of experiments.

Mutation of hcmv-miR-US33as-5p Impaired Viral Resistance to IFN

Given the effect of genome editing on the targeting region, we next investigated whether mutation would affect viral infection. Both MRC-5 and HFF cells were infected with WT or ΔmiRNA HCMV for 48 h, followed by treatment with CHX and stimulation with IFN for 24 h. The RNA and DNA were then collected for further analysis. The transcription of IFNAR1 was obviously higher in cells infected with ΔmiRNA HCMV. However, there was no difference in the transcription of IFNAR2 (**Figure 6A**). The results were furtherly determined by western blot (**Figure 6B**). In addition, the relative mRNA levels of all 6 selected ISGs in the WT HCMV-infected cells were obviously lower than those in the ΔmiRNA HCMV-infected cells in the presentation of IFN stimulation (**Figure 6C**). Furthermore, the HCMV copy number in ΔmiRNA HCMV-infected MRC-5 cells was significantly lower than that in the WT HCMV-infected MRC-5 cells in the presentation of IFN, while no obvious difference was seen between groups without IFN incubation (**Figure 6D**). These results indicate that inhibition of hcmv-miR-US33as-5p enhanced the expression of ISGs and weakened HCMV replication, which could impair the resistance of HCMV to IFN.

hcmv-miR-US33as-5p Is Essential for Latent Viral Infection and Viral Reactivation

Many researchers agree with the view that the differentiation of monocytes into macrophages *in vivo* may represent a pivotal process triggering the recurrence of the latent virion, furtherly rendering CMV to disseminate into host tissues (24–27). Among the *in vitro* cell lines to mimic the natural infection, human monocytic leukemia cells (THP-1) and differentiated THP-1 cells (d-THP-1) are universal models in which HCMV latency and reactivation are investigated (6, 28, 29). Considering that herpesvirus miRNAs play key roles in latency and reactivation, the biological function of hcmv-miR-US33as-5p was investigated in THP-1 and d-THP-1 cells.

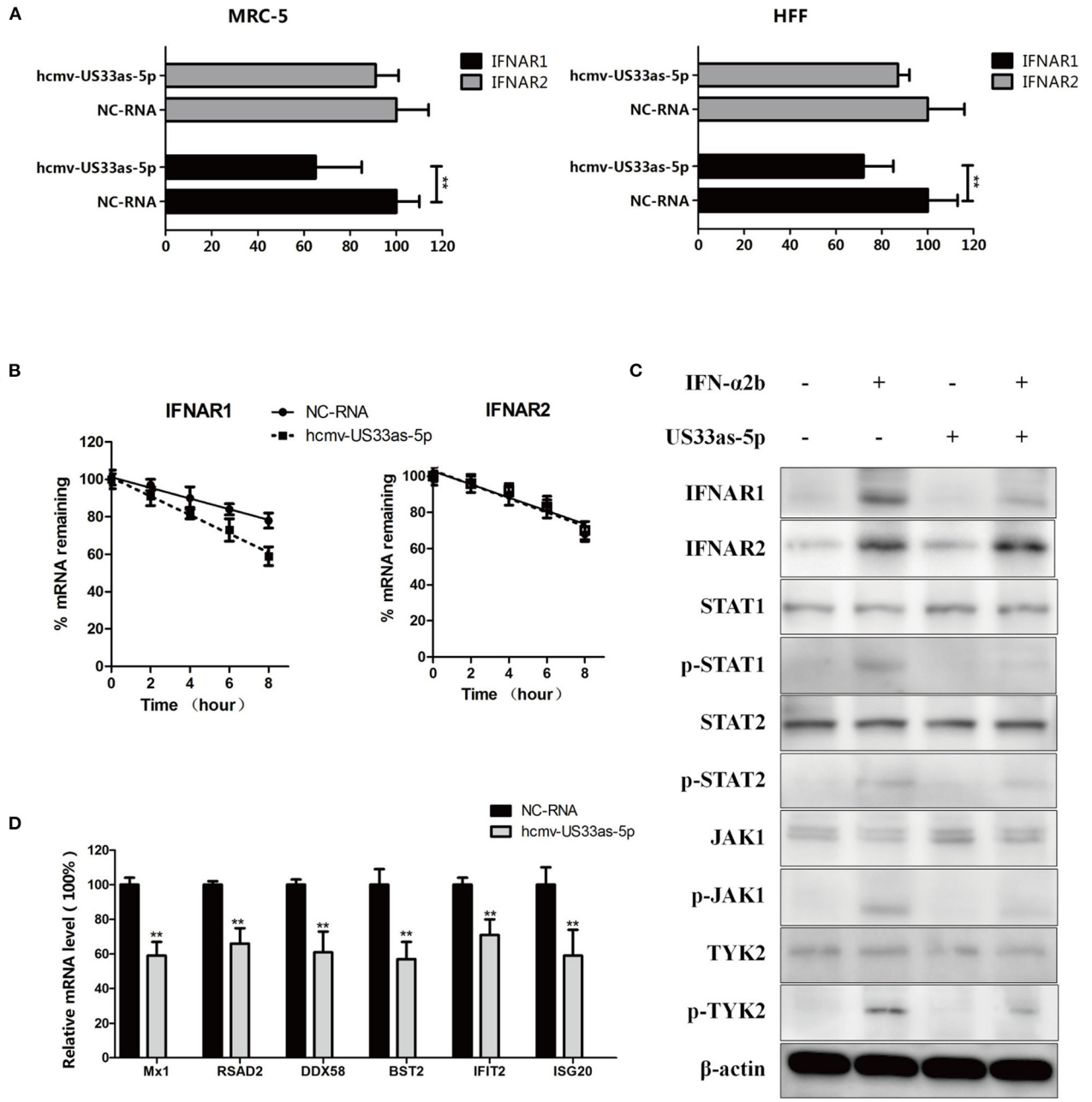

FIGURE 3 | hcmv-miR-US33as-5p downregulates IFNAR1 and Jak-STAT, limiting the release of ISGs. **(A)** MRC-5 and HFF cells were cultured in 24-well plates and transiently transfected with hcmv-US33as-5p mimics or a negative control RNA (NC-RNA) at a concentration of 100 nM for 24 h. The cells were treated with 10 μg/mL CHX, followed by stimulation by 1,500 U/mL IFNα for 1 h until harvest. The expression of IFNAR1 and IFNAR2 was determined by qPCR. **(B)** The mRNA stability of IFNAR1 and IFNAR2 in MRC-5 cells transfected with US33as-5p mimics or NC-RNA and incubated with actinomycin D to inhibit transcription is presented as the amount of mRNA detected at a given time relative to that at 0 h, which was set as 100%. **(C)** Immunoblotting of lysates from MRC-5 cells underwent treatment similar to that described in **Figure 3A** and prolonged incubation with 1,500 U/mL IFN-α for 6 h. was performed. Blots were probed for IFNAR1, IFNAR2, STAT1, STAT2, Jak1, Tyk2 and their phosphorylated forms. β-Actin was run as a loading control. **(D)** The expression of multiple ISGs (Mx1, RSAD2, DDX58, BST2, IFIT2, and ISG20) in MRC-5 cells was determined by qPCR. The RNA harvested from MRC-5 cells underwent treatment similar to that described in **Figure 3C**. GAPDH was amplified as a loading control. The assays were performed in triplicate wells, and data were collected from two different experiments and are represented as the means ± SDs; $^{**}p < 0.05$.

THP-1 cells were infected with WT or ΔmiRNA HCMV and maintained for 10 days in culture medium. Then, the THP-1 cells were stimulated with TPA at 50 ng/mL and further maintained in medium for another 5 days (**Figure 7A**). Expression of the IE gene (UL122) was not detected until 9 days post-infection (dpi) in THP-1 cells and detected after 1 day of TPA

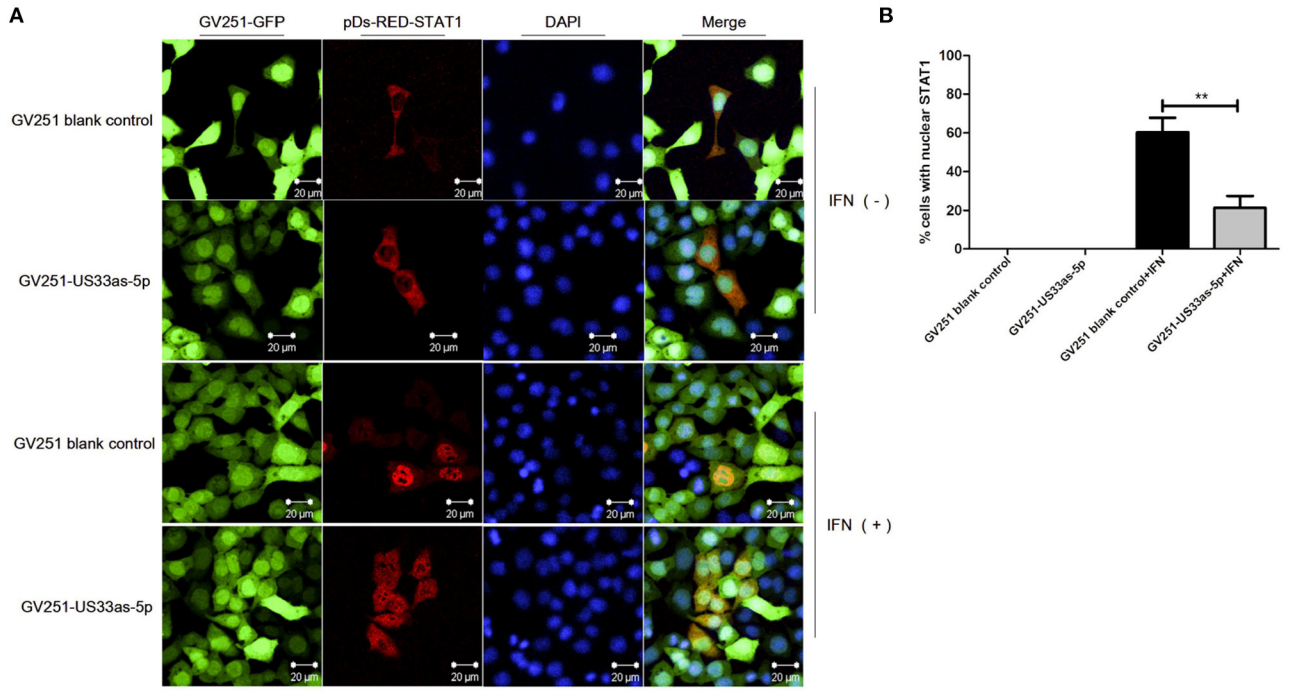

FIGURE 4 | hcmv-miR-US33as-5p inhibits the nuclear translocation of STAT1. **(A)** 293 cells were transfected with GV251 blank vector or vector containing US33as-5p, followed by selection with G418 (0.5 μg/mL) for 3 days. The cells were then transfected with the pDs-RED-STAT1 vector for 24 h and then stimulated with or without IFNα (1,500 IU/mL) for 12 h. STAT1 nuclear localization was detected by indirect immunofluorescence. **(B)** Percentage of cells with nuclear STAT1 from experiments performed in A. Cells were counted in three random fields and the results are expressed as mean ± SD (**$p < 0.01$). Fluorescence was visualized with an Olympus FluoView 1000 confocal microscope. All images were captured under a 60× objective lens.

induction in d-THP-1 cells with both WT and ΔmiRNA HCMV infection, indicating that the latency and reactivation models had been successfully established (**Figure 7B**). We next investigated the expression of hcmv-miR-US33as-5p at different stages of infection (**Figure 7C**). Samples were collected from cells at 9 dpi and 3 days after TPA induction, representative of viral latency and reactivation, respectively. As expected, the expression of hcmv-miR-US33as-5p was not detected in cells infected with ΔmiRNA HCMV. In addition, analysis of miRNA expression indicated that hcmv-miR-US33as-5p was expressed both in latency and reactivation. However, its expression exhibit a higher level in reactivation than in latency. This result suggests that miRNAs can affect viral infection in both latency and reactivation.

In another set of experiments based on the same *in vitro* models of latency and reactivation, THP-1 cells were infected with WT or ΔmiRNA HCMV and stimulated in a culture medium supplemented with or without IFN (500 IU/mL) from infection. The THP-1 cells were further stimulated with TPA to induce differentiation at 10 dpi in viral reactivation groups (see **Figure 8A** for details). The expression of hcmv-miR-US33as-5p was determined by qPCR (**Figure 8B**). The viral loads at 9 dpi or 3 days after TPA induction was then determined by qPCR. The results showed no differences in the effects of WT and ΔmiRNA HCMV during latency and reactivation without IFN stimulation and no difference in viral load between the two groups during quiescent infection in the presence of IFN. However, IFN obviously inhibited viral replication in cells infected with ΔmiRNA HCMV during reactivation compared with cells infected with WT HCMV (**Figure 8C**). The results were furtherly verified by western blot targeting viral IE proteins (**Figure 8D**). To confirm that hcmv-miR-US33as-5p plays a role in HCMV virology during latency, the mRNA level of IFNAR1 9 dpi in THP-1/d-THP-1 cells was examined (**Figure 8E**). In cells with IFN stimulation, cells in both latency and reactivation group infected with ΔmiRNA HCMV had obviously higher IFNAR1 expression levels than those infected with WT HCMV, indicating the biological function of miRNAs during quiescent infection and viral recurrence. This is furtherly determined by examination of transcription of downstream ISGs by qPCR (**Figure 8F**). Taken together, these results suggest that hcmv-miR-US33as-5p serves as a powerful mediator of viral resistance to IFN in latency.

DISCUSSION

Although the detailed mechanism remains to be fully elucidated, researchers have reported several strategies by which viruses subvert the IFN pathway, such as mechanisms involving pp65, pp71 (pUL82), US9, and IE86, all of which are proteins encoded by HCMV (19, 21–23, 30–36). To the best of our knowledge,

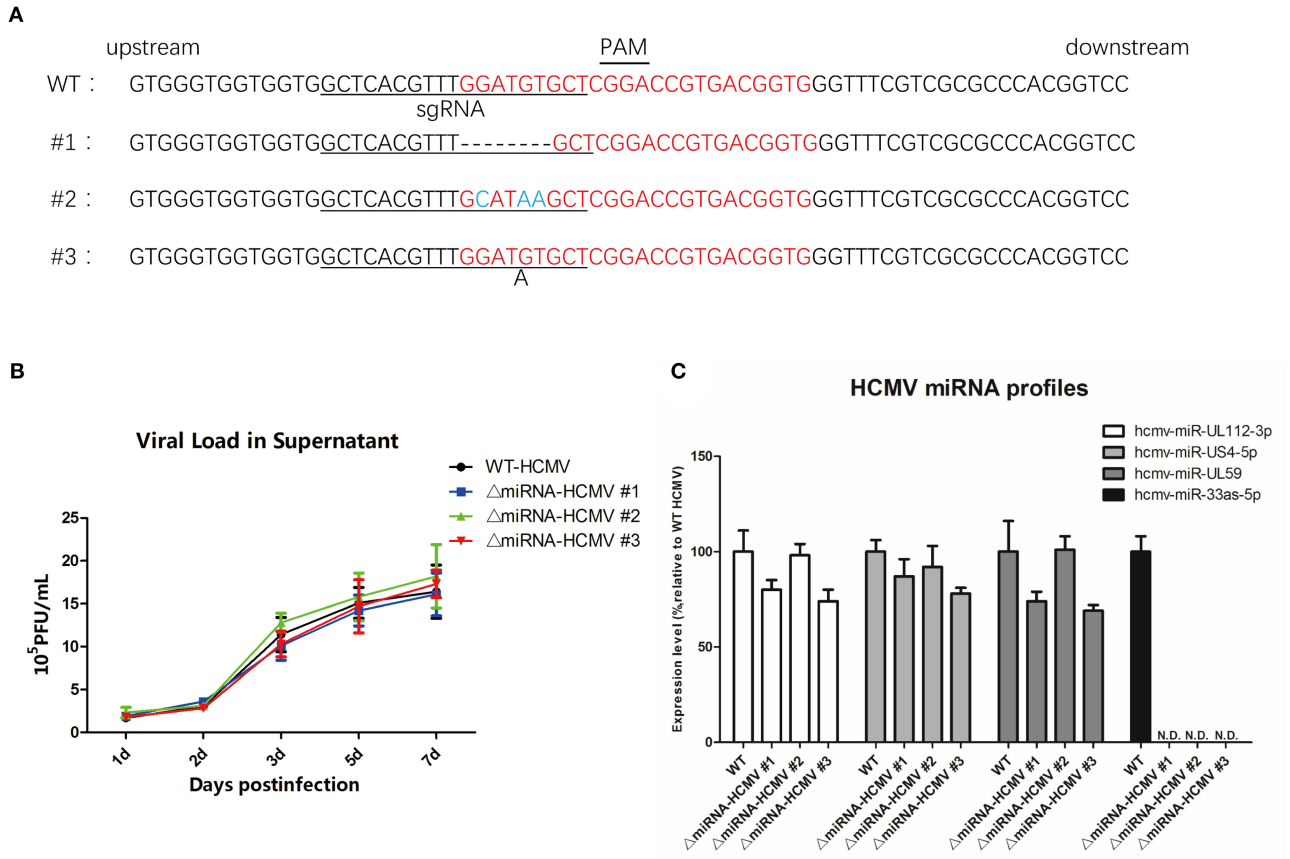

FIGURE 5 | CRISPR-Cas9 introduced mutations in hcmv-US33as-5p and did not obviously affect HCMV virology. **(A)** The DNA sequence surrounding the hcmv-US33as-5p editing region from each HCMV mutant strain was amplified by PCR and analyzed by DNA sequencing. The PAM sequence and sgRNA-targeting region are indicated with lines. The DNA sequence-editing miRNA bases are highlighted in red. Deletions and insertions are presented by dashed lines or a letter underneath the sequence, respectively. No mutations in the control samples were observed. **(B)** MRC-5 cells were infected with WT, ΔmiRNA HCMV #1, ΔmiRNA HCMV #2, or ΔmiRNA HCMV #3 at an MOI = 1, and the viral loads from the supernatants at 1d, 2d, 3d, 5d, 7d were determined by qPCR. **(C)** The expression of several miRNAs encoded by HCMV at 72 hpi was examined by qPCR.

this study is the first to report how HCMV miRNA affects the IFN signaling pathway. In this study, we found that hcmv-miR-US33as-5p encoded by HCMV is expressed during both lytic and latent infection. It binds the 3′-UTR of IFNAR1, blocks IFN stimulation, and further inactivates the JAK-STAT signaling pathway, resulting in the limited release of ISGs (**Figure 9**). HCMV with mutant miR-US33as-5p exhibited a lower resistance to IFN treatment than HCMV with WT miR-US33as-5p. Therefore, we have provided a new approach by which HCMV manipulates the expression of miRNAs to achieve immune evasion.

A total of 26 miRNAs encoded by HCMV are listed in the miRBase database (37), and many of these miRNAs target both viral and cellular genes involved in HCMV processes, such as the maintenance of latency and immune evasion (38, 39). Several miRNAs, such as miR-UL148D and miR-US22, have been reported to play a key role in latency as well as reactivation (13, 40–44). hcmv-miR-US33as-5p, the miRNA discussed in this article, is not included in the miRbase database; however, we and other investigators found that this miRNA is expressed during both lytic and especially latent infection (7). Notably, previous studies analyzed several HCMV-encoded miRNAs expressed during latent infection by directly screening 20 HCMV miRNAs, including miR-UL112-5p, miR-UL36-5p, and miR-UL22A-5p, in human peripheral blood mononuclear cells (PBMCs). However, this screen did not include hcmv-miR-US33as-5p, which maybe because it is not listed in the miRBase database (43).

By analysis with RNAhybrid, we found that hcmv-miR-US33as-5p may interact with the IFN signaling pathway, an approach by which it serves as a pivotal mediator of broad antiviral activity. In the next series of experiments, we found that hcmv-miR-US33as-5p can interact with the 3′-UTR of IFNAR1, especially by base-pairing with its seed region, leading to a reduction in the expression of IFNAR1. Interestingly, we found that the sequence of the M1 site in the miRNA plays a more important role in the biological function of this miRNA than the M2 site, which is near the 5′ end of the miRNA. The results from **Figure 3B** showed lower levels of transcripts, furtherly indicate

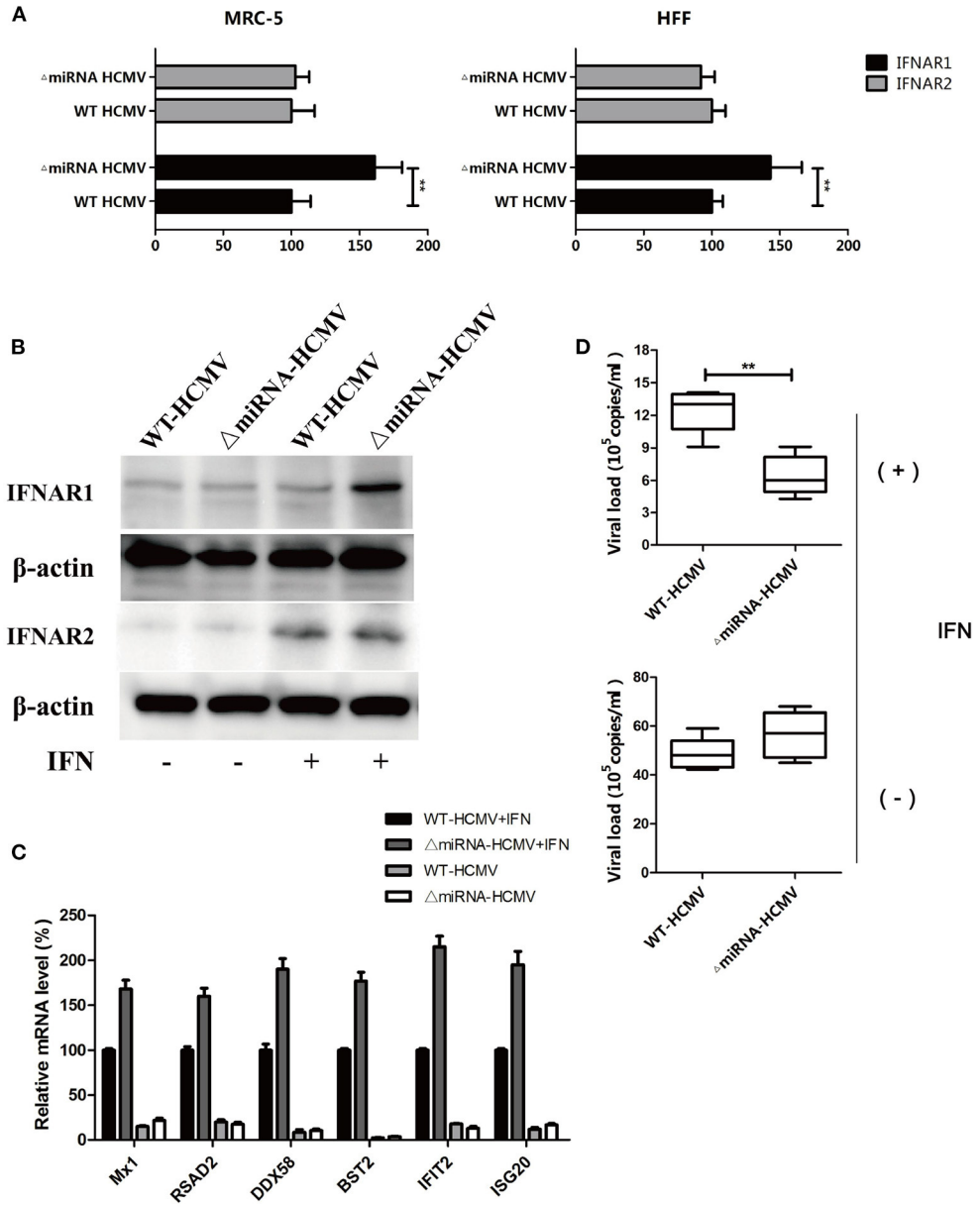

FIGURE 6 | Knockout of hcmv-US33as-5p alleviated blockade of the IFN pathway. **(A,B)** MRC-5 and HFF cells were infected with WT or ΔmiRNA HCMV for 48 h, followed by treatment with CHX and stimulation with IFN for 24 h. The expression of IFNAR1 and IFNAR2 was determined by qPCR and western blot. **(C)** The expression of multiple ISGs was examined by qPCR. **(D)** Total DNA was isolated from the supernatants, and HCMV copy numbers were determined and calculated by qPCR. $^{**}p < 0.05$.

that the hcmv-miR-US33as-5p target IFNAR1 by binding the 3′-UTR of IFNAR1 mRNA to inhibit transcription. Because HCMV can employ multiple strategies to defend against the IFN pathway (45), hcmv-miR-US33as-5p mimics in the absence of HCMV were used to investigate changes in downstream signaling pathways. Consistent with our previous hypothesis, hcmv-miR-US33as-5p mimics obviously inhibited the expression of IFNAR1, inhibiting the phosphorylation of STAT1, STAT2, Tyk2, and Jak1, further reducing the expression of downstream ISGs.

IFNs are virus-inducible cytokines that comprise a primordial and tightly regulated defense system against acute viral infection by activating the conserved Jak-STAT signal transduction pathway, which enhances antiviral function and induces immunoregulatory activities (46). IFNs bind the corresponding receptor IFNAR1, stimulating the release of hundreds of ISGs, but only a few of these ISGs have been characterized with respect to their role in antiviral activity (47). For example, the Mx1 gene encodes a guanosine triphosphate (GTP)-metabolizing

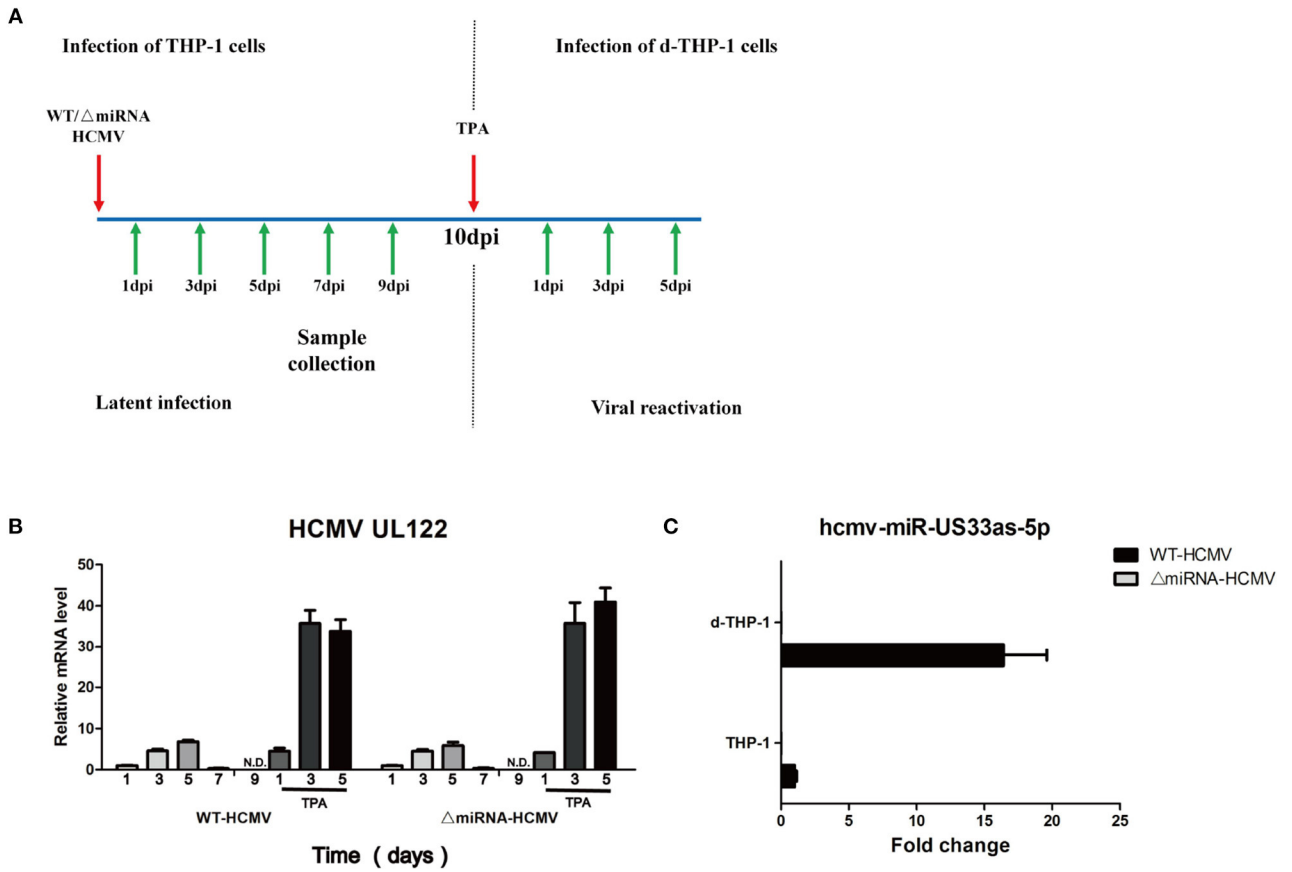

FIGURE 7 | hcmv-US33as-5p is expressed in both viral latency and reactivation. **(A)** A schematic diagram depicting *in vitro* models of latent infection and viral reactivation. THP-1 cells were infected with WT or ΔmiRNA HCMV (MOI = 10) and maintained for 10 days in the culture medium. Then, the THP-1 cells were differentiated into macrophages (d-THP-1) by stimulated with TPA in order to render the cells permissive to HCMV infection and further maintained in the medium for another 5 days. **(B)** The expression of viral UL122 at 1, 3, 5, 7, and 9 dpi and 1, 3, and 5 days after TPA stimulation was detected by qPCR. **(C)** The expression of hcmv-US33as-5p at 9 dpi and 3 days after TPA stimulation was determined by qPCR.

protein that participates in the cellular antiviral response, antagonizing the replication process in RNA or DNA viruses (48). The protein encoded by the DDX58 gene contains RNA helicase-DEAD box protein motifs and a caspase recruitment domain (CARD) involved in viral dsRNA recognition and regulation of the immune response (49). RSAD2 restricts the replication of a wide range of viruses by modulating cellular metabolic pathways essential for viral replication and/or cell proliferation and survival (50). BST2 inhibits viral replication by tethering enveloped virions to the cell surface to restrict viral release and by inducing the NF-κB-dependent antiviral immune response (51). IFITM2 and other proteins in the IFITM family prevent viruses from traversing the lipid bilayer of the cell and accessing the cytoplasm (52). IFIT2 is involved in a nonspecific antiviral program through its direct interaction with eIF3, which subsequently suppresses translation by more than 60% in cells and viruses during protein synthesis (53). HCMV invasion induces the cellular secretion of IFNs, but this strategy is far less effective than the strategy by which HCMV-encoded miR-US33as-5p targets IFNAR1, which abolishes primary immunomodulatory activity and reduces the release of ISGs. Interestingly, several recent research reported that the association between Covid-19, CMV and inflammageing which potentially leads to higher rates of Covid-19-related mortality (54–56). As miR-US33as-5p would also be active during latent infection, this may become relevant for patients who will experience an IFN response and reactivate CMV. Besides, the impact on IFN and downregulation of IFNAR1 has also been studied in cancer models (57–62). Considering that CMV found to be present in a majority of tumor cells in several forms such as brain tumors, breast, colon, prostate, ovarian cancer as well as in metastases (63–67), it is reasonable to propose that CMV miRNAs are involved in cancer progression as well as IFN treatment, by which further investigation to characterize the role of IFN in tumor invasion is required.

To investigate whether hcmv-miR-US33as-5p plays an important role in real infection, we constructed an HCMV strain in which hcmv-miR-US33as-5p was mutated by CRISPR-Cas9 technology. This genome-editing approach is a convenient way to investigate the role of specific genes in viral activity

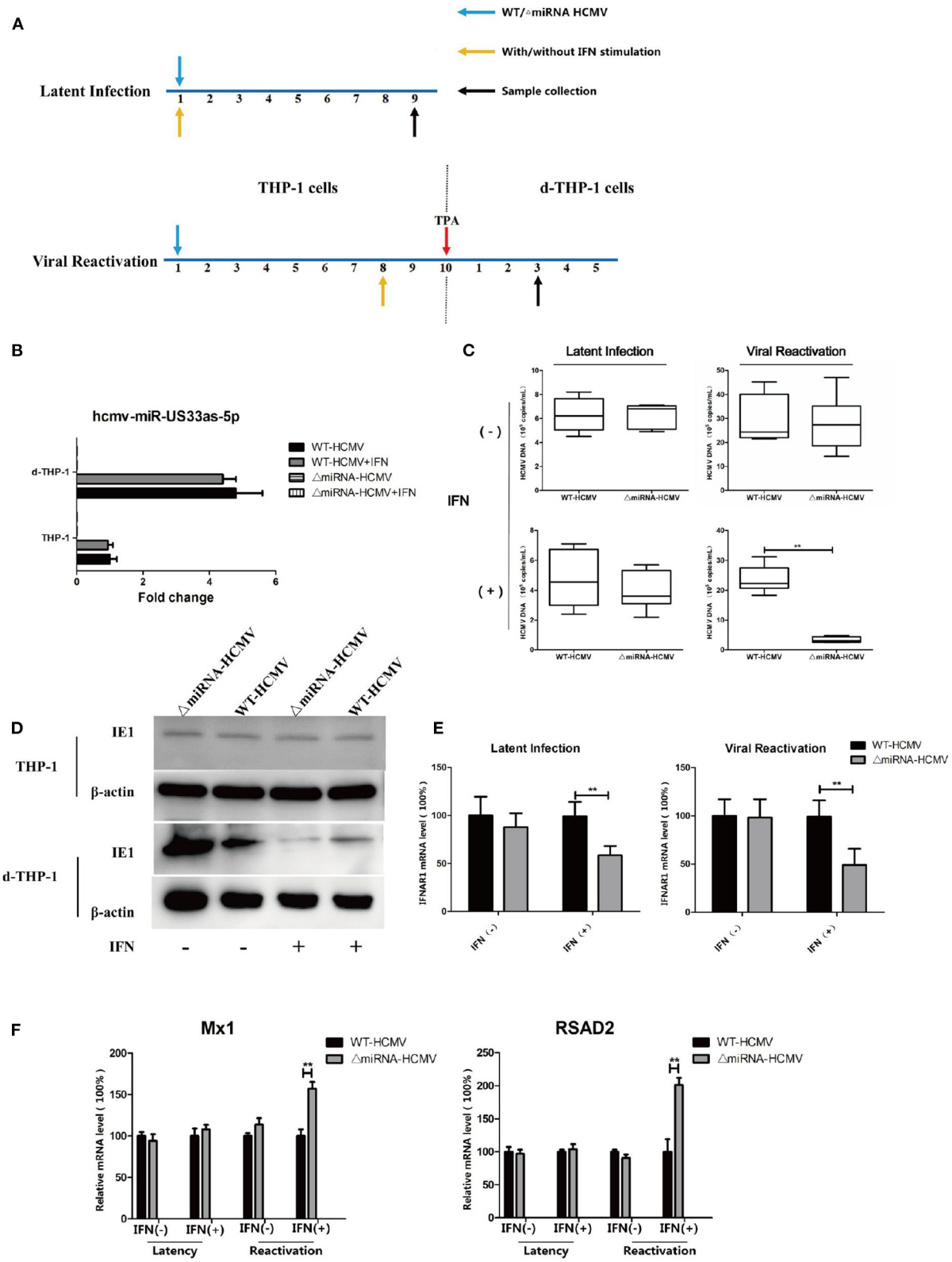

FIGURE 8 | A lack of hcmv-US33as-5p impaired viral resistance to the IFN pathway during latency and reactivation. **(A)** A schematic diagram depicting the *in vitro* models of latent infection and viral reactivation. For latent infection, THP-1 cells were infected with WT or ΔmiRNA HCMV (MOI = 10) and maintained for 9 days in a

(Continued)

FIGURE 8 | culture medium supplemented with or without IFN (500 IU/mL). For viral reactivation, THP-1 cells were infected and maintained for 10 days in the culture medium. Then, the THP-1 cells were stimulated with TPA and further maintained in the medium for another 5 days. The cells were stimulated in a culture medium supplemented with or without IFN (500 IU/mL) beginning at 8 dpi. **(B)** The expression of miRNA among groups was examined by qPCR.THP-1 **(C,D)** The viral loads at indicated days were determined by qPCR and western blot. **(E,F)** The expression of IFNAR1 and ISGs (Mx1, RSAD2) among groups were determined by qPCR. $^{**}p < 0.05$.

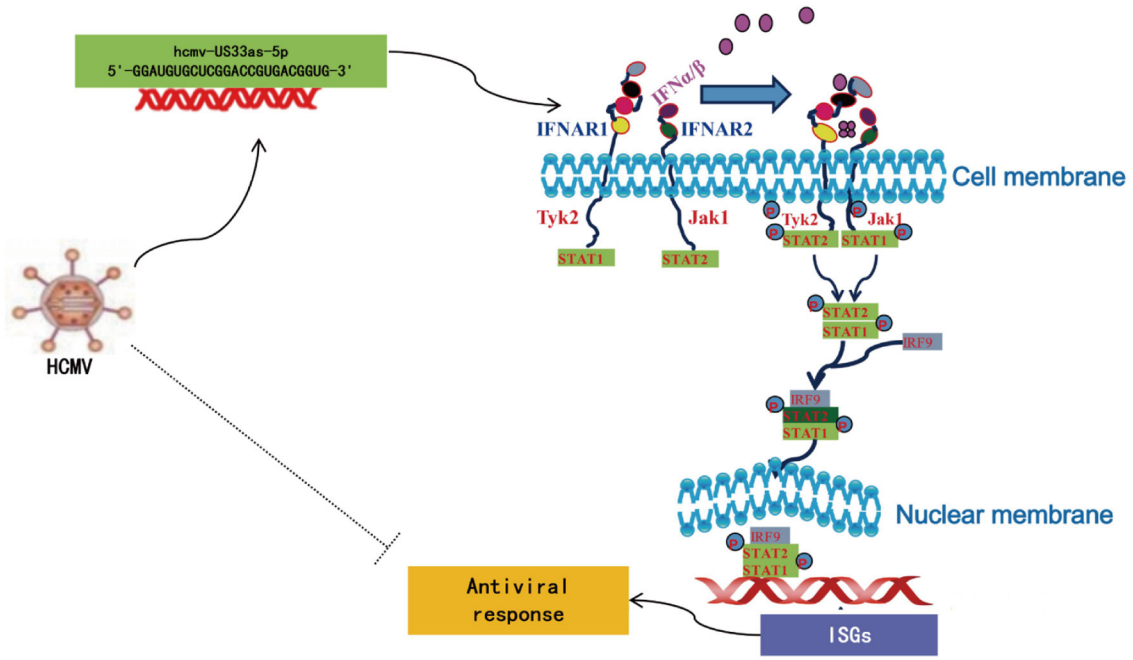

FIGURE 9 | Schematic of the mechanism by which hcmv-US33as-5p facilitates the IFN pathway.

and was reported in several recent studies (18, 68, 69). We provide evidence that this genome-editing strategy did not have an obvious effect on viral proliferation or the expression of other viral miRNAs. Based on this finding, we tested viral resistance to IFN in MRC-5 and HFF cells, THP-1 cells and d-THP-1 cells, which acted as models of primary infection, latency and reactivation, respectively. Upon the viral infection of MRC-5 and HFF cells, we observed that the expression of ISGs in cells infected with the mutant virus was higher than that in cells infected with the WT virus, and the viral load in cells infected with mutant HCMV was obviously lower than that in cells infected with WT virus, suggesting that hcmv-miR-US33as-5p knockout significantly impaired viral resistance to IFN treatment. These effects were slightly different from those on latency and reactivation. IFN treatment showed an obvious inhibitory effect against viral reactivation; however, IFN treatment failed to reduce latent HCMV infection in THP-1 cells but did alter the expression of IFNAR1. Several factors may contribute to these results: (1) Several other mechanisms may have been employed by HCMV to interact with the type I IFN pathway (30, 31, 33, 35, 36), (2) the IFN incubation period may have been too short for IFN to exhibit inhibitory effects, and (3) a lower viral load during latency may only partially reflect the antiviral effects of IFN treatment. We favor the first hypothesis. Nevertheless, the data revealed that hcmv-miR-US33as-5p is involved in evading host immune attack during latency. A previous study indicated that miR-US4-1 encoded by HCMV in human plasma serves as a biomarker for predicting the efficacy of IFN-α treatment in chronic hepatitis B patients (70). Considering the high prevalence of HCMV in the population, further investigation to reveal the clinical link between HCMV miRNAs and the type I IFN response in remains necessary.

When HCMV enters latency, the expression of major viral proteins is silenced, and the replication cycle of the virus stops. It is believed that viral proteins expressed during latency are recognized by the host immune system, while as a non-immunogenic approach, viral miRNAs benefit viral persistence in the presentation of immune surveillance. Recently, two groups separately identified miR-UL-148D and miR-US22 as playing a key role in viral reactivation, demonstrating that mutant cytomegalovirus (CMV) failed to reactivate in an *in vitro* hematopoietic progenitor cell (HPC) model. Therefore, viral-encoded miRNAs may be ideal in the environment of latent infection, in which cells harbor viruses for long periods and subtle effects on gene regulation may be effective.

In conclusion, our research provides the first details in understanding the molecule mechanisms by which HCMV-encoded miRNAs dampen type I IFN to achieve immune evasion. However, the present study has some limitations. The latency model established in THP-1 cells may be restricted to mimicking quiescent HCMV infection in the host, and more investigations in other experimental HCMV latency models, such as Kasumi-3 cells and CD34+ primary HPCs (43), as well as clinical samples are needed. Therefore, further research to illustrate the role of hcmv-miR-US33as-5p in an *in vivo* model is required and may provide insight into how CMV establishes lifelong infection in the face of the host immune response.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

QZ designed, performed, analyzed the *in vitro* experiments, and wrote the manuscript. XS designed and performed an analysis of samples. PM and LL assisted with the design of laboratory methods. YangZ designed and performed statistical analyses. JD and YanyZ designed and oversaw the project and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev Med Virol.* (2010) 20:202–13. doi: 10.1002/rmv.655
2. Britt W. Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease. *Curr Top Microbiol Immunol.* (2008) 325:417–70. doi: 10.1007/978-3-540-77349-8_23
3. Noriega V, Redmann V, Gardner T, Tortorella D. Diverse immune evasion strategies by human cytomegalovirus. *Immunol Res.* (2012) 54:140–51. doi: 10.1007/s12026-012-8304-8
4. Shukla GC, Singh J, Barik S. MicroRNAs: processing, maturation, target recognition and regulatory functions. *Mol Cell Pharmacol.* (2011) 3:83–92.
5. Lim LP, Glasner ME, Yekta S, Burge CB, Bartel DP. Vertebrate microRNA genes. *Science.* (2003) 299:1540. doi: 10.1126/science.1080372
6. Shen ZZ, Pan X, Miao LF, Ye HQ, Chavanas S, Davrinche C, et al. Comprehensive analysis of human cytomegalovirus microRNA expression during lytic and quiescent infection. *PLoS ONE.* (2014) 9:e88531. doi: 10.1371/journal.pone.0088531
7. Stark TJ, Arnold JD, Spector DH, Yeo GW. High-resolution profiling and analysis of viral and host small RNAs during human cytomegalovirus infection. *J Virol.* (2012) 86:226–35. doi: 10.1128/JVI.05903-11
8. Grey F. Role of microRNAs in herpesvirus latency and persistence. *J Gen Virol.* (2015) 96(Pt. 4):739–51. doi: 10.1099/vir.0.070862-0
9. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell Host Microbe.* (2009) 5:376–85. doi: 10.1016/j.chom.2009.03.003
10. Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M, et al. Host immune system gene targeting by a viral miRNA. *Science.* (2007) 317:376–81. doi: 10.1126/science.1140956
11. Abend JR, Ramalingam D, Kieffer-Kwon P, Uldrick TS, Yarchoan R, Ziegelbauer JM. Kaposi's sarcoma-associated herpesvirus microRNAs target IRAK1 and MYD88, two components of the toll-like receptor/interleukin-1R signaling cascade, to reduce inflammatory-cytokine expression. *J Virol.* (2012) 86:11663–74. doi: 10.1128/JVI.01147-12
12. Hook LM, Grey F, Grabski R, Tirabassi R, Doyle T, Hancock M, et al. Cytomegalovirus miRNAs target secretory pathway genes to facilitate formation of the virion assembly compartment and reduce cytokine secretion. *Cell Host Microbe.* (2014) 15:363–73. doi: 10.1016/j.chom.2014.02.004
13. Kim Y, Lee S, Kim S, Kim D, Ahn JH, Ahn K. Human cytomegalovirus clinical strain-specific microRNA miR-UL148D targets the human chemokine RANTES during infection. *PLoS Pathog.* (2012) 8:e1002577. doi: 10.1371/journal.ppat.1002577
14. Xia T, O'Hara A, Araujo I, Barreto J, Carvalho E, Sapucaia JB, et al. EBV microRNAs in primary lymphomas and targeting of CXCL-11 by ebv-mir-BHRF1-3. *Cancer Res.* (2008) 68:1436–42. doi: 10.1158/0008-5472.CAN-07-5126
15. Deng J, Xiao J, Ma P, Gao B, Gong F, Lv L, et al. Manipulation of viral MicroRNAs as a potential antiviral strategy for the treatment of cytomegalovirus infection. *Viruses.* (2017) 9:118. doi: 10.3390/v9050118
16. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods.* (2014) 11:783–4. doi: 10.1038/nmeth.3047
17. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science.* (2014) 343:84–7. doi: 10.1126/science.1247005
18. King MW, Munger J. Editing the human cytomegalovirus genome with the CRISPR/Cas9 system. *Virology.* (2019) 529:186–94. doi: 10.1016/j.virol.2019.01.021
19. Abate DA, Watanabe S, Mocarski ES. Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. *J Virol.* (2004) 78:10995–1006. doi: 10.1128/JVI.78.20.10995-11006.2004
20. Biolatti M, Dell'Oste V, Pautasso S, Gugliesi F, von EJ, Krapp C, et al. Human cytomegalovirus tegument protein pp65 (pUL83) dampens type I interferon production by inactivating the DNA sensor cGAS without affecting STING. *J Virol.* (2018) 92:e01774-17. doi: 10.1128/JVI.01774-17
21. Browne EP, Shenk T. Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. *Proc Natl Acad Sci USA.* (2003) 100:11439–44. doi: 10.1073/pnas.1534570100
22. Li T, Chen J, Cristea IM. Human cytomegalovirus tegument protein pUL83 inhibits IFI16-mediated DNA sensing for immune evasion. *Cell Host Microbe.* (2013) 14:591–9. doi: 10.1016/j.chom.2013.10.007
23. Fu YZ, Su S, Gao YQ, Wang PP, Huang ZF, Hu MM, et al. Human cytomegalovirus tegument protein UL82 inhibits STING-mediated signaling to evade antiviral immunity. *Cell Host Microbe.* (2017) 21:231–43. doi: 10.1016/j.chom.2017.01.001
24. Reeves MB, MacAry PA, Lehner PJ, Sissons JG, Sinclair JH. Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the

dendritic cells of healthy carriers. *Proc Natl Acad Sci USA*. (2005) 102:4140–5. doi: 10.1073/pnas.0408994102
25. Taylor-Wiedeman J, Sissons P, Sinclair J. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J Virol.* (1994) 68:1597–604. doi: 10.1128/JVI.68.3.1597-1604.1994
26. Sinclair J, Sissons P. Latency and reactivation of human cytomegalovirus. *J Gen Virol.* (2006) 87(Pt. 7):1763–79. doi: 10.1099/vir.0.81891-0
27. Streblow DN, Nelson JA. Models of HCMV latency and reactivation. *Trends Microbiol.* (2003) 11:293–5. doi: 10.1016/S0966-842X(03)00149-5
28. Meshesha MK, Bentwich Z, Solomon SA, Avni YS. *In vivo* expression of human cytomegalovirus (HCMV) microRNAs during latency. *Gene.* (2016) 575:101–7. doi: 10.1016/j.gene.2015.08.040
29. Arcangeletti MC, Vasile SR, Rodighiero I, De Conto F, Medici MC, Maccari C, et al. Human cytomegalovirus reactivation from latency: validation of a "switch" model *in vitro*. *Virol J.* (2016) 13:179. doi: 10.1186/s12985-016-0634-z
30. Kim JE, Kim YE, Stinski MF, Ahn JH, Song YJ. Human cytomegalovirus IE2 86 kDa protein induces STING degradation and inhibits cGAMP-mediated IFN-β induction. *Front Microbiol.* (2017) 8:1854. doi: 10.3389/fmicb.2017.01854
31. Choi HJ, Park A, Kang S, Lee E, Lee TA, Ra EA, et al. Human cytomegalovirus-encoded US9 targets MAVS and STING signaling to evade type I interferon immune responses. *Nat Commun.* (2018) 9:125. doi: 10.1038/s41467-017-02624-8
32. Miller DM, Zhang Y, Rahill BM, Waldman WJ, Sedmak DD. Human cytomegalovirus inhibits IFN-alpha-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-alpha signal transduction. *J Immunol.* (1999) 162:6107–13.
33. Baron M, Davignon JL. Inhibition of IFN-gamma-induced STAT1 tyrosine phosphorylation by human CMV is mediated by SHP2. *J Immunol.* (2008) 181:5530–6. doi: 10.4049/jimmunol.181.8.5530
34. Juckem LK, Boehme KW, Feire AL, Compton T. Differential initiation of innate immune responses induced by human cytomegalovirus entry into fibroblast cells. *J Immunol.* (2008) 180:4965–77. doi: 10.4049/jimmunol.180.7.4965
35. Ashley CL, Glass MS, Abendroth A, McSharry BP, Slobedman B. Nuclear domain 10 components upregulated via interferon during human cytomegalovirus infection potently regulate viral infection. *J Gen Virol.* (2017) 98:1795–805. doi: 10.1099/jgv.0.000858
36. Biolatti M, Dell'Oste V, De Andrea M, Landolfo S. The human cytomegalovirus tegument protein pp65 (pUL83): a key player in innate immune evasion. *New Microbiol.* (2018) 41:87–94.
37. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* (2014) 42:D68–73. doi: 10.1093/nar/gkt1181
38. Hook L, Hancock M, Landais I, Grabski R, Britt W, Nelson JA. Cytomegalovirus microRNAs. *Curr Opin Virol.* (2014) 7:40–6. doi: 10.1016/j.coviro.2014.03.015
39. Ng KR, Li JY, Gleadle JM. Human cytomegalovirus encoded microRNAs: hitting targets. *Expert Rev Anti Infect Ther.* (2015) 13:1469–79. doi: 10.1586/14787210.2015.1106939
40. Mikell I, Crawford LB, Hancock MH, Mitchell J, Buehler J, Goodrum F, et al. HCMV miR-US22 down-regulation of EGR-1 regulates CD34+ hematopoietic progenitor cell proliferation and viral reactivation. *PLoS Pathog.* (2019) 15:e1007854. doi: 10.1371/journal.ppat.1007854
41. Lau B, Poole E, Krishna B, Sellart I, Wills MR, Murphy E, et al. The Expression of human cytomegalovirus MicroRNA MiR-UL148D during latent infection in primary myeloid cells inhibits activin A-triggered secretion of IL-6. *Sci Rep.* (2016) 6:31205. doi: 10.1038/srep33771
42. Talaya A, Giménez E, Pascual MJ, Gago B, Piñana JL, Hernández-Boluda JC, et al. An investigation of the utility of plasma Cytomegalovirus (CMV) microRNA detection to predict CMV DNAemia in allogeneic hematopoietic stem cell transplant recipients. *Med Microbiol Immunol.* (2020) 209:15–21. doi: 10.1007/s00430-019-00632-7
43. Pan C, Zhu D, Wang Y, Li L, Li D, Liu F, et al. Human cytomegalovirus miR-UL148D facilitates latent viral infection by targeting host cell immediate early response gene 5. *PLoS Pathog.* (2016) 12:e1006007. doi: 10.1371/journal.ppat.1006007
44. Wang YP, Qi Y, Huang YJ, Qi ML, Ma YP, He R, et al. Identification of immediate early gene X-1 as a cellular target gene of hcmv-mir-UL148D. *Int J Mol Med.* (2013) 31:959–66. doi: 10.3892/ijmm.2013.1271
45. Biolatti M, Gugliesi F, Dell'Oste V, Landolfo S. Modulation of the innate immune response by human cytomegalovirus. *Infect Genet Evol.* (2018) 64:105–14. doi: 10.1016/j.meegid.2018.06.025
46. Zou J, Secombes CJ. Teleost fish interferons and their role in immunity. *Dev Comp Immunol.* (2011) 35:1376–87. doi: 10.1016/j.dci.2011.07.001
47. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature.* (2011) 472:481–5. doi: 10.1038/nature09907
48. Jørgensen JB, Johansen A, Hegseth MN, Zou J, Robertsen B, Collet B, et al. A recombinant CHSE-214 cell line expressing an Mx1 promoter-reporter system responds to both interferon type I and type II from salmonids and represents a versatile tool to study the IFN-system in teleost fish. *Fish Shellfish Immunol.* (2007) 23:1294–303. doi: 10.1016/j.fsi.2007.07.008
49. Liu G, Park HS, Pyo HM, Liu Q, Zhou Y. Influenza A virus panhandle structure is directly involved in RIG-I activation and interferon induction. *J Virol.* (2015) 89:6067–79. doi: 10.1128/JVI.00232-15
50. Honarmand EK. A unifying view of the broad-spectrum antiviral activity of RSAD2 (viperin) based on its radical-SAM chemistry. *Metallomics.* (2018) 10:539–52. doi: 10.1039/C7MT00341B
51. Kong N, Shan T, Wang H, Jiao Y, Zuo Y, Li L, et al. BST2 suppresses porcine epidemic diarrhea virus replication by targeting and degrading virus nucleocapsid protein with selective autophagy. *Autophagy.* (2019) 16:1737–52. doi: 10.1080/15548627.2019.1707487
52. Diamond MS, Farzan M. The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nat Rev Immunol.* (2013) 13:46–57. doi: 10.1038/nri3344
53. Zhou X, Michal JJ, Zhang L, Ding B, Lunney JK, Liu B, et al. Interferon induced IFIT family genes in host antiviral defense. *Int J Biol Sci.* (2013) 9:200–8. doi: 10.7150/ijbs.5613
54. Kadambari S, Klenerman P, Pollard AJ. Why the elderly appear to be more severely affected by COVID-19: The potential role of immunosenescence and CMV. *Rev Med Virol.* (2020) 30:e2144. doi: 10.1002/rmv.2144
55. Honore PM, Barreto GL, Kugener L, Redant S, Attou R, Gallerani A, et al. SARS-CoV-2 infection as a risk factor for herpesviridae reactivation: consider the potential influence of corticosteroid therapy. *Crit Care.* (2020) 24:623. doi: 10.1186/s13054-020-03349-9
56. Lehner GF, Klein SJ, Zoller H, Peer A, Bellmann R, Joannidis M. Correlation of interleukin-6 with Epstein-Barr virus levels in COVID-19. *Crit Care.* (2020) 24:657. doi: 10.1186/s13054-020-03384-6
57. Vitale G, van Eijck CH, van Koetsveld Ing PM, Erdmann JI, Speel EJ, van der Wansem Ing K, et al. Type I interferons in the treatment of pancreatic cancer: mechanisms of action and role of related receptors. *Ann Surg.* (2007) 246:259–68. doi: 10.1097/01.sla.0000261460.07110.f2
58. Du B, Waxman DJ. Medium dose intermittent cyclophosphamide induces immunogenic cell death and cancer cell autonomous type I interferon production in glioma models. *Cancer Lett.* (2020) 470:170–80. doi: 10.1016/j.canlet.2019.11.025
59. Gangaplara A, Martens C, Dahlstrom E, Metidji A, Gokhale AS, Glass DD, et al. Type I interferon signaling attenuates regulatory T cell function in viral infection and in the tumor microenvironment. *PLoS Pathog.* (2018) 14:e1006985. doi: 10.1371/journal.ppat.1006985
60. Katlinski KV, Gui J, Katlinskaya YV, Ortiz A, Chakraborty R, Bhattacharya S, et al. Inactivation of interferon receptor promotes the establishment of immune privileged tumor microenvironment. *Cancer Cell.* (2017) 31:194–207. doi: 10.1016/j.ccell.2017.01.004
61. Evgin L, Huff AL, Wongthida P, Thompson J, Kottke T, Tonne J, et al. Oncolytic virus-derived type I interferon restricts CAR T cell therapy. *Nat Commun.* (2020) 11:3187. doi: 10.1038/s41467-020-17011-z
62. Lu C, Klement JD, Ibrahim ML, Xiao W, Redd PS, Nayak-Kapoor A, et al. Type I interferon suppresses tumor growth through activating the STAT3-granzyme B pathway in tumor-infiltrating cytotoxic T lymphocytes. *J Immunother Cancer.* (2019) 7:157. doi: 10.1186/s40425-019-0635-8
63. Pandey RK, Ojha R, Dipti K, Kumar R, Prajapati VK. Immunoselective algorithm to devise multi-epitope subunit vaccine fighting against human cytomegalovirus infection. *Infect Genet Evol.* (2020) 82:104282. doi: 10.1016/j.meegid.2020.104282

64. Paradowska E, Jabłońska A, Studzińska M, Wilczyński M, Wilczyński JR. Detection and genotyping of CMV and HPV in tumors and fallopian tubes from epithelial ovarian cancer patients. *Sci Rep.* (2019) 9:19935. doi: 10.1038/s41598-019-56448-1
65. Joseph GP, McDermott R, Baryshnikova MA, Cobbs CS, Ulasov IV. Cytomegalovirus as an oncomodulatory agent in the progression of glioma. *Cancer Lett.* (2017) 384:79–85. doi: 10.1016/j.canlet.2016.10.022
66. Richardson AK, Walker LC, Cox B, Rollag H, Robinson BA, Morrin H, et al. Breast cancer and cytomegalovirus. *Clin Transl Oncol.* (2020) 22:585–602. doi: 10.1007/s12094-019-02164-1
67. Massara L, Khairallah C, Yared N, Pitard V, Rousseau B, Izotte J, et al. Uncovering the anticancer potential of murine cytomegalovirus against human colon cancer cells. *Mol Ther Oncolytics.* (2020) 16:250–61. doi: 10.1016/j.omto.2020.01.007
68. Kamel M, El-Sayed A. Utilization of herpesviridae as recombinant viral vectors in vaccine development against animal pathogens. *Virus Res.* (2019) 270:197648. doi: 10.1016/j.virusres.2019.197648
69. Wang D, Wang XW, Peng XC, Xiang Y, Song SB, Wang YY, et al. CRISPR/Cas9 genome editing technology significantly accelerated herpes simplex virus research. *Cancer Gene Ther.* (2018) 25:93–105. doi: 10.1038/s41417-018-0016-3
70. Pan Y, Wang N, Zhou Z, Liang H, Pan C, Zhu D, et al. Circulating human cytomegalovirus-encoded HCMV-miR-US4-1 as an indicator for predicting the efficacy of IFNα treatment in chronic hepatitis B patients. *Sci Rep.* (2016) 6:23007. doi: 10.1038/srep 23007

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Latent Cytomegalovirus-Driven Recruitment of Activated CD4+ T Cells Promotes Virus Reactivation

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Human cytomegalovirus (HCMV) infection is not cleared by the initial immune response but persists for the lifetime of the host, in part due to its ability to establish a latent infection in cells of the myeloid lineage. HCMV has been shown to manipulate the secretion of cellular proteins during both lytic and latent infection; with changes caused by latent infection mainly investigated in CD34+ progenitor cells. Whilst CD34+ cells are generally bone marrow resident, their derivative CD14+ monocytes migrate to the periphery where they briefly circulate until extravasation into tissue sites. We have analyzed the effect of HCMV latent infection on the secretome of CD14+ monocytes, identifying an upregulation of both CCL8 and CXCL10 chemokines in the CD14+ latency-associated secretome. Unlike CD34+ cells, the CD14+ latency-associated secretome did not induce migration of resting immune cell subsets but did induce migration of activated NK and T cells expressing CXCR3 in a CXCL10 dependent manner. As reported in CD34+ latent infection, the CD14+ latency-associated secretome also suppressed the anti-viral activity of stimulated CD4+ T cells. Surprisingly, however, co-culture of activated autologous CD4+ T cells with latently infected monocytes resulted in reactivation of HCMV at levels comparable to those observed using M-CSF and IL-1β cytokines. We propose that these events represent a potential strategy to enable HCMV reactivation and local dissemination of the virus at peripheral tissue sites.

Keywords: human cytomegalovirus, latency, monocytes, reactivation, CD4+ T cells

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INTRODUCTION

A characteristic of human cytomegalovirus (HCMV), common to all the herpesviruses, is an ability to establish a lifelong latent infection. In healthy individuals, primary infection and subsequent reactivation of latent HCMV rarely cause disease; whereas in immunocompromised or immune suppressed patients, it can be life-threatening (1). Persistent HCMV infection is established in the immune competent despite a broad and robust immune response and this inability of the immune response to completely clear HCMV infection is likely due to the numerous immune evasion

molecules encoded by the virus (2) as well as the ability of the virus to establish a latent infection. CD34+ progenitor cells and their monocyte derivatives are an established site of latent HCMV carriage *in vivo* (3–5) characterized by the carriage of viral genome in the absence of infectious virion production (3). However, viral gene transcription has been reported during latency (6–9) resulting in expression of numerous viral genes involved in the maintenance of viral latency, such as US28 (10–14). Carriage of HCMV in monocytes from the bone marrow to the peripheral tissue sites (15) can result in virus reactivation due to differentiation of monocytes to mature myeloid cells (16) and likely prolongs the lifespan of the infected monocyte (17). Evidence for the periodic subclinical reactivation of the virus has been surmised by the continual presence of large HCMV-specific T cell populations in infected individuals (18) and the suggested association of HCMV persistence with long-term illnesses such as vascular disease (19).

CD34+ progenitor cells – pluripotent cells that give rise to all circulating blood cells, populate the bone marrow environment (20). However, once the bone marrow resident CD34+ cell matures into monocyte derivatives, they migrate from the bone marrow to the peripheral blood (21) circulating for a day or so (22), patrolling the endothelial cell layer in an inactive state (23). The mature monocyte then either leaves the circulation to traffic to tissue sites where they may differentiate to, for instance, tissue resident macrophages (24, 25) or they die *via* apoptosis (22, 23, 26). In normal steady state conditions the type of mature myeloid cell the tissue resident monocyte differentiates into is dependent on signals from the local tissue microenvironment (20). Localized acute inflammation has been shown to recruit CD14+ monocytes in humans to the kidneys, intestine, skin, lungs and heart (25), inflammatory cytokines will provide the localized monocyte with very different signals to a bone marrow resident CD34+ cell. Latency and reactivation of HCMV is directly linked to the differentiation status of the infected cell. Latency is established in bone marrow resident CD34+ cells and the subsequent egress and terminal differentiation of CD34+ cells to macrophages and dendritic cells is concomitant with HCMV reactivation (27), reactivating HCMV has been identified in tissue resident macrophages *in vivo* (28). HCMV also manipulates the host's cellular processes prolonging the life-span of monocyte cells (17, 26, 29) and promoting the migration of monocytes from the circulation into tissue sites (15). Therefore, it is important to improve our understanding of how latent HCMV infection manipulates the host's cellular processes and immune responses in different tissue environments.

The manipulation of secreted cellular proteins (the cell secretome) by HCMV during lytic infection includes the release of factors that induce angiogenesis (30) and the release of inflammatory cytokines (31). We have previously shown that latent HCMV infection in CD34+ progenitors also modulates the cell secretome resulting in increased levels of CCL8, which recruits CD4+ T cells, as well as increased secretion of cellular IL-10 (cIL-10) and TGF-β, which suppress anti-viral functions of recruited CD4+ T cells (32). Another study of latent infection utilizing granulocyte macrophage progenitors have shown increased expression of CCL2, which enhances the migration of monocytes (33). A short-term model of latent infection in CD14+ monocytes revealed secretion of inflammatory immune mediators and promotion of differentiation to a macrophage-like phenotype (34). In a previous study, we also observed that viral IL-10 produced during latent infection of CD14+ monocytes results in upregulation of secretion of cIL-10 and CCL8 (35). However, a comprehensive assessment of the cellular secretome of latently infected monocytes has yet to be described and the effect of this latency-associated secretome on other immune cells has not been addressed.

Thus, using an established experimental model of HCMV latent infection in CD14+ monocytes (36), we have characterized the latency-associated changes in the cell secretome using chemokine and cytokine arrays. Consistent with previous studies in CD34+ and CD14+ cells (34, 35, 37), we observed upregulation of expression of cIL-10, CCL8 and CXCL10 by latently infected monocytes. We go onto show that the latency-associated secretome promoted the recruitment of immune cell subsets; in particular the recruitment of activated NK cells, CD8+ and CD4+ T cells *via* the interaction of CXCR3 expressed by the activated immune cells and CXCL10 present in the secretome. In addition, we also demonstrate that the latent secretome inhibited the production of anti-viral cytokines by stimulated CD4+ T cells. Intriguingly, the co-culture of activated CD4+ T cells with latently infected CD14+ monocytes promoted viral reactivation, likely due to the induction of differentiation pathways in the monocyte. Together, our data suggests that HCMV latently infected monocytes which have migrated to peripheral sites modulate the cellular secretome to enable reactivation but concomitantly prevent immune effector function to allow local dissemination of the virus in order to support long-term persistence of the viral infection of the host.

MATERIALS AND METHODS

Donor Sample and Ethics Statement

Ethical approval for the work on healthy human samples was obtained from the Health Research Authority (HRA) Cambridge Central Research Ethics Committee (97/092) for this study, informed written consent was obtained from all healthy donors in accordance with the Declaration of Helsinki. Heparinized peripheral blood was collected from healthy donors or cells isolated from apheresis cones (National Health Service (NHS) Blood and Transplant Service). HCMV serostatus was determined using an IgG enzyme-linked immunosorbent assay (Trinity Biotech, Co. Wicklow, Ireland). 13 HCMV-seronegative, 7 HCMV-seropositive donors and 3 HCMV-seronegative apheresis cones were used in this study.

Viruses

A low passage isolate of HCMV strain TB40/E and TB40/E UL32-GFP derived from it (a gift from Christian Sinzger, University of Ulm, Germany) and TB40/E-IE2-EYFP virus (a gift from Michael Winkler, Ulm University Hospital, Germany) were used for

infections in this study, as indicated in the text. The infectious titer of the TB40/E strain was determined using HFFF cells; the pfu/ml (plaque forming units) was used to calculate the Multiplicity of Infection used to infect monocytes. The amount of TB40/E UL32-GFP and TB40/E-IE2-EYFP virus strains used to infect monocytes was assessed by titration of a range of concentrations of individual virus stocks on monocytes and choosing the input dose which resulted in a latent infection (relative absence of GFP or EYFP signals) compared to fluorescent cells following treatment with either PMA (Sigma Aldrich, Poole, UK) or M-CSF and IL-1β (Miltenyi Biotec, Bisley, UK), in order to reactivate the virus. Ultra-violet inactivation of virus strains used in this study was performed by placing an aliquot of virus in a tissue culture plate and placing this within 10cm of a UV germicidal (254nm) lamp for 60 minutes to inactivate the virus stock. We routinely test UV inactivated virus by infecting fibroblast and looking for IE protein expression, IE is not detectable by Immunofluorescence in these confirmatory studies.

Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples or apheresis cone mononuclear cells using either Lymphoprep (Axis-shield, Alere Ltd, Stockport, UK) or Histopaque-1077 (Sigma Aldrich) density gradient centrifugation.

HCMV Latency and Infection of Monocytes

CD14+ Monocytes were isolated from donor PBMC by MACS using anti-CD14+ direct beads (Miltenyi Biotech), according to manufacturer's instructions and separated on LS columns or an AutoMACS Pro (Miltenyi Biotec). Purified monocytes were adhered to a tissue culture plate at either 0.1 x 10^6 cells per well density for 96 well plates, 0.3 x 10^6 cells per well for 48 well plates or 0.5 x 10^6 cells per well for 24 well plates, and then incubated overnight in X-VIVO 15 (Lonza, Slough, UK) supplemented with 2.5mM L-Glutamine (Sigma Aldrich) at 37°C in a humidified CO_2 atmosphere.

Monocyte latent secretomes were generated by infecting adherent monocytes with TB40/E strain at a HFFF titrated MOI of 5 or the equivalent amount of UV-inactivated virus for 3 hours at 37°C in L-glutamine supplemented X-VIVO 15. Media was then replaced following a DPBS (Sigma Aldrich) wash and the infected cells were incubated in fresh supplemented X-VIVO 15 at 37°C in a humidified CO_2 atmosphere. The supernatant (secretome) of the Mock, UV irradiated, and latently infected monocytes were collected and then replenished at days 3, 7 and 10 or 14. The collected supernatants were clarified by centrifugation. Latent infection was confirmed by harvesting RNA from the 3 cell treatments at day 7 and using RT-qPCR methods to compare relative expression of UL138 transcripts compared to the relative absence of IE72 transcripts controlled by GAPDH transcripts as explained in the supplementary methods with representative results also shown in **Figure S1**. We have demonstrated that in this experimental model of latency, that by day 7 latency is established, shown by expression of UL138 and absence of IE transcripts (38).

Latent infection of adherent monocytes in 96-well or 48-well plates with strains TB40/E UL32 GFP or TB40/E-IE2-EYFP at a pre-titrated concentration of virus (MOI was dependent on individual virus preparations) was performed for 3 hours in L-glutamine supplemented X-VIVO 15 at 37°C. Media was then replaced following a PBS wash and the infected cells were incubated in fresh supplemented X-VIVO 15 at 37°C in a humidified CO_2 atmosphere for 4 – 6 days to allow latency to establish. At this time RNA was harvested from mock and infected cells to confirm latent infection by RT-qPCR and used for reactivation of HCMV from Latency experiments.

Cytokine and Chemokine Array Analysis

The day 10 secretomes from mock, UV irradiated and Latent virus infected CD14+ monocytes were analyzed by Proteome Profiler Array – Human Chemokine Array Kit (R & D Systems, Abingdon, UK), RayBio Human Cytokine Array C1000 and RayBio Human Cytokine Array C5 (RayBiotech, supplied by Insight Biotechnology Ltd, Wembley, UK) following the manufacturer's instructions. The arrays were imaged by autoradiography and then analyzed by ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.) to measure the density of each spot and to compare the relative amount of proteins expressed in different secretomes. The fold change in proteins expressed by the latent infected secretome were calculated as: $Fold\ change = \frac{(Density\ of\ Latent\ infection\ spot - Density\ of\ Mock\ infected\ spot)}{(Density\ of\ UV\ irradiated\ spot - Density\ of\ Mock\ infected\ spot)}$. Then whether the individual proteins in the array were significantly upregulated across all 3 experiments was tested by multiple student t-tests. The results of this analysis are presented as a volcano plot (39) in **Figure 1A**.

Neutralization of Interferons During the Generation of Latent CD14+ Monocyte Secretomes

Adherent CD14+ monocytes were infected with mock, UV irradiated or TB40/E strain as described above in the presence of excess neutralizing anti-Human Interferon α [3μg; Clone: MMHA-6; EC50 20ng/ml (40)], anti-Human Interferon β [4μg/ml; Clone : MMHB-3 (41)] (both PBL Assay Science, USA) and Ultra-leaf anti-human Interferon γ [10μg/ml; Clone: B27 (42)] (BioLegend, London, UK) antibodies or Mouse IgG1 isotype control (Clone: 11711) (R & D Systems). The secretomes generated were harvested after 10 days, clarified and then analyzed by ELISA for CXCL10 and CCL8 cytokines.

Generating Secretomes From Monocytes Treated With Recombinant Human IFNγ

Uninfected adherent monocytes were treated with recombinant human IFNγ protein (R&D Systems) over a concentration range of 100IU – 3.125IU for 4 hours. The IFNγ containing medium was removed and the cells washed with DPBS twice, then L-glutamine supplemented X-VIVO 15 was added to the treated cells and the cells were incubated at 37°C in a humidified CO_2 atmosphere. Continuous application of 100IU/ml IFNγ was used as a positive control and X-VIVO 15 media alone was used as the

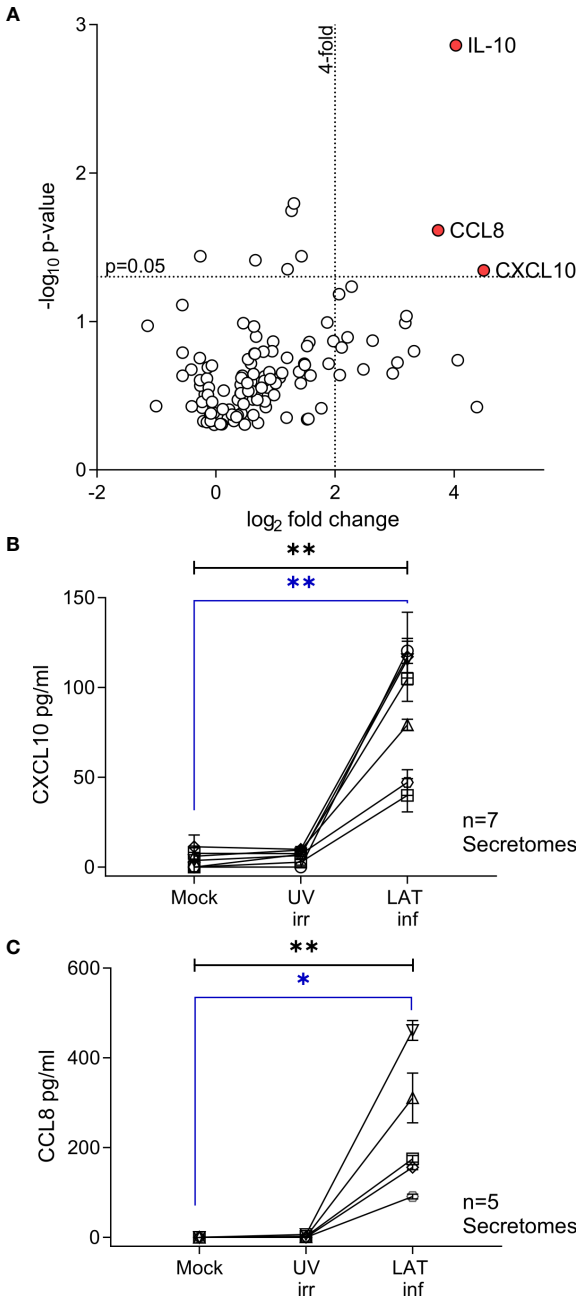

FIGURE 1 | CXCL10, CCL8 and cIL-10 are upregulated in the secretome from latently infected CD14+ monocytes. A Volcano plot summarizing the protein array results from 3 independent day 7 secretomes, significant ($p<0.05$) highly upregulated (values > 4-fold) proteins are indicated by the red circle and label. Data was analyzed as described in the methods. **(A)** Confirmatory ELISA results for the production of CXCL10 **(B)** and CCL8 **(C)** show that both cytokines are significantly upregulated in the HCMV latent infected secretome at day 7. Seven and six independent secretomes were analyzed for CXCL10 and CCL8 production respectively. Friedman 1-way ANOVA results are shown in black on the graph; CXCL10 p=0.0012** and CCL8 p=0.0031**. Post hoc Dunn's test results are shown in blue CXCL10 p=0.004** and CCL8 p=0.0133*.

negative control. Supernatants were harvested and media replenished on days 3, 6 and 10 and then analyzed by ELISA for CXCL10 production.

Cytokine Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

Human IFNγ ELISA MAX Standard and Human CXCL10 and CCL8 ELISA MAX Deluxe sets (all Biolegend) were used to quantify cytokine concentrations in secretomes and supernatants. ELISAs were performed according to the manufacturer's recommended protocols.

Isolation of T Cells, B Cells, Monocytes and NK Cells

PBMCs were sorted into cellular subpopulations by positive selection using anti-CD14 microbeads to isolate monocytes, anti-CD4 microbeads for CD4+ T cells, anti-CD8 microbeads for CD8+ T cells and anti-CD19 microbeads for B cells with either LS columns or an AutoMACS Pro (Miltenyi Biotec). NK cells were isolated by either positive selection using anti-CD56 microbeads (Miltenyi Biotec) or negative selection using the NK cell isolation kit (Miltenyi Biotec) or with the EasySep Human NK cell enrichment kit (Stem Cell Technologies, Grenoble, France) following the manufacturer's instructions.

Preparation of Activated PBMC Subsets

Activated CD4+, CD8+ T and NK cells were generated in two ways. In the first method, isolated CD4+ and CD8+ T cells were re-suspended in RPMI-1640 (Sigma Aldrich) supplemented with 100IU/ml penicillin, 100µg/ml streptomycin and 10% Fetal Calf Serum (Gibco, Paisley, UK or PanBiotech, Wimborne, UK) – RPMI-10 and stimulated with irradiated (solid source γ-irradiator) autologous PBMC and 1µl/ml PHA (Sigma Aldrich) in the presence of 50IU/ml rhIL-2 (CFAR, NIBSC). The polyclonally activated T cell lines were maintained for up to 2 weeks at 37°C in a humidified CO_2 atmosphere, with media and IL-2 replenishment every 5 days. Isolated NK cells were stimulated by an irradiated mixture of autologous PBMC and allogeneic lymphoblast cell line (BLCL) and 50IU/ml IL-2 in RPMI-10 and cultured for up to two weeks at 37°C in a humidified CO_2 atmosphere, with periodic replenishment of media and IL-2. In the other method, total PBMC were stimulated with irradiated allogeneic PBMC and 50IU/ml rhIL-2 for the polyclonal activation of NK cells and the addition of 1µg/ml anti-CD3 (clone CD3-2) and 0.5µg/ml anti-CD28 (clone CD28-A) (both Mabtech AB, Nacka Strand, Sweden) for the polyclonal activation of T cells in RPMI-10. After 5 – 8 days stimulation the activated NK cells and CD4+ and CD8+ T cells were isolated by positive selection as described in section 2.8 or by using the NK cell, CD4+ T cell and CD8+ T cell isolation kits (Miltenyi Biotec) using an AutoMACS Pro, following manufacturer instructions. Activated NK cells and activated PBMC were sorted into two populations of cells (CXCR3+ and CXCR3-) using a BD

FACSAria cell sorter by staining with Live Dead Far-Red (Thermo Fisher Scientific, Loughborough, UK) and CXCR3-PE (BioLegend).

Transwell-Migration and CXCL10 Neutralization Assay

Transwell ChemoTx plates (5-μm pore size and 30-μl well volume) (Neuro Probe Inc, USA) were used to determine cell migration to latent and control secretomes. Cell subsets were fluorescently labelled using Calcein AM (BD Biosciences, Wokingham, UK) according to the manufacturer's protocol. 2 x 10^4 labelled cells in 20μl of X-VIVO-15 per well were transferred to the transwell plate and incubated at 37°C for 2 hours with supernatants from mock, UV and latently infected CD14+ monocytes in the lower chamber. Supernatants from monocyte-derived macrophages stimulated with LPS were used as a positive control, while X-VIVO-15 alone was used as a negative control. Migrated cells were enumerated using an UV microscope, five fields of view of each well were counted and all conditions were run in triplicate. CXCL10 neutralization assays were performed using supernatants or supernatants treated with anti-CXCL10 neutralizing antibodies or IgG2a isotype control (R & D Systems) for 1 hour using the recommended neutralization procedure and dose of the manufacturer, prior to being used in the migration assays.

Flow Cytometry Methods

Phenotyping of Resting and Activated PBMC Subsets

The phenotype of resting and activated NK and T cell subsets was assessed by flow cytometry by staining with 3 antibody cocktails all containing Live Dead Far Red (Thermo Fisher Scientific); and (i) CD56 FITC, CXCR3 PE and CD3 PerCP Cy5.5; (ii) CD4 FITC, CXCR3 PE and CD3 PerCP Cy5.5; (iii) CD3 FITC, CXCR3 PE and CD8 PerCP Cy5.5 (details of antibody clones and manufacturer are listed in **Table S2**), following staining the cells were washed and fixed with 2% Paraformaldehyde in PBS solution (2% PFA (made from 4% PFA in PBS, Santa Cruz Biotechnology Inc, Dallas, USA)) and acquired on a BD Accuri C6 flow cytometer.

Further details of antibody cocktails used to assess the phenotype of resting and activated NK and T cell subsets are detailed in the supplementary methods (section 1.3 and **Table S2**), example gating analysis figures are also included (**Figure S2**).

Phenotyping of Monocytes and CXCR3+ and M-CSF Treated Co Cultured Monocytes

Latent infected monocytes and latent infected monocytes treated with either M-CSF and IL-1β or co-cultured with CXCR3+ T cells were harvested using Accutase (BioLegend). Details of the antibodies and methods used to analyze these samples can be found in the supplementary methods (Section 1.3, **Table S2** and **Figure S2**).

HCMV Reactivation Experiments

Adherent monocytes were latently infected with either TB40/E-IE2-EYFP or TB40/E UL32-GFP strain of HCMV as described above. Between 4-days – 6-days infection the latently infected CD14+ monocytes were treated with either CXCR3+ sorted PBMC, activated CD8+, CD4+ T cells, NK cells, 20ng/ml M-CSF and 10ng/ml IL-1β (both Miltenyi Biotec) or PMA (Sigma Aldrich). The treated monocytes were observed by fluorescent microscope and EYFP or GFP expressing cells enumerated on the subsequent days post treatment.

To assess whether latently infected CD14+ monocytes treated with activated CD4+ T cells and M-CSF & IL-1β fully reactivated virus, fibroblasts were overlaid onto the treated monocytes and co-cultured for up to 14 days. Lytic HCMV infected fibroblasts were observed by fluorescent microscope and photographed. Quantification of HCMV DNA level in the overlaid fibroblast cultures was performed by isolating DNA using a previously described method (43), with quality and quantity being determined using a Nanodrop 1000 (Thermo Fisher Scientific), before HCMV genomic DNA (gDNA) level was determined using HCMV gDNA-specific primers (**Table S1**) with Luna Universal SYBR Green qPCR Master Mix (NEB, Hitchin, UK) as per manufacturer's instructions on an ABI StepOnePlus (Thermo Fisher Scientific). DNA copy number was then determined by referencing to host GAPDH promoter copy number *via* the Pfaffl method (44).

Suppression Assays and Cell Proliferation Assay

PBMC were depleted of CD8+ T cells by MACS using anti-CD8+ direct beads (Miltenyi Biotec), according to manufacturer's instructions and separated on an AutoMACS Pro. The resulting CD4+ T cell & Antigen Presenting Cell (APC) PBMC were resuspended in either X-VIVO 15, X-VIVO 15 with 4ng/ml TGF-β and 10ng/ml IL-10 (both Miltenyi Biotec), neat Mock infected monocyte secretome, neat UV irradiated infected monocyte secretome or neat Latent Infected Monocyte secretome. The cells were then plated in 48-well tissue culture plates and incubated overnight at 37°C in a humidified CO_2 atmosphere. After 24 hours incubation, the cells were stimulated with 1μg/ml anti-CD3 and 0.5μg/ml anti-CD28 (both Mabtech AB) and overlapping peptide pools for HCMV proteins (43) resulting in a 1:2 dilution of the secretomes and TGF-β/IL-10 mix. Following a further 24-hour incubation at 37°C in a humidified CO_2 atmosphere, the plates were centrifuged, and supernatants harvested and then analyzed for the production of IFN-γ by ELISA. Full details of proliferation assays used to measure whether latent secretomes can suppress CD4+ T cells can be found in the supplementary material (Section 1.8).

Statistics

Statistical analysis was performed using GraphPad Prism version 8.00 and 9.00 for Windows (GraphPad Software, San Diego, CA, USA). Multiple data sets groups were compared using a 1-way ANOVA Kruskall-Wallis test or Friedman test (for matched

samples) with *post hoc* Dunn's or Sidak's multiple comparisons tests to correct for multiple testing false discovery.

RESULTS

IL-10, CCL8 and CXCL10 Are Upregulated in the Latent HCMV Infected CD14+ Monocyte Secretome

We have previously shown that experimental latent HCMV infection of CD34+ progenitor cells alters the cellular secretome resulting in the upregulation of chemokines CCL8, CCL2 and secretion of TGF-β and cellular IL-10 (cIL-10) (32). Monocytes, which arise from CD34+ progenitor cells, are also a site of latent HCMV carriage *in vivo* (3). Consequently, we wanted to investigate whether the cellular secretome is also modulated in latently infected monocytes and, additionally, how this compares with the latency associated CD34+ secretome. Using an experimental model of latent HCMV infection of CD14+ monocytes (**Figure S1**), we screened secretomes from three independently generated latent HCMV infections of CD14+ monocytes using antibody arrays. In order to identify changes specific to latent infection, the fold change of cytokines in the secretome of latently infected monocytes was expressed relative to levels seen in the secretome of monocytes infected with UV-inactivated HCMV and corrected for background protein expression in mock infected monocytes (**Figure S3**). This analysis identified three proteins, IL-10, CCL8 and CXCL10, which were significantly upregulated (more than 4-fold) in all three latency-associated secretomes (**Figure 1A**). The production of CXCL10 and CCL8 by latently infected monocytes was confirmed by ELISA (**Figures 1B, C**); the level of both chemokines in the latently infected CD14+ monocytes were significantly increased over mock and UV inactivated infection controls.

The promoters of CXCL10 and CCL8 contain both Type I and II Interferon-responsive elements (45). As such, the overexpression of these chemokines could simply represent the induction of an anti-viral interferon response to infection rather than long term effects of latent carriage of virus. To determine if this was the case, latent secretomes were generated in the presence of neutralizing antibodies for IFN-α, IFN-β, IFN-γ or isotype controls. Analysis of the mock, UV irradiated and latency-associated secretomes by ELISA for CCL8 (**Figure 2A**) and CXCL10 (**Figure 2B**) shows that both chemokines are generated by the latently infected monocytes in the presence of interferon neutralizing antibodies and the magnitude of production is not significantly different. In addition, analysis of latency-associated secretomes after sequential replacement with fresh media across multiple time points (wash out experiments), revealed that latently infected monocytes continually produce CXCL10 (**Figure 2C**). In contrast, the treatment of monocytes with exogenous IFN-γ at the beginning of culture to stimulate CXCL10 production did not result in the continuous production of CXCL10 after IFN-γ is washed out (**Figure 2D**). Taken together, these data suggest that both CCL8 and CXCL10 are produced as a result of the latent HCMV infection of monocytes.

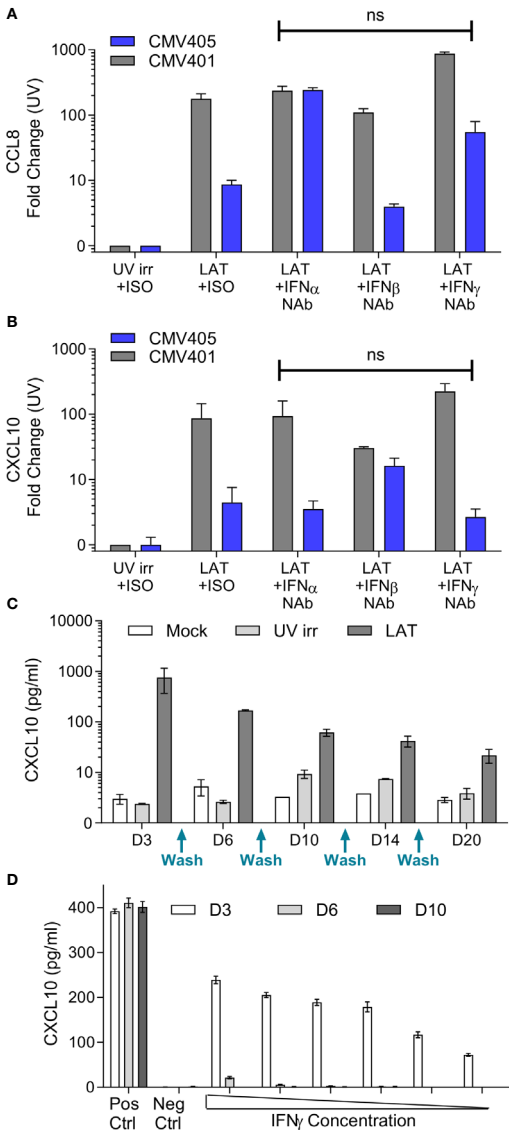

FIGURE 2 | Production of CCL8 and CXCL10 by latently infected CD14+ monocytes is independent of interferon signaling. Neutralization of IFNα, IFNβ and IFNγ does not prevent the production of either CCL8 **(A)** or CXCL10 **(B)** from latent infected CD14+ cells at day 7 post infection. CXCL10 and CCL8 was measured by ELISA, with background chemokine production in response to mock infection subtracted and fold-change results above UV irradiated infection from 2 CMV sero-negative donors CMV401 and CMV405 shown. There was no significant (ns) change in the production of CCL8 or CXCL10 in the presence of neutralizing antibodies (Kruskall-Wallis test). **(C)** Freshly isolated monocytes were infected with TB40/E virus as described, cell supernatant was removed and replaced (indicated on x-axis) over a 20-day period, error bars represent SEM. This shows that CXCL10 is only reproduced by the latent infected monocytes over this time period. **(D)** Treatment of monocytes by recombinant IFNγ protein shows CXCL10 is detectable at day 3 but lost at later time points. Freshly isolated monocytes were stimulated with decreasing amount of IFNγ from 100IU/ml for 4 hours. Supernatants were collected on days 3 (white), 6 (grey) and 10 (black). Levels of CXCL10 were assayed using ELISA. Positive control consisted of continuous 100IU/ml IFNγ and negative control was media alone. This is the representative results of three repeats, error bars represent SEM.

Latency-Associated CD14+ Monocyte Secretomes Induce Activated CXCR3+ Immune Cell Migration Mediated by CXCL10

Cellular chemotaxis can be regulated by various chemokines and cytokines. In the context of HCMV infection, we have previously demonstrated that secreted factors from latently infected CD34+ cells promoted the migration of CD14+ monocytes and resting CD4+ T cells (32). Thus, we investigated the effect of latently infected CD14+ monocyte secretomes on cellular migration. Using a transwell migration assay, we assessed the impact of latency-associated CD14+ monocyte secretomes on the migration of NK cells (**Figures 3A, D**), CD8+ T cells (**Figures 3B, E**), CD4+ T cells (**Figures 3C, F**), B cells (**Figure S4A**), activated directly ex vivo or *in vitro*, and on monocytes (**Figure S4B**). In contrast to our observations with the latency-associated CD34+ secretome (32), we observed no significant migration of the freshly isolated lymphocyte cell subsets to the monocyte latency-associated secretome in six donors tested (**Figures 3A–C**; **Figures S4A, B**), despite the presence of CCL8 in these secretomes (**Figure 1C**). However, when cells were polyclonally activated prior to the assay, we saw significant migration of activated NK cells and CD4+ T cells (**Figures 3D, F**) and an upregulation of migration of activated CD8+ T cells (**Figure 3E**) to the latent infected monocyte secretomes.

CXCR3, a receptor that interacts with CXCL10 (46), is known to be upregulated on subsets of activated CD4+ T cells, CD8+ T cells and NK cells (47–49). We, therefore, analyzed the expression of CXCR3 on both resting and polyclonally activated T and NK cell subsets. The data show that there is low level expression of CXCR3 on all three subsets isolated directly *ex vivo* (**Figures 4A–C**, left hand histogram). However, CXCR3 expressed by un-activated T and NK cells has been shown to be non-responsive to its chemokine ligands (47), possibly explaining why *ex vivo* NK and T cells did not migrate to the latent secretome. The polyclonal activation of the NK cells, CD8+ and CD4+ T cells resulted in an upregulation of CXCR3 expression in all cases (**Figures 4A–C**, right hand histogram). Furthermore, flow sorting of activated NK cells into CXCR3+ and CXCR3- populations prior to performing a transwell migration assay showed that only the CXCR3 expressing cells had the capacity to migrate (**Figure S4C**). Importantly, antibody neutralization of CXCL10 present in the latently infected monocyte secretomes significantly abrogated the migration of all three activated cellular subsets (**Figures 4D–F**).

Latent HCMV Infected CD14+ Monocyte Secretome Suppresses T Cell Function

Virus driven recruitment of activated CD4+ T cells to latently infected cells does, at first, seem counter-intuitive with respect to virus survival; CD4+ T cells can be potently anti-viral and thus, hypothetically, if HCMV-specific, could limit HCMV reactivation (50, 51). However, we also observed elevated levels of cIL-10 (**Figure 1A**), an immunomodulatory cytokine that can suppress IFN-γ production by T cells (52), in the latently infected monocyte secretomes. Therefore, we hypothesized that the latently infected monocyte secretomes may also suppress possible anti-viral activity of the recruited CD4+ T cells. We assessed the production of IFN-γ by CD4+ T cells following polyclonal stimulation in the presence or absence of latency-associated secretomes. As expected, polyclonal stimulation of CD4+ T cells induced IFN-γ production (**Figure 5A**). However, this was significantly suppressed in the presence of the latently infected monocyte secretomes for each of five donors tested (**Figure 5B**, right-hand graph). Interestingly, treatment of stimulated CD4+ T cells with TGF-β and cIL-10 only suppressed IFN-γ production in three of the five same donors (**Figure 5B**, left-hand graph). Furthermore, we also observed a suppression of both IFN-γ production and cell proliferation by the latently infected monocyte secretome when the CD4+ cells were stimulated with HCMV antigen in some donors (**Figures S5A, B**).

To determine if the secretomes produced by latently infected cells could also suppress HCMV lytic replication, we utilized viral dissemination assays and show that spread of lytic virus was not inhibited (**Figures S6A, B**). We also determined if the secretomes caused an alteration in the phenotype of bystander uninfected monocytes (**Figures S6C, D**), however there was no change in expression of myeloid differentiation markers in monocytes incubated with latency-associated secretomes compared to secretomes from untreated monocytes. This evidence suggests that while proteins secreted by the latently infected monocyte recruit activated CD4+ T cells to its location, it can also suppress known anti-viral functions, such as production of IFN-γ, from these cells.

CXCR3+ CD4+ T Cell Co-Culture Induces Reactivation From Latent HCMV Infected CD14+ Monocytes

The accumulation of CXCL10 in the latently infected monocyte secretome and the consequent recruitment of activated lymphocyte cell subsets was unexpected (32). However, virally induced supernatants from monocytes that recruited these activated lymphocyte cell subsets also simultaneously reduced their effector function. Consequently, we reasoned that recruitment of activated immune cells to the site of latent infection (as long as their effector functions were suppressed) might, in some way, have a pro-viral effect on latency and/or reactivation. To interrogate this in more detail, we initially performed a co-culture experiment with CXCR3+ PBMC and monocytes latently infected with either TB40/E UL32-GFP or TB40/E IE2-YFP tagged strains of HCMV. Both pp150 (encoded by UL32) and IE2 proteins are expressed during lytic replication of the virus and, thus, can be used as markers of HCMV reactivation. Virus reactivation from latently infected monocytes was induced by culturing them in the presence of either GM-CSF and IL-4 or M-CSF and IL-1β cytokines which differentiate monocytes into a dendritic cell or macrophage like phenotype, respectively. When latently infected monocytes were incubated with CXCR3+ PBMCs, virus reactivation (UL32-GFP expression) was observed (**Figure 6A** left-hand panel) which was shown to be statistically significant (**Figure 6A** right-hand

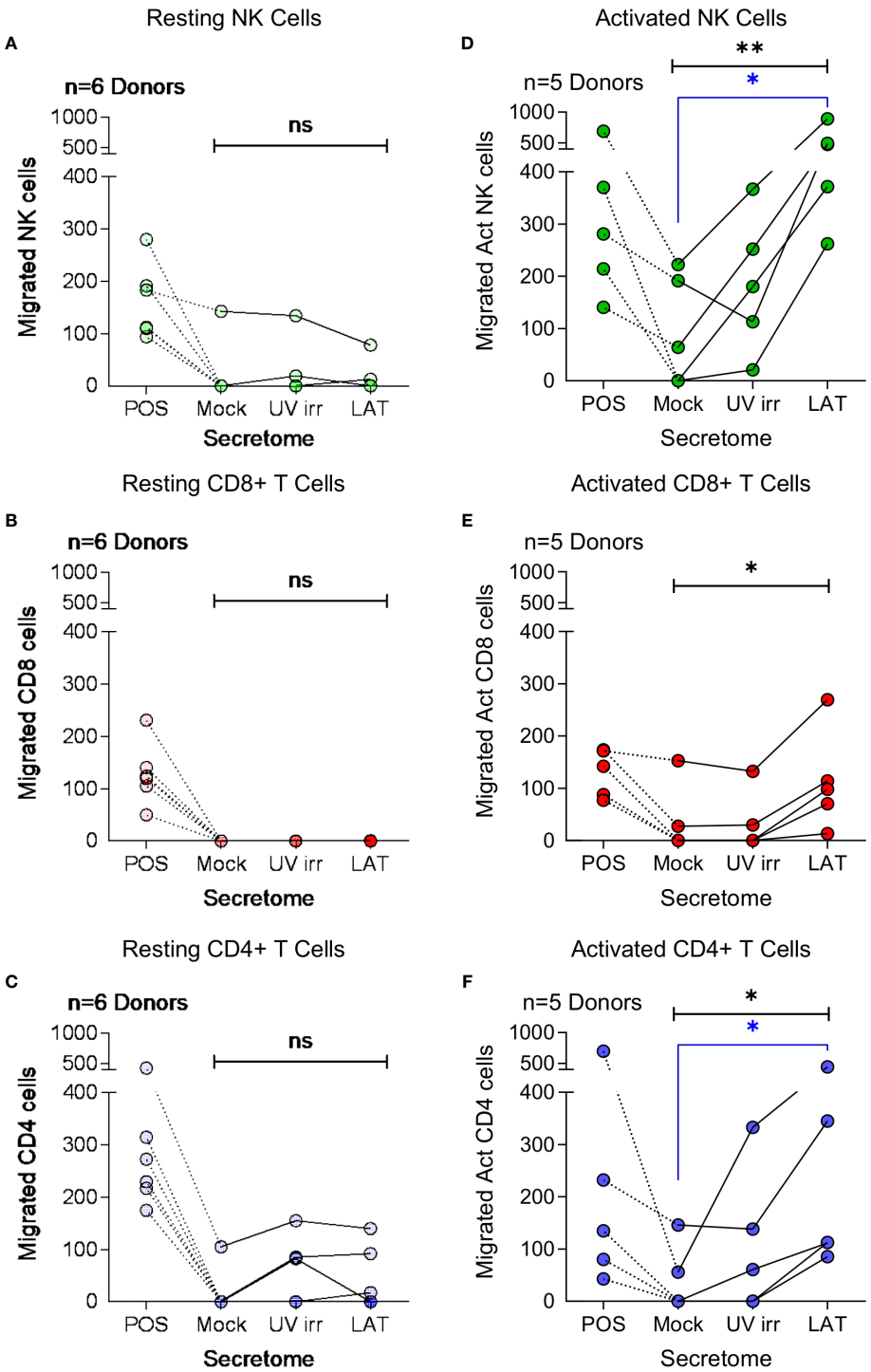

FIGURE 3 | Migration of activated immune cell subsets in response to monocyte latent secretome. Transwell migration assays were performed in response to positive control, Mock, UV irradiated (UV irr) and Latent infected (LAT) CD14+ monocyte secretomes from day 7 harvest in multiple donors (indicated on each graph). Shown are the results from migration assays performed with resting NK cells – light green **(A)**, CD8+ T cells – light red **(B)** and CD4+ T cells – light blue **(C)** subsets. Following polyclonal activation (Act) transwell migration assays were performed with NK cells – dark green **(D)**, CD8+ T cells – dark red **(E)** and CD4+ T cells – dark blue **(F)**. All tested immune cell subsets from all donors migrated to the positive control, but there was only significant migration of the activated cell subsets (Friedman's 1 way ANOVA – results indicated in black on graphs; Act NK p=0.0085**, Act CD8 p=0.0123* and Act CD4 p=0.0123*, non significant (ns) results are also indicated) to the latent infected secretomes. Activated NK cells and CD4+ T cells significantly migrated to the latent infected secretome (Dunn's post-test shown in blue on graphs; NK p=0.0133* and CD4 p=0.0342*).

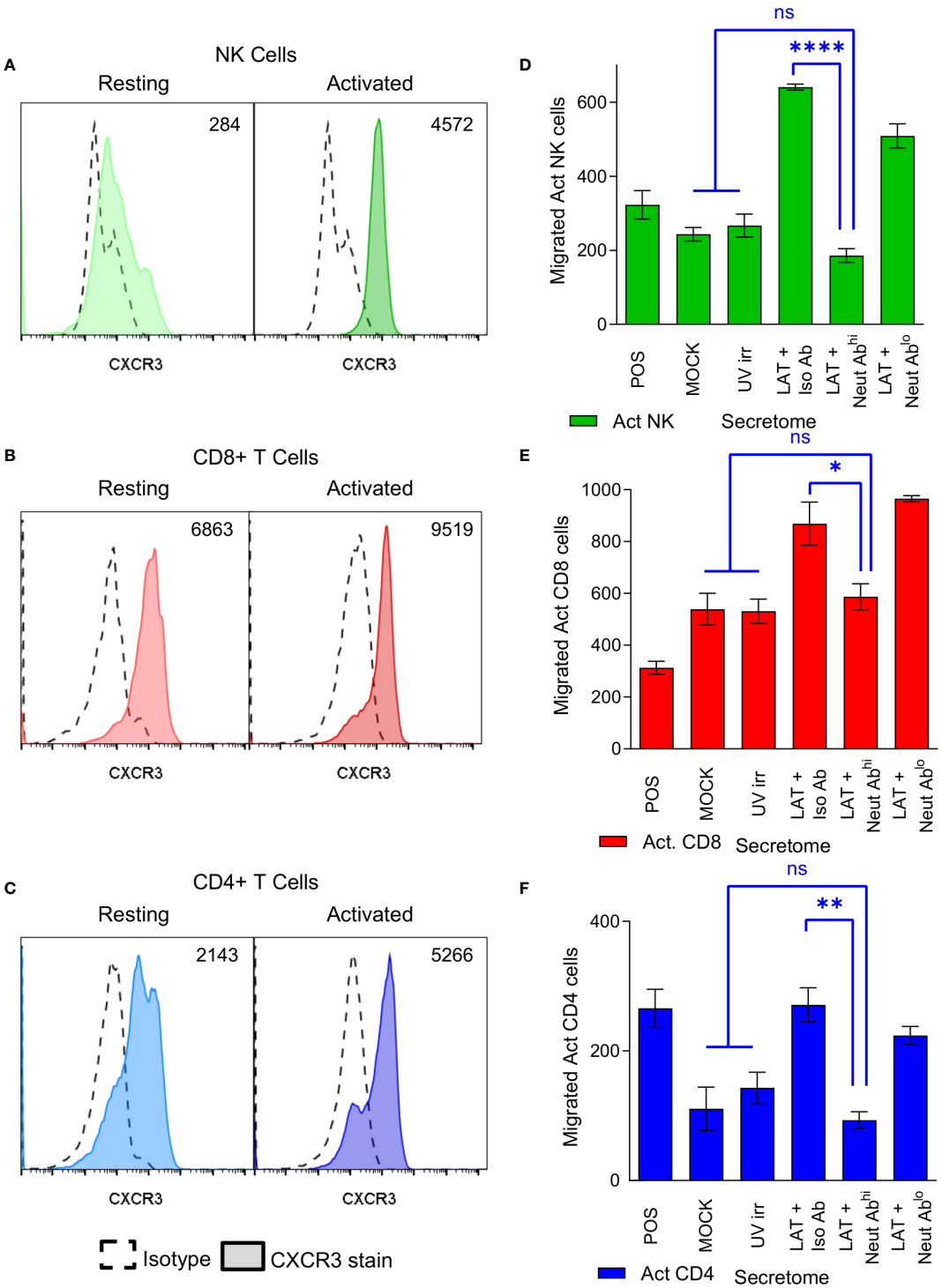

FIGURE 4 | CXCR3 is upregulated on activated immune cell subsets and neutralization of CXCL10 abrogates migration of these cells. Histograms from flow cytometry analysis of CXCR3 expression on resting (left-hand histogram) and activated (right-hand histogram) NK cells **(A)**, CD8+ T cells **(B)** and CD4+ T cells **(C)** showing increased expression of CXCR3 on activated cell subsets (value shown on graph normalized geo-mean of CXCR3 expression for all cells analyzed). Representative results from 6 individual donors shown. Transwell migration assays were performed on the latent infected monocyte secretomes in the presence of CXCL10 neutralizing antibody or isotype controls showing that neutralization of CXCL10 significantly abrogates migration of activated NK cells **(D)**, CD8+ T cells **(E)** and CD4+ T cells **(F)** (1-way ANOVA with Sidak's multiple comparison results shown in blue on the graphs; NK $p<0.0001$****, CD8 $p=0.0488$* and CD4 $p=0.0032$**, non-significant (ns) comparisons are also shown.).

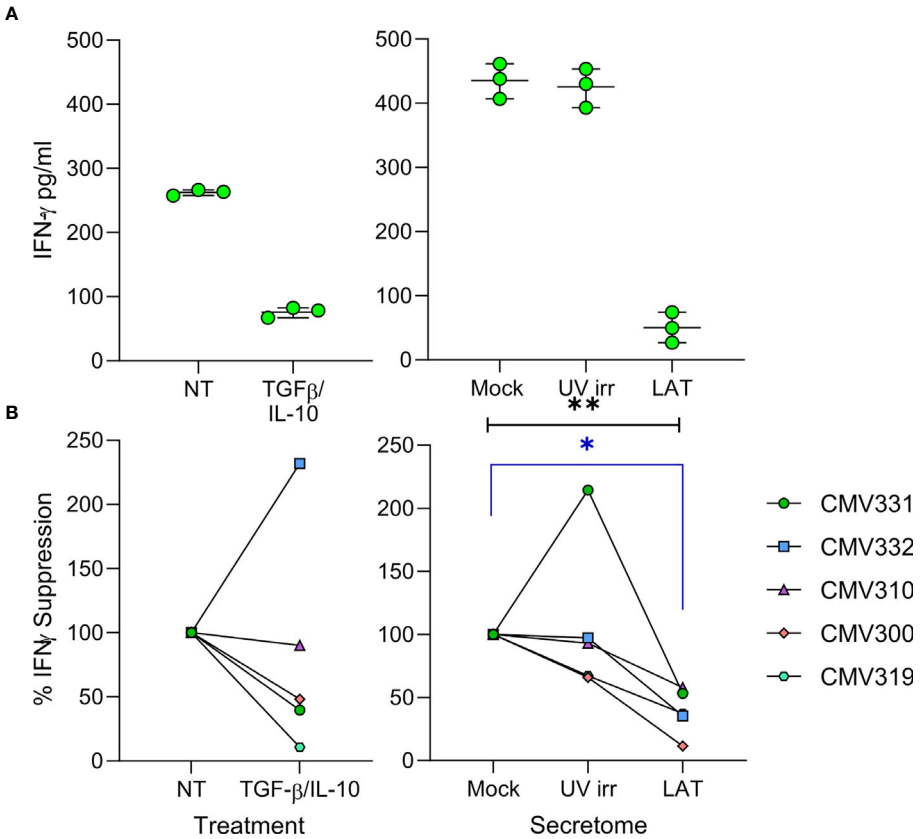

FIGURE 5 | The latent infected monocyte secretome inhibits the production of IFNγ in response to polyclonal activation. CD4+ T cells were resuspended in either X-VIVO Media (Not Treated (NT)), media with added recombinant protein TGFβ & IL-10 (TGFβ/IL-10), Mock, UV irradiated (UV irr) or Latent infected (LAT) secretomes; following 24 hours pre-treatment the CD4+ T cells were stimulated with a mixture of anti-CD3 and anti-CD28 for a further 24 hours and then supernatants harvested. Production of IFNγ in the supernatants was measured by ELISA, representative results from one donor are shown **(A)**. Summary graphs showing the percentage suppression by either TGFβ/IL-10 treatment or the Latent infected monocyte secretomes of polyclonally stimulated CD4+ T cells from 5 donors **(B)**. The Latent infected secretomes significantly suppressed the production of IFNγ by polyclonal stimulation (Friedman matched 1-way ANOVA test (black line p=0.0085**) and Dunn's corrected post-test (blue line p=0.0133*).

graph). Importantly, these levels of reactivation were comparable to that observed with the cytokine cocktails that promote dendritic (IL-4/GM-CSF) and macrophage (IL-1β/M-CSF) differentiation (**Figure 6A**). To determine which CXCR3 expressing cells could drive HCMV reactivation, we assessed the contribution of individual cell populations. A comparison of co-cultures of separate CXCR3+ populations of NK cells, CD8+ T cells and CD4+ T cells with latently infected monocytes alongside positive control for reactivation M-CSF and IL-1β treatment of infected monocytes was performed. The results show that co-culture with purified activated NK cells or CD8+ T cells did not result in virus reactivation in three separate donors tested (**Figure S7A**). Co-culture with activated NK cells may result in killing of latently infected monocytes, as the number of reactivating cells is lower than in the other conditions, this is not however a significant repression of infected monocyte numbers. In contrast, co-culture of activated CD4+ T cells with latently infected monocytes promoted virus reactivation at levels that were comparable to those observed following monocyte differentiation with M-CSF and IL-1β cytokines (**Figure 6B**). We also demonstrated that expression of IE2-YFP in monocytes, is indicative of the production of infectious virions, as the addition of fibroblasts to the reactivating monocyte culture results in infection of the fibroblast cell layer. Fibroblast overlaid on monocytes treated with M-CSF and IL-1β cytokines and co-cultured with CXCR3+ CD4+ T cells formed IE2-YFP positive infectious plaques (**Figure 6C** left-hand panel). This observation was quantified by the measurement of genomic HCMV DNA present in the fibroblast overlaid cultures (**Figure 6C** right-hand graph), showing the presence of HCMV DNA in all reactivating conditions (PMA and cytokine treated and activated CD4+ T cell co-culture).

Cytokines produced by allogeneically stimulated T cells have been demonstrated to promote virus reactivation in supernatant transfer experiments (53) and, thus, we asked whether cytokines produced by polyclonally stimulated CD4+ cells were similarly able to induce virus reactivation. Supernatants derived from activated immune cell subsets were co-cultured with a THP-1 monocytic cell line stably transfected with an integrated MIEP driven GFP expression cassette which act as a model of

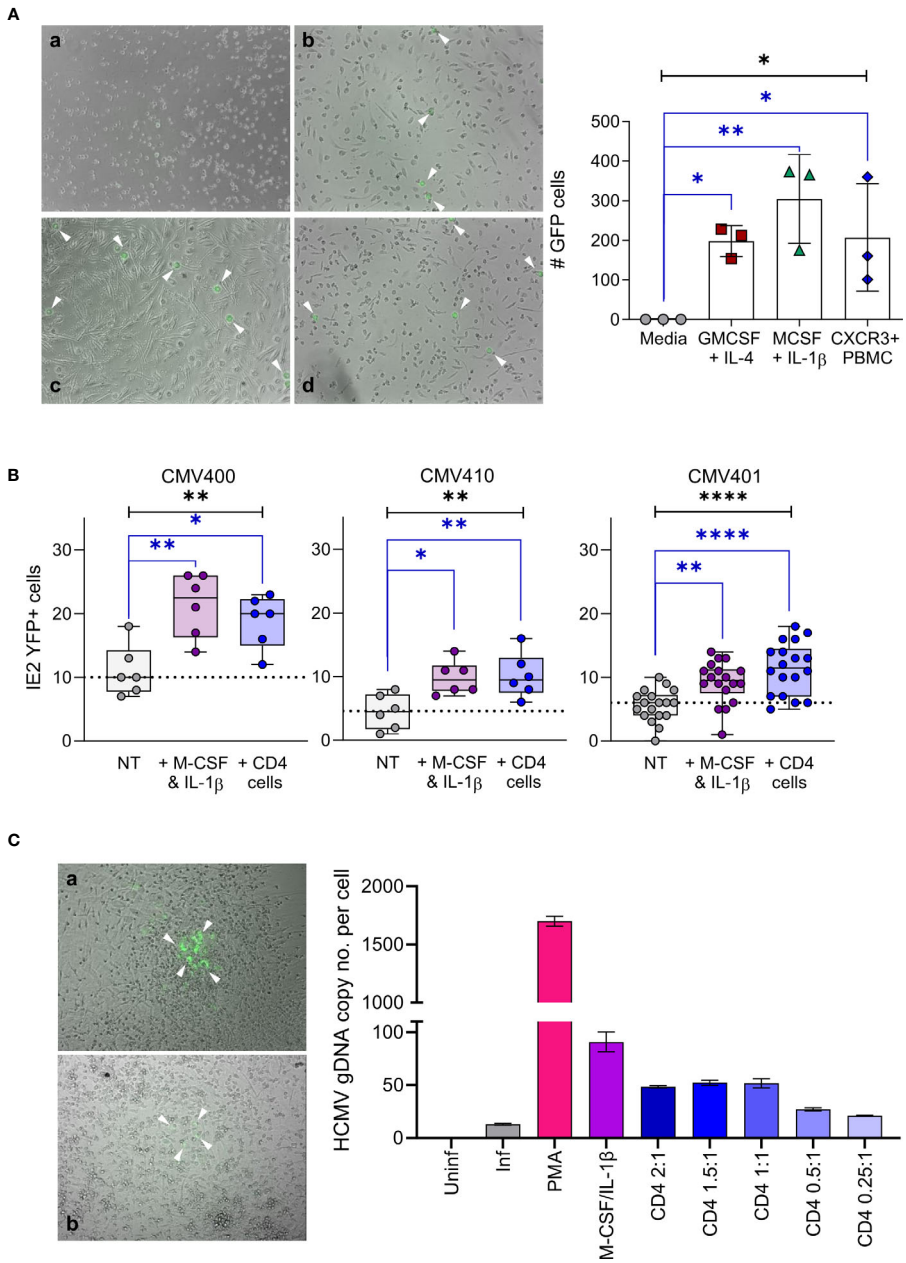

FIGURE 6 | Activated CD4+ T cells can reactivate HCMV infection of latent infected monocytes. **(A)** CD14+ monocytes from a CMV sero-negative donor were infected with TB40/E UL32-GFP virus and co-cultured with activated PBMC or treated with cytokine cocktails (GM-CSF & IL-4 or M-CSF & IL-1β), virus reactivating GFP positive cells (white arrows) were visualized by microscope (right-hand panel – a Media, b M-CSF, c CXCR3+ PBMC & d GM-CSF) and enumerated (left-hand graph). All treatment conditions results in significant levels of GFP expressing cells representing reactivated virus (1-way ANOVA (black line: p=0.0112*) with corrected Dunnets post-test (blue lines: GM-CSF p=0.044*, M-CSF p=0.0048**, PBMC p=0.0363*). Shown is one representative donor of 4 different donors analyzed. **(B)** CD14+ monocytes latently infected with TB40/E IE2-YFP virus were co-cultured with activated CD4+ T cells or treated with M-CSF & IL-1β in 3 CMV sero-negative donors, reactivating YFP positive cells were enumerated. Both activated CD4+ T cells and M-CSF & IL-1β significantly increased reactivation of HCMV from latently infected autologous CD14+ monocytes (Kruskal Wallis 1-way ANOVA (Black line: CMV400 p=0.0049**; CMV410 p=0.0078**; CMV401 p<0.0001****) and Dunn's post-test (Blue line CMV400 MCSF p=0.003**, CD4 p=0.03*; CMV410 M-CSF p=0.0133*, CD4 p=0.0098**; CMV401 MCSF p=0.0086**, CD4 p<0.0001****)). **(C)** CD14+ monocytes latently infected with TB40/E IE2-YFP virus were co-cultured with activated CD4+ T cells, treated with M-CSF & IL-1β or treated with PMA, then the cultures were overlaid with fibroblasts to measure the production of infectious virions. The resulting plaques were visualized by microscope (left-hand panel – a M-CSF & b Act CD4+ Treated) and IE2-YFP infected fibroblast plaques are indicated (white arrows). The infection of fibroblasts was quantified by measurement of genomic HCMV DNA present in each condition (right-hand graph), HCMV genome copy number was determined by qPCR of UL44 promoter relative to host GAPDH promoter. This demonstrates that detection of IE2-YFP positive monocytes can be used as a surrogate for the production of infectious virions in this system.

differentiation dependent induction of MIEP activity - when the THP-1 cells differentiate GFP is induced. Using this model cell line, we observed that supernatants derived from polyclonally activated CD4+ T cells do not promote increased MIEP expression whereas co-culture with activated CD4+ T cells do increase GFP expression (**Figure S7B**). This suggests that a physical interaction between the activated CD4+ T cell and monocyte is required. Phenotype analysis of monocytes co-cultured with CD4+ T cells showed that monocytes increased expression of T cell co-stimulation molecules CD80 and CD86 whereas monocytes differentiated with M-CSF and IL-1β increased expression of macrophage associated markers CD64 and CD68 (**Figures S8A, B**), all these upregulated markers are consistent with monocyte differentiation to myeloid derivatives. CD4+ T cell activation was confirmed by increased expression of CD40L, CXCR4 and 4-1BB alongside increased CXCR3 expression. In addition, MHC Class II (HLA-DR) was robustly upregulated on CXCR3+ CD4+ T cells in multiple donors (**Figure S8C**).

It has been reported that ligation of CD4 expressed on monocytes, by MHC Class II molecules expressed on other cells, promotes differentiation to macrophages (54) *via* Src family kinase (SFK), mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways. Given the importance of these pathways in HCMV reactivation from dendritic cells (55, 56) we attempted to investigate whether inhibitors of ERK-MAPK signaling (U0126) and Src Family Kinases (PP2) prevented reactivation in our co-culture model. Unfortunately, treatment of latently infected monocytes with the inhibitors for the 96 hours incubation required for activated CD4+ T cells and M-CSF and IL-1β to trigger expression of IE2 protein was toxic. We investigated if by detecting IE72 mRNA by RT-qPCR we could perform these reactivation experiments over a shorter time period when these inhibitors would be less toxic to the cells. Latently infected monocytes were stimulated with either PMA (which induces rapid reactivation and IE72 expression) or M-CSF & IL-1β in the presence of U0126 inhibitor or its inactive control. The results at 24 hours post stimulation show PMA drives IE72 transcripts and this is partially inhibited by 10μm U0126 and not U0124 (the inactive analog), however M-CSF & IL-1β did not induce IE72 at this time point. By 48 hours PMA drive IE72 was no longer inhibited by U0126, as such inhibitions of these signaling pathways in an experimental set up that takes 96 hours to cause reactivation is not tractable.

DISCUSSION

Taken together, our analyses of the latency-associated secretome of monocytes is consistent with the view that latent HCMV infection results in modulation of the cellular secretome of the myeloid lineage which profoundly affects the latent cell microenvironment and modulates host immune responses to the latent reservoir (57, 58). Whilst carriage of latent HCMV genome by monocytes is likely to be short lived due to the limited lifespan of monocytes once they have migrated to the periphery (15, 22, 24), viral genomes can be detected in CD14+ monocytes isolated from healthy HCMV infected individuals (43, 59) and, importantly, the virus can be reactivated from these cells (3, 16, 53, 60). Therefore, consideration of the impact of latent infection on the local micro-environment in peripheral tissue sites, not just bone marrow sites of latency, is crucial for a full understanding of latency and reactivation *in vivo* and may be particularly helpful in the development of therapeutic measures to target HCMV reactivation in transplantation patients or pregnant women, the latter of which can lead to congenital HCMV (cCMV) sequalae in the new-born.

Previously, we have shown that experimental latent infection of CD34+ progenitor cells alters the cellular secretome to induce migration of CD4+ T cells and subsequent suppression of their effector function (32). That study revealed the impact of HCMV latent carriage on the CD34+ progenitor cellular microenvironment in the bone marrow but did not consider the very different environment encountered at peripheral tissue sites by the HCMV infected CD34+ myeloid derivatives. Here, we have shown that experimental latent infection of CD14+ monocytes, an established *in vitro* model system for latent infection *in vivo* (36), also results in changes to the cellular secretome causing upregulation of cIL-10, CCL8 and, in particular, CXCL10. Our analyses are consistent with another study of short-term latency in monocytes (34), but that study did not address the functional consequences of increases in latency-associated CCL8 and CXCL10 and did not identify an increase in cIL-10. These differences in latent secretome analyses may be attributable to the fact that, in our study, latency-associated secretomes were analysed at much later times (up to 14 days latency). The bioactivity of the CD14+ monocyte latent secretome differs to that observed for CD34+ progenitor cells in that monocytes and CD4+ T cells isolated directly *ex vivo* did not specifically migrate to the CD14+ secretome. Instead, we observed a significant recruitment of activated NK cells, CD8+ and CD4+ T cells, all with increased CXCR3 expression, to the CD14+ monocyte latency-associated secretome. Migration of these immune cell subsets was abrogated by neutralisation of CXCL10, a known ligand of CXCR3, in the CD14+ latency-associated secretome. Suggesting that the migration of activated CD8+ and CD4+ T cells as well as NK cells is mediated *via* interaction of CXCL10 present in the latently infected monocyte secretomes and that this effect is specific to CXCR3 expressing immune cells. Clear parallels from our study can be drawn with other infections, for example, the expression of CXCL10 was IFN independent – an observation also made with Hepatitis A infection (61). Furthermore, herpes simplex virus promotes CXCR3-mediated migration of CD4+ T cells to sites of infection (62) and CD8+ T cells to sites of latent infection (63). Recruitment of NK cells in the lung during Influenza A infection is similarly dependent on CXCR3 expression (64). These results could represent host driven anti-viral responses although, at least in the case of HCMV, the recruitment of CXCR3+ cells appears to be virus driven.

Activated antigen specific CD4+ T cells directed against HCMV have been demonstrated to be highly anti-viral (50, 51).

Thus, it seems counterintuitive for the latent infection to modulate the cellular secretome to produce chemokines that attract activated and potentially antigen-specific immune cells to latently infected monocytes. However, our previous studies of HCMV latent infection in CD34+ progenitors showed that the latent secretome was also able to suppress inflammatory cytokine production by CD4+ T cells due to the presence of immunomodulatory cytokines including cIL-10 and TGF-β (32) as well as HCMV vIL-10 (35). This observation also holds true for the monocyte secretome, which we demonstrated has a significant inhibition on the production of IFNγ by CD4+ T cells following polyclonal activation. Whilst the suppression of activated CD4+ T cell anti-viral activity at the local site of latent monocytes would enable persistence of the virus infection, it does not entirely explain why activated immune cells are recruited. It may be that the secretion of CXCL10 and the recruitment of activated T cells is an unintended, and unwanted, consequence of the latent infection. Alternatively, the cellular secretome from latently infected monocyte might be promoting the recruitment of activated CD4+ T cells to the local tissue microenvironment for a pro-viral purpose.

While the establishment of cellular latency in reservoir sites such as CD34+ progenitor cells within the bone marrow is a long-term survival strategy for HCMV within the infected host, virus has to be able to reactivate in order for viral replication and dissemination to occur with a potential to transmit to a new host. In order to achieve this, the virus needs to be able to react to favourable changes in the cellular environment and external signals in order to initiate reactivation and provide a permissive cellular environment for virus replication. It is well established that HCMV reactivation is closely linked to myeloid cell differentiation and inflammatory environments (16, 53, 56, 65, 66). Utilisation of the monocyte, a derivative of CD34+ progenitor cells, to export the latent HCMV infection from the bone marrow to the periphery is one possible strategy for the virus to employ in order to reach an appropriate environment for virus replication. To overcome the short lifespan of monocytes in the periphery (15, 22, 24) the virus has been shown to manipulate cellular processes to promote a pro-survival state (17). Therefore, we hypothesized that the latent viral infection may be promoting a pro-inflammatory environment at peripheral sites by inducing production of CXCL10, rather than needing to relocate to a pre-existing inflammatory environment. The recruitment of activated CD4+ T cells by CXCL10 in the local environment may, then, trigger myeloid cell differentiation and successful virus reactivation, despite the concomitant recruitment of activated NK cells which may kill latently infected monocytes. Consistent with this, co-culture of latently infected monocytes with activated CD4+ T cells results in reactivation of virus and increased expression of myeloid differentiation markers. Further, our evidence suggests that direct interaction between the activated T cell and infected monocytes is required, possibly *via* ligation of HLA-DR, upregulated on CXCR3+ CD4+ T cells, with CD4 expressed on monocytes and other myeloid cell subsets (67). This CXCR3-mediated signalling to the monocyte has been shown to result in monocyte differentiation to a mature myeloid population (54) and is known to involve the ERK-MAPK pathway. Interestingly, the ERK-MAPK signalling pathway is also utilised by the M-CSF receptor (68) and Src family kinases are also implicated in reactivation of HCMV (55, 56). Unfortunately, due to the toxicity of the inhibitors to the ERK-MAPK signalling pathway over the time required to observe reactivation induced by both CD4+ T cell co-culture as well as M-CSF & IL-1β treatment we were not able to confirm this hypothesis. Other approaches to investigate the mechanisms involved in the reactivation of virus by activated CD4+ T cells include the use of neutralising antibodies to both CD4 (expressed by the monocytes) and HLA-DR (expressed by the T cells) or the use of recombinant HLA-DR to stimulate monocytes. These experimental approaches are limited to known ligand interactions, however, it is possible that reactivation of virus by activated CD4+ T cells or by differentiation with M-CSF and IL-1β employs similar signalling mechanisms with both resulting in monocyte differentiation to terminally differentiated myeloid cells.

Taken together, our observations support a model whereby latent HCMV promotes the recruitment of activated CD4+ T cells to monocyte sites of latency to promote viral reactivation which, *in vivo*, may support local dissemination of the virus at peripheral tissue sites. Importantly, HCMV simultaneously down-regulates effector functions of CD4+ T cells thereby "cherry picking" the effects of this CD4+ T cell recruitment by obtaining the benefit of T cell inflammation and contact-driven myeloid differentiation of the monocytes but preventing any T cell-mediated anti-viral immune response. In interpreting the evidence from this study it is important to note that this is an *ex vivo* model system, utilised because of the low frequency of latently infected monocytes in peripheral blood (69). There is evidence from many studies that subclinical reactivation of HCMV likely occurs in persistently infected, healthy, individuals; for instance there is a strong association of CMV infection with vascular disease in population studies (19) and CMV DNA has been identified in atherosclerotic plaques in a number of studies (70–72). It has been shown that HCMV latent infection results in profound changes in the latently infected cells besides just secreted cellular proteins - it also manipulates apoptotic pathways (17, 73) and modulates expression of cellular microRNAs (35, 74). HCMV latent infection of monocytes is also known to manipulate the recruitment of neutrophils (75) as well as the motility of latently infected monocytes to increase their trans endothelial migration (76). The association of persistent HCMV infection with vascular disease could also be partly explained by recruitment of activated T cells to peripheral sites of latency, as demonstrated here, as it is long established that CXCR3 expressing CD4+ T cells are recruited to atherosclerotic plaque sites (77). Therefore, the evidence from this study using an *ex vivo* model of latently infected monocytes, suggests potential avenues for future investigations looking for CMV reactivation in peripheral tissue sites in association with CXCL10 production and recruitment of CXCR3 expressing activated T cells.

In conclusion, the work presented here shows that latent HCMV carriage in monocytes at peripheral tissue sites results in manipulation of the cellular secretome of the infected cell in order to recruit activated immune cells resulting in reactivation and potential dissemination of the virus. This underscores how dynamic latent HCMV infection is and the extraordinary range of cellular processes that are manipulated by latent infection. We believe that identifying and understanding such latency-associated changes can only improve the development of therapeutic agents for use in clinical situations where CMV reactivation can cause significant morbidity and mortality such as in post-transplant or other immunosuppressed patients.

DATA AVAILABILITY STATEMENT

The protein array data summarised in **Figures 1** and **S2** of this study are publicly available. This data can be found here: https://www.ebi.ac.uk/biostudies/studies/S-BSST619.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Research Authority (HRA) Cambridge Central Research Ethics Committee (97/092). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SJ, MW, JS, EP, IG and MR designed research. SJ, KC, IG, GS, AG, CH, EP, IM, GM, GO and MW performed research. SJ, KC, IG, GS, AG, IM, CH and MW analyzed data. SJ, MR and MW wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.657945/full#supplementary-material

REFERENCES

1. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev* (2009) 22(1):76–98. doi: 10.1128/CMR.00034-08
2. Jackson SE, Redeker A, Arens R, van Baarle D, van den Berg SPH, Benedict CA, et al. CMV immune evasion and manipulation of the immune system with aging. *Geroscience* (2017) 39(3):273–91. doi: 10.1007/s11357-017-9986-6
3. Sinclair J. Human cytomegalovirus: Latency and reactivation in the myeloid lineage. *J Clin Virol* (2008) 41(3):180–5. doi: 10.1016/j.jcv.2007.11.014
4. Hahn G, Jores R, Mocarski ES. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc Natl Acad Sci U S A* (1998) 95(7):3937–42. doi: 10.1073/pnas.95.7.3937
5. Rossetto CC, Tarrant-Elorza M, Pari GS. Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and CD34 (+) cells. *PloS pathogens* (2013) 9(5):e1003366. doi: 10.1371/journal.ppat.1003366
6. Shnayder M, Nachshon A, Krishna B, Poole E, Boshkov A, Binyamin A, et al. Defining the Transcriptional Landscape during Cytomegalovirus Latency with Single-Cell RNA Sequencing. *MBio* (2018) 9(2):18. doi: 10.1128/mBio.00013-18
7. Cheng S, Caviness K, Buehler J, Smithey M, Nikolich-Zugich J, Goodrum F. Transcriptome-wide characterization of human cytomegalovirus in natural infection and experimental latency. *Proc Natl Acad Sci U S A* (2017) 114(49):E10586–E95. doi: 10.1073/pnas.1710522114
8. Jenkins C, Abendroth A, Slobedman B. A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. *J Virol* (2004) 78(3):1440–7. doi: 10.1128/JVI.78.3.1440-1447.2004
9. Goodrum FD, Jordan CT, High K, Shenk T. Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. *Proc Natl Acad Sci U S A* (2002) 99(25):16255–60. doi: 10.1073/pnas.252630899
10. Krishna BA, Poole EL, Jackson SE, Smit MJ, Wills MR, Sinclair JH. Latency-Associated Expression of Human Cytomegalovirus US28 Attenuates Cell Signaling Pathways To Maintain Latent Infection. *MBio* (2017) 8(6):17. doi: 10.1128/mBio.01754-17
11. Humby MS, O'Connor CM. Human Cytomegalovirus US28 Is Important for Latent Infection of Hematopoietic Progenitor Cells. *J Virol* (2015) 90(6):2959–70. doi: 10.1128/JVI.02507-15
12. Crawford LB, Caposio P, Kreklywich C, Pham AH, Hancock MH, Jones TA, et al. Human Cytomegalovirus US28 Ligand Binding Activity Is Required for Latency in CD34(+) Hematopoietic Progenitor Cells and Humanized NSG Mice. *MBio* (2019) 10(4):e01889–19. doi: 10.1128/mBio.01889-19
13. Elder EG, Krishna BA, Williamson J, Lim EY, Poole E, Sedikides GX, et al. Interferon-Responsive Genes Are Targeted during the Establishment of Human Cytomegalovirus Latency. *mBio* (2019) 10(6):e02574–19. doi: 10.1128/mBio.02574-19
14. Krishna BA, Humby MS, Miller WE, O'Connor CM. Human cytomegalovirus G protein-coupled receptor US28 promotes latency by attenuating c-fos. *Proc Natl Acad Sci U S A* (2019) 116(5):1755–64. doi: 10.1073/pnas.1816933116
15. Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C, Yurochko AD. Human cytomegalovirus (HCMV) infection of endothelial cells promotes naive monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. *J Virol* (2006) 80(23):11539–55. doi: 10.1128/JVI.01016-06
16. Reeves MB, MacAry PA, Lehner PJ, Sissons JG, Sinclair JH. Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc Natl Acad Sci U S A* (2005) 102(11):4140–5. doi: 10.1073/pnas.0408994102
17. Collins-McMillen D, Chesnokova L, Lee BJ, Fulkerson HL, Brooks R, Mosher BS, et al. HCMV Infection and Apoptosis: How Do Monocytes Survive HCMV Infection? *Viruses* (2018) 10(10):533. doi: 10.3390/v10100533
18. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells

dominate the memory compartments of exposed subjects. *J Exp Med* (2005) 202(5):673–85. doi: 10.1084/jem.20050882
19. Savva GM, Pachnio A, Kaul B, Morgan K, Huppert FA, Brayne C, et al. Cytomegalovirus infection is associated with increased mortality in the older population. *Aging Cell* (2013) 12(3):381–7. doi: 10.1111/acel.12059
20. Wolf AA, Yanez A, Barman PK, Goodridge HS. The Ontogeny of Monocyte Subsets. *Front Immunol* (2019) 10:1642. doi: 10.3389/fimmu.2019.01642
21. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* (2009) 27(1):669–92. doi: 10.1146/annurev.immunol.021908.132557
22. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med* (2017) 214(7):1913–23. doi: 10.1084/jem.20170355
23. Ozanska A, Szymczak D, Rybka J. Pattern of human monocyte subpopulations in health and disease. *Scand J Immunol* (2020) 92(1): e12883. doi: 10.1111/sji.12883
24. Guilliams M, Mildner A, Yona S. Developmental and Functional Heterogeneity of Monocytes. *Immunity* (2018) 49(4):595–613. doi: 10.1016/j.immuni.2018.10.005
25. Coillard A, Segura E. In vivo Differentiation of Human Monocytes. *Front Immunol* (2019) 10:1907. doi: 10.3389/fimmu.2019.01907
26. Stevenson EV, Collins-McMillen D, Kim JH, Cieply SJ, Bentz GL, Yurochko AD. HCMV reprogramming of infected monocyte survival and differentiation: a Goldilocks phenomenon. *Viruses* (2014) 6(2):782–807. doi: 10.3390/v6020782
27. Soderberg-Naucler C, Streblow DN, Fish KN, Allan-Yorke J, Smith PP, Nelson JA. Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent. *J Virol* (2001) 75(16):7543–54. doi: 10.1128/JVI.75.16.7543-7554.2001
28. Sinzger C, Plachter B, Grefte A, The TH, Jahn G. Tissue macrophages are infected by human cytomegalovirus in vivo. *J Infect Dis* (1996) 173(1):240–5. doi: 10.1093/infdis/173.1.240
29. Chan G, Nogalski MT, Stevenson EV, Yurochko AD. Human cytomegalovirus induction of a unique signalsome during viral entry into monocytes mediates distinct functional changes: a strategy for viral dissemination. *J leukocyte Biol* (2012) 92(4):743–52. doi: 10.1189/jlb.0112040
30. Dumortier J, Streblow DN, Moses AV, Jacobs JM, Kreklywich CN, Camp D, et al. Human cytomegalovirus secretome contains factors that induce angiogenesis and wound healing. *J Virol* (2008) 82(13):6524–35. doi: 10.1128/JVI.00502-08
31. Fiorentini S, Luganini A, Dell'Oste V, Lorusso B, Cervi E, Caccuri F, et al. Human cytomegalovirus productively infects lymphatic endothelial cells and induces a secretome that promotes angiogenesis and lymphangiogenesis through interleukin-6 and granulocyte-macrophage colony-stimulating factor. *J Gen Virol* (2011) 92(Pt 3):650–60. doi: 10.1099/vir.0.025395-0
32. Mason GM, Jackson SE, Okecha G, Poole E, Sissons JG, Sinclair J, et al. Human cytomegalovirus latency-associated proteins elicit immune-suppressive IL-10 producing CD4(+) T cells. *PloS Pathog* (2013) 9(10): e1003635. doi: 10.1371/journal.ppat.1003635
33. Stern JL, Slobedman B. Human cytomegalovirus latent infection of myeloid cells directs monocyte migration by up-regulating monocyte chemotactic protein-1. *J Immunol* (2008) 180(10):6577–85. doi: 10.4049/jimmunol.180.10.6577
34. Noriega VM, Haye KK, Kraus TA, Kowalsky SR, Ge Y, Moran TM, et al. Human cytomegalovirus modulates monocyte-mediated innate immune responses during short-term experimental latency in vitro. *J Virol* (2014) 88 (16):9391–405. doi: 10.1128/JVI.00934-14
35. Poole E, Avdic S, Hodkinson J, Jackson SE, Wills M, Slobedman B, et al. Latency-associated viral interleukin-10 (IL-10) encoded by human cytomegalovirus modulates cellular IL-10 and CCL8 Secretion during latent infection through changes in the cellular microRNA hsa-miR-92a. *J Virol* (2014) 88(24):13947–55. doi: 10.1128/JVI.02424-14
36. Reeves MB, Sissons JGP, Sinclair JH. Reactivation of human cytomegalovirus in dendritic cells. *Discovery Med* (2005) 5(26):170–4.
37. Mason GM, Poole E, Sissons JG, Wills MR, Sinclair JH. Human cytomegalovirus latency alters the cellular secretome, inducing cluster of differentiation (CD)4+ T-cell migration and suppression of effector function. *Proc Natl Acad Sci U S A* (2012) 109(36):14538–43. doi: 10.1073/pnas.1204836109
38. Groves IJ, Jackson SE, Poole EL, Nachshon A, Rozman B, Schwartz M, et al. Bromodomain proteins regulate human cytomegalovirus latency and reactivation allowing epigenetic therapeutic intervention. *Proc Natl Acad Sci USA* (2021) 118(9):e2023025118. doi: 10.1073/pnas.2023025118
39. Kuyuk SA, Ercan I. Commonly used statistical methods for detecting differential gene expression in microarray experiments. *Biostatistics Epidemiol Int J* (2017) 1(1):1–8. doi: 10.30881/beij.00001
40. Cabatu SC, Pandya Y, Digioia G, Xu J, Esposito J, Pestka S, et al. PS3-69 Binding and neutralization of monoclonal antibodies to human interferon alpha subtypes. *Cytokine* (2010) 52(1-2):96. doi: 10.1016/j.cyto.2010.07.408
41. Schaeffer J, Carnec X, Reynard S, Mateo M, Picard C, Pietrosemoli N, et al. Lassa virus activates myeloid dendritic cells but suppresses their ability to stimulate T cells. *PloS Pathog* (2018) 14(11):e1007430. doi: 10.1371/journal.ppat.1007430
42. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* (2007) 8(9):942–9. doi: 10.1038/ni1496
43. Jackson SE, Sedikides GX, Okecha G, Poole EL, Sinclair JH, Wills MR. Latent Cytomegalovirus (CMV) Infection Does Not Detrimentally Alter T Cell Responses in the Healthy Old, But Increased Latent CMV Carriage Is Related to Expanded CMV-Specific T Cells. *Front Immunol* (2017) 8:733. doi: 10.3389/fimmu.2017.00733
44. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* (2001) 29(9):e45. doi: 10.1093/nar/29.9.e45
45. Rusinova I, Forster S, Yu S, Kannan A, Masse M, Cumming H, et al. Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res* (2013) 41(Database issue):D1040–6. doi: 10.1093/nar/gks1215
46. Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med* (1996) 184(3):963–9. doi: 10.1084/jem.184.3.963
47. Loetscher M, Loetscher P, Brass N, Meese E, Moser B. Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. *Eur J Immunol* (1998) 28(11):3696–705. doi: 10.1002/(SICI)1521-4141(199811)28:11<3696::AID-IMMU3696>3.0.CO;2-W
48. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* (1998) 101(4):746–54. doi: 10.1172/JCI1422
49. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. *Immunol Rev* (2007) 220:169–82. doi: 10.1111/j.1600-065X.2007.00563.x
50. Jackson SE, Sedikides GX, Mason GM, Okecha G, Wills MR. Human Cytomegalovirus (HCMV)-Specific CD4+ T Cells Are Polyfunctional and Can Respond to HCMV-Infected Dendritic Cells In Vitro. *J Virol* (2017) 91 (6):16. doi: 10.1128/JVI.02128-16
51. Crompton L, Khan N, Khanna R, Nayak L, Moss PAH. CD4(+) T cells specific for glycoprotein B from cytomegalovirus exhibit extreme conservation of T-cell receptor usage between different individuals. *Blood* (2008) 111(4):2053–61. doi: 10.1182/blood-2007-04-079863
52. Castillo P, Kolls JK. IL-10: A Paradigm for Counterregulatory Cytokines. *J Immunol* (2016) 197(5):1529–30. doi: 10.4049/jimmunol.1601192
53. Soderberg-Naucler C, Fish KN, Nelson JA. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* (1997) 91(1):119–26. doi: 10.1016/S0092-8674(01)80014-3
54. Zhen A, Krutzik SR, Levin BR, Kasparian S, Zack JA, Kitchen SG. CD4 ligation on human blood monocytes triggers macrophage differentiation and enhances HIV infection. *J Virol* (2014) 88(17):9934–46. doi: 10.1128/JVI.00616-14
55. Reeves MB. Cell signaling and cytomegalovirus reactivation: what do Src family kinases have to do with it? *Biochem Soc Trans* (2020) 48(2):667–75. doi: 10.1042/BST20191110
56. Dupont L, Du L, Poulter M, Choi S, McIntosh M, Reeves MB. Src family kinase activity drives cytomegalovirus reactivation by recruiting MOZ histone acetyltransferase activity to the viral promoter. *J Biol Chem* (2019) 294 (35):12901–10. doi: 10.1074/jbc.RA119.009667

57. Wills MR, Poole E, Lau B, Krishna B, Sinclair JH. The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies? *Cell Mol Immunol* (2015) 12(2):128–38. doi: 10.1038/cmi.2014.75
58. Poole E, Sinclair J. Sleepless latency of human cytomegalovirus. *Med Microbiol Immunol* (2015) 204(3):421–9. doi: 10.1007/s00430-015-0401-6
59. Parry HM, Zuo J, Frumento G, Mirajkar N, Inman C, Edwards E, et al. Cytomegalovirus viral load within blood increases markedly in healthy people over the age of 70 years. *Immun Ageing* (2016) 13(1):1. doi: 10.1186/s12979-015-0056-6
60. Reeves MB, Sinclair JH. Circulating dendritic cells isolated from healthy seropositive donors are sites of human cytomegalovirus reactivation in vivo. *J Virol* (2013) 87(19):10660–7. doi: 10.1128/JVI.01539-13
61. Sung PS, Hong SH, Lee J, Park SH, Yoon SK, Chung WJ, et al. CXCL10 is produced in hepatitis A virus-infected cells in an IRF3-dependent but IFN-independent manner. *Sci Rep* (2017) 7(1):6387. doi: 10.1038/s41598-017-06784-x
62. Zhang M, Deng X, Guan X, Geng L, Fu M, Zhang B, et al. Herpes Simplex Virus Type 2 Infection-Induced Expression of CXCR3 Ligands Promotes CD4 (+) T Cell Migration and Is Regulated by the Viral Immediate-Early Protein ICP4. *Front Immunol* (2018) 9:2932. doi: 10.3389/fimmu.2018.02932
63. Srivastava R, Khan AA, Chilukuri S, Syed SA, Tran TT, Furness J, et al. CXCL10/CXCR3-Dependent Mobilization of Herpes Simplex Virus-Specific CD8(+) TEM and CD8(+) TRM Cells within Infected Tissues Allows Efficient Protection against Recurrent Herpesvirus Infection and Disease. *J Virol* (2017) 91(14):JVI.00278–17. doi: 10.1128/JVI.00278-17
64. Carlin LE, Hemann EA, Zacharias ZR, Heusel JW, Legge KL. Natural Killer Cell Recruitment to the Lung During Influenza A Virus Infection Is Dependent on CXCR3, CCR5, and Virus Exposure Dose. *Front Immunol* (2018) 9:781. doi: 10.3389/fimmu.2018.00781
65. Reeves MB, Compton T. Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J Virol* (2011) 85(23):12750–8. doi: 10.1128/JVI.05878-11
66. Dupont L, Reeves MB. Cytomegalovirus latency and reactivation: recent insights into an age old problem. *Rev Med Virol* (2016) 26(2):75–89. doi: 10.1002/rmv.1862
67. Lynch GW, Turville S, Carter B, Sloane AJ, Chan A, Muljadi N, et al. Marked differences in the structures and protein associations of lymphocyte and monocyte CD4: resolution of a novel CD4 isoform. *Immunol Cell Biol* (2006) 84(2):154–65. doi: 10.1111/j.1440-1711.2005.01403.x
68. Rodriguez RM, Suarez-Alvarez B, Lavin JL, Ascension AM, Gonzalez M, Lozano JJ, et al. Signal Integration and Transcriptional Regulation of the Inflammatory Response Mediated by the GM-/M-CSF Signaling Axis in Human Monocytes. *Cell Rep* (2019) 29(4):860–72.e5. doi: 10.1016/j.celrep.2019.09.035
69. Roback JD, Hillyer CD, Drew WL, Laycock ME, Luka J, Mocarski ES, et al. Multicenter evaluation of PCR methods for detecting CMV DNA in blood donors. *Transfusion* (2001) 41(10):1249–57. doi: 10.1046/j.1537-2995.2001.41101249.x
70. Yaiw KC, Ovchinnikova O, Taher C, Mohammad AA, Davoudi B, Shlyakhto E, et al. High prevalence of human cytomegalovirus in carotid atherosclerotic plaques obtained from Russian patients undergoing carotid endarterectomy. *Herpesviridae* (2013) 4(1):3. doi: 10.1186/2042-4280-4-3
71. Beyaz MO, Ugurlucan M, Oztas DM, Meric M, Conkbayir C, Agacfidan A, et al. Evaluation of the relationship between plaque formation leading to symptomatic carotid artery stenosis and cytomegalovirus by investigating the virus DNA. *Arch Med Sci - Atherosclerotic Dis* (2019) 4(1):19–24. doi: 10.5114/amsad.2019.83304
72. Xenaki E, Hassoulas J, Apostolakis S, Sourvinos G, Spandidos DA. Detection of Cytomegalovirus in Atherosclerotic Plaques and Nonatherosclerotic Arteries. *Angiology* (2009) 60(4):504–8. doi: 10.1177/0003319708322390
73. Poole E, Lau JC, Sinclair J. Latent infection of myeloid progenitors by human cytomegalovirus protects cells from FAS-mediated apoptosis through the cellular IL-10/PEA-15 pathway. *J Gen Virol* (2015) 96(8):2355–9. doi: 10.1099/vir.0.000180
74. Poole E, McGregor Dallas SR, Colston J, Joseph RS, Sinclair J. Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in CD34(+) progenitors. *J Gen Virol* (2011) 92(Pt 7):1539–49. doi: 10.1099/vir.0.031377-0
75. Elder E, Krishna B, Williamson J, Aslam Y, Farahi N, Wood A, et al. Monocytes Latently Infected with Human Cytomegalovirus Evade Neutrophil Killing. *iScience* (2019) 12:13–26. doi: 10.1016/j.isci.2019.01.007
76. Aslam Y, Williamson J, Romashova V, Elder E, Krishna B, Wills M, et al. Human Cytomegalovirus Upregulates Expression of HCLS1 Resulting in Increased Cell Motility and Transendothelial Migration during Latency. *iScience* (2019) 20:60–72. doi: 10.1016/j.isci.2019.09.016
77. Szentes V, Gazdag M, Szokodi I, Dezsi CA. The Role of CXCR3 and Associated Chemokines in the Development of Atherosclerosis and During Myocardial Infarction. *Front Immunol* (2018) 9:1932. doi: 10.3389/fimmu.2018.01932

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Stochastic Episodes of Latent Cytomegalovirus Transcription Drive CD8 T-Cell "Memory Inflation" and Avoid Immune Evasion

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Acute infection with murine cytomegalovirus (mCMV) is controlled by $CD8^+$ T cells and develops into a state of latent infection, referred to as latency, which is defined by lifelong maintenance of viral genomes but absence of infectious virus in latently infected cell types. Latency is associated with an increase in numbers of viral epitope-specific $CD8^+$ T cells over time, a phenomenon known as "memory inflation" (MI). The "inflationary" subset of $CD8^+$ T cells has been phenotyped as $KLRG1^+CD62L^-$ effector-memory T cells (iTEM). It is agreed upon that proliferation of iTEM requires repeated episodes of antigen presentation, which implies that antigen-encoding viral genes must be transcribed during latency. Evidence for this has been provided previously for the genes encoding the MI-driving antigenic peptides IE1-YPHFMPTNL and m164-AGPPRYSRI of mCMV in the *H-2*^{d} haplotype. There exist two competing hypotheses for explaining MI-driving viral transcription. The "reactivation hypothesis" proposes frequent events of productive virus reactivation from latency. Reactivation involves a coordinated gene expression cascade from immediate-early (IE) to early (E) and late phase (L) transcripts, eventually leading to assembly and release of infectious virus. In contrast, the "stochastic transcription hypothesis" proposes that viral genes become transiently de-silenced in latent viral genomes in a stochastic fashion, not following the canonical IE-E-L temporal cascade of reactivation. The reactivation hypothesis, however, is incompatible with the finding that productive virus reactivation is exceedingly rare in immunocompetent mice and observed only under conditions of compromised immunity. In addition, the reactivation hypothesis fails to explain why immune evasion genes, which are regularly expressed during reactivation in the same cells in which epitope-encoding genes are expressed, do not prevent antigen presentation and thus MI. Here we show that IE, E, and L genes are transcribed during latency, though stochastically, not following the IE-E-L temporal cascade. Importantly, transcripts that encode MI-driving antigenic peptides rarely coincide with those that encode immune evasion proteins. As immune evasion can operate only in *cis*, that is, in a cell that simultaneously expresses antigenic peptides,

the stochastic transcription hypothesis explains why immune evasion is not operative in latently infected cells and, therefore, does not interfere with MI.

Keywords: antigen presentation, effector memory CD8+ T cells, gene expression, immune evasion, latency, latent infection, memory inflation, virus reactivation

INTRODUCTION

Mouse models of experimental high-dose systemic cytomegalovirus (CMV) infection, using murine cytomegalovirus (mCMV) to account for host-species specificity of CMVs (reviewed in (1)), have revealed an unconventional kinetics of the immune response. After clearance of productive infection, transient contraction of the viral-epitope specific pool of $CD8^+$ T cells is followed by pool expansion selectively for certain viral epitopes during non-productive, latent infection (2–6). This phenomenon is known as "memory inflation" (MI) (for reviews, see (7–11)). MI has prompted the promising idea to use CMVs as vaccine vectors by replacing endogenous MI-driving epitopes with epitopes of unrelated pathogens or tumors to generate enduring and self-enhancing immunological memory [(12–16), reviewed in (17–19)].

The expanding $CD8^+$ T-cell population is characterized in the mouse model by the cell surface marker phenotype $KLRG1^+CD62L^-$ and was originally classified as short-lived effector cells (SLEC) (20). A recent study has shown an extended life span of these cells, based on IL15-mediated increased expression of the anti-apoptotic protein Bcl-2, which makes them memory cell-like (21). We have therefore suggested naming these cells inflationary T effector-memory cells (iTEM) to distinguish them from $KLRG1^-CD62L^-$ conventional T effector-memory cells (cTEM) (22).

Although MI is not consistently observed in human studies on the development and maintenance of the memory $CD8^+$ T-cell response to natural infections with human cytomegalovirus (hCMV), large T-cell responses can be elicited that remain high or even increase over time, and display a phenotype characterized by an advanced differentiation stage (for recent reviews, see (23, 24)). A difference between experimental models and human infection may relate to latent viral genome load, which is determined by the extent and duration of virus replication and spread, based on the history of primary infection in terms of age at the time of infection, route of infection, initial virus dose, and immune status (25–27). As discussed and proposed by Adler and Reddehase (26), congenital infection that is characterized by an extended period of persistent virus replication and shedding due to an immature immune system, is to be expected to generate a high load of latent viral genomes favoring MI. Clinical investigations to test this hypothesis are pending, not least because of ethical concerns.

Local infection, which is rapidly controlled by the immune response (28), failed to support MI of iTEM in immunocompetent mice (22, 29). In contrast, despite the same virus dose and site of infection, transient immunodeficiency in a model of hematopoietic cell transplantation (HCT) (reviewed in (30)) led to systemic acute infection and eventually to a high latent viral genome load supporting MI of iTEM after $CD8^+$ T-cell reconstitution (2, 3, 22, 31). While these parameters can be experimentally preset to support MI in animal models, they are given and mostly unknown variables in humans who have individual histories of natural hCMV infections.

Although $CD8^+$ T-cell priming determines the magnitude of MI by generating the epitope-specific cells that can later be re-stimulated (32), MI is programmed by viral latency. Maintained expression of the lead marker of iTEM, namely KLRG1, requires persistent or at least repetitive antigen stimulation. KLRG1 is known to be expressed by $CD8^+$ T cells during chronic infections but lost in resolved infections (33). While the immunology of MI is well-characterized (7–11), the source of the viral antigens that drive MI is still under debate. CMV infections are not chronic infections with persistent, though low-level, virus production continuously providing antigen for $CD8^+$ T-cell re-stimulation, but become latent as defined by maintenance of the viral genome in certain cell types in absence of virus production [(34), reviewed in (35)]. This definition of latency by Roizman and Sears (36) applies to all members of the herpesvirus family. However, CMVs, like all other herpesviruses, can reactivate from latency to productive, recurrent infection. It has been proposed that frequent reactivation events drive MI by re-expression of antigens that then re-stimulate cells to generate a growing pool of iTEM (37).

This "reactivation hypothesis" of MI is, however, not compatible with reports that showed absence of infectious virus in tissues of latently infected mice, including the lungs that represent the major organ site of CMV pathogenesis, latent viral genome load, and reactivation in the model of neonatal infection and in the HCT model (25, 38, 39). Importantly, absence of infectious virus was confirmed by ultrasensitive detection methods that by far exceeded the sensitivity achieved by methods used for routine quantitation of infectious virus (34). Productive reactivation was in fact never observed to occur spontaneously in latently infected, immunocompetent mice, but only after experimental depletion of immune cell subsets or after general hematoablation (25, 40, 41), and the incidence of induced reactivation correlated with latent viral genome load (25, 38, 39). Notably, MI was observed also in mice latently infected with a single-cycle recombinant mCMV unable to reactivate to production of infectious virions because of genetic deletion of glycoprotein L (29). Thus, experimental data do not support the hypothesis of frequent events of productive reactivation being the driver of MI.

However, a modification of the "reactivation hypothesis", assuming incomplete reactivation under conditions of immune surveillance, remained valid. So, one might argue that inflationary $CD8^+$ T cells sense reactivation events by recognizing antigens expressed in the course of reactivation, and terminate the productive viral cycle before the assembly and release of infectious virions (42). The productive viral cycle is

characterized by coordinated gene expression defined for all members of the herpesvirus family as a temporal transcription cascade that is divided into three kinetic classes progressing from the immediate-early (IE) phase, to the early (E) phase, and finally to the late (L) phase (43–45). Thus, if the hypothesis applies, the analysis of transcripts in tissues of latently infected mice should be in conformity with coordinated gene expression.

Here we provide evidence in support of an alternative hypothesis for explaining MI, the "stochastic transcription hypothesis" (8) proposing sporadic episodes of transient de-silencing of genes in latent viral genomes in a stochastic fashion, not following the IE-E-L temporal cascade of productive cycle transcription. Notably, only the "stochastic transcription hypothesis" can explain why expression of viral immune evasion genes does not prevent the presentation of MI-driving antigenic peptides.

MATERIALS AND METHODS

Viruses and Mice

Bacterial artificial chromosome (BAC)-cloned virus MW97.01, derived from BAC plasmid pSM3fr (46, 47), is herein referred to as mCMV-WT. Cell culture-derived high titer virus stocks were generated by a standard protocol (48).

Female BALB/c (8-week-old) mice were purchased from Harlan Laboratories and were housed under specified pathogen-free (SPF) conditions in the Translational Animal Research Center (TARC) of the University Medical Center Mainz.

Experimental HCT and Establishment of Latent mCMV Infection

Syngeneic hematopoietic cell transplantation (HCT) with 9-week-old female BALB/c mice as bone marrow cell (BMC) donors and recipients was described previously (48, 49). In brief, hematoablative conditioning was performed by total-body γ-irradiation with a single dose of 6.5 Gy. At 6 h after irradiation, $5x10^6$ BMC were infused into the tail vein of the recipients, followed by intraplantar infection with $1x10^5$ PFU of mCMV-WT. Organ infectivity was followed up to 4 months post-HCT by a high-sensitivity plaque assay performed under conditions of "centrifugal enhancement of infectivity" [(34, 48) and references therein].

Quantitation of Latent Viral Genomes in Lung Tissue

To determine the latent viral DNA load in lung tissue of latently infected mice, DNA from the postcaval lobe was extracted with the DNeasy blood and tissue kit (catalog no. 69504; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral and cellular genomes were quantitated in absolute numbers by *M55*-specific and *pthrp*-specific qPCRs normalized to a $\log_{10}$-titration of standard plasmid pDrive_gB_PTHrP_Tdy (50, 51).

In Vitro Transcripts

For generation of *in vitro* transcripts, the sequences encompassing the open reading frames (ORFs) M86, M105, M112/E1, and m152 were amplified by PCR with the respective oligonucleotides (**Table S1**) from viral DNA (strain Smith ATCC VR-1399) as template. The resulting products were inserted *via* UA-cloning into the pDrive vector (Qiagen) to generate pDrive-M86, pDrive-M105, pDrive-E1, and pDrive-m152, respectively. For generation of *in vitro* transcripts for the viral ORFs m04 and m06, the respective sequences were amplified by PCR and inserted *via* the HindIII and XmaI restriction site into vector pSP64 Poly(A) (Promega, Madison, WI). All vectors were linearized with EcoRI (ThermoFisher Scientific, Langenselbold, Germany) and used as template for *in vitro* transcription with the MAXIscript SP6/T7 Transcription Kit (catalog no. AM1320, ThermoFisher Scientific). *In vitro* transcripts IE1 and m164 were described previously (52, 53).

Analysis and Quantitation of Transcripts

Viral transcripts in latently infected lungs were detected by quantitative reverse transcriptase PCR (RT-qPCR) as described previously (50). Briefly, lungs of latently infected HCT recipients were cut into pieces followed by shock-freezing in liquid N2. Total RNA was isolated with the RNeasy Mini Kit (catalog no. 7410, Qiagen) according to the manufacturer's instructions, including an on-column DNAase I (catalog no. 79254, Qiagen) digestion. Synthesis of cDNA and transcript quantification were performed using 100 ng of RNA per sample and the OneStep RT-PCR Kit (catalog no. 210212, Qiagen). For absolute quantitation, dilution series of specific *in vitro* transcripts served as standards (50). In parallel, cellular β-actin transcripts were quantified for normalization. For primers and probes, see **Table S2**.

Separation of Lung Endothelial Cells

Endothelial cells (EC) from latently infected mice were sort-purified from single-cell suspensions of lung tissue by cytofluorometric cell sorting. Single-cell suspensions were prepared essentially as described previously (22, 48), though with modifications. In brief, lungs were perfused with PBS supplemented with 10U/ml Heparin (Ratiopharm, Ulm, Germany). Lungs were excised, tracheae, bronchi, and pulmonary lymph nodes were discarded, and the lung lobes were minced. The tissue of 4-5 lungs was digested in 15 ml Gey's Balanced Salt Solution (GBSS), containing collagenase A (1.6 mg/mL; catalog no. 10 103 586 001, Roche, Mannheim, Germany) and DNase I (50 μg/mL; catalog no. DN-25, Sigma-Merck, Darmstadt, Germany), for 1 h at 37°C with constant stirring. The resulting cell suspension was washed with GBSS, and after lysis of erythrocytes washed again with GBSS. After blocking of Fc receptors with CD16/CD32 monoclonal antibody (mAb), cells were labeled with R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD31 mAb (clone 390, AbD Serotec, Kidlington, United Kingdom). The cell pellet was resuspended in phosphate-buffered saline (PBS) containing 1% FCS, and cells were labeled with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse mAb CD146 (clone ME-9F1, Miltenyi Biotec, Bergisch Gladbach, Germany). $CD31^+CD146^+$ ECs were isolated at the FACS core facility (IMB Mainz, Germany) by cytofluorometric cell sorting using BD FACS Aria (BD Bioscience, San Jose, CA).

Cytofluorometric Analyses

Single-cell suspensions were prepared from lung tissue as described (22, 48). Unspecific staining was blocked with unconjugated anti-FcγRII/III antibody (anti-CD16/CD32; clone 2.4G2, BD Pharmingen, Heidelberg, Germany). Cells were specifically stained with the following antibodies for multi-color cytofluorometric analyses: ECD-conjugated anti-CD8α (clone 53-6.7; Beckman Coulter, Krefeld, Germany), FITC-conjugated anti-KLRG1 (clone 2F1; eBioscience, Frankfurt), PE-Cy5-conjugated anti-CD127 (clone A7R34; eBioscience, Frankfurt), and PE-Cy7-conjugated anti-CD62L (clone MEL-14; Beckman Coulter). Phenotypic characterization of peptide-specific $CD8^+$ T cells was performed using PE-conjugated dextramers H-2Ld/YPHFMPTNL (IE1), H-2Dd/AGPPRYSRI (m164), and H-2Kd/TYWPVVSDI (M105) (22, 31). H-2Kb/SIINFEKL served as the control for excluding unspecific staining (Immudex, Copenhagen, Denmark). For the analyses, a "live gate" was routinely set on leukocytes in the forward scatter (FSC) versus sideward scatter (SSC) plot. All cytofluorometric analyses were performed with flow cytometer FC500 and CXP analysis software (Beckman Coulter).

Statistical Calculations

Statistical significance of differences between two independent sets of data was evaluated using the two-sided unpaired *t*-test with Welch's correction for unequal variances. In the case of genome quantification, log-normally distributed data were log-transformed to enter the *t*-test. Differences were considered as statistically significant for p-values of <0.05 (*), <0.01 (**) and <0.001 (***). Graph Pad Prism 6.04 (Graph Pad Software, San Diego, CA) was used for the calculations. Frequencies of transcriptional events were estimated from the fraction of transcript-negative pieces by using the Poisson distribution function as described (54, 55). Detection limits for viral transcripts and the corresponding 95% confidence intervals (CI) were determined by limiting dilution analysis as described (54–56). The null hypothesis of independent distribution of pairs of gene expression events was evaluated by organizing data in 2x2 contingency tables for calculation by Fisher's exact probability test (https://www.socscistatistics.com/tests/fisher/default2.aspx) (57). The null hypothesis is not refuted, and thus a correlation not assumed, if p >0.05.

RESULTS

Viral Transcription in Latently Infected Lungs Comprises Genes of the Three Kinetic Classes IE-E-L of the Viral Replication Cycle and Declines Over Time

Lungs represent a major organ site of CMV pathogenesis in clinical HCT (reviewed in (58)) and in the mouse model of experimental HCT (59). They were identified as a site of high latent viral DNA load and high risk of reactivation to recurrent infection in mouse models of neonatal infection (25, 38) and infection under conditions of HCT (34, 39). In addition, the phenomenon of MI was originally observed for $CD8^+$ T cells in persistent pulmonary infiltrates in the mouse model of HCT and mCMV infection (2, 59). We thus focus here on studying viral transcription and MI in latently infected lungs in the well-established HCT model (**Figures 1A, B**). In accordance with previous work in this model (60), productive viral replication was cleared by 4 months after HCT and infection, based on the high-sensitivity assay of "centrifugal enhancement of infectivity" (34) (**Figure 1C**). Viral genome remained present in the lungs over the entire observation period of 8 months with a statistically significant decline only between 4 months and 6 months (**Figure 1D**). So, the definition of viral latency, namely presence of viral genome in absence of infectious virus (36), is fulfilled in our study, which is a precondition for linking MI to viral latency.

Already at a time before MI was discovered, we showed stochastic activity of mCMV IE-phase gene *ie1* during latent infection of the lungs as a first example of a "transcript expressed in latency" (TEL) [(52), reviewed in (8)], and soon later stochastic and independent expression of genes *ie1* and *ie2* was described (54). These two genes flank the major IE promoter-enhancer and form a bidirectional gene pair governed by independent promoters (61, 62). As gene *ie1* encodes protein IE1/pp89, which contains the antigenic peptide IE1-YPHFMPTNL that is presented by the MHC class-I molecule L^d (63), we proposed episodes of antigen presentation during latent infection in absence of completion of the viral productive cycle (52). The IE1 peptide was the first peptide shown to drive MI of $CD8^+CD62L^-$ T effector-memory cells (TEM) (2). A role for IE1-specific $CD8^+$ T cells in immune sensing and surveillance of latent mCMV infection was proposed in the original report on antigenicity of IE proteins (64), and an antigenicity loss mutant coding for peptide IE1-YPHFMPTNA provided evidence for this (42). Only recently, transcripts coding for the second MI-driving peptide of mCMV in the $H\text{-}2^d$ haplotype, namely peptide m164-AGPPRYSRI presented by the MHC class-I molecule D^d (3), were detected in latently infected lungs (56). To our knowledge, in other mouse models of MI, such as MI in the $H\text{-}2^b$ haplotype, transcription during viral latency of genes coding for MI-driving antigenic peptides has not been studied.

Here we have addressed the question if transcription during latency follows the coordinated and temporal gene expression cascade of the productive viral cycle, characterized by directed IE-E-L phase progression (see the Introduction). For a clonal analysis, we used the established method of quantitating viral transcripts in individual tissue pieces of latently infected lungs (41, 42, 51, 52, 54). We chose transcripts to represent viral proteins characterizing the three kinetic phases. The MI-driving protein IE1 represents the IE phase, the MI-driving protein m164 is expressed in both the IE and the E phase (56), the E phase proteins E1 (65–67) and M105 (68) are both critically involved in viral DNA replication, and the L phase protein M86 is the major capsid protein (MCP) essential for virion structure and assembly (69). We quantitated transcripts by RT-qPCR at 4, 6, and 8 months after HCT and infection. The analysis was performed for 5 latently infected mice per time and for 8 lung tissue pieces p1-p8 per lung, derived from the three lobes of the right lung and the postcaval lobe (for a

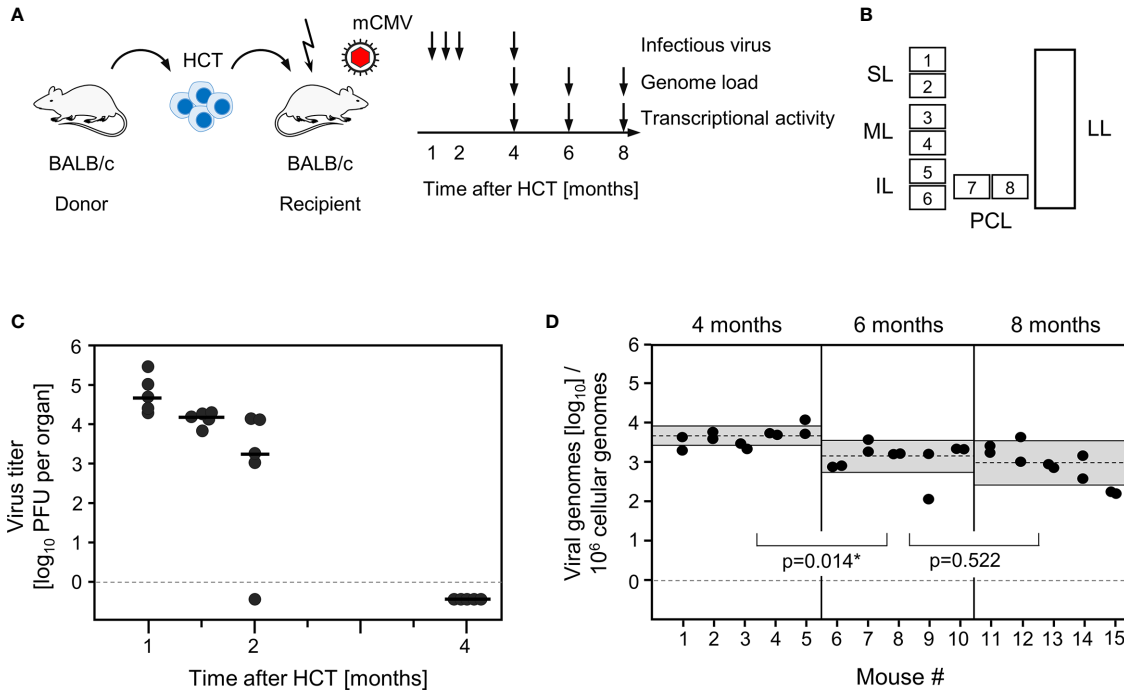

FIGURE 1 | Outline of the model and verification of the establishment of viral latency in the lungs. **(A)** Sketch of the experimental model of syngeneic hematopoietic cell transplantation (HCT) with BALB/c mice as hematopoietic cell donors and recipients, and schedule of assays. The flash symbol indicates hematoablative treatment of the recipients by total-body γ-irradiation with a single dose of 6.5 Gy prior to performing HCT and infection with mCMV. **(B)** Scheme of the lungs in anatomical view with tissue pieces p1-p6 used for quantitation of viral transcripts, p7 and p8 used for quantitation of viral transcripts and latent viral DNA load simultaneously, and the left lung used for cytofluorometric analyses. SL, superior lobe; ML, middle lobe, IL, inferior lobe; PCL, postcaval lobe, LL, left lung. **(C)** Virus titers expressed as plaque-forming units (PFU) per organ, were determined under conditions of centrifugal enhancement of infectivity. Routinely, 1% aliquots of lung homogenate were tested. Negative results were confirmed by plating the homogenate in total to avoid a sampling error. Symbols represent data from individual mice. Median values are marked. **(D)** Latent viral DNA load determined for lung tissue pieces p7 and p8 of the PCLs of mice #1-to-#5 (at 4 months), #6-to-#10 (at 6 months), and #11-to-#15 (at 8 months). Each single symbol represents the mean value from triplicate determinations. The shaded areas indicate the 95% confidence intervals for the log-normally distributed data. (*) Data sets are considered as being significantly different if $p < 0.05$.

scheme, see **Figure 1B**). Thus, 40 pieces altogether were analysed per time. The detection limits for the 5 phase-marker transcripts as well as for the transcript of the house-keeping gene standard *β-actin* in the respective RT-qPCRs were determined by limiting dilution analysis of synthetic transcripts, and were found to be comparable with overlapping 95% confidence limits (55) (**Figure S1**). At a glance, all phase-marker genes were found to be expressed in latently infected lungs, although also transcript-negative pieces existed for each of them (**Figures 2A–C**). This already indicated critical gaps in the IE-E-L cascade. The overall transcriptional activity declined over time, with the strongest recession between 4 and 6 months (**Figures 2A, B**), corresponding to a loss of latent viral genomes in this period (**Figure 1D**).

Viral Transcription in Latently Infected Lungs Follows Stochastic Patterns Incompatible With the Temporal Gene Expression Cascade During Productive Reactivation

The quantitative expression data (**Figure 2**) were categorized into tissue pieces being positive or negative for TEL from the five chosen phase-marker genes, and the resulting contextual expression patterns are shown for the 40 lung tissue pieces per time of analysis to reveal the genes expressed in the individual pieces (**Figure 3**). At 4 months after HCT and infection, the overall high transcriptional activity prevented a formal exclusion of productive infection in the 9 out of 40 pieces (#1p3/4/5, #2p6, #3p2/3/4/6, and #4p5) in which all of the five chosen phase-marker genes were found to be expressed. However, as mCMV has a coding capacity of about 200 open readings frames specifying many more essential E and L phase genes (70, 71), these 9 pieces most likely have unidentified other critical gaps in the gene expression cascade. The stochastic mode of gene expression without completion of the productive cycle becomes more evident at 6 months and 8 months when the overall transcriptional activity has declined. Notably, there even existed pieces in which the late gene *M86* was expressed, although the E phase genes *e1* and *M105*, which are essential for progression to the L phase, were both not expressed. Examples are pieces #9p6, #11p5, and #12p3.

The existence of negative pieces for any of the five phase-marker genes indicates stochastic events that follow the Poisson distribution function, from which one can calculate the number

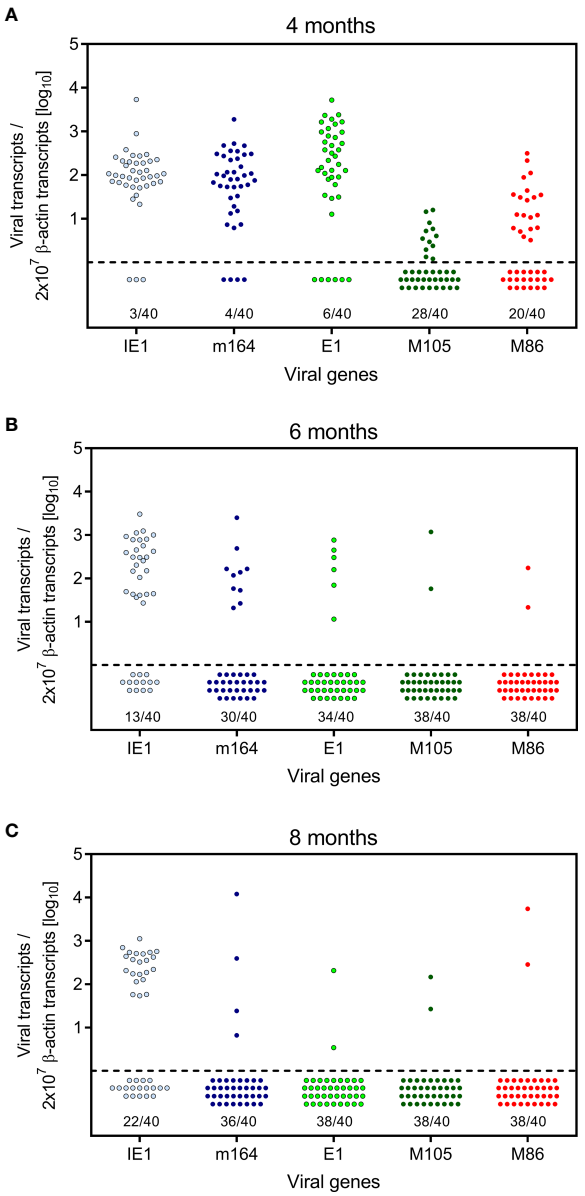

FIGURE 2 | Kinetics of viral transcriptional activity in latently infected lungs. Transcripts from the indicated IE, E, and L phase-marker genes were quantitated by RT-qPCR at **(A)** 4 months, **(B)** 6 months, and **(C)** 8 months after HCT and infection. Symbols represent data from lung tissue pieces p1-to-p8 of 5 mice, that is altogether 40 pieces per time of analysis. Pieces negative in the respective RT-qPCR are shown below the dashed line. The proportion F(0) of negative samples is indicated.

of transcription events (54). This number is higher than the number of positive pieces, because a positive piece necessarily reflects at least one clonal transcription event but may comprise also more than one transcription event. The fraction of negative pieces F(0) (**Figures 2A–C**) defines the Poisson distribution parameter lambda (λ) = - lnF(0) and allows to calculate the fraction F(n) of tissue pieces with 1,2, …n transcription events according to the formula F(n) = λ/n x F(n-1) (41, 42, 51, 54) (**Figure 4A**). For an illustration, the occupancies of tissue pieces with TEL events are shown for 8 pieces of statistically averaged lungs by down-extrapolating data from 40 pieces from five mice per time of analysis (**Figure 4B**). This illustrates that TEL are mostly of clonal origin at 6 and 8 months, with the exception of IE1 for which biclonal and triclonal transcription existed.

Memory Inflation and Deflation Reflect Preceding Events of Transcription During Viral Latency

In an attempt to relate MI to transcription of epitope-encoding genes, we used the left lung of the mice, corresponding to the TEL analyses performed with the three lobes of the right lung and the postcaval lobe (for a scheme, see **Figure 1B**), to isolate lung-infiltrating lymphocytes for cytofluorometric analyses (**Figures 5, 6**). We quantitated lung-infiltrate $CD8^+$ T-lymphocytes specific for the known antigenic peptides IE1, m164, and M105 in the *H-2^d* haplotype (reviewed in (72)), shown exemplarily for 6 months (**Figure 5A**). The full kinetics of frequencies, normalized to lung-infiltrating lymphocytes (**Figure 5B**), is compared to TEL activity in terms of transcriptional events determined in parallel in the same cohort of mice (**Figures 4A** and **5C**). As the bottom-line message, MI peaking at 6 months is preceded by high TEL activity, and the deflation seen at 8 months is preceded by a decline in TEL activity.

Latent mCMV genomes reside in non-hematopoietic tissue cells in organs, specifically in endothelial cell (EC) types [(73), reviewed in (8, 35)], including $CD31^+CD146^+$ EC in the lungs (**Figure S2**). Previous work in chimera models has shown that MI depends on antigen presentation by non-hematopoietic cells (60, 74). In models of MI after systemic infections that result in a high latent virus genome load in organs, the expanding $CD8^+$ T-cell pool is made up primarily of inflationary effector-memory T cells (iTEM) characterized by the cell surface phenotype $KLRG1^+CD62L^-$ (20, 60, 74). In contrast, these cells decline over time after local primary infection that leads to an only low latent virus genome load in organs (22). Here we have included the marker molecule CD127 (IL-7Rα) to further distinguish between $KLRG1^+CD127^-$ iTEM and $KLRG1^+CD127^+$ double-positive effector cells (DPEC) (75, 76) as well as $KLRG^-CD127^+$ conventional effector-memory T cells (cTEM) within $CD8^+CD62L^-$ cells (**Figure 6A**). As we have shown previously that the majority of cells of long-term cytolytic T-lymphocyte lines (CTLL) propagated in cell culture assume DPEC phenotype (72), we surmised that repetitive antigen restimulation by latently infected cells might generate CTLL *in vivo*. However, the kinetics revealed an MI predominantly made up by iTEM with just minimal contributions from DPEC and cTEM. As expected, the pool of $KLRG1^-CD127^+CD62L^+$ central memory T cells (TCM) does not expand at the non-lymphoid site of lung tissue (**Figure 6B**). A triple-negative population of $KLRG1^-CD127^-CD62L^-$ cells, discussed as representing early effector cells (EEC) (75, 76), is not further considered here as it does not participate in MI (data not shown).

While our focus was here on the lungs as the most prominent site of CMV pathogenesis in HCT recipients, EC or EC-related

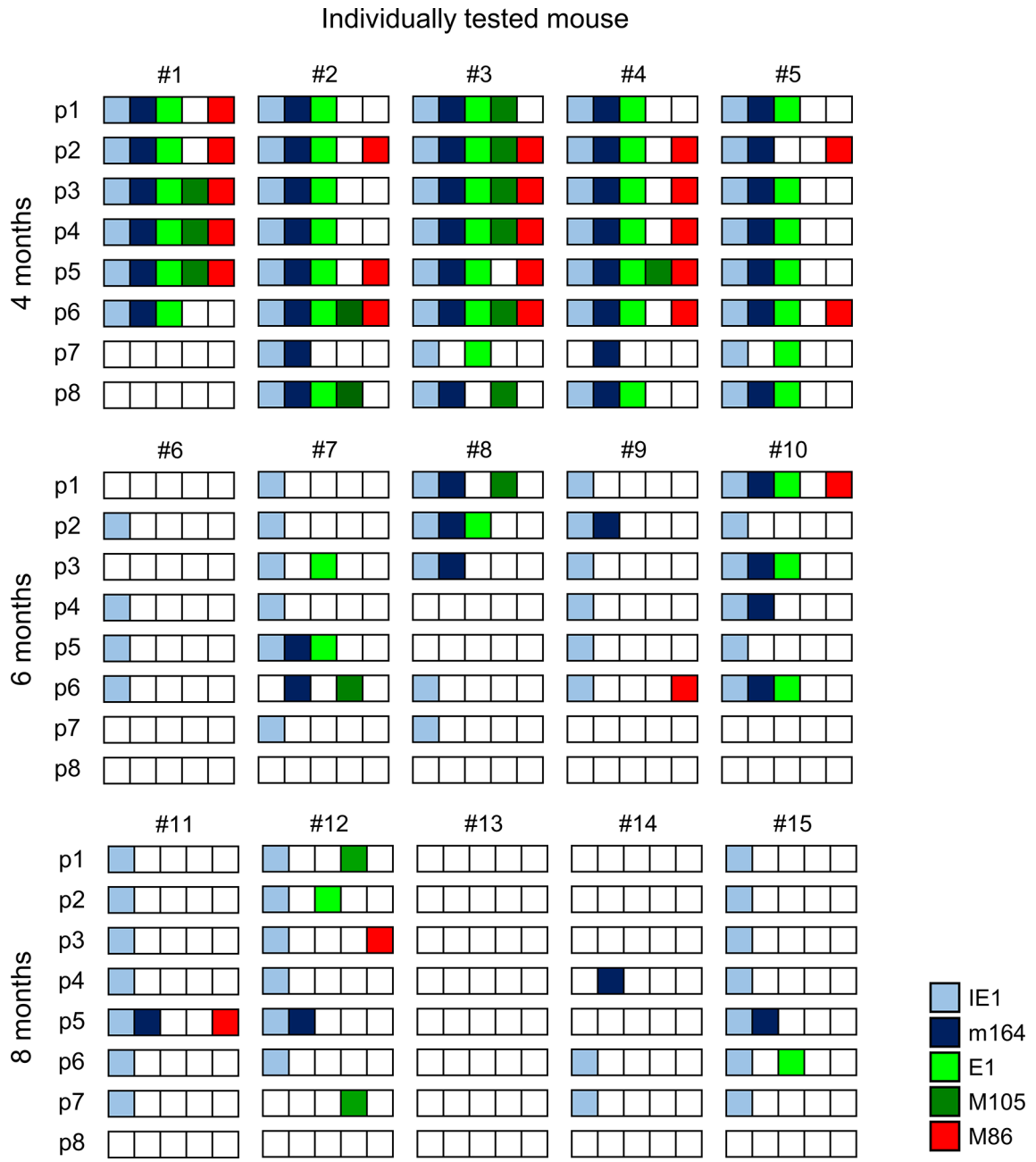

FIGURE 3 | Stochastic gene expression patterns in latently infected lungs. The quantitative gene expression data shown in **Figure 2** were categorized into positive or negative for transcripts from the respective viral gene and were assigned to the lung tissue pieces p1-to-p8 of mice #1-to-#15. Boxes negative for the respective transcripts are left blank, boxes positive for the respective transcripts are shown color-coded as specified in the internal legend.

cells are cellular sites of latent mCMV infection also in other organs, including the spleen (reviewed in (35)), and thus likely contribute to MI. As a central lymphoid organ and organ site of mCMV latency, the spleen can harbor recirculating T cells that have received an antigen signal during the patrolling of non-lymphoid tissue sites, but can also provide an antigen signal locally. We have therefore studied MI in the spleen in parallel in the same experiment for which lung data are shown above (**Figure S3**, corresponding to **Figures 5**, **6**). In essence, like in the lungs, MI in the population of $CD8^+$ T cells is predominantly made up of iTEM with the same hierarchy of viral epitopes and similar kinetics, though with some distinctive differences. Specifically, the relative decline in numbers of iTEM between 6 months and 8 months was less for epitopes IE1 and m164 compared to the lungs, whereas the number of iTEM specific for epitope M105 was even slightly increasing. Notably, TCM

A

4 months	IE1		m164		E1		M105		M86	
Events/piece	Pieces	Events	Pieces	Events	Pieces	Events	Pieces	Events	Pieces	Events
0	3	0	4	0	6	0	28	0	20	0
1	8	8	9	9	11	11	10	10	14	14
2	10	20	11	22	11	22	2	4	5	10
3	9	27	8	24	7	21	0	0	1	3
4	6	24	5	20	3	12	0	0	0	0
5	3	15	2	10	2	10	0	0	0	0
6	1	6	1	6	0	0	0	0	0	0
Sum of positive	27	100	36	91	34	76	12	14	20	27

6 months	IE1		m164		E1		M105		M86	
Events/piece	Pieces	Events	Pieces	Events	Pieces	Events	Pieces	Events	Pieces	Events
0	13	0	30	0	34	0	38	0	38	0
1	15	15	9	9	6	6	2	2	2	2
2	8	16	1	2	0	0	0	0	0	0
3	3	9	0	0	0	0	0	0	0	0
4	1	4	0	0	0	0	0	0	0	0
Sum of positive	27	44	10	11	6	6	2	2	2	2

8 months	IE1		m164		E1		M105		M86	
Events/piece	Pieces	Events	Pieces	Events	Pieces	Events	Pieces	Events	Pieces	Events
0	18	0	36	0	38	0	38	0	38	0
1	14	14	4	4	2	2	2	2	2	2
2	6	12	0	0	0	0	0	0	0	0
3	2	6	0	0	0	0	0	0	0	0
Sum of positive	22	32	4	4	2	2	2	2	2	2

B

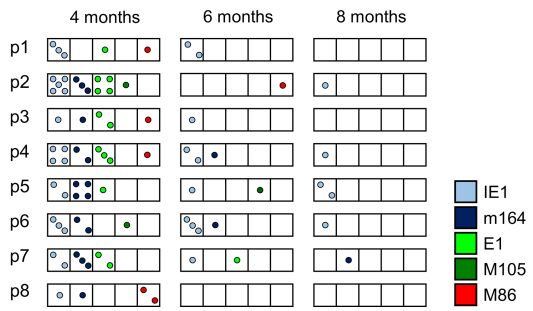

FIGURE 4 | Quantitation of transcriptional episodes in latently infected lungs. **(A)** Poisson distribution analysis of clonality. Based on the fraction of tissue pieces negative for transcripts from the respective viral gene (**Figure 2**), the Poisson distribution parameter $\lambda = -\ln F(0)$ allows the calculation of the numbers of clonal F(n=1), biclonal F(n=2) and oligoclonal F(n >2) transcription events according to the formula $F(n) = \lambda/n \times F(n-1)$. **(B)** Illustration of clonality for a "statistical lung", representing the average of lungs derived from 5 mice per time of analysis.

stayed at low-level throughout the observation time. Based on previous findings on secondary iTEM pool contraction at late times due to exhaustion of frequently re-sensitized high-avidity cells (22), we speculate that re-stimulation by local TEL activity is generally less frequent in the spleen compared to the lungs, and is particularly rare for epitope M105 for which the iTEM pool continued to expand instead of contract.

Stochasticity of Viral Gene Expression During Latency Allows MI by Avoiding Immune Evasion

CMVs express immune evasion proteins that interfere with cell surface trafficking of peptide-loaded MHC class-I (pMHC-I) complexes in the MHC class-I pathway of antigen processing and presentation (reviewed in (77, 78)). In the case of mCMV, three "viral regulators of antigen presentation" (vRAP) operate in the E phase. The negative vRAP m06/gp48 (79, 80) and the positive vRAP m04/gp34 (81–83) compete for pMHC-I cargo in post-Golgi network sorting to the lysosome and the cell surface, respectively. They thus oppose each other when co-expressed (84). In consequence, during productive infection when both are expressed, immune evasion is primarily determined by the negative vRAP m152/gp40 that traps pMHC-I complexes in a *cis*-Golgi/ER intermediate-Golgi compartment (85, 86).

Given the overall low transcriptional activity of viral epitope-encoding genes during latency, one wonders why the mechanisms of immune evasion do not interfere with antigen presentation and thus do not prevent MI. If the "reactivation hypothesis" applies, reactivation originating from viral genomes in a latently infected cell would proceed along the programmed IE-E-L phase progression (see the Introduction) and must inevitably reach the point at which the E-phase vRAP m152/gp40 is expressed to interfere with pMHC-I cell surface presentation and thus also with MI. If, however, the "stochastic

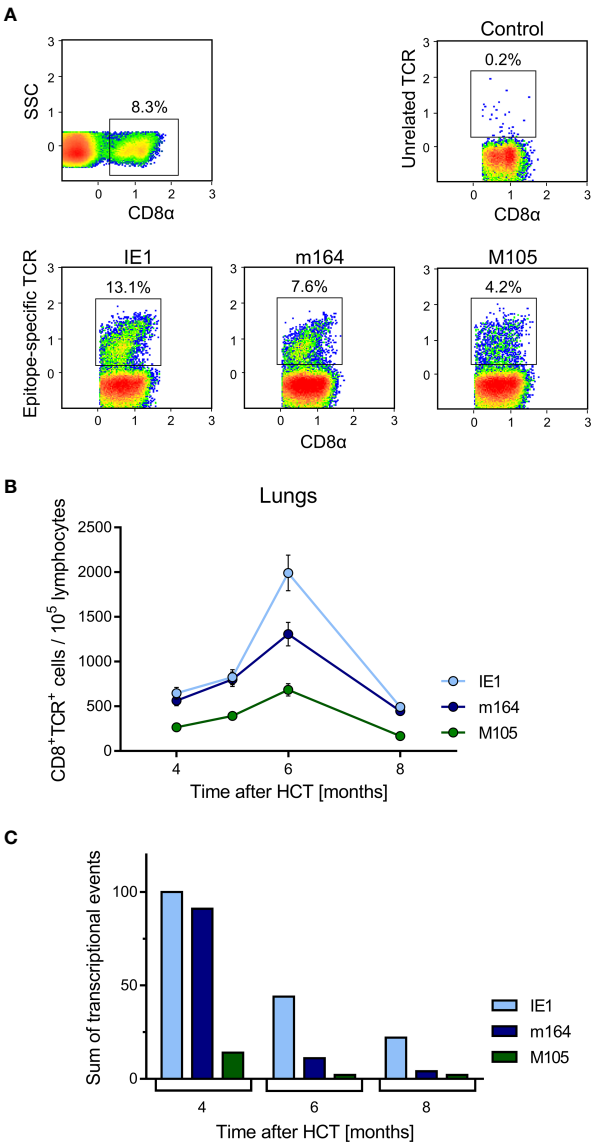

FIGURE 5 | Time course of MI of viral epitope-specific $CD8^+$ T cells in the lungs. **(A)** Gating strategy for the cytofluorometric quantitation of pulmonary $CD8^+$ T cells expressing T-cell receptors specific for the pMHC-I complexes IE1-L^d, m164-D^d, and M105-K^d. Control, PE-conjugated pMHC-I dextramer H-2Kb/SIINFEKL. SSC, sideward scatter. Data refer to 6 months after HCT and infection. **(B)** Response kinetics of viral epitope-specific $CD8^+$ T cells isolated from pulmonary infiltrates of the LL at the indicated times after HCT and infection. Note that, so far, antigenic peptides are not identified for proteins E1 and M86. Shown are median values and range for five mice per time of analysis. **(C)** Corresponding total numbers of transcriptional events as determined in SL, ML, IL, and PCL (recall **Figure 4A**).

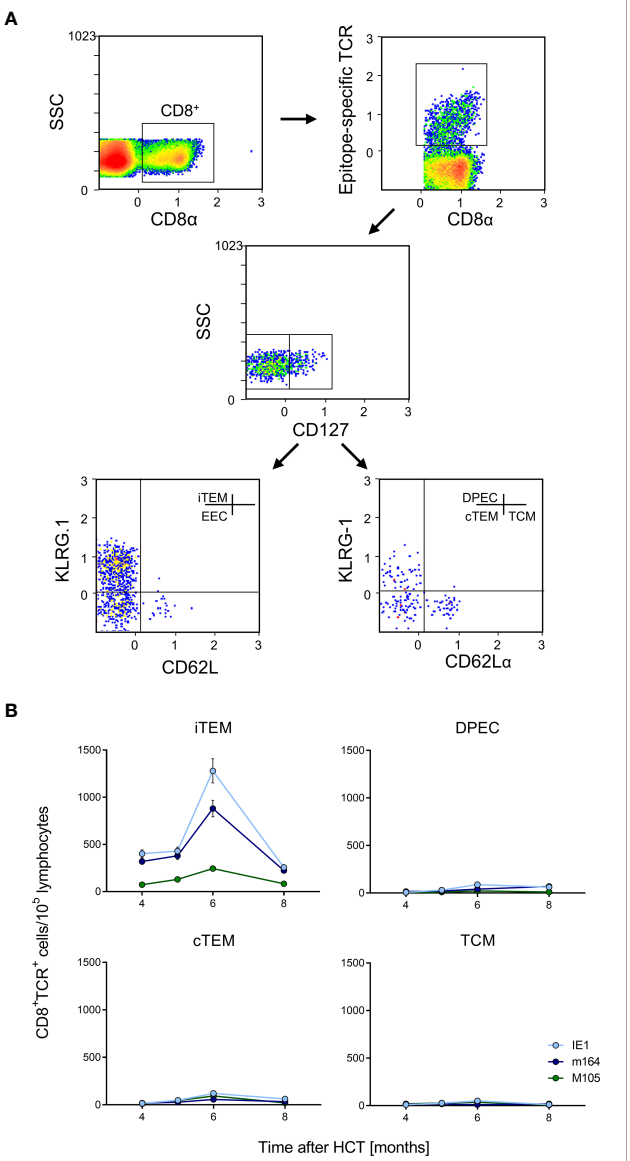

FIGURE 6 | Identification of iTEM as the "inflationary" subset of viral epitope-specific $CD8^+$ T cells in the lungs. **(A)** Gating strategy for the cytofluorometric quantitation of viral epitope-specific $KLRG1^+CD62L^-CD127^-$ inflationary effector-memory cells (iTEM), $KLRG1^-CD62L^-CD127^-$ early effector cells (EEC), $KLRG1^+CD62L^-CD127^+$ double-positive effector cells (DPEC), $KLRG1^-CD62L^-CD127^+$ conventional effector-memory cells (cTEM), and $KLRG1^-CD62L^+CD127^+$ central memory cells (TCM). SSC, sideward-scatter. Data refer to IE1-specific cells measured at 6 months after HCT and infection. **(B)** Response kinetics of the indicated subsets of viral epitope-specific $CD8^+$ T cells isolated from pulmonary infiltrates of the LL at the indicated times after HCT and infection. Shown are median values and range for five mice per time of analysis.

transcription hypothesis" applies, epitope-encoding viral genes and immune evasion molecule-encoding genes are not necessarily expressed in the same cell and thus do not meet each other.

To decide between these two hypotheses, we studied transcription of MI-driving genes and of vRAP-encoding genes in latently infected lungs of five mice at 6 months after HCT and primary infection (**Figure 7**, for the experimental protocol of HCT, see **Figure 7A**). To enhance statistical resolution, lungs were subdivided into 18 pieces (**Figure 7B**). The two pieces of the postcaval lobe, p10 and p11, served to determine the latent viral

DNA load (**Figure 7C**). Pieces p1-p9 of the three lobes of the right lung and pieces p12-p18 of the left lung, altogether 80 pieces of the lungs of 5 mice, were used to detect transcripts encoding MI-driving proteins IE1 and m164, as well as transcripts encoding vRAPs m04, m06, and m152 (**Figure 7D**).

At a glance, for each of the lungs of the 5 mice tested, the summarized expression patterns reveal tissue pieces in which either or both of the MI-driving antigens IE1 and m164 are expressed in absence of both inhibitory vRAPs m06 and m152 (**Figure 8A**). Examples are pieces #1p4/7/12, #2p2/12/13/14, #3p6/8, #4p14/18, and #5p1/6/8/14/16/18. The positive vRAP m04 was rarely expressed, specifically only in pieces #2p16 and #3p8, so that it can be neglected in this particular experiment. Altogether, there apparently existed quite a number of tissue pieces in which absence of immune evasion allowed the presentation of MI-driving antigenic peptides.

Moreover, inhibitory vRAPs can only operate when expressed in the same cell that expresses a MI-driving antigen. They cannot inhibit antigen presentation in a neighboring cell. In consequence, double occupancy of a tissue piece with MI-driving transcription events and immune evasion-mediating transcription events does not necessarily imply that immune evasion is operative. If cases of co-expression in the same cell were frequent, the number of pieces simultaneously positive for antigen-encoding and inhibitory vRAP-encoding transcripts should be higher than expected by the null hypothesis of independent distribution of these transcriptional events, that is, expression in different cells. As revealed by Fisher's Exact Probability Test comparing the observed 2x2 contingency table with the one expected for independent distribution (57), the hypothesis of independence was accepted with $p > 0.05$ (**Figure 8B**).

In conclusion, viral immune evasion does not prevent MI-driving antigen presentation, because antigens and inhibitory vRAPs are rarely co-expressed in the same cell.

DISCUSSION

It was the aim of our study to contribute to the open question of how MI-driving antigens are provided during viral latency, a state defined by absence of infectious virus despite presence of viral genomes in latently infected cells, from which reactivation

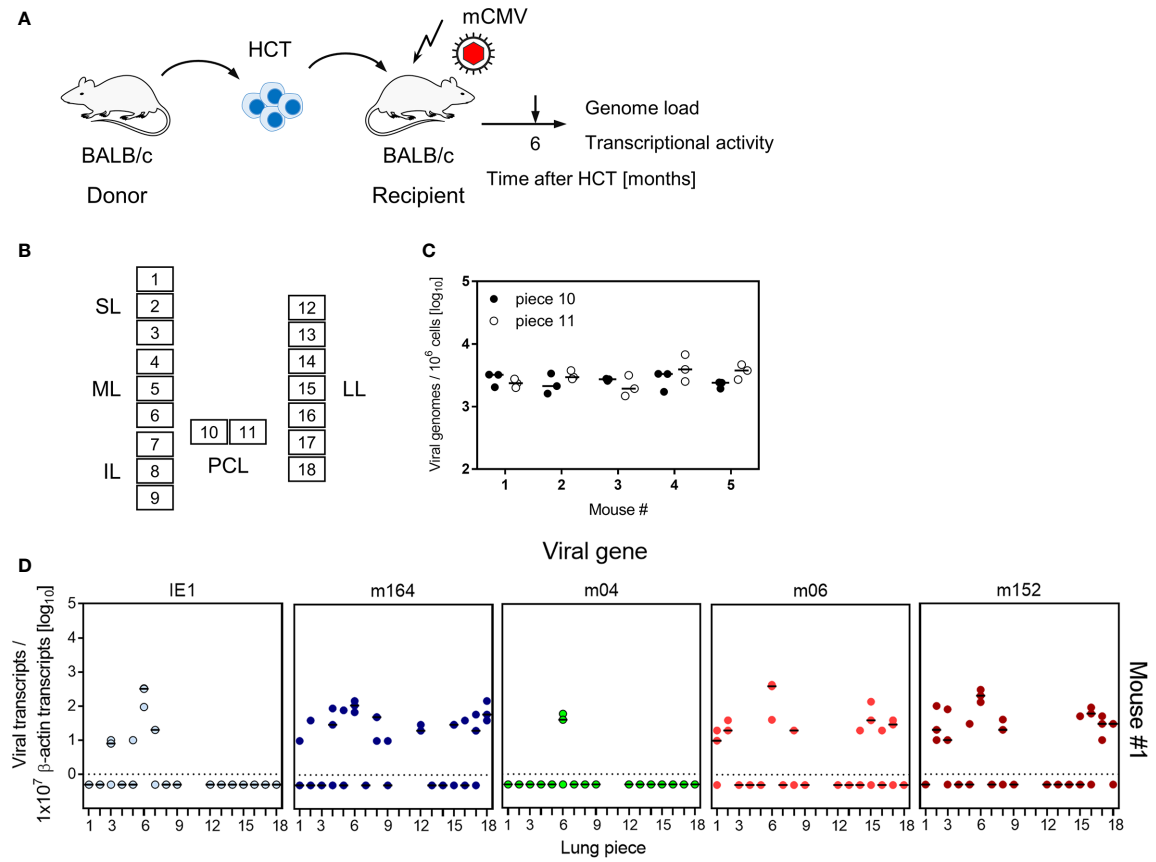

FIGURE 7 | Transcriptional activity of MI-driving and of vRAP-encoding genes in latently infected lungs. **(A)** Sketch of the experimental model of HCT and infection (for more explanation, see **Figure 1**). **(B)** Scheme of the lungs in anatomical view with tissue pieces p1-to-p9 and p12-to-p18 used for quantitation of viral transcripts. Pieces p10 and p11 were used to determine the latent viral DNA load. SL, superior lobe; ML, middle lobe, IL, inferior lobe; PCL, postcaval lobe, LL, left lung. **(C)** Viral DNA load determined in triplicates. The median values are marked. **(D)** Quantitation of transcripts from the indicated genes shown exemplarily for lung tissue pieces from mouse #1. Mice #2-to-#5 were analysed accordingly. Symbols represent triplicate measurements. The median values are marked.

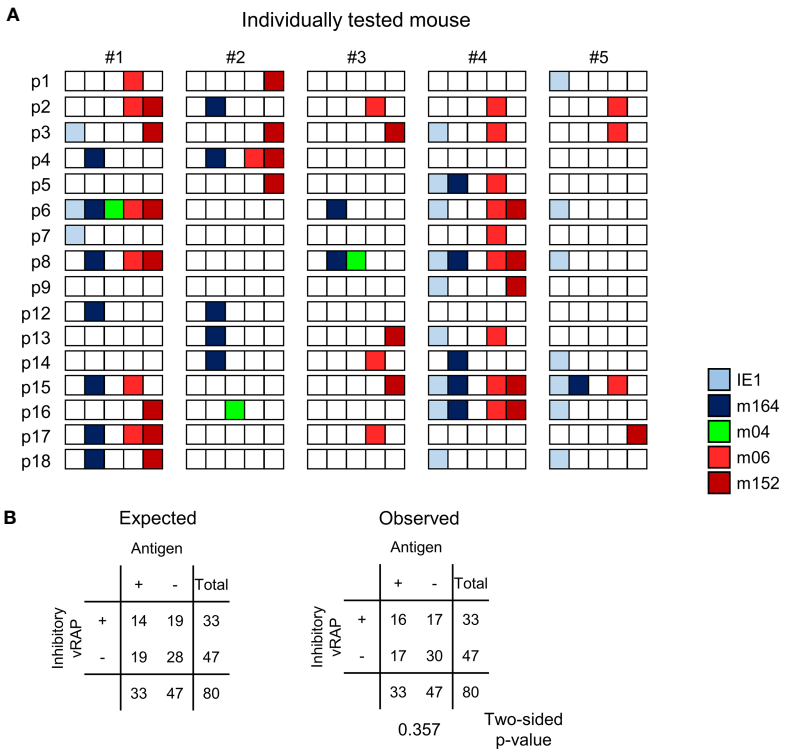

FIGURE 8 | Stochastic gene expression patterns of MI-driving and of vRAP-encoding genes in latently infected lungs. **(A)** The quantitative gene expression data, shown in **Figure 7D** exemplarily for mouse #1, were categorized for all five mice into positive or negative for transcripts from the respective viral gene and were assigned to the lung tissue pieces p1-to-p9 and p12-to-p18 of mice #1-to-#5. Boxes negative for the respective transcripts are left blank, boxes positive for the respective transcripts are shown color-coded as specified in the internal legend. **(B)** Expected and observed 2 x 2 contingency tables for the expression of MI-driving antigens (IE1 and m164 combined) and of immunoevasive vRAPs (m06 and m152 combined) for testing the null hypothesis of independent distribution with Fisher's Exact Probability Test. The null hypothesis is accepted for p >0.05.

of the full transcriptional program to productive infection can be re-initiated [for reviews, see (35, 36)]. Roizman's definition of herpesvirus latency, originally proposed for alpha-herpesviruses (36), has long been disputed with the alternative hypothesis of "low-level persistent infection" below the detection limit of assays for infectious virus. Obviously, low-level persistent infection would elegantly explain sustained provision of antigens for driving MI. However, epigenetic silencing of essential genes of the productive/lytic viral cycle and latency-specific patterns of latency-associated transcription argued for the existence of true molecular latency of the beta-herpesvirus hCMV in hematopoietic progenitor cells committed to the myeloid lineage [for reviews, see (35, 87)]. Specifically, as IE genes code for essential transactivator proteins in the viral program of replication, absence of lytic cycle IE transcripts long served as molecular evidence for latency. In turn, presence of lytic cycle IE transcripts was taken as indicating productive infection, though it is hardly possible to draw a clear distinction between a continual 'persistent' infection and frequent episodes of reactivation from latency that mimic persistence the better the shorter the intervals are. On the organismal level, latent infection and truly persistent or intermittent reactivated productive infections can co-exist compartmentalized to different cell types and organs. For instance, after acute mCMV infection, virus replication persists for some time in glandular epithelial cells of salivary glands when latency is already established in cells of other organs (25), such as in liver sinusoidal endothelial cells (LSECs) (73). Likewise, experimentally provoked reactivation of latent mCMV in organs was found to be a stochastic process that can take place in any one organ that harbors latent viral genomes, while other organs stay latently infected (25, 26). Similarly, during clinical hCMV latency, children can shed low levels of virus from infected epithelial cells in the salivary glands or kidneys for months to years, while, at the same time, infection is already latent in hematopoietic myeloid lineage progenitor cells [for reviews, see (26, 88)].

Epigenetic switches are thought to determine the transition of the viral genome into and out of latency [for reviews, see (87, 89)]. The binary view of viral gene silencing during molecular latency and coordinated de-silencing upon productive reactivation has been challenged for hCMV by highly sensitive assays that detected low levels of viral transcripts from each of the three kinetic gene classes IE, E, and L in latently infected myeloid lineage hematopoietic cells [reviewed in (90)]. This led Collins-McMillen and Goodrum to propose an equilibrium

between "true latency" characterized by a pattern of restricted latency-associated transcription and "dynamic latency" where IE, E, and L genes of the lytic program are expressed sporadically not following the canonical temporal IE-E-L cascade of productive reactivation (90). More recent work on the transcriptome of latent hCMV determined by single-cell RNA sequencing (scRNAseq) arrived at the conclusion that latently infected $CD34^+$ hematopoietic progenitor cells (HPCs) as well as $CD14^+$ monocytes express a broad spectrum of canonical viral lytic cycle genes at a low level [(91), reviewed in (92)]. Based on these findings, cells latently infected with hCMV almost certainly express antigen-encoding viral genes and thus could potentially present antigenic peptides driving MI. However, a retrospect on studies of the immune response to hCMV revealed only limited evidence supportive of MI occurring in humans (23). One may speculate that missing or inhibited MI in latently infected humans may relate to the virally encoded interleukin-10, a form of which is expressed in cells latently infected with hCMV [(93, 94), reviewed in (95)]. Alternatively, in view of the broad spectrum of transcripts revealed by scRNAseq in latently infected myeloid lineage hematopoietic cells, expression of immune evasion genes interfering with the MHC/HLA class-I pathway of antigen presentation (77) might prevent MI.

In contrast to hCMV, the predominant cell types in which mCMV latency is established are not myeloid lineage hematopoietic cells but are EC, as shown for LSECs in the liver (73) and for EC of the capillary bed of the lungs [this report]. In accordance with viral latency in ECs, MI of $KLRG1^+CD62L^-$ iTEM during latency has been shown to be driven by non-hematopoietic cells (60, 74), and the finding that iTEM proliferate in response to viral antigen presented by cells that are accessible to the blood supply (96) is compatible with antigen presentation by latently-infected EC of the lung microvasculature. Notably, our previous work has shown that genes coding for the MI-driving antigenic peptides IE1 and m164 are expressed in latently infected lungs (52, 54, 56).

It was the aim of our study to decide between two models of MI-driving viral gene expression during latency (**Figure 9A**). The "reactivation hypothesis" proposes episodes of productive virus reactivation characterized by progression of the canonical IE-E-L gene expression cascade within a cell. In contrast, the "stochastic expression hypothesis" proposes stochastic events of viral gene de-silencing that can also generate transcripts of the three kinetic classes IE, E, and L, although not in the temporal order and not necessarily all in one cell. In both models, high latent viral genome load favors high transcriptional activity, because the number of viral genomic DNA molecules determines the probability of productive cycle reactivation as well as of stochastic gene de-silencing. In accordance with this, we found here that the loss of latent viral genomes between 4 and 6 months after infection led to a drop in the transcriptional activity.

Previous work by Snyder and colleagues (29) has shown MI after high-dose systemic infection with a single-cycle recombinant mCMV lacking the essential glycoprotein L. This finding implies that MI does not depend on release of infectious virus and thus modulates the "reactivation hypothesis" in the sense that completion of the lytic cycle is not a demand for driving MI. The interpretation of MI not being driven by recurrence of infectious virus is also strongly supported by the already discussed finding of MI depending on direct antigen presentation by latently infected non-hematopoietic tissue cells (60, 74). This is in accordance with the observation that MI-inducing epitopes do not depend on the immunoproteasome for antigen processing (97). In contrast, virus released after productive reactivation, like virus produced during acute infection (60), should involve hematopoietic lineage antigen-presenting cells, such as dendritic cells, which constitutively express the immunoproteasome.

The conclusion that MI does not depend on productive reactivation was still compatible with the assumption of a canonical IE-E-L gene expression cascade of non-productive

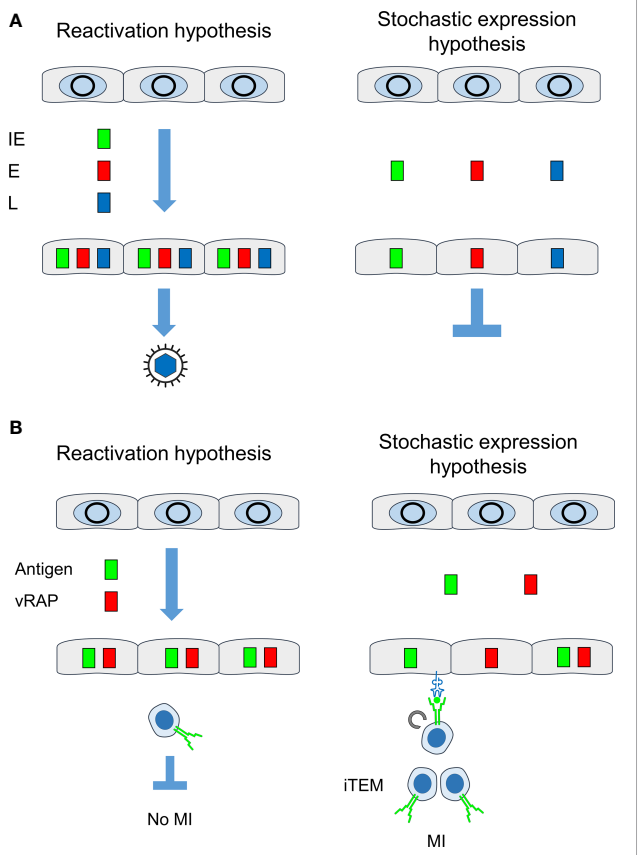

FIGURE 9 | Comparison between "reactivation hypothesis" and "stochastic expression hypothesis". **(A)** Consequences on the temporal order of viral gene expression and on recurrence of infectious virus. **(B)** Consequences on antigen presentation for driving MI. IE, immediate-early phase or protein; E, early phase or protein; L, late phase or protein; vRAP, (negative) viral regulator of antigen presentation, iTEM, inflationary effector-memory $CD8^+$ T cells; MI, memory inflation. Grey circles, silenced circularized viral genomes in the nucleus of latently infected non-hematopoietic tissue cells. Color-coded boxes: viral transcripts and proteins. Receptor symbols on tissue cells and on iTEM represent antigenic peptide-presenting MHC class-I molecules and T-cell receptors, respectively.

reactivation interrupted at a stage before virion assembly and release. However, our data showing stochastic expression patterns of genes of the three kinetic classes falsify the "reactivation hypothesis" and, instead, strongly support the "stochastic expression hypothesis". Intriguingly, gaps in the expression patterns for essential transcripts provide an immediate explanation for maintenance of latency despite expression of genes of all three kinetic classes. Note that experimentally induced reactivation does, in fact, follow the canonical IE-E-L gene expression cascade and proceeds to the production of infectious virus (41).

It is important to understand that stochastic expression of viral genes during latency means that epigenetic switches between viral chromatin opening and closing, and thus between gene desilencing and silencing, respectively, can be described by the Poisson distribution function, which allows us to calculate the frequency of transcription events at a certain time (54). It is understood that off-on-off states generate expression patterns that fluctuate in the time course, so that what we observe are snapshots. Stochastic expression, however, is not the same as spontaneous expression. As we have shown previously, TNFα signaling to the major immediate-early enhancer of mCMV enhances the transcription from gene *ie1*, which encodes the IE1 protein and MI-driving antigenic peptide (51, 61). In accordance with the stochastic nature of enhancer action (98), this enhancement was caused by increasing the frequency of still stochastic transcription events (51, 61). It is current understanding that signaling increases the probability of transcription initiation, rather than the duration of transcription once it is initiated, although one can recognize the stochastic nature of gene activity only when on-states are rare, as it is the case in viral latency. The frequencies of on-states, as we saw them in the stochastic gene expression patterns, differ significantly between different viral genes (see the numbers of transcription events in **Figure 4A**). As an extreme example, m04 was rarely expressed (**Figure 8**). These differences likely relate to different promoter activities, although experimental proof is pending. The finding that IE1 transcription stands out is reasonably explained by the fact that a strong transcriptional enhancer regulates it. Interestingly, Smith and colleagues recently presented data indicating that stochastic encounters with antigen account for the clonal dynamics during MI (99). Our data provide a molecular explanation for these immunological findings, namely that stochastic encounters of inflationary iTEM with antigen are based on stochastic expression of the corresponding viral genes during latency.

IE1 is the prototype of an MI-driving antigenic peptide (2–4), and this likely relates to the high frequency of transcription during mCMV latency, which corresponds to frequent presentation of IE1 peptide-L^d complexes on the surface of latently infected cells for re-stimulating cognate iTEM. At first glance, it may surprise that one of our experiments by chance picked up a case with overall low IE1 transcriptional activity and even absence of IE1 transcripts in 2 out of 5 mice tested individually (**Figure 8**). This brings us to consider two aspects: (i) Transcription patterns represent just snapshots, whereas the iTEM pool "samples" preceding transcription events and memorizes antigen presentation over longer periods. This explains the phase shift between a high rate of transcription at 4 months and the peak number of iTEM at 6 months when transcriptional activity had already declined (compare **Figures 3**, **4** with **Figures 5**, **6**). (ii) iTEM sense antigen presentation while patrolling in tissues for immune surveillance, and then terminate viral gene expression by their effector functions. We concluded this previously from a low frequency of IE1 transcription events in lungs during latent infection with virus mCMV-YPHFMPTNL, which expresses the epitope, and a high frequency after infection with the L9A epitope loss mutant mCMV-YPHFMPTNA (42). Thus, low transcriptional activity might reflect a high level of iTEM activity.

Stochastic expression patterns also help us to understand why reduction of transcription by immune sensing of latently infected cells is epitope-selective. If, for instance, IE1 and m164 were co-expressed in the same cells due to coordinated gene expression during productive reactivation, IE1-specific iTEM should not only reduce IE1 but also m164 transcription events. Apparently, this was not the case (**Figures 7**, **8**). Finally, one should keep in mind that transcriptional activity of epitope-encoding viral genes during latency is a prime condition for MI to occur, but other parameters represent bottlenecks. These include the amount and stability of the antigenic protein (for a recent review, see (100)), the efficacy of its proteasomal processing and the affinity with which the peptide binds to the presenting MHC-I molecule (42, 101), and the functional avidity of the tissue-patrolling $CD8^+$ T cells (22).

Our data also offer an elegant answer to the so far pending question of why viral immune evasion proteins/vRAPs do not prevent MI, although they strongly reduce cell surface presentation of pMHC-I complexes (78, 102). Very early work on the role of immune evasion in MI by Gold and colleagues (103) arrived at the conclusion that interference with antigen presentation has little effect on the size of the iTEM pool. For quite some time, this correct finding was mistaken as an evidence against an *in vivo* relevance of immune evasion in general, although soon thereafter a crucial role of immune evasion in the effector phase of the antiviral $CD8^+$ T-cell response was demonstrated in many models (for a review, see (104)). Recently, it has been shown that immune evasion is the reason for a failure in preventing lethal virus spread and histopathology in mouse models of allogeneic HCT and CMV infection (105, 106).

This apparent discrepancy is now explained by the stochastic expression of MI-driving genes and immune evasion genes during viral latency. Whereas every cell in which antigens are expressed in the course of reactivation inevitably also reaches the point at which vRAPs become expressed, stochastic gene expression rarely leads to a "by chance co-expression" of MI-driving antigens and vRAPs in the same cell (**Figure 9B**). In consequence, there always exist latently infected cells in which the presentation of MI-driving antigenic peptides is not inhibited by immune evasion.

In an attempt to provide a mathematical model, Gabel and colleagues (37) studied the dynamics of MI in individual mice and found that curve fitting is best when intervals between iTEM re-stimulations are short enough to level the oscillation between expansion and contraction of the iTEM pool. With this

understanding, the authors proposed frequent episodes of productive virus reactivation with bursts of virus release providing the antigen for frequent re-stimulation of iTEM. Such a view on MI is, however, incompatible with experimental data showing that productive reactivation is exceedingly rare in latently infected immunocompetent mice (34, 41, 42, 51) and is also incompatible with critical gaps in viral gene expression [this report] as well as with MI induced by the single-cycle virus mutant (29). Instead, the mathematical modeling of MI is in perfect accordance with an almost continuous re-stimulation of iTEM by antigenic peptides derived from episodes of stochastic gene expression during viral latency.

Overall, our data provide reasonable evidence to conclude that stochastic gene expression during viral latency is the viral driver of MI.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of the 'Landesuntersuchungsamt Rheinland-Pfalz' according to German federal law §8 Abs. 1 TierSchG (animal protection law), permission numbers 177-07/G 10-1-052 and 177-07/G14-1-015.

AUTHOR CONTRIBUTIONS

MR and NL are responsible for conception and design of the study, analysis, and interpretation of the data. MG, AR, KF, CS, and NL conducted the work and analysed the data. MR wrote the first draft of the manuscript. NL wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.668885/full#supplementary-material

Supplementary Figure 1 | Detection limits of RT-qPCRs. Limiting dilution analysis (55) based on the experimentally determined fractions of negative replicates F(0) for log_2 graded numbers of the indicated synthetic *in vitro* transcripts (starting with 8 transcripts) subjected to RT-qPCRs in 16 replicates each. The plots of lnF(0) on the ordinate and the number of transcripts on the abscissa show the Poisson distribution regression lines calculated with the maximum likelihood method. Color-shaded areas indicate the 95% confidence intervals (CI) for the estimated most probable number (MPN). The MPN is the number of transcripts revealed as the abscissa coordinate of the point of intersection between the regression line and a line at the ordinate value lnF(0) = ln1/e = -1 (dashed lines). The null hypothesis of Poisson distribution is accepted for $p > 0.05$.

Supplementary Figure 2 | Localization of latent viral genome to EC isolated from the lungs. $CD31^+CD146^+$ EC were isolated by digestion of latently infection lung tissue followed by cytofluorometric cell sorting. Latent viral genome load was determined by qPCR specific for gene *M55*. The latent viral genome is found enriched in the sorted EC compared to all lung cells. Symbols represent triplicate measurements with the median values indicated. The analysis was performed for five latently infected mice analysed individually.

Supplementary Figure 3 | Time course of MI and identification of iTEM as the "inflationary" subset of viral epitope-specific $CD8^+$ T cells in the spleen. **(A)** Gating strategy for the cytofluorometric quantitation of spleen-derived $CD8^+$ T cells expressing T-cell receptors specific for the pMHC-I complexes IE1-L^d, m164-D^d, and M105-K^d. Control, PE-conjugated pMHC-I dextramer H-2Kb/SIINFEKL. SSC, sideward-scatter. Data refer to 6 months after HCT and infection. **(B)** Response kinetics of viral epitope-specific $CD8^+$ T cells isolated from the spleen at the indicated times after HCT and infection. Shown are median values and range for five mice per time of analysis. **(C)** Response kinetics of the indicated subsets of viral epitope-specific $CD8^+$ T cells isolated from the spleen at the indicated times after HCT and infection. Shown are median values and range for five mice per time of analysis. For further explanation, see the legend of **Figure 6**.

REFERENCES

1. Reddehase MJ, Lemmermann NAW. Mouse Model of Cytomegalovirus Disease and Immunotherapy in the Immunocompromised Host: Predictions for Medical Translation That Survived the "Test of Time". *Viruses* (2018) 10:693. doi: 10.3390/v10120693
2. Holtappels R, Pahl-Seibert MF, Thomas D, Reddehase MJ. Enrichment of Immediate-Early 1 (m123/pp89) Peptide-Specific CD8 T Cells in a Pulmonary CD62L(lo) Memory-Effector Cell Pool During Latent Murine Cytomegalovirus Infection of the Lungs. *J Virol* (2000) 74:11495–503. doi: 10.1128/jvi.74.24.11495-11503.2000
3. Holtappels R, Thomas D, Podlech J, Reddehase MJ. Two Antigenic Peptides From Genes m123 and m164 of Murine Cytomegalovirus Quantitatively Dominate CD8 T-Cell Memory in the H-2d Haplotype. *J Virol* (2002) 76:151–64. doi: 10.1128/jvi.76.1.151-164.2002
4. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, et al. Memory Inflation: Continuous Accumulation of Antiviral CD8+ T Cells Over Time. *J Immunol* (2003) 170:2022–9. doi: 10.4049/jimmunol.170.4.2022
5. Munks MW, Cho KS, Pinto AK, Sierro S, Klenerman P, Hill AB. Four Distinct Patterns of Memory CD8 T Cell Responses to Chronic Murine Cytomegalovirus Infection. *J Immunol* (2006) 177:450–8. doi: 10.4049/jimmunol.177.1.450
6. Munks MW, Gold MC, Zajac AL, Doom CM, Morello CS, Spector DH, et al. Genome-Wide Analysis Reveals a Highly Diverse Cd8 T Cell Response to Murine Cytomegalovirus. *J Immunol* (2006) 1766:3760–6. doi: 10.4049/jimmunol.176.6.3760
7. Klenerman P, Oxenius A. T Cell Responses to Cytomegalovirus. *Nat Rev Immunol* (2016) 16:367–77. doi: 10.1038/nri.2016.38
8. Seckert CK, Griessl M, Büttner JK, Scheller S, Simon CO, Kropp KA, et al. Viral Latency Drives 'Memory Inflation': A Unifying Hypothesis Linking Two Hallmarks of Cytomegalovirus Infection. *Med Microbiol Immunol* (2012) 201:551–66. doi: 10.1007/s00430-012-0273-y

9. Seckert CK, Grießl M, Büttner JK, Freitag K, Lemmermann NA, Hummel MA, et al. "Immune Surveillance of Cytomegalovirus Latency and Reactivation in Murine Models: Link to 'Memory Inflation'," In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. I. Norfolk, UK: Caister Academic Press (2013). p. 374–416.
10. Cicin-Sain L. Cytomegalovirus Memory Inflation and Immune Protection. *Med Microbiol Immunol* (2019) 208:339–47. doi: 10.1007/s00430-019-00607-8
11. Welten SPM, Baumann NS, Oxenius A. Fuel and Brake of Memory T Cell Inflation. *Med Microbiol Immunol* (2019) 208:329–38. doi: 10.1007/s00430-019-00587-9
12. Karrer U, Wagner M, Sierro S, Oxenius A, Hengel H, Dumrese T, et al. Expansion of Protective CD8+ T-Cell Responses Driven by Recombinant Cytomegaloviruses. *J Virol* (2004) 78:2255–64. doi: 10.1128/jvi.78.5.2255-2264.2004
13. Hansen SG, Piatak M, Ventura AB, Hughes CM, Gilbride RM, Ford JC, et al. Immune Clearance of Highly Pathogenic SIV Infection. *Nature* (2013) 502:100–4. doi: 10.1038/nature12519
14. Hansen SG, Zak DE, Xu G, Ford JC, Marshall EE, Malouli D, et al. Prevention of Tuberculosis in Rhesus Macaques by a Cytomegalovirus-Based Vaccine. *Nat Med* (2018) 24:130–43. doi: 10.1038/nm.4473
15. Hansen SG, Marshall EE, Malouli D, Ventura AB, Hughes CM, Ainslie E, et al. A Live-Attenuated RhCMV/SIV Vaccine Shows Long-Term Efficacy Against Heterologous SIV Challenge. *Sci Transl Med* (2019) 11:eaaw2607. doi: 10.1126/scitranslmed.aaw2607
16. Caposio P, van den Worm S, Crawford L, Perez W, Kreklywich C, Gilbride RM, et al. Characterization of a Live-Attenuated HCMV-based Vaccine Platform. *Sci Rep* (2019) 9:19236. doi: 10.1038/s41598-019-55508-w
17. Jarvis MA, Hansen SG, Nelson JA, Picker LJ, Früh K. "Vaccine Vectors Using the Unique Biology and Immunology of Cytomegalovirus". In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. II. Norfolk, UK: Caister Academic Press (2013). p. 450–62.
18. Früh K, Picker L. CD8+ T Cell Programming by Cytomegalovirus Vectors: Applications in Prophylactic and Therapeutic Vaccination. *Curr Opin Immunol* (2017) 47:52–6. doi: 10.1016/j.coi.2017.06.010
19. Méndez AC, Rodríguez-Rojas C, Del Val M. Vaccine Vectors: The Bright Side of Cytomegalovirus. *Med Microbiol Immunol* (2019) 208:349–63. doi: 10.1007/s00430-019-00597-7
20. Snyder CM, Cho KS, Bonnett EL, van Dommelen S, Shellam GR, Hill AB. Memory Inflation During Chronic Viral Infection is Maintained by Continuous Production of Short-Lived, Functional T Cells. *Immunity* (2008) 29:650–9. doi: 10.1016/j.immuni.2008.07.017
21. Baumann NS, Torti N, Welten SPM, Barnstorf I, Borsa M, Pallmer K, et al. Tissue Maintenance of CMV-Specific Inflationary Memory T Cells by IL-15. *PLoS Pathog* (2018) 14:e1006993. doi: 10.1371/journal.ppat.1006993
22. Holtappels R, Freitag K, Renzaho A, Becker S, Lemmermann NAW, Reddehase MJ. Revisiting CD8 T-Cell 'Memory Inflation': New Insights With Implications for Cytomegaloviruses as Vaccine Vectors. *Vaccines* (2020) 8:402. doi: 10.3390/vaccines8030402
23. Jackson SE, Sedikides GX, Okecha G, Wills MR. Generation, Maintenance and Tissue Distribution of T Cell Responses to Human Cytomegalovirus in Lytic and Latent Infection. *Med Microbiol Immunol* (2019) 208:375–89. doi: 10.1007/s00430-019-00598-6
24. van den Berg SP, Pardieck IN, Lanfermeijer J, Sauce D, Klenerman P, van Baarle D, et al. The Hallmarks of CMV-Specific CD8 T-Cell Differentiation. *Med Microbiol Immunol* (2019) 208:365–73. doi: 10.1007/s00430-019-00608-7
25. Reddehase MJ, Balthesen M, Rapp M, Jonjić S, Pavić I, Koszinowski UH. The Conditions of Primary Infection Define the Load of Latent Viral Genome in Organs and the Risk of Recurrent Cytomegalovirus Disease. *J Exp Med* (1994) 179:185–93. doi: 10.1084/jem.179.1.185
26. Adler SP, Reddehase MJ. Pediatric Roots of Cytomegalovirus Recurrence and Memory Inflation in the Elderly. *Med Microbiol Immunol* (2019) 208:323–8. doi: 10.1007/s00430-019-00609-6
27. Redeker A, Welten SP, Arens R. Viral Inoculum Dose Impacts Memory T-Cell Inflation. *Eur J Immunol* (2014) 44:1046–57. doi: 10.1002/eji.201343946
28. Böhm V, Simon CO, Podlech J, Seckert CK, Gendig D, Deegen P, et al. The Immune Evasion Paradox: Immunoevasins of Murine Cytomegalovirus Enhance Priming of CD8 T Cells by Preventing Negative Feedback Regulation. *J Virol* (2008) 82:11637–50. doi: 10.1128/JVI.01510-08
29. Snyder CM, Cho KS, Bonnett EL, Allan JE, Hill AB. Sustained CD8+ T Cell Memory Inflation After Infection With a Single-Cycle Cytomegalovirus. *PLoS Pathog* (2011) 7:e1002295. doi: 10.1371/journal.ppat.1002295
30. Reddehase MJ. Mutual Interference Between Cytomegalovirus and Reconstitution of Protective Immunity After Hematopoietic Cell Transplantation. *Front Immunol* (2016) 7:294. doi: 10.3389/fimmu.2016.00294
31. Holtappels R, Lemmermann NA, Podlech J, Ebert S, Reddehase MJ. Reconstitution of CD8 T Cells Protective Against Cytomegalovirus in a Mouse Model of Hematopoietic Cell Transplantation: Dynamics and Inessentiality of Epitope Immunodominance. *Front Immunol* (2016) 7:232. doi: 10.3389/fimmu.2016.00232
32. Baumann NS, Welten SPM, Torti N, Pallmer K, Borsa M, Barnstorf I, et al. Early Primed KLRG1- CMV-specific T Cells Determine the Size of the Inflationary T Cell Pool. *PLoS Pathog* (2019) 15:e1007785. doi: 10.1371/journal.ppat.1007785
33. Thimme R, Appay V, Koschella M, Panther E, Roth E, Hislop AD, et al. Increased Expression of the NK Cell Receptor KLRG1 by Virus-Specific CD8 T Cells During Persistent Antigen Stimulation. *J Virol* (2005) 79:12112–6. doi: 10.1128/JVI.79.18.12112-12116.2005
34. Kurz S, Steffens HP, Mayer A, Harris JR, Reddehase MJ. Latency Versus Persistence or Intermittent Recurrences: Evidence for a Latent State of Murine Cytomegalovirus in the Lungs. *J Virol* (1997) 71:2980–7. doi: 10.1128/JVI.71.4.2980-2987.1997
35. Reddehase MJ, Lemmermann NA. Cellular Reservoirs of Latent Cytomegaloviruses. *Med Microbiol Immunol* (2019) 208:391–403. doi: 10.1007/s00430-019-00592-y
36. Roizman B, Sears AE. An Inquiry Into the Mechanisms of Herpes Simplex Virus Latency. *Annu Rev Microbiol* (1987) 41:543–71. doi: 10.1146/annurev.mi.41.100187.002551
37. Gabel M, Baumann NS, Oxenius A, Graw F. Investigating the Dynamics of MCMV-Specific CD8+ T Cell Responses in Individual Hosts. *Front Immunol* (2019) 10:1358. doi: 10.3389/fimmu.2019.01358
38. Balthesen M, Messerle M, Reddehase MJ. Lungs are a Major Organ Site of Cytomegalovirus Latency and Recurrence. *J Virol* (1993) 67:5360–6. doi: 10.1128/JVI.67.9.5360-5366.1993
39. Steffens HP, Kurz S, Holtappels R, Reddehase MJ. Preemptive CD8 T-Cell Immunotherapy of Acute Cytomegalovirus Infection Prevents Lethal Disease, Limits the Burden of Latent Viral Genomes, and Reduces the Risk of Virus Recurrence. *J Virol* (1998) 72:1797–04. doi: 10.1128/JVI.72.3.1797-1804.1998
40. Polić B, Hengel H, Krmpotić A, Trgovcich J, Pavić I, Luccaronin P, et al. Hierarchical and Redundant Lymphocyte Subset Control Precludes Cytomegalovirus Replication During Latent Infection. *J Exp Med* (1998) 188:1047–54. doi: 10.1084/jem.188.6.1047
41. Kurz SK, Reddehase MJ. Patchwork Pattern of Transcriptional Reactivation in the Lungs Indicates Sequential Checkpoints in the Transition From Murine Cytomegalovirus Latency to Recurrence. *J Virol* (1999) 73:8612–22. doi: 10.1128/JVI.73.10.8612-8622.1999
42. Simon CO, Holtappels R, Tervo HM, Böhm V, Däubner T, Oehrlein-Karpi SA, et al. CD8 T Cells Control Cytomegalovirus Latency by Epitope-Specific Sensing of Transcriptional Reactivation. *J Virol* (2006) 80:10436–56. doi: 10.1128/JVI.01248-06
43. Honess RW, Roizman B. Regulation of Herpesvirus Macromolecular Synthesis. I. Cascade Regulation of the Synthesis of Three Groups of Viral Proteins. *J Virol* (1974) 14:8–19. doi: 10.1128/JVI.14.1.8-19.1974
44. Fortunato EA, Spector DH. Regulation of Human Cytomegalovirus Gene Expression. *Adv Virus Res* (1999) 54:61–128. doi: 10.1016/s0065-3527(08)60366-8
45. Keil GM, Ebeling-Keil A, Koszinowski UH. Temporal Regulation of Murine Cytomegalovirus Transcription and Mapping of Viral RNA Synthesized at Immediate Early Times After Infection. *J Virol* (1984) 50:784–95. doi: 10.1128/JVI.50.3.784-795.1984
46. Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. Cloning and Mutagenesis of a Herpesvirus Genome as an Infectious Bacterial Artificial Chromosome. *Proc Natl Acad Sci USA* (1997) 94:14759–63. doi: 10.1073/pnas.94.26.14759
47. Wagner M, Jonjic S, Koszinowski UH, Messerle M. Systematic Excision of Vector Sequences From the BAC-Cloned Herpesvirus Genome During Virus Reconstitution. *J Virol* (1999) 73:7056–60. doi: 10.1128/JVI.73.8.7056-7060.1999

48. Podlech J, Holtappels R, Grzimek NK, Reddehase MJ. "Animal Models: Murine Cytomegalovirus". In: SHE Kaufmann, D Kabelitz, editors. *Methods in Microbiology. Immunology of Infection*. London, UK: Academic Press (2002). p. 493–525. doi: 10.1016/S0580-9517(02)32103-2
49. Renzaho A, Podlech J, Kühnapfel B, Blaum F, Reddehase MJ, Lemmermann NA. Cytomegalovirus-Associated Inhibition of Hematopoiesis is Preventable by Cytoimmunotherapy With Antiviral CD8 T Cells. *Front Cell Infect Microbiol* (2020) 10:138. doi: 10.3389/fcimb.2020.00138
50. Lemmermann NA, Podlech J, Seckert CK, Kropp KA, Grzimek NK, Reddehase MJ, et al. "CD8 T Cell Immunotherapy of Cytomegalovirus Disease in the Murine Model". In: D Kabelitz, SHE Kaufmann, editors. *Methods in Microbiology*, vol. 37 . London, UK: Immunology of infection. Academic Press (2010). p. 369–420. doi: 10.1016/S0580-9517(10)37016-4
51. Simon CO, Seckert CK, Dreis D, Reddehase MJ, Grzimek NK. Role for Tumor Necrosis Factor Alpha in Murine Cytomegalovirus Transcriptional Reactivation in Latently Infected Lungs. *J Virol* (2005) 79:326–40. doi: 10.1128/JVI.79.1.326-340.2005
52. Kurz SK, Rapp M, Steffens HP, Grzimek NK, Schmalz S, Reddehase MJ. Focal Transcriptional Activity of Murine Cytomegalovirus During Latency in the Lungs. *J Virol* (1999) 73:482–94. doi: 10.1128/JVI.73.1.482-494.1999
53. Holtappels R, Grzimek NK, Simon CO, Thomas D, Dreis D, Reddehase MJ. Processing and Presentation of Murine Cytomegalovirus pORFm164-derived Peptide in Fibroblasts in the Face of All Viral Immunosubversive Early Gene Functions. *J Virol* (2002) 76:6044–53. doi: 10.1128/jvi.76.12.6044-6053.2002
54. Grzimek NK, Dreis D, Schmalz S, Reddehase MJ. Random, Asynchronous, and Asymmetric Transcriptional Activity of Enhancer-Flanking Major Immediate-Early Genes ie1/3 and ie2 During Murine Cytomegalovirus Latency in the Lungs. *J Virol* (2001) 75:2692–705. doi: 10.1128/JVI.75.6.2692-2705.2001
55. Lefkovits I, Waldman H. "Limiting Dilution Analysis". In: *Limiting Dilution Analysis of Cells in the Immune System*. Cambridge, UK: Cambridge University Press (1979). p. 60–82. doi: 10.1016/B978-0-12-442750-1.50032-2
56. Renzaho A, Schmiedeke JK, Griessl M, Kühnapfel B, Seckert CK, Lemmermann NA. Transcripts Expressed in Cytomegalovirus Latency Coding for an Antigenic IE/E Phase Peptide That Drives "Memory Inflation". *Med Microbiol Immunol* (2019) 208:439–46. doi: 10.1007/s00430-019-00615-8
57. Lefkovits I, Waldman H. "Statistical Test, Limitations and Reproducibility". In: *Limiting Dilution Analysis of Cells in the Immune System*. Cambridge, UK: Cambridge University Press (1979). p. 93–114. doi: 10.1016/B978-0-12-442750-1.50032-2
58. Seo S, Boeckh M. "Clinical Cytomegalovirus Research: Haematopoietic Cell Transplantation". In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. 2. Norfolk, UK: Caister Academic Press (2013). p. 335–51.
59. Podlech J, Holtappels R, Pahl-Seibert MF, Steffens HP, Reddehase MJ. Murine Model of Interstitial Cytomegalovirus Pneumonia in Syngeneic Bone Marrow Transplantation: Persistence of Protective Pulmonary CD8-T-Cell Infiltrates After Clearance of Acute Infection. *J Virol* (2000) 74:7496–507. doi: 10.1128/jvi.74.16.7496-7507.2000
60. Seckert CK, Schader SI, Ebert S, Thomas D, Freitag K, Renzaho A, et al. Antigen-Presenting Cells of Haematopoietic Origin Prime Cytomegalovirus-Specific CD8 T-Cells But are Not Sufficient for Driving Memory Inflation During Viral Latency. *J Gen Virol* (2011) 92:1994–2005. doi: 10.1099/vir.0.031815-0Xxx
61. Simon CO, Kühnapfel B, Reddehase MJ, Grzimek NK. Murine Cytomegalovirus Major Immediate-Early Enhancer Region Operating as a Genetic Switch in Bidirectional Gene Pair Transcription. *J Virol* (2007) 81:7805–10. doi: 10.1128/JVI.02388-06
62. Kropp KA, Simon CO, Fink A, Renzaho A, Kühnapfel B, Podlech J, et al. Synergism Between the Components of the Bipartite Major Immediate-Early Transcriptional Enhancer of Murine Cytomegalovirus Does Not Accelerate Virus Replication in Cell Culture and Host Tissues. *J Gen Virol* (2009) 90:2395–401. doi: 10.1099/vir.0.012245-0
63. Reddehase MJ, Rothbard JB, Koszinowski UH. A Pentapeptide as Minimal Antigenic Determinant for MHC Class I-Restricted T Lymphocytes. *Nature* (1989) 337:651–3. doi: 10.1038/337651a0
64. Reddehase MJ, Koszinowski UH. Significance of Herpesvirus Immediate Early Gene Expression in Cellular Immunity to Cytomegalovirus Infection. *Nature* (1984) 312:369–71. doi: 10.1038/312369a0
65. Bühler B, Keil GM, Weiland F, Koszinowski UH. Characterization of the Murine Cytomegalovirus Early Transcription Unit E1 That is Induced by Immediate-Early Proteins. *J Virol* (1990) 64:1907–19. doi: 10.1128/JVI.64.5.1907-1919.1990
66. Ciocco-Schmitt GM, Karabekian Z, Godfrey EW, Stenberg RM, Campbell AE, Kerry JA. Identification and Characterization of Novel Murine Cytomegalovirus M112-113 (e1) Gene Products. *Virology* (2002) 294:199–208. doi: 10.1006/viro.2001.1311
67. Schumacher U, Handke W, Jurak I, Brune W. Mutations in the M112/M113-Coding Region Facilitate Murine Cytomegalovirus Replication in Human Cells. *J Virol* (2010) 84:7994–8006. doi: 10.1128/JVI.02624-09
68. Morello CS, Kelley LA, Munks MW, Hill AB, Spector DH. DNA Immunization Using Highly Conserved Murine Cytomegalovirus Genes Encoding Homologs of Human Cytomegalovirus UL54 (Dna Polymerase) and UL105 (Helicase) Elicits Strong CD8 T-Cell Responses and is Protective Against Systemic Challenge. *J Virol* (2007) 81:7766–75. doi: 10.1128/JVI.00633-07
69. Hui WH, Tang Q, Liu H, Atanasov I, Liu F, Zhu H, et al. Protein Interactions in the Murine Cytomegalovirus Capsid Revealed by CryoEM. *Protein Cell* (2013) 4:833–45. doi: 10.1007/s13238-013-3060-7
70. Rawlinson WD, Farrell HE, Barrell BG. Analysis of the Complete DNA Sequence of Murine Cytomegalovirus. *J Virol* (1996) 70:8833–49. doi: 10.1128/JVI.70.12.8833-8849.1996
71. Redwood AJ, Shellam GR, Smith LM. "Molecular Evolution of Murine Cytomegalovirus Genomes". In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. 1. Norfolk, UK: Caister Academic Press (2013). p. 23–37.
72. Ebert S, Podlech J, Gillert-Marien D, Gergely KM, Büttner JK, Fink A, et al. Parameters Determining the Efficacy of Adoptive CD8 T-Cell Therapy of Cytomegalovirus Infection. *Med Microbiol Immunol* (2012) 201:527–39. doi: 10.1007/s00430-012-0258-x
73. Seckert CK, Renzaho A, Tervo HM, Krause C, Deegen P, Kühnapfel B, et al. Liver Sinusoidal Endothelial Cells are a Site of Murine Cytomegalovirus Latency and Reactivation. *J Virol* (2009) 83:8869–84. doi: 10.1128/JVI.00870-09
74. Torti N, Walton SM, Brocker T, Rülicke T, Oxenius A. Non-Hematopoietic Cells in Lymph Nodes Drive Memory CD8 T Cell Inflation During Murine Cytomegalovirus Infection. *PLoS Pathog* (2011) 7:e1002313. doi: 10.1371/journal.ppat.1002313
75. Obar JJ, Jellison ER, Sheridan BS, Blair DA, Pham QM, Zickovich JM, et al. Pathogen-Induced Inflammatory Environment Controls Effector and Memory CD8+ T Cell Differentiation. *J Immunol* (2011) 187:4967–78. doi: 10.4049/jimmunol.1102335
76. Obar JJ, Lefrançois L. Memory CD8+ T Cell Differentiation. *Ann N Y Acad Sci* (2010) 1183:251–66. doi: 10.1111/j.1749-6632.2009.05126.x
77. Reddehase MJ. Antigens and Immunoevasins: Opponents in Cytomegalovirus Immune Surveillance. *Nat Rev Immunol* (2002) 2:831–44. doi: 10.1038/nri932
78. Lemmermann NA, Fink A, Podlech J, Ebert S, Wilhelmi V, Böhm V, et al. Murine Cytomegalovirus Immune Evasion Proteins Operative in the MHC Class I Pathway of Antigen Processing and Presentation: State of Knowledge, Revisions, and Questions. *Med Microbiol Immunol* (2012) 201:497–512. doi: 10.1007/s00430-012-0257-y
79. Reusch U, Muranyi W, Lucin P, Burgert HG, Hengel H, Koszinowski UH. A Cytomegalovirus Glycoprotein Re-routes MHC Class I Complexes to Lysosomes for Degradation. *EMBO J* (1999) 18:1081–91. doi: 10.1093/emboj/18.4.1081
80. Fink A, Mikuličić S, Blaum F, Reddehase MJ, Florin L, Lemmermann NA. Function of the Cargo Sorting Dileucine Motif in a Cytomegalovirus Immune Evasion Protein. *Med Microbiol Immunol* (2019) 208:531–42. doi: 10.1007/s00430-019-00604-x
81. Kleijnen MF, Huppa JB, Lucin P, Mukherjee S, Farrell H, Campbell AE, et al. A Mouse Cytomegalovirus Glycoprotein, gP34, Forms a Complex With Folded Class I Mhc Molecules in the ER Which is Not Retained But is Transported to the Cell Surface. *EMBO J* (1997) 16:685–94. doi: 10.1093/emboj/16.4.685
82. Fink A, Blaum F, Babic Cac M, Ebert S, Lemmermann NA, Reddehase MJ. An Endocytic YXXΦ (YRRF) Cargo Sorting Motif in the Cytoplasmic Tail of Murine Cytomegalovirus AP2 'Adapter Adapter' Protein m04/gp34 Antagonizes Virus Evasion of Natural Killer Cells. *Med Microbiol Immunol* (2015) 204:383–94. doi: 10.1007/s00430-015-0414-1

83. Holtappels R, Gillert-Marien D, Thomas D, Podlech J, Deegen P, Herter S, et al. Cytomegalovirus Encodes a Positive Regulator of Antigen Presentation. *J Virol* (2006) 80:7613–24. doi: 10.1128/JVI.00723-06
84. Becker S, Fink A, Podlech J, Giese I, Schmiedeke JK, Bukur T, et al. Positive Role of the MHC Class-I Antigen Presentation Regulator m04/gp34 of Murine Cytomegalovirus in Antiviral Protection by CD8 T Cells. *Front Cell Infect Microbiol* (2020) 10:454. doi: 10.3389/fcimb.2020.00454
85. Ziegler H, Thale R, Lucin P, Muranyi W, Flohr T, Hengel H, et al. A Mouse Cytomegalovirus Glycoprotein Retains MHC Class I Complexes in the ERGIC/Cis-Golgi Compartments. *Immunity* (1997) 6:57–66. doi: 10.1016/s1074-7613(00)80242-3
86. Fink A, Renzaho A, Reddehase MJ, Lemmermann NA. The p36 Isoform of Murine Cytomegalovirus m152 Protein Suffices for Mediating Innate and Adaptive Immune Evasion. *Viruses* (2013) 5:3171–91. doi: 10.3390/v5123171
87. Reeves M, Sinclair J. "Epigenetic Regulation of Human Cytomegalovirus Gene Expression: Impact on Latency and Reactivation". In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. 1. Norfolk, UK: Caister Academic Press (2013). p. 330–46.
88. Britt W. Manifestations of Human Cytomegalovirus Infection: Proposed Mechanisms of Acute and Chronic Disease. *Curr Top Microbiol Immunol* (2008) 325:417–70. doi: 10.1007/978-3-540-77349-8_23
89. Elder E, Sinclair J. HCMV Latency: What Regulates the Regulators? *Med Microbiol Immunol* (2019) 208:431–8. doi: 10.1007/s00430-019-00581-1
90. Collins-McMillen D, Goodrum FD. The Loss of Binary: Pushing the Herpesvirus Latency Paradigm. *Curr Clin Microbiol Rep* (2017) 4:124–31. doi: 10.1007/s40588-017-0072-8
91. Shnayder M, Nachshon A, Krishna B, Poole E, Boshkov A, Binyamin A, et al. Defining the Transcriptional Landscape During Cytomegalovirus Latency With Single-Cell RNA Sequencing. *mBio* (2018) 9:e00013–18. doi: 10.1128/mBio.00013-18
92. Schwartz M, Stern-Ginossar N. The Transcriptome of Latent Human Cytomegalovirus. *J Virol* (2019) 93:e00047–19. doi: 10.1128/JVI.00047-19
93. Jenkins C, Garcia W, Godwin MJ, Spencer JV, Stern JL, Abendroth A, et al. Immunomodulatory Properties of a Viral Homolog of Human Interleukin-10 Expressed by Human Cytomegalovirus During the Latent Phase of Infection. *J Virol* (2008) 82:3736–50. doi: 10.1128/JVI.02173-07
94. Poole E, Avdic S, Hodkinson J, Jackson S, Wills M, Slobedman B, et al. Latency-Associated Viral Interleukin-10 (IL-10) Encoded by Human Cytomegalovirus Modulates Cellular IL-10 and CCL8 Secretion During Latent Infection Through Changes in the Cellular MicroRNA hsa-miR-92a. *J Virol* (2014) 88:13947–55. doi: 10.1128/JVI.02424-14
95. Poole E, Neves TC, Oliveira MT, Sinclair J, da Silva MCC. Human Cytomegalovirus Interleukin 10 Homologs: Facing the Immune System. *Front Cell Infect Microbiol* (2020) 10:245. doi: 10.3389/fcimb.2020.00245
96. Smith CJ, Turula H, Snyder CM. Systemic Hematogenous Maintenance of Memory Inflation by MCMV Infection. *PLoS Pathog* (2014) 10:e1004233. doi: 10.1371/journal.ppat.1004233
97. Hutchinson S, Sims S, O'Hara G, Silk J, Gileadi U, Cerundolo V, et al. A Dominant Role for the Immunoproteasome in CD8+ T Cell Responses to Murine Cytomegalovirus. *PLoS One* (2011) 6:e14646. doi: 10.1371/journal.pone.0014646
98. Fiering S, Whitelaw E, Martin DI. To Be or Not to Be Active: The Stochastic Nature of Enhancer Action. *Bioessays* (2000) 22:381–7. doi: 10.1002/(SICI)1521-1878(200004)22:4<381::AID-BIES8>3.0.CO;2-E
99. Smith CJ, Venturi V, Quigley MF, Turula H, Gostick E, Ladell K, et al. Stochastic Expansions Maintain the Clonal Stability of CD8+ T Cell Populations Undergoing Memory Inflation Driven by Murine Cytomegalovirus. *J Immunol* (2020) 204:112–21. doi: 10.4049/jimmunol.1900455
100. Yewdell JW, Hollý J. Drips Get Molecular. *Curr Opin Immunol* (2020) 64:130–6. doi: 10.1016/j.coi.2020.05.009
101. Dekhtiarenko I, Ratts RB, Blatnik R, Lee LN, Fischer S, Borkner L, et al. Peptide Processing is Critical for T-Cell Memory Inflation and May be Optimized to Improve Immune Protection by CMV-Based Vaccine Vectors. *PLoS Pathog* (2016) 12:e1006072. doi: 10.1371/journal.ppat.1006072
102. Lemmermann NA, Gergely K, Böhm V, Deegen P, Däubner T, Reddehase MJ. Immune Evasion Proteins of Murine Cytomegalovirus Preferentially Affect Cell Surface Display of Recently Generated Peptide Presentation Complexes. *J Virol* (2010) 84:1221–36. doi: 10.1128/JVI.02087-09
103. Gold MC, Munks MW, Wagner M, McMahon CW, Kelly A, Kavanagh DG, et al. Murine Cytomegalovirus Interference With Antigen Presentation has Little Effect on the Size or the Effector Memory Phenotype of the CD8 T Cell Response. *J Immunol* (2004) 172:6944–53. doi: 10.4049/jimmunol.172.11.6944
104. Lemmermann NA, Böhm V, Holtappels R, Reddehase MJ. In Vivo Impact of Cytomegalovirus Evasion of CD8 T-Cell Immunity: Facts and Thoughts Based on Murine Models. *Virus Res* (2011) 157:161–74. doi: 10.1016/j.virusres.2010.09.022
105. Holtappels R, Schader SI, Oettel O, Podlech J, Seckert CK, Reddehase MJ, et al. Insufficient Antigen Presentation Due to Viral Immune Evasion Explains Lethal Cytomegalovirus Organ Disease After Allogeneic Hematopoietic Cell Transplantation. *Front Cell Infect Microbiol* (2020) 10:157. doi: 10.3389/fcimb.2020.00157
106. Gezinir E, Podlech J, Gergely KM, Becker S, Reddehase MJ, Lemmermann NAW. Enhancement of Antigen Presentation by Deletion of Viral Immune Evasion Genes Prevents Lethal Cytomegalovirus Disease in Minor Histocompatibility Antigen-Mismatched Hematopoietic Cell Transplantation. *Front Cell Infect Microbiol* (2020) 10:279. doi: 10.3389/fcimb.2020.00279

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REVIEW
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Is It Feasible to Use CMV-Specific T-Cell Adoptive Transfer as Treatment Against Infection in SOT Recipients?

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During the last decade, many studies have demonstrated the role of CMV specific T-cell immune response on controlling CMV replication and dissemination. In fact, it is well established that transplanted patients lacking CMV-specific T-cell immunity have an increased occurrence of CMV replication episodes and CMV-related complications. In this context, the use of adoptive transfer of CMV-specific T-cells has been widely investigated and applied to Hematopoietic Stem Cell Transplant patients and may be useful as a therapeutic alternative, to reconstitute the CMV specific T-cell response and to control CMV viremia in patients receiving a transplantation. However, only few authors have explored the use of T-cell adoptive transfer in SOT recipients. We propose a novel review in which we provide an overview of the impact of using CMV-specific T-cell adoptive transfer on the control of CMV infection in SOT recipients, the different approaches to stimulate, isolate and expand CMV-specific T-cells developed over the years and a discussion of the possible use of CMV adoptive cellular therapy in this SOT population. Given the timeliness and importance of this topic, we believe that such an analysis will provide important insights into CMV infection and its treatment/prevention.

Keywords: cytomegalovirus, CMV-specific immune response, T-cell adoptive transfer, CMV treatment, cellular therapy

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INTRODUCTION

Viral infection, including cytomegalovirus (CMV), BK virus and Epstein-Barr virus, remains a major cause of morbidity and mortality in immunocompromised individuals (1–5). While in immunocompetent individuals latent CMV infection is controlled by the immune system (6), in transplant recipients, both hematopoietic stem cell (HSCT) and solid organ transplantation (SOT), CMV infection is one of the main infectious complications. CMV seropositive allogeneic HSCT patients presents the highest risk of recurrent infections, followed by CMV seronegative SOT recipients that receive a graft from a seropositive donor (R-/D+), HIV patients, and patients who have received T-cell depletion therapies (alemtuzumab, antithymocyte globulin, or post-transplant cyclophosphamide) (6, 7). The incidence of CMV reactivation/reinfection in SOT is 16–56% (8–12), with a median value of 30%, while in HSCT has been reported to be 30–70%, with a median value of

37% (13–15). In addition to the direct effects of CMV proliferation causing viral syndrome with clinical manifestations such as gastroenteritis, pneumonitis, hepatitis, uveitis, retinitis, encephalitis and graft rejection, CMV infection also cause indirect effects related with increased incidence of graft rejection and opportunistic infections or decreased recipient survival (16–18).

Cell mediated immune response is considered the most important arm of the immune system against CMV infection with increasing evidences demonstrating a role of CMV-specific T-cells in protecting from infection, which can contribute to improve clinical care after transplantation (19–27).

A few authors have suggested the importance of monitoring patient's CMV-specific immunity using standardized tools for individualizing the risk of CMV infection after transplantation (21, 28–31). Thus, using both immunological and virological patient monitoring may provide a wider knowledge of patients' clinical situation that may facilitate clinical decisions during follow-up of SOT recipients (32).

Although the antiviral drugs to treat CMV infection have highly improved during the years, there are still some issues associated with the use of the available antivirals (ganciclovir, foscarnet, cidofovir and more recently letermovir) such as undesirable side effects (nephrotoxicity) and selection of resistance mutations in addition to the high cost. Consequently, strong efforts have been made to search for new therapeutic approaches (33).

In this context, the use of cellular therapy may be useful to reconstitute the CMV specific T-cell response and to control CMV viremia in SOT recipients. Here we provide a synthesis of recent data regarding the impact of using CMV-specific T-cell adoptive transfer on the control of CMV infection in SOT recipients, the different approaches to stimulate, isolate and expand CMV-specific T-cells developed over the years and a discussion of the possible use of CMV adoptive cellular therapy in these patients.

USE OF ADOPTIVE TRANSFER OF CMV-SPECIFIC T-CELLS IN THE CONTEXT OF SOLID ORGAN TRANSPLANTATION

The use of CMV-specific T-cell adoptive transfer is currently being evaluated for clinical application, with promising results as a treatment for CMV infection and disease in ulcerative enteritis in primary immunodeficiency (34) or in pediatric retinitis caused by CMV (35).

In the context of transplantation, CMV-specific T-cell transfer has been widely investigated and applied to Hematopoietic Stem Cell Transplant (HSCT) patients, both prophylactically, to reconstitute protective antiviral immunity, and as a treatment in patients with refractory CMV infection (36–38). In contrast, in SOT recipients it has been less investigated probably due to the T-cell response attenuation produced by the administration of the immunosuppressive therapy. In addition, SOT recipients may not tolerate donor-derived cytolytic T lymphocytes (CTLs) due to the activation of cytokine-mediated stimulation of the alloreactive T-cells causing direct alloimmune injury (39, 40).

Few authors have explored the use of T-cell adoptive transfer in SOT recipients during the last decade (**Table 1**). In 2009, Brestrich et al. (41) performed a study in a lung transplanted recipient with a severe and persistent CMV pneumonia resistant to ganciclovir and foscarnet. Patient´s peripheral blood mononuclear cells (PBMCs) were stimulated with overlapping peptide pools covering the whole protein IE-1 and pp65 and CMV-specific INF-γ positive cells were subsequently selected and infused. The patient was treated with two infusions of $1 \times 10^7/m^2$ CMV-specific T-cells. After the first infusion, the patient developed an overall improvement, with a decrease of the viral load and pneumonia symptoms and an increase of the CMV-specific T-cell levels. Four weeks after the first infusion, a second infusion was administered due to a worsening of the disease, testing positive for CMV. However, the patient died due to graft failure with a negative biopsy for CMV antigen (41).

Since then, a number of authors have explored the potential of T-cell adoptive transfer as a therapy in SOT recipients (42). A renal transplant recipient (D+/R-) with refractory CMV infection received partially HLA-compatible (at three of six HLA loci A, B and DRB1) CMV-specific T-cells at a dose of 1.6×10^7 T-cells/m^2, successfully generated from a third donor. Nineteen days following the infusion, a fifty fold decreased of the CMV DNA viral load was observed and plasma exchange was ceased due to resolution of hematological features of thrombotic microangiopathy (platelets 269×109/L, LDH 369 IU/L, no red cell fragments on blood film). Patients was discharged from hospital four weeks after the infusion (43). The authors highlighted the effective application of CMV-specific CTLs from third donors, suggesting that creating donor cell-banks could be useful as a therapeutic alternative in SOT recipients (43–45). In a later study, the same group successfully expanded autologous CMV-specific T-cells from a seronegative recipient that received a seropositive lung allograft and that developed a CMV disease due to ganciclovir resistant CMV infection (46). CMV-specific T-cells were isolated and stimulated with autologous PBMCs coated with HLA class I-restricted CMV peptide epitopes, based on patient´s HLA class I typing. The *in vitro* expanded T-cells showed an increase in HLA epitopes (A1, B7 and B35) and in the proportion of IFN-γ+ CD107a+ cells that indicates the granule-dependent (perforin/granzyme) pathway of cytotoxic CD8+ T-cells. The patient received four infusions of 3×10^7 autologous T-cells. After the infusion of the *in vitro* expanded T-cells no adverse events occurred, the CMV viral load became undetectable, the patient's usual immunosuppression regime was resumed, hepatic and bone marrow function remained normal with no evidence of acute rejection. These results indicated that adoptive therapy can contribute to immune control of CMV infection (46). Pierucci et al. (47) employed autologous T-cell transfer in a seronegative lung transplant recipient with a ganciclovir and foscarnet resistant CMV infection, who also developed cidofovir-related nephrotoxicity. Cells were obtained from patient´s peripheral blood and expanded using epitopes of

synthetic HLA-compatible peptides (pp65, pp50 and IE-1). Around 42% of the obtained CD8+ T-cells were CMV-specific and T-cells were restricted to three HLA Class I alleles: HLA-A1, HLA-B7 and HLA-B8. The patient received 2 infusions (1.9×10^7 T cells/infusion) 2 weeks apart, with no side effects and with low CMV titers during two months after which a relapse of the viral load occurred. The patient received a third infusion (22.2×10^6 T-cells) showing some therapeutic benefit, with further significant reduction in CMV titers, which was maintained for 2 months. The patient did not have any documented rejection or acute change in lung function after the T-cell infusions. However, the patient died due to clinical complications unrelated to CMV infection (47).

The most ambitious study carried out to date was performed in a cohort of 21 SOT recipients (13 kidney, 8 lung and 1 heart) who developed recurrent ganciclovir resistant CMV infections. Thirteen of these patients (8 D+/R-, 3 D+/R+ and 2 D-/R-) were subjected to T-cell (ranging from $22.2\text{-}245 \times 10^6$ T-cells) adoptive transfer receiving a maximum of 6 doses one of which discontinued therapy after a single dose. Adverse events attributable to T-cell infusion were grade 1 or 2 (fatigue and malaise) with no adverse events associated with a change in the graft status. Eleven of the 13 showed objective improvement in their symptoms including a reduction (with a median drop of 1.2×10^3 CMV copies/mL) or resolution of CMV reactivation and resolution of CMV disease symptoms. In addition, the use of antiviral drug therapy was either completely stopped (in 5 of 11 patients) or significantly reduced (in 6 of 11patients). Evidences of immunological reconstitution was associated with control of viremia (48).

Based on these promising results, several clinical studies are currently been conducted: (i) A clinical trial (NCT03665675) including 20 patients, both HSCT recipients and SOT recipients is been conducted, to study the effect of transferring allogeneic CMV-specific T lymphocytes on CMV infection or reactivation. The first results will be available at the end of 2021. (ii) A clinical trial (NCT02779439) with 25 patients enrolled, to elucidate the biological efficacy of therapeutically administered most closely HLA-matched third-party donor-derived specific cytotoxic T lymphocytes (CTLs) targeting CMV, following allogeneic blood or marrow stem cell or SOT. (iii) A clinical trial (NCT04364178) including 25 patients assessing whether partially matched, ≥2/6 HLA-matched, viral specific T-cells have efficacy against CMV in subjects who have previously received any type of allogeneic HSCT or SOT. (iv) A clinical trial (NCT03266640) with 20 participants investigating the therapeutic role of CMV CTLs in children, adolescents and young adults (CAYA) with refractory CMV infection post allogeneic HSCT or SOT.

Together these results suggest that, although there is still space for improvement, the use of CMV-specific T-cell adoptive transfer is promising in SOT recipients with limited options for CMV-infection treatment.

CELLULAR THERAPIES AVAILABLE

During the last years a better understanding of the CMV-specific T-cell immunology such as the conserved T-cell epitopes (49), has led to the improvement of the methods for *ex vivo* T-cell culture (50). In addition, rapid tests to evaluate the effector function of the CMV-specific T-cells have become available (51, 52). In this section, we describe the features of the methodologies available to generate CMV specific T-cells, which are summarized in **Figure 1** and **Table 2**.

T-Cell Expansion

To successfully generate and expand CMV-specific T-cells, it is crucial to define the most immunogenic epitopes used by the antigen presenting cells (APC) to promote the activation and proliferation of peptide-specific T-cells (53). A large number of antigens expressed at different stages during viral replication participate in the activation of both CMV-specific CD8+ and CD4+ T-cells, known to mediate the immune response against the virus (50). IE-1 and pp65 proteins are two of the most immunodominant CMV antigens and have been widely used to stimulate the CMV-specific immune response (50, 54–56).

Different approaches have been carried out for *in vivo* expansion and generation of CMV-specific T-cells (57). In the initial studies, CMV-specific CD8+ T-cell clones were generated by stimulating donor peripheral blood mononuclear cells (PBMC) with CMV-infected fibroblasts (23). However, this approach was discontinued because of the risk of producing infection in patients. Later, CMV lysates or pp65-NLV peptide were used to stimulate CMV-specific T-cells (58–60). Using the pp65-NLV peptide only stimulated adoptive immunity against a single viral epitope (50) and its application may be limited for HLA-A2 patients/donors (60). To overcome this problem, "poly-specific" products targeting multiple antigens were generated by incubating allogeneic T-cells *in vitro* with clusters of 15-mer peptides spanning the entire pp65 antigen to generate CMV-specific oligoclonal T-cells (61). Adoptive transfer of the oligoclonal T-cells were able to eliminate viremia, and infused cells persisted for up to two years (61, 62).

The improvement of the methodology for *ex vivo* expansion has reduced the presence of alloreactive or naive T-cells in the final product (63). In addition, T-cell *ex vivo* stimulation and expansion requires a small blood volume to establish the T-cell culture, making possible the generation of CMV-specific T-cells from low levels of circulating T-cells and naive donor sources (51).

Direct Selection Using Specific Peptide–MHC (pMHC)

Using pMHC multimers allows to isolate T-cells based on the T-cells receptor (TCR) ability to bind a complex mixture of peptide-loaded recombinant HLA molecules (53). Since this method is restricted by HLA type, a previous knowledge about the immunodominance of the epitopes is necessary. HLA-peptide tetramers from pp65 and IE-1 proteins have been previously used to select CD8+ T-cells that were further isolated using magnetic beads (64).

This method allows to reduce the time and improve the quality of the final product, minimizing alloreactivity (57). However, the main disadvantages of this technique are related with the limitation of the method to isolate only CD8+ or CD4 T-cell populations, and the irreversibility of the binding that can

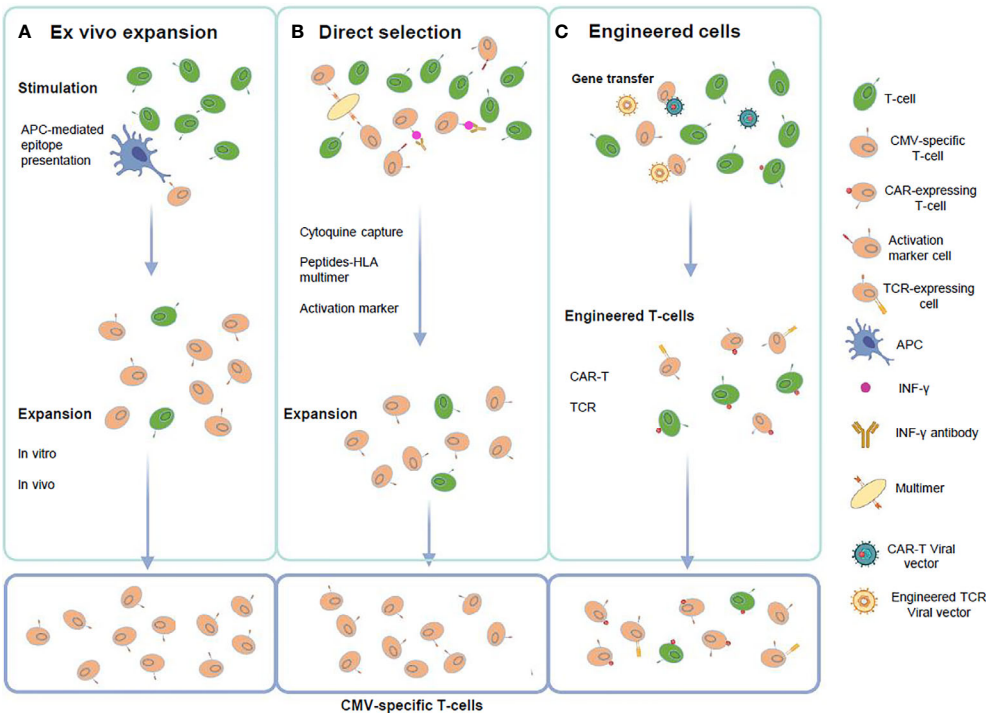

FIGURE 1 | Strategies for the generation of CMV-specific T-cells. **(A)** *Ex vivo* T-cell expansion requires the *in vitro* stimulation and expansion of T-cells using APCs presenting viral peptides or proteins. **(B)** Direct selection employs virus-derived peptide specific multimers in the setting of a HLA class-I molecule, viral antigen T-cell stimulation followed by cytokine expressing T-cell selection using antibody coated immunomagnetic beads or activation marker selection based on the detection of specific surface molecules that are selectively expressed or strongly up-regulated after T-cell activation. **(C)** Genetic manipulation requires gene transfer of high affinity CMV-specific T-cell receptors (TCR) or chimeric-antigen receptors (CAR) to change specificity of T-cells to CMV antigens. This figure was created using BioRender.com.

cause changes in the T-cell phenotype, leading to functional alterations of the purified T-cell population (such as TCR internalization, activation, overstimulation and cell death) (65–67). It has been shown that pMHC multimer binding interferes with the functional status of epitope-specific T-cell population *in vivo*, causing epitope-specific tolerance in a dose-dependent manner (68, 69). This intrinsic characteristic of pMHC multimer binding substantially limits the clinical application of this technology.

This issue has been further solved with the development of the Streptamer technology in which the binding of the HLA peptide and the antigen-specific TCR is reversed, by competing with a molecule that causes the Streptamer to monomerize, causing no alteration of the phenotype or the functional status of the T-cells (70–72). However, the selected T-cells are limited by the HLA restriction imposed by the Streptamer, and this may be a limitation for CD8+ T-cells survival when CD4+ T-cells are absent (73). Some authors have used this new technology to isolate CD8+ T-cells from CMV seropositive donors, demonstrating both immune reconstitution, as well as antiviral safety and efficacy after HSCT (74, 75). The results obtained with this technology are promising however, further studies are necessary to demonstrate efficacy in SOT recipients.

In the context of SOT, p-MHC multimers has been previously used using autologous T-cells harvested from lifelong immunosuppressed patients (while healthy donors were used in HSCT). In these patients, deficiencies in T-cell differentiation, longevity, as well as the use of immunosuppressive regimen can affect to long-term survival of the transplanted cells limiting its use for adoptive therapy (76). The associated challenges of this method could be minimized by using partially HLA-matched CMV-specific T-cells obtained from a third party donor (43). This approach was shown to be safe to treat CMV infection in SOT patients, however, more research is needed (43).

Direct Selection Using Cytokine Capture System (CCS)

CMV-specific T-cells can also be selected using IFN-γ cytokine capture system (CCS), a rapid assay that allows to select and enrich CD8+ and CD4+ INF-γ secreting T-cells that have been previously stimulated using viral antigens (77). This strategy allows T-cell selection that in contrast with pMHC has no HLA restriction and as an additional benefit, stimulating and capturing a polyclonal population of CD4+ and/or CD8+ T-cells depending on the antigen used for stimulation, not achieved using the Streptamer strategy. Different authors have successfully isolated functional CMV-specific T-cells using this method. Two of these studies stimulated donors PBMC using pp65 that were administrated to patients after HSCT who were able to expand the CMV-specific T-cells and reduced the CMV load in blood (78, 79). More recently,

TABLE 1 | List of available works of CMV-specific T-cell transfer in SOT.

Method	Organ and D/R status	Number of infused cells	Number of infusions	Cell line phenotype	Stimulation method	Post-infusion clinical outcomes
Direct selection by IFN-γ capture Brestrich et al. (41)	1 Lung +/+	Fresh 1 × 10^7 T-cells/m^2	2	95% CD3+ cells with 2.7% and 92.3% CD4+ and CD8+ cells. No CD16+ natural killer cells and only 0.1% CD19+ B cells	Overlapping IE-1/pp65 peptide pools	No side effects occurred after the infusion. The number of CMV-specific T-cells increased, while viral load decreased. The patient died from graft failure
Ex vivo expansion from a third party donor (43)	1 Kidney +/−	Frozen 1.6 x10^7 T-cells/m^2	1	16.6% CD4+ and 79.4% CD8+ cells	Overlapping pp65 peptide pool	The patient developed a mild fever but no other adverse effects were noted and within 4 months his CMV viral load decreased from >5×10^6 copies to 682 copies/mL and remained controlled up to 1 year
Autologous *Ex vivo* expansion (46)	1 Lung +/−	Fresh 3 × 10^7 T-cells	4	82.6% CD3+ cells, including 14% CD4+ and 73.8% CD8+ cells	PBMC coated with HLA class I-restricted CMV epitopes	Decrease in viral load. No graft rejection
Autologous *Ex vivo* expansion (47)	1 Lung +/−	Frozen two of 1.9 x10^7 cells and one of 22.2 x 10^6 T-cells	3	Two first infusions 41.6% CD8+ cells Third infusion 4.43% CD8+ cells	HLA Class I restricted epitopes from pp65, pp50 and IE-1	The patient did not have any documented rejection or acute change in lung function after the T-cell infusions but finally died due to clinical complications unrelated to CMV
Autologous *Ex vivo* expansion (48)	13 kidney, 8 lung and 1 heart +/− +/+ −/−	Frozen 22.2-245 × 10^6 T-cells	6	20% CD4+ and 70% CD8+ cells	HLA class I– and class II–restricted epitopes from pp65, pp50, IE-1, gH, and gB	None of the patients who received adoptive CMV-specific T-cell therapy showed treatment-related grade 3, 4, or 5 adverse events. Reduction or resolution of CMV reactivation and/or disease and improved response to antiviral drug therapy

Kim et al. (80) used the automated CliniMACS Prodigy platform to generate pp65-specific CTL that exhibited functional activity, including efficient proliferation, sustained antigen-specific IFN-γ secretion, and cytotoxicity against pp65-pulsed target T-cells. Although little clinical experience is available, this approach has the potential to be applicable to any type of patients with a clinical emergency due to CMV-related diseases including SOT recipients (80, 81).

Other selection strategy is to isolate and enrich activated viral-specific T-cells after antigen stimulation based on the detection of specific surface molecules that are selectively expressed or strongly up-regulated after T-cell activation, such as CD25, CD69, CD137 and CD154 (82–84). In this sense, several publications have shown results using CD137 as a specific activation marker due to its predominant expression on T lymphocytes after activation, including CD8+ and CD4+ cells (85, 86). This approach allows simultaneous targeting of antiviral T-helper and effector cells. Other data showed the feasibility of isolating CMV-specific T-cells from PBMCs through the use of CD25 and CD154 activation marker expression (82, 87). However, as both markers are predominantly expressed in CD4+ T-cells, these strategies do not allow the enrichment of CD8+ cytotoxic T-cells.

T-Cell Generation Using Activation Marker and Engineered T-Cells (CAR, TCR)

Other interesting strategies based on the successful performance for cancer treatment (88–90) is the gene modification of patient's lymphocytes with tumor-specific T-cell receptors (TCRs) or chimeric antigen receptors (CAR). The generation of autologous CAR T-cells which has also been explored as immunotherapy against CMV (91, 92, 93) enables antigen recognition in a MHC independent manner and can be designed to specifically target conserved and essential epitopes of the selected antigen (94), overcoming pathogen escape mechanisms. In a nutshell, CAR consists of a defined antigen-binding domain represented by a single-chain fragment variable (scFv) antibody, an extracellular spacer region, a transmembrane domain, and an intracellular domain that triggers T-cell activation, mainly by the T-cell receptor signaling domain CD3ζ (94). Several groups have recently generated gB-targeted CAR T-cells using scFvs derived from gB-specific NAb antibody (SM5-1) fused to CARs with 4-1BB (BBL) or CD28 (28S) costimulatory domains and subcloned into retroviral vectors (95, 96). In a recent study, CD4+ and CD8+ T-cells obtained from blood or cord blood of CMV-seronegative donors were transduced showing efficacy in preclinical models (96). Further clinical studies will be necessary to demonstrate *in vivo* efficacy.

The other TCR strategy uses heterodimers integrated by alpha and beta peptide chains to recognize specific polypeptide fragments presented by MHC complexes. While CAR-T-cell therapy identifies exclusively antigens located in the cell surface, TCR can also recognize intracellular antigenic fragments presented by MHC molecules (97). However, TCR T-cell therapy is restricted to MHC presentation, which represents a limitation of the strategy. The main goal of TCR T-cells is to modify the TCR binding to the pathogen antigens. Naturally, the affinity of TCRs for the pathogen antigens is

TABLE 2 | Characteristics of the T-cell therapies available.

Method	System	Advantages	Disadvantages
***Ex vivo* expansion**		No restricted by HLA type; small blood volume required; naïve donor can be used; generation of polyclonal T-cells	Extensive culture period; seropositive donors required
Direct Selection	pMHC multimer	No needed extensive *ex vivo* manipulation and undergo rapid expansion *in vivo*	Restricted by HLA type and streptamer; seropositive donors required; high frequency of specific T-cells needed; select for a limited repertoire of CD8+ cells
	Cytoquine capture	No needed extensive ex vivo manipulation and undergo rapid expansion *in vivo*; not restricted by HLA type; isolation of polyclonal CD4+ and CD8+ cells	Requires seropositive donors; large blood volumes needed
	Activation marker	Rapid detection and enrichment of T-cells; broader repertoire of antigen-specific T-cells; Compatible with other assay formats; not restricted by HLA; not needed previous information of immunodominant epitopes; no specialized APC such as dendritic cells are needed	Time-consuming and difficulty to isolate and expand functional cells; identification of novel T-cell epitopes often requires screening of a high number of epitopes
Genetically engineered cells	CAR-T	Recognize antigens in an HLA-independent manner; target conserved and essential epitopes; infused to a broad range of patients irrespective of HLA	Only surface antigens can be targeted; restricted by epitope; expensive; Several toxicities
	TCR-T	Wider range of targets; high affinity for specific antigens through genetic engineering; strong activation when a small amount of antigen is present; use of natural T-cell signaling mechanisms	Expensive; time- and labor-consuming; MHC restricted and depends on presentation by MHC molecules to recognize targets and activate T cell function; risk of hybridization (mismatch) between exogenous and endogenous chains

very low, which difficult the recognition. To overcome this problem, modifications of the TCR using genetic engineering technology has been able to enhance the specificity and affinity of the recognition of the antigens by T-cells (98).

Cell Therapy Limitations and Alternatives in SOT Patients

The intensity and long-term immunosuppression requirement to prevent allograft rejection pre-disposes SOT recipients to a wide range of viral complications (1). In addition, antiviral treatment can generate side effects such as nephrotoxicity (99), and the selection of drug-resistant mutant CMV strains (100), limiting treatment capability in SOT recipients. Based on these limitations, cell therapy may be an appropriate and effective alternative antiviral treatment. However, as pointed out previously, deficiencies in T-cell differentiation and lifelong immunosuppression can affect to long-term survival of the transfused cells, interfering in the antiviral functionality and limiting its use for adoptive therapy in SOT recipients (76). Here, we analyze the alternatives available to overcome these limitations.

Different authors have demonstrated that *in vitro* generated CMV -specific CTL are highly sensitive to immunosuppressive drugs (such as cyclosporin A and FK506) impairing the production of effector cytokines (101, 102). A possible solution in order to overcome this problem, is to genetically modify the *in vitro* generated CTL to confer resistance to these drugs (103, 104). Alternatively, decreasing patient´s immunosuppression during a period post-infusion may allow the expansion and functionality of the CMV-specific T-cells. As an example, Macesic et al. used third-party T-cells to infuse a kidney transplant patient who had ganciclovir resistant persistent CMV viremia, and decreased the levels of immunosuppressive drugs. A significant decrease of the patient CMV DNA viral load, from $>5x10^6$ copies to 682 copies/mL, was observed within 4 months after transfusion and remained controlled up to 1 year, leading to clearance of the infection (43). These results suggested that the use of third-party CMV-specific T-cells could be used in patients that admit a reduction of the immunosuppression regimen without compromising the allograft stability.

Another limitation is associated with deficiencies of T-cell differentiation in SOT recipients receiving immunosuppression. Most of the studies have used the viral antigens UL123 (IE1) and UL83 (pp65), known to promote a strong T-cellular response, for T-cell ex vivo stimulation to generate CMV-specific oligoclonal T-cells. Few studies have provided information regarding the cell mediated response to other viral multiple antigens in addition to IE1 and pp65 (54, 105–107). Thus, efforts should be made to promote the generation of CD8+ and CD4+ T-cells displaying multiple polyfunctional effector functions that may be more effective in controlling CMV infection (50, 54–56).

As previously mentioned infusion of donor derive T-cells from donors may also transfer alloreactive T cells in numbers sufficient that could trigger episodes of rejection, particularly if the donor and the host differ in one or more HLA alleles, due to sensitization to specific non-self HLA alleles present on the donor T-cells. A way of assessing this issue is to extensive culturing T cells or even establishing T-cell clones to eradicate alloreactive T cells but may also result in replicative senescence of the *ex vivo*-manipulated virus-specific T cells (108).

The creation of third-party cell banks as well as third party donor registries has emerged as a new possibility of treatment that employs T-cells derived from partially HLA-matched third-party donors (109). The use of this method allows to achieve a rapid "off the shelf" product that could be used in a broader range of patients. Furthermore, it offers the potential advantage of targeting multiple viral epitopes rather than a monospecific approach, potentially increasing the antiviral effect (109). Over the past years third party donor T-cell banks have been established. Such banks permit selection of T-cells on the basis of HLA allele phenotype, viral specificity and HLA restriction, which may provide distinct advantages, particularly in the treatment of HLA non-identical recipients. Although it is still under study, the obtained results to date are highly promising.

FUTURE DIRECTIONS AND PERSPECTIVES

CMV is a major cause of severe complications in SOT recipients such as graft loss especially in patients that develop CMV infection with antiviral refractory CMV strains (110, 111). The period early after the transplant is considered critical due to the high risk of infections associated with a high incidence of CMV (42). The role of CMV-specific T-cell immune reconstitution after SOT have demonstrated several benefits, including lower risk of CMV infection and graft rejection. Thus, the development and improvement of new CMV-specific T-cell transfer based therapies could be a useful to adjust the therapeutic interventions (112–114). However, despite the increasing interest on adoptive CMV specific T-cell transfer, most of the information available comes from studies in HSCT recipients (23, 53, 115). Only few reports including a small number of SOT recipients have used T-cell adoptive immunotherapy as a treatment of CMV infection or disease (41, 43, 46–48). These studies enrolled SOT recipients that previously failed to conventional treatment, with low survival rate. Although promising results were obtained, further development have been limited due to difficulties of T-cell expansion in SOT that are receiving immunosuppressive regimens, and the risk of graft rejection after T-cell administration. One possible approach to overcome these limitations is generating ready to use third-party CMV-specific T-cell banks to ensure the availability of well characterized the T-cell products (57, 116). In addition, better results should be obtained using T-cell adoptive immunotherapy in SOT recipients that had optimal clinical outcomes. Results from the ongoing clinical trial analyzing the safety and feasibility of administering CMV specific- CTLs from haploidentical donors in transplant patients would be of importance to implement T-cell adoptive therapy in SOT recipients.

CONCLUSIONS

Recent studies have significantly increased our knowledge about the protective role of CMV-specific T-cell immune response against CMV infection and disease. And thus the use of T-cell adoptive therapy may help to restore the CMV-specific immunity for preventing CMV infection in addition to serve as a treatment for CMV infections in SOT individuals who do not respond to conventional therapies, such as patients infected with antiviral resistant strains with no alternative treatment available. Recent findings regarding the development of new techniques to select, isolate and enrich functional CMV-specific T-cells and the possible generation of third party donor cell banks may help to use CMV-specific adoptive transfer as an alternative therapy for SOT recipients. However, further work is clearly needed in order to fully understand and assess the clinical utility of these techniques in SOT recipients.

AUTHOR CONTRIBUTIONS

MN and FM: writing and revision of the manuscript. EG-R: conceptualization, writing, revision, and editing of the manuscript and supervision. PP-R: project funding and administration, conceptualization, writing, revision and editing of the manuscript, and supervision. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Fishman JA. Infection in Solid-Organ Transplant Recipients. *N Engl J Med* (2007) 357:2601–14. doi: 10.1056/NEJMra064928
2. Fishman JA, Costa SF, Alexander BD. Infection in Kidney Transplant Recipients. In: *Kidney Transplantation - Principles and Practice*. Philadelphia p. 517–38. doi: 10.1016/B978-0-323-53186-3.00031-0
3. Carratalà J, Montejo M, Pérez-Romero P. Infections Caused by Herpes Viruses Other Than Cytomegalovirus in Solid Organ Transplant Recipients. *Enferm Infecc Microbiol Clin* (2012) 30:63–9. doi: 10.1016/S0213-005X(12)70084-8
4. Vanichanan J, Udomkarnjananun S, Avihingsanon Y, Jutivorakool K. Common Viral Infections in Kidney Transplant Recipients. *Kidney Res Clin Pract* (2018) 37:323–37. doi: 10.23876/j.krcp.18.0063
5. Fishman JA. Infection in Organ Transplantation. *Am J Transplant* (2017) 17:856–79. doi: 10.1111/ajt.14208
6. Styczynski J. Who is the Patient At Risk of CMV Recurrence: A Review of the Current Scientific Evidence With a Focus on Hematopoietic Cell Transplantation. *Infect Dis Ther* (2018) 7:1–16. doi: 10.1007/s40121-017-0180-z
7. Cho SY, Lee DG, Kim HJ. Cytomegalovirus Infections After Hematopoietic Stem Cell Transplantation: Current Status and Future Immunotherapy. *Int J Mol Sci* (2019) 20:2666. doi: 10.3390/ijms20112666
8. Nagai S, Mangus RS, Anderson E, Ekser B, Kubal CA, Fridell JA, et al. Cytomegalovirus Infection After Intestinal/Multivisceral Transplantation: A Single-Center Experience With 210 Cases. *Transplantation* (2016) 100:451–60. doi: 10.1097/TP.0000000000000832
9. Kamar N, Mengelle C, Esposito L, Guitard J, Mehrenberger M, Lavayssière L, et al. Predictive Factors for Cytomegalovirus Reactivation in Cytomegalovirus-Seropositive Kidney-Transplant Patients. *J Med Virol* (2008) 80:1012–7. doi: 10.1002/jmv
10. Selvey LA, Lim WH, Boan P, Swaminathan R, Slimings C, Harrison AE, et al. Cytomegalovirus Viraemia and Mortality in Renal Transplant Recipients in

the Era of Antiviral Prophylaxis. Lessons From the Western Australian Experience. *BMC Infect Dis* (2017) 17:1–10. doi: 10.1186/s12879-017-2599-y
11. Ramanan P, Razonable RR. Cytomegalovirus Infections in Solid Organ Transplantation: A Review. *Infect Chemother* (2013) 45:260–71. doi: 10.3947/ic.2013.45.3.260
12. Natori Y, Humar A, Husain S, Rotstein C, Renner E, Singer L, et al. Recurrence of CMV Infection & the Effect of Prolonged Antivirals in Organ Transplant Recipients. *Transplantation* (2017) 101:1449–54. doi: 10.1097/TP.0000000000001338
13. Green ML, Leisenring W, Xie H, Mast CT, Cui Y, Sandmaier BM, et al. CMV Viral Load and Mortality After Hematopoietic Cell Transplantation: A Cohort Study in the Era of Preemptive Therapy. *Lancet Haematol* (2016) 3:e119–127. doi: 10.1016/S2352-3026(15)00289-6.CMV
14. Styczynski J, Czyzewski K, Wysocki M, Gryniewicz-Kwiatkowska O, Kolodziejczyk-Gietka A, Salamonowicz M, et al. Increased Risk of Infections and Infection-Related Mortality in Children Undergoing Haematopoietic Stem Cell Transplantation Compared to Conventional Anticancer Therapy: A Multicentre Nationwide Study. *Clin Microbiol Infect* (2016) 22:179.e1–e10. doi: 10.1016/j.cmi.2015.10.017
15. Teira P, Battiwalla M, Ramanathan M, Barrett AJ, Ahn KW, Chen M, et al. Early Cytomegalovirus Reactivation Remains Associated With Increased Transplant-Related Mortality in the Current Era: A CIBMTR Analysis. *Blood* (2016) 127:2427–38. doi: 10.1182/blood-2015-11-679639
16. Chan ST, Logan AC. The Clinical Impact of Cytomegalovirus Infection Following Allogeneic Hematopoietic Cell Transplantation: Why the Quest for Meaningful Prophylaxis Still Matters. *Blood Rev* (2017) 31:173–83. doi: 10.1016/j.blre.2017.01.002
17. Stern M, Hirsch H, Cusini A, Van Delden C, Manuel O, Meylan P, et al. Cytomegalovirus Serology and Replication Remain Associated With Solid Organ Graft Rejection and Graft Loss in the Era of Prophylactic Treatment. *Transplantation* (2014) 98:1013–8. doi: 10.1097/TP.0000000000000160
18. Litjens NHR, van der Wagen L, Kuball J, Kwekkeboom J. Potential Beneficial Effects of Cytomegalovirus Infection After Transplantation. *Front Immunol* (2018) 9:389. doi: 10.3389/fimmu.2018.00389
19. Mena-Romo JD, Pérez Romero P, Martín-Gandul C, Gentil MÁ, Suárez-Artacho G, Lage E, et al. CMV-specific T-Cell Immunity in Solid Organ Transplant Recipients At Low Risk of CMV Infection. Chronology and Applicability in Preemptive Therapy. *J Infect* (2017) 75:336–45. doi: 10.1016/j.jinf.2017.05.020
20. Blanco-Lobo P, Cordero E, Martín-Gandul C, Gentil MA, Suárez-Artacho G, Sobrino M, et al. Use of Antibodies Neutralizing Epithelial Cell Infection to Diagnose Patients At Risk for CMV Disease After Transplantation. *J Infect* (2016) 72:597–607. doi: 10.1016/j.jinf.2016.02.008
21. López-Oliva MO, Martinez V, Buitrago Á, Jiménez C, Rivas B, Escuin F, et al. Pretransplant CD8 T-Cell Response to IE-1 Discriminates Seropositive Kidney Recipients At Risk of Developing CMV Infection Posttransplant. *Transplantation* (2014) 97:839–45. doi: 10.1097/01.TP.0000438025.96334.eb
22. Picarda G, Benedict CA. Cytomegalovirus: Shape-Shifting the Immune System. *J Immunol* (2018) 200:3881–9. doi: 10.4049/jimmunol.1800171
23. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, et al. Reconstitution of Cellular Immunity Against Cytomegalovirus in Recipients of Allogeneic Bone Marrow by Transfer of T-Cell Clones From the Donor. *N Engl J Med* (1995) 333:1038–44. doi: 10.1056/NEJM199510193331603
24. Gamadia LE, Rentenaar RJ, Baars PA, Remmerswaal EBM, Surachno S, Weel JFL, et al. Differentiation of Cytomegalovirus-Specific CD8 + T Cells in Healthy and Immunosuppressed Virus Carriers. *Blood* (2001) 98:754–61. doi: 10.1182/blood.V98.3.754
25. Sester M, Sester U, Gartner B, Heine G, Girndt M, Mueller-Lantzsch N, et al. Levels of Virus-Specific CD4 T Cells Correlate With Cytomegalovirus Control and Predict Virus-Induced Disease After Renal Transplantation. *Transplantation* (2001) 71:1287–94. doi: 10.1097/00007890-200105150-00018
26. Gabanti E, Bruno F, Lilleri D, Fornara C, Zelini P, Cane I, et al. Human Cytomegalovirus (HCMV)-specific CD4+and CD8+ T Cells are Both Required for Prevention of HCMV Disease in Seropositive Solid-Organ Transplant Recipients. *PloS One* (2014) 9:e106044. doi: 10.1371/journal.pone.0106044
27. Lim EY, Jackson SE, Wills MR. The CD4+ T Cell Response to Human Cytomegalovirus in Healthy and Immunocompromised People. *Front Cell Infect Microbiol* (2020) 10:202. doi: 10.3389/fcimb.2020.00202
28. Yong MK, Lewin SR, Manuel O. Immune Monitoring for CMV in Transplantation. *Curr Infect Dis Rep* (2018) 20(4):1–9. doi: 10.1007/s11908-018-0610-4
29. Rittà M, Costa C, Sidoti F, Balloco C, Ranghino A, Messina M, et al. Pre-Transplant Assessment of CMV-specific Immune Response by Elispot Assay in Kidney Transplant Recipients. *New Microbiol* (2015) 38:329–35.
30. Cantisán S, Rodelo-Haad C, Páez-Vega A, Nieto A, Vaquero JM, Poyato A, et al. Factors Related to the Development of CMV-specific CD8+ T Cell Response in CMV-seropositive Solid Organ Transplant Candidates. *Am J Transplant* (2015) 15:715–22. doi: 10.1111/ajt.13012
31. Bestard O, Lucia M, Crespo E, Van Liempt B, Palacio D, Melilli E, et al. Pretransplant Immediately early-1-specific T Cell Responses Provide Protection for CMV Infection After Kidney Transplantation. *Am J Transplant* (2013) 13:1793–805. doi: 10.1111/ajt.12256
32. Hoon Han S. Immunological Prediction of Cytomegalovirus (CMV) Replication Risk in Solid Organ Transplantation Recipients: Approaches for Regulating the Targeted Anti-CMV Prevention Strategies. *Infect Chemother* (2017) 49:161–75. doi: 10.3947/ic.2017.49.3.161
33. Meesing A, Razonable RR. New Developments in the Management of Cytomegalovirus Infection After Transplantation. *Drugs* (2018) 78:1085–103. doi: 10.1007/s40265-018-0943-1
34. Ciccocioppo R, Comoli P, Gallia A, Basso S, Baldanti F, Corazza GR. Autologous Human Cytomegalovirus-Specific Cytotoxic T Cells as Rescue Therapy for Ulcerative Enteritis in Primary Immunodeficiency. *J Clin Immunol* (2014) 34:681–5. doi: 10.1007/s10875-014-0060-1
35. Seo S, Smith C, Fraser C, Patheja R, Shah SP, Rehan S, et al. Adoptive T-Cell Therapy for Pediatric Cytomegalovirus-Associated Retinitis. *Blood Adv* (2019) 3:1774–7. doi: 10.1182/bloodadvances.2019000121
36. Hill GR, Tey SK, Beagley L, Crough T, Morton JA, Clouston AD, et al. Successful Immunotherapy of HCMV Disease Using Virus-Specific T Cells Expanded From an Allogeneic Stem Cell Transplant Recipient: Case Report. *Am J Transplant* (2010) 10:173–9. doi: 10.1111/j.1600-6143.2009.02872.x
37. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, et al. Off-the-Shelf Virus-Specific T Cells to Treat BK Virus, Human Herpesvirus 6, Cytomegalovirus, Epstein-Barr Virus, and Adenovirus Infections After Allogeneic Hematopoietic Stem-Cell Transplantation. *J Clin Oncol* (2017) 35:3547–57. doi: 10.1200/JCO.2017.73.0655
38. Pei XY, Zhao XY, Chang YJ, Liu J, Xu LP, Wang Y, et al. Cytomegalovirus-Specific T-Cell Transfer for Refractory Cytomegalovirus Infection After Haploidentical Stem Cell Transplantation: The Quantitative and Qualitative Immune Recovery for Cytomegalovirus. *J Infect Dis* (2017) 216:945–56. doi: 10.1093/infdis/jix357
39. Kaminski H, Fishman JA. The Cell Biology of Cytomegalovirus: Implications for Transplantation. *Am J Transplant* (2016) 16:2254–69. doi: 10.1111/ajt.13791
40. Stern A, Papanicolaou GA, Prevention CMV. And Treatment in Transplantation: What's New in 2019. *Curr Infect Dis Rep* (2019) 21:1–11. doi: 10.1007/s11908-019-0699-0
41. Brestrich G, Zwinger S, Fischer A, Schm M, Schmück M, Röhmhild A, et al. Adoptive T-Cell Therapy of a Lung Transplanted Patient With Severe CMV Disease and Resistance to Antiviral Therapy: Brief Communication. *Am J Transplant* (2009) 9:1679–84. doi: 10.1111/j.1600-6143.2009.02672.x
42. Haidar G, Boeckh M, Singh N. Cytomegalovirus Infection in Solid Organ and Hematopoietic Cell Transplantation: State of the Evidence. *J Infect Dis* (2020) 221:S23–31. doi: 10.1093/infdis/jiz454
43. Macesic N, Langsford D, Nicholls K, Hughes P, Gottlieb DJ, Clancy L, et al. Adoptive T Cell Immunotherapy for Treatment of Ganciclovir-Resistant Cytomegalovirus Disease in a Renal Transplant Recipient. *Am J Transplant* (2015) 15:827–32. doi: 10.1111/ajt.13023
44. Blyth E, Clancy L, Simms R, Ma CKK, Burgess J, Deo S, et al. Donor-Derived CMV-specific T Cells Reduce the Requirement for CMV-directed Pharmacotherapy After Allogeneic Stem Cell Transplantation. *Blood* (2013) 121:3745–58. doi: 10.1182/blood-2012-08-448977
45. Clancy LE, Blyth E, Simms RM, Micklethwaite KP, Ma CKK, Burgess JS, et al. Cytomegalovirus-Specific Cytotoxic T Lymphocytes Can be Efficiently

Expanded From Granulocyte Colony-Stimulating Factor-Mobilized Hemopoietic Progenitor Cell Products Ex Vivo and Safely Transferred to Stem Cell Transplantation Recipients to Facilitate Imm. *Biol Blood Marrow Transplant* (2013) 19:725–34. doi: 10.1016/j.bbmt.2013.01.021
46. Holmes-Liew C-L, Holmes M, Beagley L, Hopkins P, Chambers D, Smith C, et al. Adoptive T-Cell Immunotherapy for Ganciclovir-Resistant CMV Disease After Lung Transplantation. *Clin Transl Immunol* (2015) 4:e35. doi: 10.1038/cti.2015.5
47. Pierucci P, Malouf M, Glanville AR, Beagley L, Smith C, Khanna R. Novel Autologous T-Cell Therapy for Drug-Resistant Cytomegalovirus Disease After Lung Transplantation. *J Hear Lung Transplant* (2016) 35:685–7. doi: 10.1016/j.healun.2015.12.031
48. Smith C, Beagley L, Rehan S, Neller MA, Crooks P, Solomon M, et al. Autologous Adoptive T-Cell Therapy for Recurrent or Drug-Resistant Cytomegalovirus Complications in Solid Organ Transplant Recipients: A Single-Arm Open-Label Phase I Clinical Trial. *Clin Infect Dis* (2019) 68:632–40. doi: 10.1093/cid/ciy549
49. Peggs KS, Verfuerth S, Pizzey A, Khan N, Guiver M, Moss PA, et al. Adoptive Cellular Therapy for Early Cytomegalovirus Infection After Allogeneic Stem-Cell Transplantation With Virus-Specific T-Cell Lines. *Lancet* (2003) 362:1375–7. doi: 10.1016/S0140-6736(03)14634-X
50. Crough T, Khanna R. Immumobiology of Human Cytomegalovirus: From Bench to Bedside. *Clin Microbiol Rev* (2009) 22:76–98. doi: 10.1128/CMR.00034-08
51. Gerdemann U, Keirnan JM, Katari UL, Yanagisawa R, Christin AS, Huye LE, et al. Rapidly Generated Multivirus-Specific Cytotoxic T Lymphocytes for the Prophylaxis and Treatment of Viral Infections. *Mol Ther* (2012) 20:1622–32. doi: 10.1038/mt.2012.130
52. Vera JF, Brenner LJ, Gerdemann U, Ngo MC, Sili U, Liu H, et al. Accelerated Production of Antigen-Specific T-Cells for Pre-Clinical and Clinical Applications Using Gas-Permeable Rapid Expansion Cultureware (G-Rex). *J¢Immunother* (2010) 33:305–15. doi: 10.1097/CJI.0b013e3181c0c3cb. Accelerated
53. Bollard CM, Heslop HE. T Cells for Viral Infections After Allogeneic Hematopoietic Stem Cell Transplant. *Blood* (2016) 127:3331–40. doi: 10.1182/blood-2016-01-628982
54. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly Targeted Human Cytomegalovirus-Specific CD4+ and CD8+ T Cells Dominate the Memory Compartments of Exposed Subjects. *J Exp Med* (2005) 202:673–85. doi: 10.1084/jem.20050882
55. Jackson SE, Mason GM, Okecha G, Sissons JGP, Wills MR. Diverse Specificities, Phenotypes, and Antiviral Activities of Cytomegalovirus-Specific CD8+ T Cells. *J Virol* (2014) 88:10894–908. doi: 10.1128/jvi.01477-14
56. Jackson SE, Sedikides GX, Mason GM, Okecha G, Wills MR. Human Cytomegalovirus (HCMV)-Specific CD4+ T Cells are Polyfunctional and Can Respond to HCMV-Infected Dendritic Cells in Vitro. *J Virol* (2017) 91:1–20. doi: 10.1128/jvi.02128-16
57. Houghtelin A, Bollard CM. Virus-Specific T Cells for the Immunocompromised Patient. *Front Immunol* (2017) 8:1272. doi: 10.3389/fimmu.2017.01272
58. Peggs K, Verfuerth S, Mackinnon S. Induction of Cytomegalovirus (CMV)-specific T-Cell Responses Using Dendritic Cells Pulsed With CMV Antigen: A Novel Culture System Free of Live CMV Virions. *Blood* (2001) 97:994–1000. doi: 10.1182/blood.V97.4.994
59. Einsele H, Roosnek E, Rufer N, Sinzger C, Riegler S, Löffler J, et al. Infusion of Cytomegalovirus (CMV)-specific T Cells for the Treatment of CMV Infection Not Responding to Antiviral Chemotherapy. *Blood* (2002) 99:3916–22. doi: 10.1182/blood.V99.11.3916
60. Micklethwaite K, Hansen A, Foster A, Snape E, Antonenas V, Sartor M, et al. Ex Vivo Expansion and Prophylactic Infusion of CMV-pp65 Peptide-Specific Cytotoxic T-Lymphocytes Following Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2007) 13:707–14. doi: 10.1016/j.bbmt.2007.02.004
61. Koehne G, Hasan A, Doubrovina E, Prockop S, Tyler E, Wasilewski G. J. Et al. Immunotherapy With Donor T-Cells Sensitized With Overlapping Pentadecapeptides for Treatment of Persistent CMV Infection or Viremia. *Biol Blood Marrow Transplant* (2015) 21:1663–78. doi: 10.1016/j.bbmt.2015.05.015
62. Bao L, Cowan MJ, Dunham K, Horn B, McGuirk J, Gilman A, et al. Adoptive Immunotherapy With CMV-specific Cytotoxic T Lymphocytes for Stem Cell Transplant Patients With Refractory CMV Infections. *J Immunother* (2012) 35:293–8. doi: 10.1097/CJI.0b013e31824300a2
63. Leen AM, Heslop HE, Brenner MK. Antiviral T-Cell Therapy. *Immunol Rev* (2014) 258:12–29. doi: 10.1111/imr.12138
64. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, et al. Adoptive Transfer of Cytomegalovirus-Specific CTL to Stem Cell Transplant Patients After Selection by HLA-peptide Tetramers. *J Exp Med* (2005) 202:379–86. doi: 10.1084/jem.20040613
65. Xu XN, Purbhoo MA, Chen N, Mongkolsapaya J, Cox JH, Meier UC, et al. A Novel Approach to Antigen-Specific Deletion of CTL With Minimal Cellular Activation Using α3 Domain Mutants of MHC Class I/Peptide Complex. *Immunity* (2001) 14:591–602. doi: 10.1016/S1074-7613(01)00133-9
66. Daniels MA, Jameson SC. Critical Role for CD8 in T Cell Receptor Binding and Activation by Peptide/Major Histocompatibility Complex Multimers. *J Exp Med* (2000) 191:335–45. doi: 10.1084/jem.191.2.335
67. Whelan J, Dunbar PR, Price D, Purbhoo M, Lechner F, Ogg GS, et al. Specificity of CTL Interactions With Peptide-MHC Class I Tetrameric Complexes is Temperature Dependent. *J Immunol* (1999) 163:4342–8.
68. O'Herrin SM, Slansky JE, Tang Q, Markiewicz MA, Gajewski TF, Pardoll DM, et al. Antigen-Specific Blockade of T Cells in Vivo Using Dimeric MHC Peptide. *J Immunol* (2001) 167:2555–60. doi: 10.4049/jimmunol.167.5.2555
69. Maile R, Wang B, Schooler W, Meyer A, Collins EJ, Frelinger JA. Antigen-Specific Modulation of an Immune Response by in Vivo Administration of Soluble MHC Class I Tetramers. *J Immunol* (2001) 167:3708–14. doi: 10.4049/jimmunol.167.7.3708
70. Knabel M, Franz TJ, Schiemann M, Wulf A, Villmow B, Schmidt B, et al. Reversible MHC Multimer Staining for Functional Isolation of T-Cell Populations and Effective Adoptive Transfer. *Nat Med* (2002) 8:631–7. doi: 10.1038/nm0602-631
71. Schiemann M, Holzapfel G, Schmidt T, Germeroth L, Wagner H, Peschel C, et al. Reversible HLA Multimers (Streptamers) for the Isolation of Human Cytotoxic T Lymphocytes Functionally Active Against Tumor- and Virus-Derived Antigens. *J Immunol Methods* (2007) 320:119–31. doi: 10.1016/j.jim.2007.01.001
72. Tischer S, Kaireit T, Figueiredo C, Hiller O, Maecker-kolhoff B, Geyeregger R. I Et al. Establishment of the Reversible Peptide-Major Histocompatibility Complex (PMHC) Class I Histamer Technology: Tool for Visualization and Selection of Functionally Active Antigen-Specific CD8+ T Lymphocytes. *Int Immunol* (2012) 24:561–72. doi: 10.1093/intimm/dxs059
73. Novy P, Quigley M, Huang X, Yang Y. CD4 T Cells are Required for CD8 T Cell Survival During Both Primary and Memory Recall Responses. *J Immunol* (2007) 179:8243–51. doi: 10.4049/jimmunol.179.12.8243
74. Schmitt A, Tonn T, Busch DH, Grigoleit GU, Einsele H, Odendahl M, et al. Adoptive Transfer and Selective Reconstitution of Streptamer-Selected Cytomegalovirus-Specific CD8+ T Cells Leads to Virus Clearance in Patients After Allogeneic Peripheral Blood Stem Cell Transplantation. *Transfusion* (2011) 51:591–9. doi: 10.1111/j.1537-2995.2010.02940.x
75. Neuenhahn M, Albrecht J, Odendahl M, Schlott F, Dössinger G, Schiemann M, et al. Transfer of Minimally Manipulated CMV-speci Fi C T Cells From Stem Cell or Third-Party Donors to Treat CMV Infection After. *Leukemia* (2017) 31:2161–71. doi: 10.1038/leu.2017.16
76. Brestrich G, Zwinger S, Roemhild A, Noutsias M, Rohde M, Keeren K, et al. Generation of HCMV-specific T-Cell Lines From Seropositive Solid-Organ-Transplant Recipients for Adoptive T-Cell Therapy. *J Immunother* (2009) 32:932–40. doi: 10.1097/CJI.0b013e3181b88fda
77. Campbell JDM. Detection and Enrichment of Antigen-Specific CD4+ and CD8+ T Cells Based on Cytokine Secretion. *Methods* (2003) 31:150–9. doi: 10.1016/S1046-2023(03)00125-7
78. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive Transfer of pp65-specific T Cells for the Treatment of Chemorefractory Cytomegalovirus Disease or Reactivation After Haploidentical and Matched Unrelated Stem Cell Transplantation. *Blood* (2010) 116:4360–7. doi: 10.1182/blood-2010-01-262089
79. Peggs KS, Thomson K, Samuel E, Dyer G, Armoogum J, Chakraverty R, et al. Directly Selected Cytomegalovirus-Reactive Donor T Cells Confer Rapid and Safe Systemic Reconstitution of Virus-Specific Immunity Following

Stem Cell Transplantation. *Clin Infect Dis* (2011) 52:49–57. doi: 10.1093/cid/ciq042
80. Kim N, Nam YS, Il I, JY L, YW J, Song Y, et al. Robust Production of Cytomegalovirus Pp65-Specific T Cells Using a Fully Automated IFN-γ Cytokine Capture System. *Transfus Med Hemother* (2018) 45:13–22. doi: 10.1159/000479238
81. Priesner C, Esser R, Tischer S, Marburger M, Aleksandrova K, Maecker-Kolhoff B, et al. Comparative Analysis of Clinical-Scale IFN-γ-Positive T-Cell Enrichment Using Partially and Fully Integrated Platforms. *Front Immunol* (2016) 7:393. doi: 10.3389/fimmu.2016.00393
82. Samuel ER, Newton K, MacKinnon S, Lowdell MW. Successful Isolation and Expansion of CMV-reactive T Cells From G-CSF Mobilized Donors That Retain a Strong Cytotoxic Effector Function. *Br J Haematol* (2013) 160:87–100. doi: 10.1111/bjh.12082
83. Frentsch M, Arbach O, Kirchhoff D, Moewes B, Worm M, Rothe M, et al. Direct Access to CD4+ T Cells Specific for Defined Antigens According to CD154 Expression. *Nat Med* (2005) 11:1118–24. doi: 10.1038/nm1292
84. Wolfl M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, et al. Activation-Induced Expression of CD137 Permits Detection, Isolation, and Expansion of the Full Repertoire of CD8+ T Cells Responding to Antigen Without Requiring Knowledge of Epitope Specificities. *Blood* (2007) 110:201–10. doi: 10.1182/blood-2006-11-056168
85. Wehler TC, Karg M, Distler E, Konur A, Nonn M, Meyer RG, et al. Rapid Identification and Sorting of Viable Virus-Reactive CD4+ and CD8+ T Cells Based on Antigen-Triggered CD137 Expression. *J Immunol Methods* (2008) 339:23–37. doi: 10.1016/j.jim.2008.07.017
86. Han S, Huang Y, Liang Y, Ho Y, Wang Y. Phenotype and Functional Evaluation of Ex Vivo Generated Antigen-Specific Immune Effector Cells With Potential for Therapeutic Applications. *J Hematol Oncol* (2009) 2:1–16. doi: 10.1186/1756-8722-2-34
87. Samuel ER, Beloki L, Newton K, Mackinnon S, Lowdell MW. Isolation of Highly Suppressive CD25 +FoxP3+ T Regulatory Cells From G-CSF-mobilized Donors With Retention of Cytotoxic Anti-Viral CTLs: Application for Multi-Functional Immunotherapy Post Stem Cell Transplantation. *PloS One* (2014) 9:1–12. doi: 10.1371/journal.pone.0085911
88. Seif M, Einsele H, Löffler J. CAR T Cells Beyond Cancer: Hope for Immunomodulatory Therapy of Infectious Diseases. *Front Immunol* (2019) 10:2711. doi: 10.3389/fimmu.2019.02711
89. Zhang Y, Li Y. T Cell Receptor-Engineered T Cells for Leukemia Immunotherapy. *Cancer Cell Int* (2019) 19:1–7. doi: 10.1186/s12935-018-0720-y
90. Zhao L, Cao YJ. Engineered T Cell Therapy for Cancer in the Clinic. *Front Immunol* (2019) 10:2250. doi: 10.3389/fimmu.2019.02250
91. Full F, Lehner M, Thonn V, Goetz G, Scholz B, Kaufmann KB, et al. T Cells Engineered With a Cytomegalovirus-Specific Chimeric Immunoreceptor. *J Virol* (2010) 84:4083–8. doi: 10.1128/JVI.02117-09
92. Proff J, Walterskirchen C, Brey C, Geyeregger R, Full F, Ensser A, et al. Cytomegalovirus-Infected Cells Resist T Cell Mediated Killing in an HLA-recognition Independent Manner. *Front Microbiol* (2016) 7:1–15. doi: 10.3389/fmicb.2016.00844
93. Gottlieb DJ, Clancy LE, Withers B, Mcguire HM, Luciani F, Singh M, et al. Prophylactic antigen-specific T-cells targeting seven viral and fungal pathogens after allogeneic haemopoietic stem cell transplant. *Clin Transl Immunol* (2021) 10:1–18. doi: 10.1002/cti2.1249
94. Sadelain M, Brentjens R, Riviere I. The Basic Principles of Chimeric Antigen Receptor (CAR) Design. *Cancer Discovery* (2013) 3:388–98. doi: 10.1158/2159-8290.CD-12-0548
95. Proff J, Brey CU, Ensser A, Holter W, Lehner M. Turning the Tables on Cytomegalovirus: Targeting Viral Fc Receptors by CARs Containing Mutated CH2-CH3 Igg Spacer Domains. *J Transl Med* (2018) 16:1–12. doi: 10.1186/s12967-018-1394-x
96. Olbrich H, Theobald SJ, Slabik C, Gerasch L, Schneider A, Mach M, et al. Adult and Cord Blood-Derived High-Affinity Gb-CAR-T Cells Effectively React Against Human Cytomegalovirus Infections. *Hum Gene Ther* (2020) 31:423–39. doi: 10.1089/hum.2019.149
97. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T Cells Mediate Sustained Antigen-Specific Antitumor Effects in Myeloma. *Nat Med* (2015) 21:914–21. doi: 10.1038/nm.3910
98. Barrett DM, Grupp SA, June CH. Chimeric Antigen Receptor– and TCR-Modified T Cells Enter Main Street and Wall Street. *J Immunol* (2015) 195:755–61. doi: 10.4049/jimmunol.1500751
99. Jacobsen T, Sifontis N. Drug Interactions and Toxicities Associated With the Antiviral Management of Cytomegalovirus Infection. *Am J Heal Pharm* (2010) 67:1417–25. doi: 10.2146/ajhp090424
100. Limaye AP. Ganciclovir-Resistant Cytomegalovirus in Organ Transplant Recipients. *Clin Infect Dis* (2002) 35:866–72. doi: 10.1086/342385
101. Taylor AL, Watson CJE, Bradley JA. Immunosuppressive Agents in Solid Organ Transplantation: Mechanisms of Action and Therapeutic Efficacy. *Crit Rev Oncol* (2005) 56:23–46. doi: 10.1016/j.critrevonc.2005.03.012
102. Egli A, Kumar D, Broscheit C, Shea DO, Humar A. Comparison of the Effect of Standard and Novel Immunosuppressive Drugs on CMV-Specific T-Cell Cytokine Profiling. *Transplantation* (2013) 95:448–55. doi: 10.1097/TP.0b013e318276a19f
103. De Angelis B, Dotti G, Quintarelli C, Huye LE, Zhang L, Zhang M, et al. Generation of Epstein-Barr Virus – Specific Cytotoxic T Lymphocytes Resistant to the Immunosuppressive Drug Tacrolimus (FK506). *Blood* (2009) 114:5–7. doi: 10.1182/blood-2009-07-230482
104. Ricciardelli I, Blundell MP, Brewin J, Thrasher A, Pule M, Amrolia PJ. Towards Gene Therapy for EBV-associated Posttransplant Lymphoma With Genetically Modi Fi Ed EBV-specific Cytotoxic T Cells. *Blood* (2014) 124:2514–22. doi: 10.1182/blood-2014-01-553362
105. Borysiewicz LK, Hickling JK, Graham S, Sinclair J, Cranage MP, Smith GL, et al. Human Cytomegalovirus-Specific Cytotoxic T Cells: Relative Frequency of Stage-Specific CTL Recognizing the 72-KD Immediate Early Protein and Glycoprotein B Expressed by Recombinant Vaccinia Viruses. *J Exp Med* (1988) 168:919–31. doi: 10.1084/jem.168.3.919
106. Crough T, Burrows JM, Fazou C, Walker S, Davenport MP, Khanna R. Contemporaneous Fluctuations in T Cell Responses to Persistent Herpes Virus Infections. *Eur J Immunol* (2005) 35:139–49. doi: 10.1002/eji.200425548
107. Ouyang Q, Wagner WM, Wikby A, Walter S, Aubert G, Dodi AI, et al. Large Numbers of Dysfunctional CD8+ T Lymphocytes Bearing Receptors for a Single Dominant CMV Epitope in the Very Old. *J Clin Immunol* (2003) 23:247–57. doi: 10.1023/A:1024580531705
108. Rauser G, Einsele H, Sinzger C, Wernet D, Kuntz G, Assenmacher M, et al. Rapid Generation of Combined CMV-specific CD4+ and CD8 + T-Cell Lines for Adoptive Transfer Into Recipients of Allogeneic Stem Cell Transplants. *Blood* (2004) 103:3565–72. doi: 10.1182/blood-2003-09-3056
109. Eiz-Vesper B, Maecker-Kolhoff B, Blasczyk R. Adoptive T-Cell Immunotherapy From Third-Party Donors: Characterization of Donors and Set Up of a T-Cell Donor Registry. *Front Immunol* (2012) 3:410. doi: 10.3389/fimmu.2012.00410
110. Kotton CN, Kumar D, Caliendo AM, Åsberg A, Chou S, Danziger-Isakov L, et al. Updated International Consensus Guidelines on the Management of Cytomegalovirus in Solid-Organ Transplantation. *Transplantation* (2013) 96:333–60. doi: 10.1097/TP.0b013e31829df29d
111. Amini L, Wagner DL, Rössler U, Zarrinrad G, Wagner LF, Vollmer T, et al. CRISPR-Cas9-Edited Tacrolimus-Resistant Antiviral T Cells for Advanced Adoptive Immunotherapy in Transplant Recipients. *Mol Ther* (2021) 29:32–46. doi: 10.1016/j.ymthe.2020.09.011
112. Abate D, Saldan A, Fiscon M, Cofano S, Paciolla A, Furian L, et al. Evaluation of Cytomegalovirus (CMV)-specific T Cell Immune Reconstitution Revealed That Baseline Antiviral Immunity, Prophylaxis, or Preemptive Therapy But Not Antithymocyte Globulin Treatment Contribute to CMV-specific T Cell Reconstitution in Kidney Tra. *J Infect Dis* (2010) 202:585–94. doi: 10.1086/654931
113. Kumar D, Chernenko S, Moussa G, Cobos I, Manuel O, Preiksaitis J, et al. Cell-Mediated Immunity to Predict Cytomegalovirus Disease in High-Risk Solid Organ Transplant Recipients. *Am J Transplant* (2009) 9:1214–22. doi: 10.1111/j.1600-6143.2009.02618.x
114. Abate D, Fiscon M, Saldan A, Cofano S, Mengoli C, Sgarabotto D, et al. Human Cytomegalovirus-Specific T-Cell Immune Reconstitution in Preemptively Treated Heart Transplant Recipients Identifies Subjects At Critical Risk for Infection. *J Clin Microbiol* (2012) 50:1974–80. doi: 10.1128/JCM.06406-11

115. Basso S, Compagno F, Zelini P, Giorgiani G, Boghen S, Bergami E, et al. Harnessing T Cells to Control Infections After Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol* (2020) 11:567531. doi: 10.3389/fimmu.2020.567531
116. Tischer S, Priesner C, Heuft HG, Goudeva L, Mende W, Barthold M, et al. Rapid Generation of Clinical-Grade Antiviral T Cells: Selection of Suitable T-Cell Donors and GMP-compliant Manufacturing of Antiviral T Cells. *J Transl Med* (2014) 12:336. doi: 10.1186/s12967-014-0336-5

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Latent CMV Infection Is Associated With Lower Influenza Virus-Specific Memory T-Cell Frequencies, but Not With an Impaired T-Cell Response to Acute Influenza Virus Infection

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Latent infection with cytomegalovirus (CMV) is assumed to contribute to the age-associated decline of the immune system. CMV induces large changes in the T-cell pool and may thereby affect other immune responses. CMV is expected to impact especially older adults, who are already at higher risk of severe disease and hospitalization upon infections such as influenza virus (IAV) infection. Here, we investigated the impact of CMV infection on IAV-specific $CD8^+$ T-cell frequencies in healthy individuals (n=96) and the response to IAV infection in older adults (n=72). IAV-specific memory T-cell frequencies were lower in healthy CMV^+ older individuals compared to healthy CMV^- older individuals. Upon acute IAV infection, CMV serostatus or CMV-specific antibody levels were not negatively associated with IAV-specific T-cell frequencies, function, phenotype or T-cell receptor repertoire diversity. This suggests that specific T-cell responses upon acute IAV infection are not negatively affected by CMV. In addition, we found neither an association between CMV infection and inflammatory cytokine levels in serum during acute IAV infection nor between cytokine levels and the height of the IAV-specific T-cell response upon infection. Finally, CMV infection was not associated with increased severity of influenza-related symptoms. In fact, CMV infection was even associated with increased IAV-specific T-cell responses early upon acute IAV infection. In conclusion, although associated with lower frequencies of memory IAV-specific T cells in healthy individuals, CMV infection does not seem to hamper the induction of a proper T-cell response during acute IAV infection in older adults.

Keywords: influenza infection, T cell, immune response, ageing, cytomegalovirus infection

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INTRODUCTION

The worldwide population is ageing rapidly. With age, deleterious changes in the immune system arise, referred to as 'immunosenescence', which impair responses against infectious diseases and vaccinations (1, 2). Age-related changes of the immune system mainly occur in the T-cell pool, including an increase in the number of $CD45RA^+$ memory T cells (3, 4) and decreased diversity of

the T-cell receptor repertoire (5, 6). About 2 decades ago, latent infection with cytomegalovirus (CMV) was implicated as a possible driving force of these age-related changes (7–9). CMV seropositivity was identified as part of the so-called 'immune risk profile', predictive of early mortality in older adults (7, 10). Moreover, CMV seropositivity is the largest non-heritable factor influencing differences among humans in the immune profile (11). CMV is generally thought to establish this large effect by its frequent attempts to reactivate during life-long carriage, thereby gradually affecting the immune system (12). Since the 2000s, it is often hypothesized that CMV might impair the human immune response to a heterologous challenge (6, 12, 13), as was shown in mice (14–16).

With age, the risk for serious complications and hospitalization after influenza virus (IAV) infection increases (17). Vaccination is an important tool to prevent infection, however also the efficacy of influenza vaccination decreases with age (2, 18). The role of CMV infection on influenza vaccination efficacy has been well studied. These studies have yielded conflicting results as a negative effect of CMV (19–21), a positive effect (1) as well as no effect of CMV on the vaccine response have been reported (22, 23). A systematic review by our group including a meta-analysis showed no clear evidence for a negative effect of CMV infection on the antibody response to influenza vaccination (24).

Both CMV and ageing primarily affect the T-cell compartment (3, 4, 25–27). During IAV infection, an effect of CMV would therefore mainly be expected on the T-cell response. T cells play an important role in clearance of the IAV (28). These T-cell responses are predominantly specific for the internal viral proteins, such as matrix protein-1 (29), which are conserved among influenza strains that have undergone antigenic drift. Indeed, pre-existing and early IAV-specific T-cell responses are associated with lower disease severity of influenza (30, 31), while delayed T-cell responses to IAV are thought to induce prolonged inflammation and delayed viral clearance and recovery (31, 32). Upon activation, IAV-specific $CD8^+$ T cells show increased expression of activation markers (33, 34), produce proinflammatory cytokines and kill virus-infected cells by releasing perforin and granzyme B (29, 35).

Whether CMV infection attenuates the immune response to IAV infection in older adults remains unclear. There are two major hypotheses explaining how CMV infection may affect the immune response to a heterologous virus. First, it has been proposed that large clonal expansions of terminally differentiated CMV-specific $CD8^+$ T cells, which are a hallmark of CMV infection and which can take up to 30%-90% of the $CD8^+$ T-cell memory pool (36–38), may fill the 'immunological space' (10, 13, 39). Thereby CMV infection may hamper the induction of other immune responses (10, 13, 39). IAV-specific T cells may thus be outcompeted by CMV-specific T cells in their competition for proliferation and survival factors (13). Secondly, it has been suggested that CMV is linked to 'inflammaging', the lingering low-graded level of inflammation occurring with ageing (40). The production of pro-inflammatory mediators has been shown to enable CMV reactivation (41, 42). Upregulation of TNF-α, IL-6 and CRP have been observed in CMV-infected individuals, as well as increased production of IL-10 (11, 43, 44). Especially the increase of IL-10 combined with a decrease of IFNγ has been associated with reduced cytolytic capacity of $CD8^+$ T cells responsible for clearing IAV, which also fits with the observed lower levels of granzyme B (45–47). Importantly, even though CMV has been suggested to diminish the T-cell response to IAV, there is no clinical evidence of a direct link between CMV infection and the T-cell response against IAV infection in humans.

We had the unique opportunity to study the effect of CMV infection on IAV in humans in the in a relatively large cohort of natural influenza infected older adults. We first investigated the effect of CMV infection on the presence of IAV-specific memory T cells in healthy young and healthy older individuals. Next we assessed the effect of latent CMV infection on the IAV-specific T-cell response in older adults undergoing an acute IAV infection. Our data show that CMV^- infection is associated with reduced frequencies of IAV-specific T cells in healthy older adults, whereas in healthy younger adults no association with CMV infection is observed. Nevertheless, CMV infection does not hamper the T-cell response to acute IAV infection in older adults.

MATERIALS AND METHODS

Study Design

Healthy Young and Older Adults

Samples of healthy individuals covering a broad age range were combined from two cohorts. Samples of young adults (n=34), between 18 and 52 years of age, from unvaccinated controls or pre-vaccination participants were selected based on age and sex from a study carried out in 2009-2011 (the Pandemic influenza vaccination trial, Netherlands Trial Register NL1952) (48). The study was approved by the Central Committee on Research Involving Human Subjects of the Netherlands. Samples of older adults (N=65), ≥60 years of age, were control samples from a study carried out in 2014-2015 (Influenza-like-illness-3, Netherlands Trial Register NL4666) (Kaaijk et al., submitted). This study was approved by the acknowledged ethical committee METC Noord Holland. Both studies were carried out in accordance with the recommendations of Good Clinical Practice with written informed consent from all subjects, in accordance with the Declaration of Helsinki.

IAV A Infected Older Adults During IAV Infection

Laboratory-confirmed Influenza virus A infected older adults were selected from the same study as the healthy older adults. In this prospective observational study participants were monitored for influenza-like-illness (ILI) in the influenza season of 2014-2015 (NL4666, Kaaijk et al., submitted). Study design of the Influenza-like-illness-3 study was comparable to previous studies as described in Van Beek et al. (49). In short, participants were instructed about

influenza-like-illness (ILI) symptoms according to the Dutch Pel criteria, defined by fever (≥37.8°C) with at least 1 other symptom of headache, muscle pain, sore throat, coughing, runny nose, or chest pain (50) and to report ILI as soon as possible after onset. Nasopharyngeal and oropharyngeal samples were obtained within 72 hours of reporting ILI by standard procedures (49). IAV infection was laboratory confirmed, and subtyped by PCR and sequencing in n=72 individuals by methods described previously (49). The 72 IAV confirmed patients were included in the current study. The H3N2 strain was detected in the majority of patients (n=64, of which n=20 clade 3C.3b, n=37 clade 3C.2a, n=7 not determined), and the H1N1 strain in the remaining individuals (n=8). Blood samples were collected within the first 72 hours of fever onset, and followed up after 2 weeks and 8 weeks.

PBMC and Serum Isolation

Peripheral blood mononuclear cells were obtained by Lymphoprep (Progen) density gradient centrifugation from heparinized blood, according to the manufacturer's instructions. PBMCs were frozen in 90% fetal calf serum and 10% dimethyl sulfoxide at -135°C until further use. Serum was isolated out of tubes with clot-activation factor and stored at -80° C until further use.

Cytomegalovirus (CMV)-Specific Antibodies

Anti-CMV IgG antibody concentrations were measured either using a commercial ELISA (IBL international GMBH) according to manufacturer's instructions or by an in-house-developed multiplex immunoassay (51), depending on the cohort. For healthy young adults, CMV-specific antibody levels were measured using a commercial ELISA. Recommended cutoffs of the commercial ELISA kit were followed. Participants with a CMV antibody level of ≥12 U/ml or higher were considered CMV^+, those with a level of ≤8 U/ml were considered CMV^-, and those with a level between 8 and 12 U/ml were considered inconclusive and hence excluded for further analysis. For older healthy adults and IAV-infected individuals, CMV-specific antibody levels were measured in serum by our in-house-developed multiplex immunoassay. Cutoff were based on previous calculations: Individuals with a CMV-specific antibody level of ≤4 arbitrary units/ml were considered to be CMV^- and individuals with an antibody level > 7.5 RU/ml were considered CMV^+, and those with a level between 4 and 7.5 arbitrary units/ml were considered inconclusive and hence excluded from further analysis (52). To reduce inter-assay variation, all samples from the same individual were measured on the same plate.

Antigen-Specific T Cells by Flow Cytometry

Healthy Individuals

HLA-A2 positive healthy individuals were selected based on availability from young and old healthy individuals for subsequent IAV-specific T-cell analysis, by staining PBMCs for expression of HLA-A2 with the HLA-A2(BB7.2)-V450 antibody (BD Bioscience). Of the HLA-A2 positive individuals, ± 4 million PBMC's were stained using the HLA-class I dextramer for epitope GILG of the M1 protein of IAV (A*0201/GILGFVFTL-APC, Immudex) for 20 minutes at room temperature. Surface staining was performed for 30 minutes at 4°C with the following antibodies: Fixable Viability Staining-780 (BD bioscience), CD3 (SK7)-AF700(BD bioscience), CD8(RPA-T8)-BrilliantViolet510, CD45RO(UCHL1)-BrilliantViolet711, CD27(O323)-BrilliantViolet786, CCR7(150503)-BrilliantUV395 (BD bioscience), KLRG-1(13F12F2)-PE-Cy7 (eBioscience), PD-1 (EH12.2H7)-PerCP Cy5.5, CD95(DX2)-BrilliantViolet421 (BD Biosciences), CD127(A019D5)-BrilliantViolet650, CD57 (HCD57)-PE and CXCR3(G025H7)-PE-Dazzle. All antibodies were purchased from Biolegend, unless stated otherwise. Acquisition was performed on a LSRFortessaX20 and data analysis was performed using FlowJo (Treestar). tSNE-analyses were performed using Cytobank (www.cytobank.org) (53) with for every donor 10.000 $CD8^+$ T cells. Donors with less than 10.000 $CD8^+$ T cells were excluded from t-SNE analysis (n=3).

IAV-Infected Individuals

Of all HLA-A2 positive individuals undergoing IAV infection (n=36), both IAV-specific T cells and CMV-specific T cells were assessed within the first 72 hours 2 and 8 weeks after fever onset. PBMCs were stained using the HLA-A2 dextramer for the GILG epitope of the M1 protein of IAV (A*0201/GILGFVFTL-APC, Immudex) (± 8 mln PBMCs) (detected in n=17) and the HLA-A2 dextramer for the NLV epitope of the pp65protein of CMV (A*0201/NLVPMVATV)-APC (Immudex) (1 mln PBMCs) for 20 minutes at room temperature. Extracellular staining was performed for 30 minutes at 4°C with the following antibodies: Fixable Viability Staining-780 (BD bioscience), CD3 (SK7)-AF700(BD bioscience), CD8(RPA-T8)-BrilliantViolet510 (Biolegend), CD45RO(UCHL1)-BrilliantViolet711 (Biolegend), CD27(O323)-BrilliantViolet786 (Biolegend), CCR7(150503)-BrilliantUV395(BD bioscience), KLRG-1(13F12F2)-PE-Cy7 (eBioscience), CD127(A019D5)-BrilliantViolet650 (Biolegend), CD57(HCD57)-PE (Biolegend) and CXCR3(G025H7)-PE-Dazzle (Biolegend), CD38(HIT2)-PE-Dazzle (BD bioscience) and HLA-DR(TU39)-BrilliantUV737 (BD Bioscience). Analysis was performed on an LSRFortessaX20.

IAV-Specific and CMV^-Specific IFNγ T-Cell Response by ELISpot

Virus-specific T-cell responses were quantified using the IFNγ enzyme-linked immunospot (ELISPOT) assay at all three time points for those of which enough cells were available (respectively n=66, n=61, n=58 out of n=72). Briefly, 400.000 PBMCs were stimulated with a 15-mer peptide-pool with 11 amino acids overlap, covering the total influenza M1 protein (1 μg/ml) (JPT) and incubated for 18 hours at 37°C on 96-well membrane-bottomed plates (PVDF plate MSIPS4510, Millipore) coated with anti-IFNγ mAbs (Mabtech). If indicated, IFNγ responses were corrected for the percentage of T cells in

lymphocytes based on flow cytometry data. For CMV responses, 100,000 PBMCs were stimulated with a 15-mer peptide-pool with 11 amino acids overlap, covering either the UL55 (1µg/ml) (JPT), the IE-1 (1µg/ml) (JPT), or the pp65 (1µg/ml) (JPT) CMV protein. The sum of the response to these three CMV peptide pools is presented in this study.

Cytokine and Chemokine Levels in Serum

Cytokines and chemokine levels in serum were assessed for all IAV-infected individuals within 72 hours, and 2 and 8 weeks after fever onset. Levels were measured by bead-based multiplex LEGENDplex™ (BioLegend) according to the manufacturer's instructions. The pro-inflammatory cytokines IL-6, IFNy, IL-10 and CRP were analyzed for this study. Stimulations were performed in duplicate. Analysis was performed on a Canto II flowcytometer. Data were analyzed *via* Legendplex V8.0 software (Biolegend). All data were transformed into averages of the logarithms of two measurements, and each data point was corrected by subtraction of the intra-assay averages to correct for batch effects.

Isolation of IAV-Specific T-Cells for T-Cell Receptor Analysis

$CD8^+$ T cells were isolated from PBMCs using a negative selection microbeads kit (Miltenyi Biotec). Next, $CD8^+$ T cells were labeled at room temperature for 20 minutes with the A*0201/GILGFVFTL-APC dextramer (Immudex) (GILG). Subsequently surface staining was added with the following mAbs: CD3(UCHT1)-PerCP (Biolegend), CD4(OKT4)-BV510 (Biolegend) and CD8(RPA-T8)-FITC (Biolegend) and $CD3^+CD4^-CD8^+GILG^+$ cells were sorted by FACS Melody (BD) directly into RNAlater (Ambion Inc. Applied Biosystems) and stored at -80°C for subsequent TCRβ clonotype analysis.

Preparing TCRbeta cDNA Libraries for High Throughput Sequencing

T-cell receptor analysis was performed as described previously (54), with minor modifications. Briefly, mRNA was isolated with the RNA microkit (Qiagen) according to manufacturer's protocol. Isolated mRNA was used for cDNA synthesis with 5'RACE template switch technology to introduce a universal primer binding site, and unique molecular identifiers (UMI's) were added at the 5' end of the cDNA molecules using the SMARTScribe Reverse Transcriptase (TaKaRa). cDNA synthesis was followed by an AMPure XP bead-based cleanup (Beckman Coulter). Purified cDNA molecules were amplified in two subsequent PCR steps using the Q5® High-Fidelity DNA Polymerase (New England BioLabs), with an AMPure XP bead-based cleanup in between. PCR products were size selected on gel and purified using the Nucleospin PCR cleanup kit (Machery-Nagel). The PCR products were sequenced *via* Illumina MiSeq paired end using 2x250 bp sequencing.

Analysis of TCRbeta Clonotype Analysis

Raw sequencing data was processed using the 12nt UMIs to correct for amplification biases and error-correction of reads. RTCR (55) was used to identify both the UMI sequence and clonotype information from the reads. Because of the relatively small number of cells per sample, additional filtering steps were followed to minimize cross-sample contamination and biases introduced by errors in the UMI sequence. Sequences were only accepted if their UMI was observed in at least 40 sequencing reads. Sequences with identical UMIs in multiple samples were removed if they did not occur in at least 1000 sequencing reads and also if their absolute frequency was lower than 10% of the maximum frequency in the other sample. UMIs were clustered within each sample within a Hamming Distance of 3.

Severity of Symptoms Assessment

Symptom assessment was performed by self-reporting by the participants. As soon as a fever occurred, participants reported the already experienced symptoms up until then and subsequently reported the last day of each symptom until full recovery up until a maximum of timepoint three (8 weeks after start of fever). This way, the presence and duration (start date and end date) of the following symptoms were collected: fever (≥37.8°C), cough, sore throat, runny nose, headache, pain while breathing and muscle pain. Symptoms of an IAV-infected individual up until ten days before onset of fever were considered to be IAV infection related and included in the analysis in this paper. The duration of each symptom in days was normalized by transformation of these values to Z-values, e.g. the number of standard deviations by which the value of the score of an individual is above or below the mean value. Finally, to assess the overall severity of symptoms during IAV infection, the average of the Z-values of the seven symptoms was calculated.

Statistical Analysis

Differences between groups (for example CMV^- compared to CMV^+) were assessed using Mann-Whitney *U* test, and comparisons within the same individuals (for example to compare time points in response upon influenza virus infection) with the Wilcoxon singed-rank test. Differences between groups in categorical variables were tested by chi-square test and corrected for multiple testing if applicable and indicated in figure legends. Correlations were tested with Spearman's rank correlation coefficient. For all analyses p values < 0.05 were considered statistically significant. Data were analyzed using GraphPad Prism 8.3 and SPSS statistics 22 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of Study Population

Healthy individuals were on average 59.2 years old (range 21-82 year) (n=99). They were categorized into young (21-52 years old) (n=34) and old (>60 years old) (n=65) individuals, of whom respectively 55.9% and 58.5% were CMV-infected (**Table 1**). No significant differences in age or sex were observed between CMV^- and CMV^+ individuals (**Table 1**). In addition to healthy individuals, older adults (average 69.9

years, range 60-88 years) with confirmed IAV infection were included in this study (n=72). Also for the IAV-infected older adults, no significant differences in age or sex were observed between CMV^+ and CMV^- individuals (**Table 1**). The majority of individuals were infected with the H3N2 strain (n=64/72), while some were infected with the H1N1 strain of influenza (n=8/72). All individuals in the H1N1 infected group turned out to be CMV^-.

CMV Induces an Increase in Senescence-Associated Markers in the T-Cell Pool in Older Adults

We assessed the effect of latent CMV infection on the $CD8^+$ T-cell pool of all healthy individuals, by performing a cluster analysis (tSNE) based on memory T-cell markers CD27, CCR7, CD95, CD45RO, and CXCR3, and senescence-associated T-cell markers CD57 and KLRG-1, known to be altered in CMV infection (56). Cluster analysis conformed the large differences between the CMV^- (n=40) and CMV^+ (n=56) group (**Figure 1A**), and six different clusters were identified. Clusters 1-3 containing non-senescent $CD27^{high}CCR7^{high}$ $CD57^{low}$ cells were predominantly present in CMV^- individuals (**Figure 1B** and **Supplementary Figure 1A**). In contrast, clusters 4-6 containing the more differentiated cells expressing $KLRG\text{-}1^{high}CD57^{medium}$ were more pronounced in CMV^+ individuals (**Figure 1B** and **Supplementary Figure 1A**). We next performed more detailed analysis on the expression of senescence-associated markers within the two age groups: young adults (20-52 years) and older adults (older than 60 years). CMV infection was associated with significantly increased expression of CD57 and KLRG-1 among older, but not in young, adults (**Figures 1C, D**). Likewise, expression of PD-1 was only significantly reduced in CMV^+ individuals (as compared to CMV^-) in the older group, and not in the younger group (**Figure 1E**). In line with this, CMV infection was associated with significantly increased frequencies of T_{EMRA} cells specifically in older individuals (**Figure 1F**). Together, this indicates that CMV infection establishes large changes in the $CD8^+$ T-cell pool by inducing terminally differentiated and senescent T cells in older adults.

Frequency of IAV-Specific T-Cells Is Decreased in CMV^+ Individuals, but Only in Older Adults

To investigate the hypothesis that CMV infection may negatively influence the immune response to other pathogens by outcompeting other antigen-specific T cells, the frequency of IAV-specific T cells in healthy individuals was determined using HLA-A2 dextramers containing the matrix protein-1 GILG-epitope. The frequency of IAV-specific T cells was significantly lower in CMV^+ compared to CMV^- individuals (P=0.0005) (**Figure 1G**). Importantly, this lower percentage of IAV-specific T cells was solely explained by a lower frequency in the older group, where some donors even had no detectable HLA-A2 GILG-specific T cells. Among the young adults no differences in the frequencies of IAV-specific T cells between CMV^- and CMV^+ individuals were observed (**Figure 1H**). Thus, CMV infection results in lower frequencies of IAV-specific memory T cells, but only in older adults.

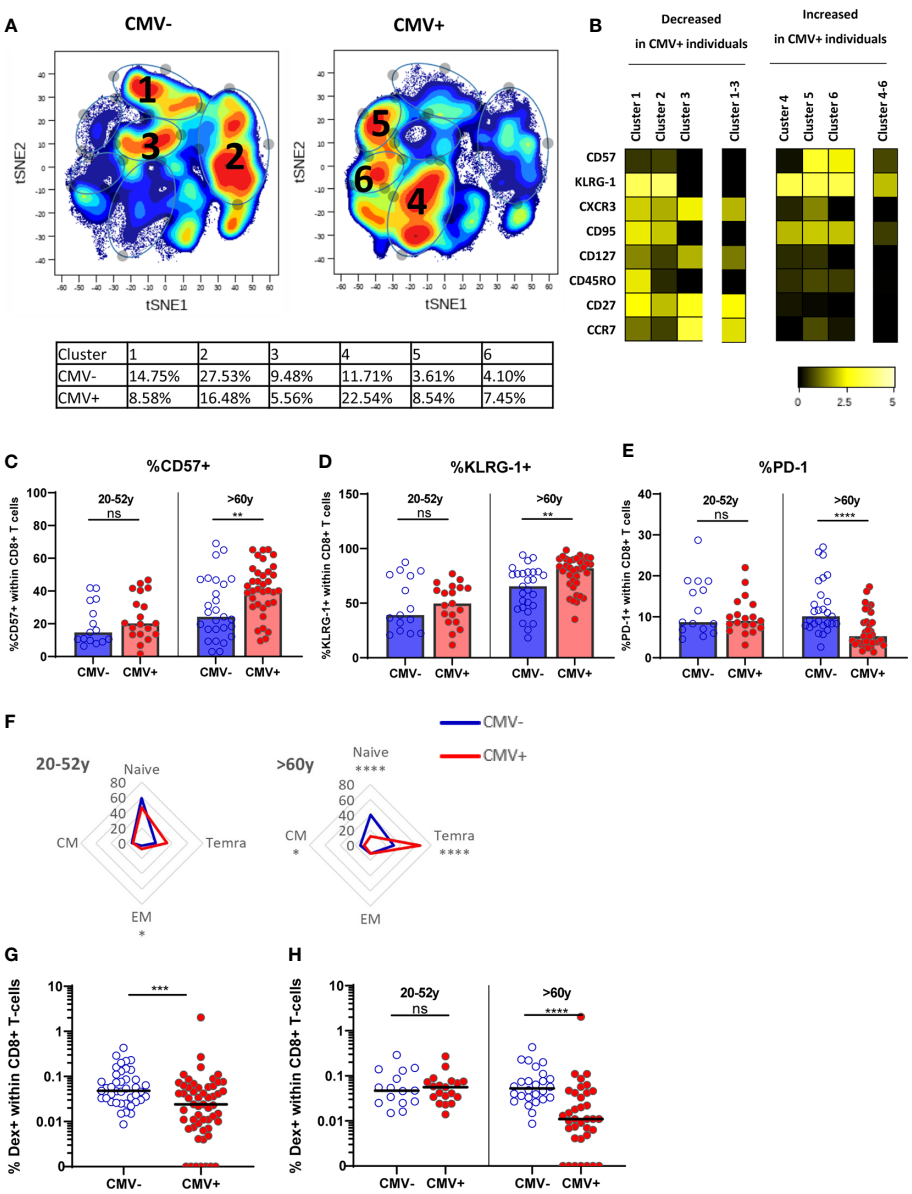
Characterization of the T-Cell Response During IAV Infection in Older Adults

To investigate the effect of CMV infection on the T-cell response during IAV infection, we characterized the T-cell response to IAV infection during infection in older adults (N=72), i.e. within 72 hours after start of fever, and 2 and 8 weeks later. The frequency of IAV-specific $CD8^+$ T cells was determined dextramer staining for the HLA-A2 GILG-epitope. IAV-specific $CD8^+$ T-cell frequencies were increased upon IAV infection at the 2 week time point compared to <72 hours after fever onset (median 0.03% to 0.15% of total $CD8^+$ T cells respectively), after which the response contracted (8 weeks post infection, median of 0.08/total $CD8^+$ T cells)

TABLE 1 | Characteristics of the study population.

Healthy young adults				
	Total (n=34)	**CMV- (n=15)**	**CMV+ (n=19)**	**Statistics**
Age (mean ± SD)	35.9 ± 10.3	35.3 ± 10.8	36.4 ± 10.1	ns
Sex (% women)	61.8%	53.3%	68.4%	ns
CMV-serostatus (CMV+)	55.9%	.	.	.
Healthy older adults				
	Total (n=65)	***CMV- (n=25)***	***CMV+ (n=37)***	***Statistics***
Age (mean ± SD)	71.7 ± 6.6	70.9 ± 6.8	72.5 ± 6.5	ns
Sex (% women)	38.5%	33.3%	44.7%	ns
CMV-serostatus (CMV+)	58.5%	.	.	.
Influenza virus infected older adults				
	Total (n=72)	***CMV- (n=35)***	***CMV+ (n=37)***	***Statistics***
Age (mean ± SD)	69.9 ± 6.1	69.2 ± 5.3	70.4 ± 6.9	ns
Sex (% women)	41.7%	37.1%	45.9%	ns
CMV-serostatus (CMV+)	51.4%	.	.	.
Influenza virus strain (%H1N1)	11.1%	22.9%	0.0%	P=0.002

Differences were tested with Mann-Whitney U test for continues variables and chi-square test for categorical variables.
ns stands for non significant.



Cluster	1	2	3	4	5	6
CMV-	14.75%	27.53%	9.48%	11.71%	3.61%	4.10%
CMV+	8.58%	16.48%	5.56%	22.54%	8.54%	7.45%

FIGURE 1 | The effect of CMV on memory CD8$^+$ T cells and IAV-specific CD8$^+$ T cells in older adults. **(A)** t-SNE analysis of total CD8$^+$ T cells based on MFI of CD57, KLRG-1, CXCR3, CD95, CD127, CD45RO, CD27 and CCR7 in CMV$^-$ and CMV$^+$ individuals (total of n = 96). **(B)** Heatmap of expression of markers of t-SNE clusters. Differences between CMV$^-$ and CMV$^+$ individuals is tested in **Supplementary Figure 1A**. **(C–E)** Percentage of CD57$^+$ **(C)**, KLRG-1$^+$ **(D)** or PD-1$^+$ **(E)** CD8$^+$ T cells in young and old CMV$^-$ and CMV$^+$ individuals. **(F)** Distribution of CD8$^+$ T cells over naive and memory subpopulation in CMV$^-$ and CMV$^+$ individuals categorized in young (left panel) and old (right panel) adults. **(G)** Percentage of IAV-specific CD8$^+$ T cells in all CMV$^-$ and CMV$^+$ individuals. **(H)** Percentage of IAV-specific CD8$^+$ T cells in CMV$^-$ and CMV$^+$ individuals categorized in young and old individuals. Median is presented in each figure. Differences between groups were compared by Mann Whitney U test. **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, non significant.

(**Figure 2A**). The increase in IAV-specific CD8$^+$ T-cell frequencies was mainly explained by the expansion of IAV-specific effector memory T cells (T_{EM}) (**Figure 2B**), while the frequencies in the other subsets (i.e. naïve (T_N), central memory (T_{CM}) and T_{EMRA}) did not increase (data not shown). IFNγ responses after *in vitro* stimulation of PBMCs with peptide pools covering the influenza matrix-protein-1 revealed similar results (**Figure 2C**).

This was true for both H3N2 influenza-infected and H1N1 influenza-infected individuals, and no difference in increase in the IFNγ response between the two strains was observed (data not shown). When the IAV-specific IFNγ response was calculated as percentage of CD3$^+$ T cells instead of PBMCs, the IAV-specific IFNγ response also tended to be higher in response to IAV infection (2 weeks) compared to steady state (8 weeks), albeit not significant (p=0.059) (**Supplementary Figure 2A** left panel).

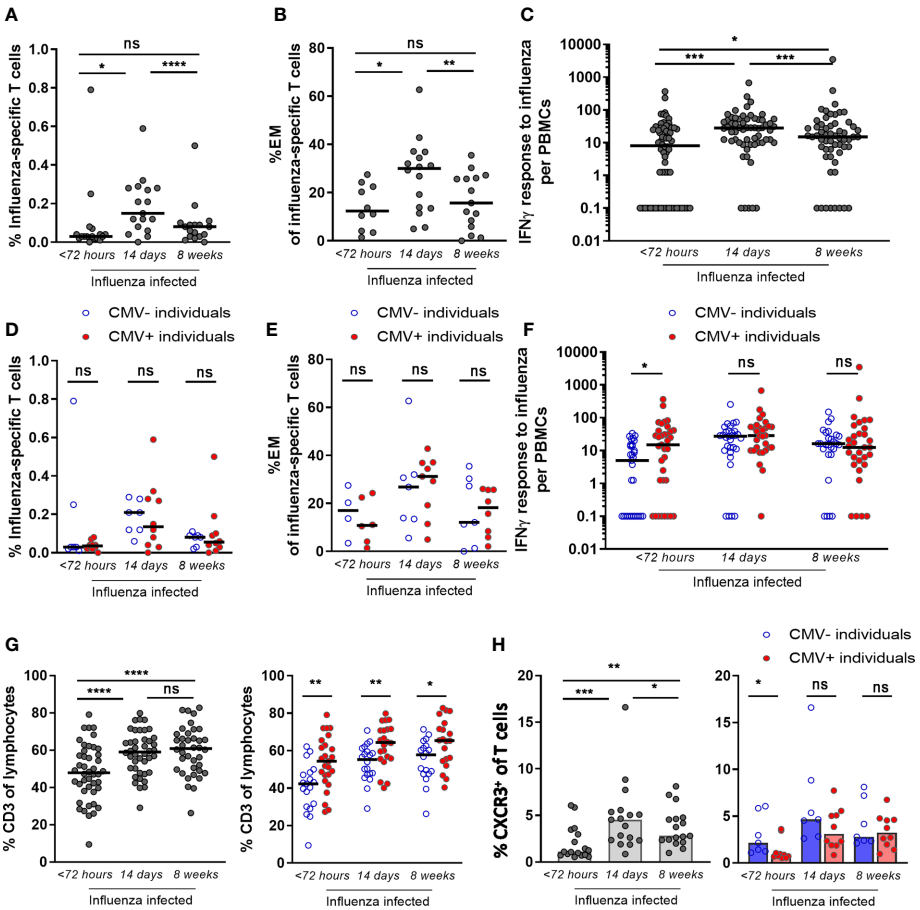


FIGURE 2 | IAV-specific CD8$^+$ T-cell response after infection shows no impairment by CMV seropositivity, but enhancement of the early IAV-specific CD8$^+$ T-cell response. **(A)** Percentage of IAV-specific CD8$^+$ T cells upon influenza infection. Dextramer for matrix protein-1 GILG-epitope was used **(B)** The percentage effector memory (EM) cells of IAV-specific CD8$^+$ T cells upon influenza infection. All HLA-A2-positive influenza-infected individuals (n =36) were stained for dextramers; IAV-specific T cells were detected at least at one time point in 17 individuals. **(C)** IAV-specific IFNγ T-cell response upon influenza infection. **(D)** Percentage of IAV-specific T cells upon influenza infection for CMV$^-$ and CMV$^+$ individuals. **(E)** The percentage effector memory (EM) cells of IAV-specific T cells upon influenza virus infection for CMV$^+$ and CMV$^-$ individuals. **(F)** IAV-specific IFNγ T-cell response upon influenza infection for CMV$^-$ and CMV$^+$ individuals. **(G)** Frequencies of CD3$^+$ T cells in the blood of IAV-infected individuals at < 72 hours, 2 weeks and 8 weeks after infection (left panel) and in CMV$^-$ and CMV$^+$ individuals (right panel). **(H)** Percentage of CXCR3$^+$ within total CD8$^+$ T cells upon infection (left panel) and in CMV$^-$ and CMV$^+$ individuals (right panel). Wilcoxon test was used to compare T-cell responses of individuals in time and Mann-Whitney U test was used to compare CMV$^-$ and CMV$^+$ individuals. Correlation between CMV-specific immune responses and IFNγ response to influenza virus was tested by Spearman correlation. T-cell IFNγ responses are presented per 1 x 10^6 PBMCs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, non significant.

T-Cell Response to IAV Infection in Older Adults Is Not Impaired by CMV Infection

To investigate whether the T-cell response during IAV infection (N=72) is influenced by CMV infection, we analyzed CMV$^+$ and CMV$^-$ individuals separately. No significant association of CMV$^-$ seropositivity on IAV-specific CD8$^+$ T-cell percentages or on the percentage of T_{EM} cells among the IAV-specific T cells was observed (**Figures 2D, E**). Likewise, IAV-specific IFNγ responses were not negatively associated with CMV seropositivity (**Figure 2F**) or with the level of CMV-specific antibodies in CMV$^+$ individuals at any of the time points (**Supplementary Figure 2B**). Surprisingly, in the acute phase (<72 hours after fever onset), CMV$^+$ individuals even showed a significantly higher IAV-specific IFNγ T-cell response than CMV$^-$ individuals (P=0.013) (**Figure 2F**). Also when corrected for the percentage of T cells among all lymphocytes, the same trend was observed (**Supplementary Figure 2A** right panel). This higher IAV-specific IFNγ T-cell response in CMV$^+$ individuals could not be explained by the moment of sampling within the 72 hours after fever onset (data not shown).

A possible explanation for the higher IAV-specific IFNγ T-cell response in CMV$^+$ individuals might be related to differences in T-cell migration. We therefore also investigated the total frequency of T cells in the blood and their expression of activation and migration markers upon IAV infection in CMV$^-$ and CMV$^+$ individuals. The frequency of total CD3$^+$ T cells in the blood of influenza-infected individuals was significantly lower early after infection compared to 2 or 8

weeks later (**Figure 2G** left panel). Comparing CMV^+ individuals with CMV^- individuals, the $CD3^+$ T-cell frequency was also consistently lower in CMV^- individuals (**Figure 2G** right panel). Next, the migration of $CD3^+$ T cells outside the blood, early after influenza infection, was further assessed by comparing the relative decrease within CMV^- and CMV^+ individuals. The drop in CD3+ T-cell frequency early after IAV infection was significantly larger in CMV^- than in CMV^+ individuals (**Supplementary Figure 3A**). Thus, the higher IFNγ IAV-specific responses in blood in CMV^+ individuals early after influenza virus infection (**Figure 2F**) may at least partly be explained by higher frequencies of total T cells in CMV^+ as compared to CMV^- individuals. The higher frequencies of total T cells may be a result of less migration of T cells. Investigating migratory capacity of T cells, we indeed found that early after IAV infection CXCR3 expression by $CD8^+$ T cells, which is associated with migratory capacity of these cells to the lungs, was lower in CMV^+ individuals compared to CMV^- individuals (**Figure 2H**). These data suggest that T cells in CMV^+ individuals may be less prone to be recruited to the lungs early after IAV infection and thereby accumulate in the blood leading to higher frequencies. However, on IAV-specific $CD8^+$ T cells, only a small trend of increased CXCR3 expression on (p=0.11) was observed in CMV^- compared to CMV^+ individuals (**Supplementary Figure 3B**) early after IAV infection. In addition, the activation status of IAV-specific T cells by the frequencies of $HLA^-DR^+CD38^+$ or $CD127^-KLRG^-1^+$ revealed no difference between CMV^- and CMV^+ individuals. IAV-specific T cells from CMV^- and CMV^+ individuals both showed increased expression of these activation markers early after IAV infection (<72 hours after start fever) as compared to 2 and 8 weeks later, irrespective of CMV serostatus. This pattern was not seen for CMV-specific T cells, which served as a control. This suggests that activation and rapid expansion of IAV-specific memory T cells early after infection occurs irrespective of CMV serostatus (**Supplementary Figure 3C**). Overall, these data suggest that migration of T cells to the lungs might play a role in the enhanced IAV-specific IFNγ response observed in CMV^+ individuals early after IAV infection (**Figure 2F**) although evidence remains circumstantial.

Large CMV^-Specific T-Cell Responses Are Not Associated With Impaired IAV-Specific T-Cell Responses

As CMV might only affect the immune system through competition for "limited immunological space" in individuals with large CMV-specific T-cell responses, we next investigated the association between CMV infection and the T-cell response to IAV by taking into account the magnitude of the IFNγ CMV-specific T-cell responses within CMV^+ individuals. First, we assessed the IFNγ T-cell response to IAV infection. No negative correlation between expanded CMV-specific T-cell responses and T-cell response to IAV infection was observed at any of the three time points (**Figure 3A**). Surprisingly, we even observed a significant positive correlation between the height of the CMV-specific IFNγ T-cell response and the height of the IAV-specific IFNγ T-cell response at 2 and 8 weeks after fever onset (**Figure 3A**) (R: 0.52, p=0.016 and R:0.45, p=0.014 respectively. In addition, we further assessed the quality of the IAV-specific T-cell response by investigating TCR diversity of the IAV-specific T-cell response, by sorting the HLA-A2-GILG-specific T cells and sequencing the TCRβ chain. The clonotype distribution was analyzed over time after IAV infection in two CMV^- and two CMV^+ individuals. In general it was observed that 89% of the different TCR sequences expressed the Vβ-19 segment, of which 74% consisted of the highly conserved -RS- motif (See **Supplementary Tables 1A, B** for the V and J segments). Interestingly, all donors showed a shift in dominance after IAV infection, however large heterogeneity was observed between the donors with some repertoires becoming more diverse and other less diverse over time. No indication for CMV-related differences were present, as no differences between the CMV^- and CMV^+ individuals were observed. Based on these data no differences were observed in the TCR diversity of the IAV-specific T-cell repertoire between CMV^- and CMV^+ individuals (**Figure 3B**). These lines of evidence do not support the competition between CMV-specific memory T cells and a proper IAV-specific T cells during IAV infection.

Cytokine Levels in Serum of Influenza-Infected Individuals Are Not Affected by CMV and Not Associated With IAV-Specific T-Cell Responses

As it is suggested that CMV is linked to "inflammaging", we questioned whether pro-inflammatory mediators may be enhanced in CMV^+ individuals during IAV-infection. We investigated the levels of pro-inflammatory cytokines in serum of CMV^+ and CMV^- individuals, and their potential association with the IAV-specific T-cell response. At the early phase of IAV infection, the inflammation-associated factors IL-6 and CRP were elevated in serum compared to 2 and 8 weeks later (**Figure 4A**). No significant differences in IL-6 and CRP levels were observed between CMV^+ and CMV^- individuals, also at the peak of the T-cell response (2 weeks later) and at steady state (week 8 after fever onset) (**Figure 4A**). We also measured the IFNγ:IL-10 ratio, as it was suggested that a shift in this ratio leads to a decline in IAV-specific T-cell responses with age and is associated with decreased protection against IAV (46, 57). No difference was observed (**Figure 4B**) in the IFNγ:IL-10 ratio between CMV^+ and CMV^- individuals at any of the time points, even though both IFNγ and IL-10 levels were elevated at the earliest timepoint (**Supplementary Figure 4**). Although increased levels of the cytokines IL-6, CRP, IFNγ and IL-10 were observed in the acute phase of IAV infection, no significant association between these cytokine levels and the height of the IAV-specific T-cell response was observed <72 hours after start of fever (**Figure 4C**) or 2 and 8 weeks later (data not shown). Together, this suggests that CMV infection in older adults does not affect cytokine levels in serum upon IAV infection.

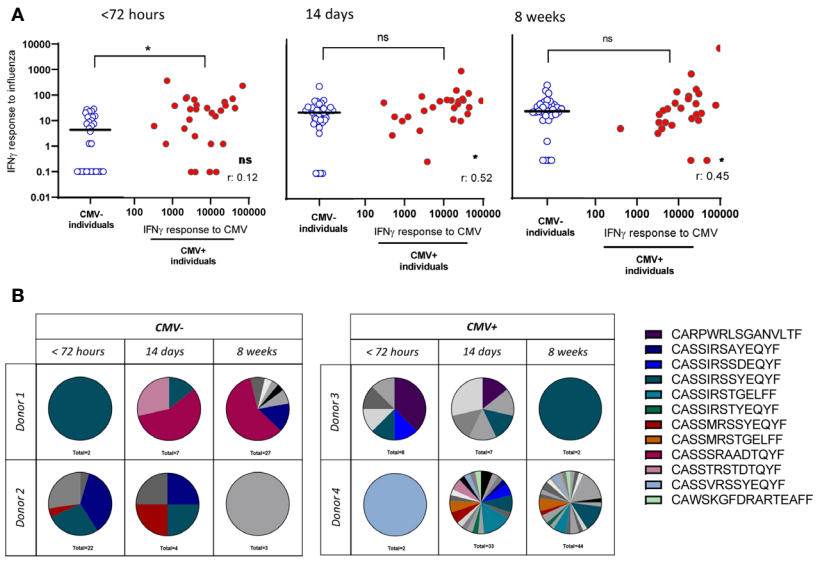


FIGURE 3 | No reduced IAV-specific T-cell response in number of T cells or clonal diversity due to large expansions of CMV-specific T cells. **(A)** IAV-specific IFNγ T-cell response in CMV$^-$ and CMV$^+$ individuals differentiated on the CMV-specific T-cell response. IAV-specific IFNγ T-cell responses are depicted in blue open circles in scatter plot for CMV$^-$ individuals and in red solid circles in correlation of CMV-specific IFNγ T-cell responses for CMV$^+$ individuals. Differences were tested using Mann-Whitney to compare CMV$^+$ and CMV$^-$ individuals. Correlation between CMV-specific T-cell responses and IFNγ response to influenza was tested by Spearman correlation. T-cell IFNγ responses are presented per 1 x 10^6 PBMCs. **(B)** T-cell repertoire of sorted HLA-A2-GILG–specific CD8$^+$ T cells, detected by PCR of two CMV$^-$ and two CMV$^+$ individuals. Each pie-chart depicts the repertoire of a donor at a certain timepoint (< 72hours, 14 days or 8 weeks after infection). Colors represent shared CDR3 sequences between timepoints and donors. Grey scales depict unique CDR3 sequences. *P < 0.05. ns, non significant.

Severity of Symptoms of IAV Virus Infection Is Not Increased by CMV

Since early T-cell responses during IAV infection play an important role in limiting disease severity, we also investigated whether the height of the IAV-specific T-cell response was associated with severity of symptoms of IAV infection. In IAV-infected older adults, the number and duration of the following symptoms was assessed: fever (≥37.8°C), cough, sore throat, runny nose, headache, pain while breathing and muscle pain. The severity of symptoms of IAV infection was positively associated with the height of the IAV-specific T-cell response at timepoint 3 (8 weeks after start of fever) (**Figure 5A**), but not within 72 hours or 2 weeks after start of fever. This association was mainly based on the number of symptoms, and not on their duration (**Supplementary Figure 5**). Thus, the IAV-specific T-cell response 8 weeks after IAV infection, and not the early T-cell response, was linked to the severity of symptoms of the IAV infection in this cohort.

We next investigated whether CMV infection was associated with more severe symptoms during IAV infection. No significant differences between CMV$^-$ and CMV$^+$ individuals were observed in the number of symptoms during IAV infection (**Figure 5B**), total duration of symptoms (**Figure 5C**), or severity of symptoms as assessed by a combination of duration and number of symptoms (**Figure 5D**). When investigating the IAV infection symptoms individually, CMV$^+$ individuals tend to suffer less from muscle pain (**Figure 5E**) and although frequency of coughing was not different between CMV$^+$ and CMV$^-$, CMV$^+$ individuals tended to suffer less long from coughing as a symptom compared to CMV$^-$ individuals (data not shown). When the frequency of individuals coughing was plotted over time, we indeed found a indication for a faster decline in the frequency of coughing individuals amongst CMV$^+$ individuals compared to CMV$^-$ individuals (**Figure 5F**). Of note, both the increased frequency of muscle pain and the longer persistence of coughing among CMV$^-$ individuals could not be ascribed to a difference in IAV strain infection between CMV$^-$ and CMV$^+$ individuals (data not shown). The severity of symptoms was also not associated with CMV$^-$specific antibody levels (data not shown). Together, this suggests that CMV infection at least does not worsen the number and duration of IAV infection symptoms.

DISCUSSION

In this study we investigated the impact of CMV infection on the immune response to IAV. We found that CMV infection is associated with a more differentiated and senescent phenotype of CD8$^+$ T cells. In healthy younger individuals, no difference in IAV-specific T-cell frequencies were observed, but, CMV$^+$ older individuals had lower frequencies of IAV-specific memory CD8$^+$ T cells compared to CMV$^-$ older individuals. Nevertheless, the induction of an IAV-specific T-cell response during active IAV infection in older adults was not impaired. Also, severity of IAV-associated symptoms was not negatively affected by CMV

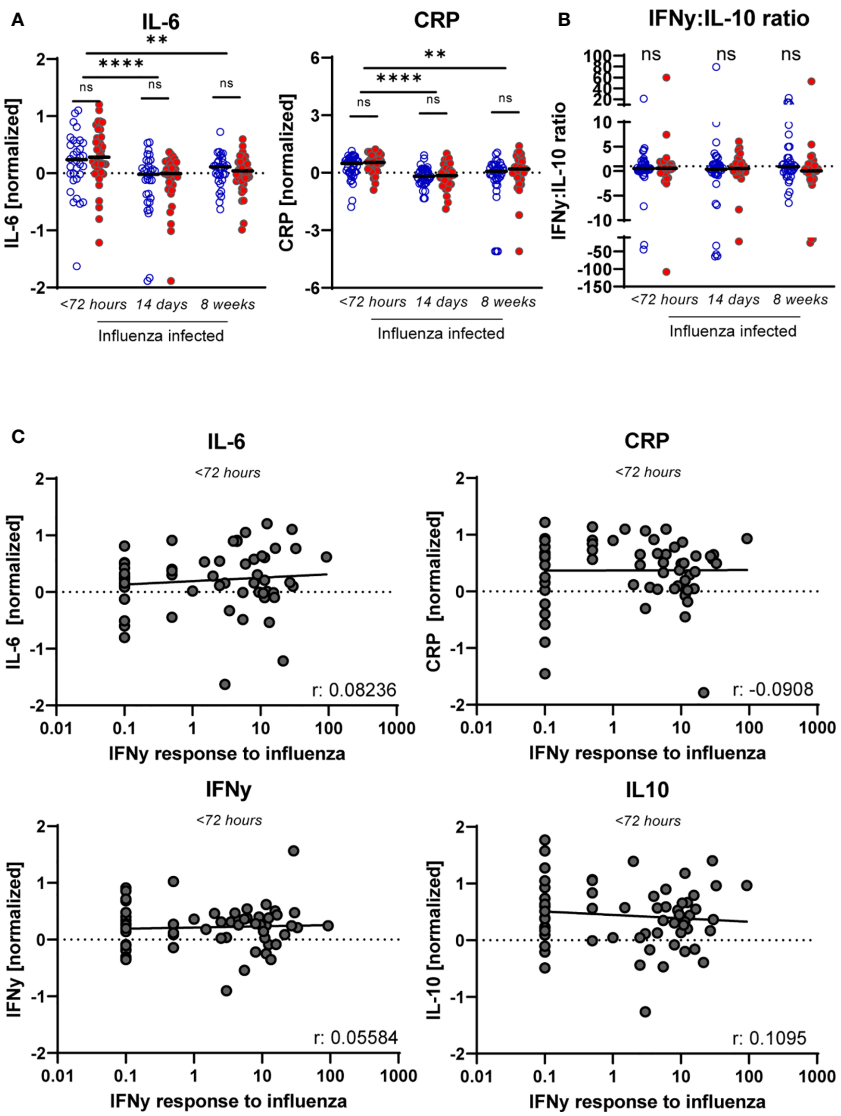


FIGURE 4 | No sign of increased inflammation in CMV$^+$ individuals based on cytokines levels in serum nor in relation to the IAV-specific T-cell response. **(A)** Serum levels of "inflammaging markers" CRP and IL-6 upon influenza infection for CMV$^+$ and CMV$^-$ individuals at < 72 hours after fever onset, and 2 and 8 weeks later. **(B)** The IFNγ:IL-10 ratio for CMV$^-$ and CMV$^+$ individuals upon influenza infection. **(C)** Magnitude of the IAV-specific IFNγ response at < 72 hours upon infection with influenza virus are not associated with the level of CRP, IL-6, IFNγ or IL-10 in serum. Serum levels of the cytokines were measured by multiplex assays and normalized based on subtracting the mean per plate. Differences were tested using unpaired T-test to compare CMV$^-$ and CMV$^+$ individuals. Correlations between IAV-specific IFNγ response and the different cytokines levels in serum were assessed by Spearman correlation. T-cell IFNγ responses are presented per 1 x 10^6 PBMCs. **P < 0.01, ****P < 0.0001. ns, non significant.

infection. We did not find any evidence for a negative effect of CMV infection on the IAV immune response by 'limited immunological space' or by increased levels of pro-inflammatory mediators. In contrast, there seemed to be a positive association between CMV infection and the IAV-specific T-cell response early after IAV infection (<72 hours after fever onset).

To the best of our knowledge, we are the first to investigate the potential effect of CMV infection on the T-cell response to a heterologous infection in humans. A negative effect of CMV infection on the functioning of the immune system is often intuitively explained by competition between T cells for 'limited immunological space'. Indeed, CMV infection has a profound impact on the composition of the overall CD8$^+$ T-cell pool in healthy individuals, by increasing the number of highly differentiated memory cells, especially in older adults (25). We show that CMV infection leads to a decrease in the frequency of memory IAV-specific T cells in healthy older adults. Relatively low frequencies of IAV-specific T cells in older adults compared to younger adults have been reported before (58, 59) and are considered to be a key determinant of a diminished T-cell response in IAV infection (60). Note, that the observed

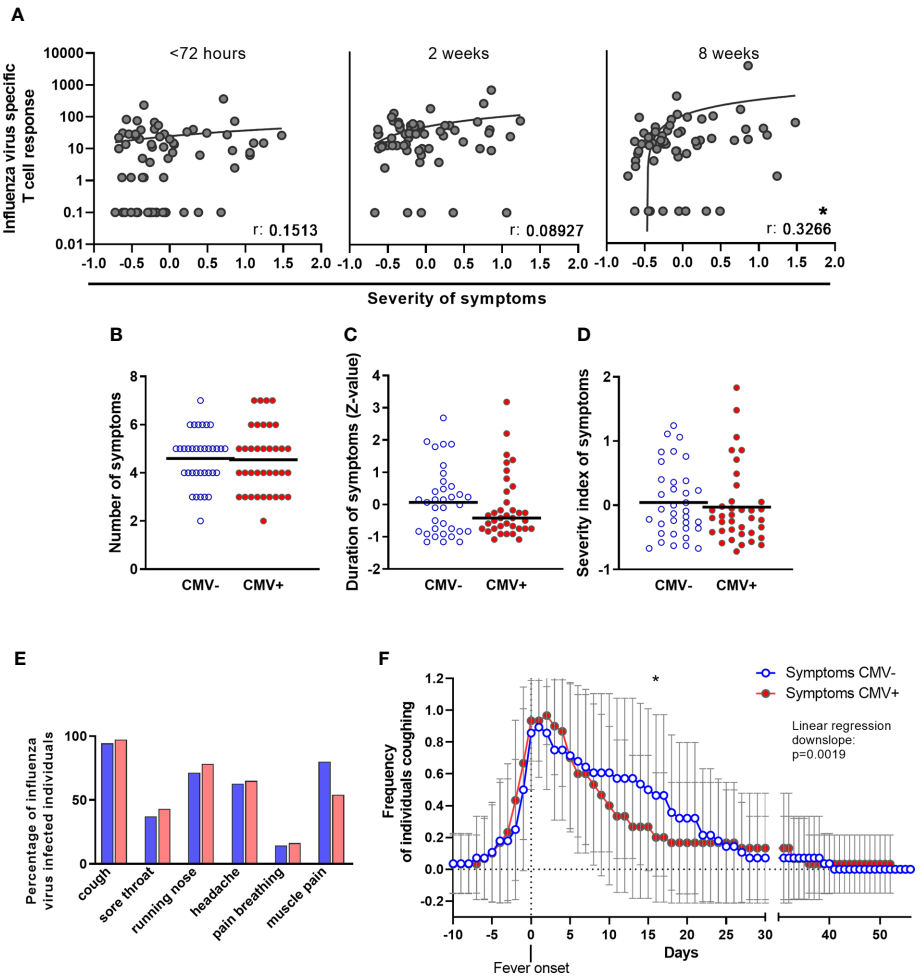


FIGURE 5 | No negative effect of CMV-infection on number and duration of symptoms of influenza virus infection in older adults. **(A)** IAV-specific T-cell responses upon influenza virus infection at < 72 hours after fever onset, and 2 and 8 weeks later was associated with the severity of symptoms of influenza infection. Association was tested by Spearman correlation**. (B)** Number of symptoms during influenza virus infection in CMV^- and CMV^+ individuals. Due to study design, participants had a minimal of two symptoms; fever (≥ 37.8°C) and at least 1 other symptom. Statistical differences between CMV^- and CMV^+ were tested by χ-square test. **(C)** Duration of having symptoms, regardless which one and how many, of influenza virus infection in CMV^- and CMV^+ individuals. Duration in days was calculated to Z-values. Difference between CMV^- and CMV^+ individuals was tested by student-t test. **(D)** Severity of symptoms, taking along both duration and number of symptoms was assessed by taking the mean of Z-values of the six symptoms (cough, sore throat, runny nose, headache, pain while breathing or muscle pain). **(E)** Percentage of CMV^- and CMV^+ individuals suffering from one of the six symptoms. Statistical differences between CMV^- and CMV^+ were tested by χ-square test and corrected for multiple testing by Bonferroni correction. **(F)** Frequency of CMV^- and CMV^+ individuals coughing during influenza virus infection. Difference between CMV^- and CMV^+ individuals was tested by linear regression analysis on the downslope (starting at day of fever onset, day 0 on the x-axis) and comparison of the slope of CMV^- individuals and CMV^+ individuals. *P < 0.05.

frequencies and characteristics of the IAV-specific response are based on one HLA-A2 epitope (GILGFVFTL). Although this epitope is immunodominant and conserved between most influenza strains, it remains to be determined whether it reflects the T-cell total IAV-specific T-cell response.

Unfortunately, absolute T-cell numbers could not be investigated in this study, leaving the possibility that the decreased frequency of IAV-specific T cells merely reflected a relative increase in CMV-specific T cells in older adults. A decline in the frequency -and not in the number- of IAV-specific T cells may explain why decreased IAV-specific T-cell frequencies in healthy older adults do not seem to result in reduced T-cell responses upon acute influenza virus infection. This was also observed in the MCMV mouse model, were reduced frequencies were observed, but the absolute counts of $CD8^+$ T cell against an heterologous virus infection were maintained in MCMV infected mice (61). Alternatively, the number of IAV-specific T cells may have been lower in CMV^+ older adults. A similar effect has previously been reported for EBV-specific T cells (37). During IAV infection, we observed that IAV-specific T cells in CMV^+ and CMV^- individuals responded equally well and no substantial changes in IAV-specific T-cell repertoire were observed in CMV^- and CMV^+ individuals. Previously, to influenza vaccination, other studies reported

impaired IAV-specific T-cell responses by CMV infection (20, 62, 63). In contrast, other groups showed a positive effect of CMV on the vaccine-induced IAV-specific T-cell response in humans (64). In addition, in mice, MCMV infection was shown to enhance the diversity of the T-cell repertoire against Listeria monocytogenes expressing OVA (65), whereas we found no differences in the repertoire diversity between CMV^- and CMV^+ individuals. This could probably be explained by the small sample size of our repertoire analyses, or by the difference in epitope, as the HLA-A2-GILG specific T-cell repertoire is suggested to be more restricted compared to other antigen-specific repertoires (66). We also found that the magnitude of the CMV-specific T-cell response was positively associated with the magnitude of the IAV-specific T-cell response 2 weeks and 8 weeks after fever onset of IAV infection. Together, this suggests that CMV^+ individuals have a sufficient amount of IAV-specific T cells able to respond to IAV infection.

Pre-existing T cells and timing of the IAV-specific $CD8^+$ T-cell response are thought to play an important role in the reduction of severity of IAV-related symptoms (30, 31). An early T-cell response has been proposed to accelerate viral clearance, whereas a delayed and prolonged T-cell response may lead to high and prolonged levels of inflammation and increased severity of disease (31, 32). Unfortunately, pre-existing T-cell responses could not be investigated in this study. Our data suggest that CMV^+ individuals have an increased early or accelerated IAV-specific T-cell response compared to CMV^- individuals. We speculate that this may lead to earlier viral clearance, and thereby might explain the faster recovery of coughing and decreased frequency of muscle pain. When we assessed severity of symptoms of symptoms, we found a significant positive correlation between the magnitude of the IAV-specific T-cell response and severity of symptoms 8 weeks after fever onset, and not early after fever onset or 2 weeks later. This suggests that a prolonged T-cell response, still present 8 weeks after fever onset, is increasing symptom severity of IAV infection.

As CMV infection was suggested to induce a more inflammatory environment (67), we also studied the potential association between CMV and inflammatory markers and how this might be related to IAV-specific T-cell responses. Although the levels of pro-inflammatory cytokines and chemokines measured here were significantly increased in the acute phase of IAV infection (<72 hours after fever onset), they were similar between CMV^+ and CMV^- individuals. Since pro-inflammatory responses were similar between CMV^+ and CMV^- individuals, it may (partly) explain the lack of difference in the severity of clinical symptoms between these groups. Furthermore, these levels did not seem to be associated with the IAV-specific T-cell response at any time point, although, we cannot rule out the possibility that other immune modulatory factors may play a role.

Most studies claiming a negative effect of CMV on an immune response to a heterologous challenge have been performed in mice. Several mouse studies have shown that only lifelong infection with MCMV leads to decreased immunity against heterologous infections (14–16). The magnitude of the effect of CMV infection might thus be linked to the duration of CMV infection and the experienced amount of viral reactivation in an infected host. We found no evidence for decreased immunity against a heterologous infection in humans infected with CMV. Also, high CMV-specific antibody levels were not associated with the height of the IAV-specific T-cell response. One of the reasons for the observed differences between mice and men, could be the order of infections by CMV and IAV. In mouse studies, mice are typically first infected with MCMV long before they are challenged with a heterologous acute infection. Many humans, in contrast, may have undergone their first IAV infection before they were infected with CMV, which may lead to the presence of IAV-specific memory T cells before the CMV-specific immune response is established. A potential harmful effect of CMV might be less pronounced in a host who already has a proper immune response against IAV. Furthermore, as mouse models of CMV are almost exclusively done in specific pathogen free mice, and humans are exposed to dozens of infections and triggers during life, it might be that the effect of CMV is magnified in mice. Even if CMV can modulate other immune responses, there is no substantial evidence that CMV impacts the function of the immune system by hampering immune responses against heterologous infections in humans.

The increased IFNγ T-cell response to IAV infection that we observed in CMV^+ older individuals remains partially unexplained. Previously, enhanced influenza vaccine responses in humans and mice were explained by an increase in IFNγ in serum (1). However, we did not observe a difference in IFNγ levels in serum between CMV^+ and CMV^- individuals in our study. However, an important difference between our study and the study by Furman et al. is the age of the individuals, as the latter observed this increase in IFNy levels mainly in younger adults. As the positive effect of CMV was observed in IAV IFNγ ELIspot assays, we cannot exclude the possibility that CMV infection may only affect $CD4^+$ T cells, which may respond in the assay as well. Another explanation for the difference in the early IAV-specific T-cell response between CMV^- and CMV^+ individuals could be related to the migratory capacity of the responding T cells, as we observed a trend towards enhanced CXCR3 expression of IAV-specific $CD8^+$ T cells in CMV^- individuals. This may lead to early migration towards tissues such as the lungs. The effect of CMV on IAV-specific T-cell responses at the site of infection instead of the blood would be of great interest, and requires further research. Also, other factors like apoptosis or proliferation of the IAV-specific T cells may play a role in the difference in IAV-specific T-cell response between CMV^- and CMV^+ individuals.

In conclusion, identification of the driving forces that induce age-related changes in the immune system is important to protect the growing population of older adults against infectious diseases. Especially IAV leads to more increased disease burden in older adults, e.g. severe

symptoms and higher risk of complications, hospitalization and mortality. Our study shows that despite the lower frequency of IAV-specific memory T-cell responses in older adults, CMV infection does not seem to impair the T-cell response against acute IAV infection. This work supports the view that CMV acts, if anything, as an immune modulatory mediator, rather than having a true negative impact on the immune system.

DATA AVAILABILITY STATEMENT

Datasets are available on request: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethical committee METC Noord Holland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The original idea for this study was from DV. SV performed the majority of experiments, gathered data and analysis. JL performed a large part of the experiments, gathered data and analysis. RJ, MH, MV, NN, and RV performed and evaluated experiments. JV designed the original study of IAV infected individuals. JD with help from JB and DV supervised the project. SV and JL prepared figures and wrote the manuscript with contributions and review from JD, DV, and JB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.663664/full#supplementary-material

REFERENCES

1. Furman D, Jojic V, Sharma S, Shen-Orr SS, Angel CJ, Onengut-Gumuscu S, et al. Cytomegalovirus Infection Enhances the Immune Response to Influenza. *Sci Transl Med* (2015) 7(281):281ra43. doi: 10.1126/scitranslmed.aaa2293
2. Goronzy JJ, Fulbright JW, Crowson CS, Poland GA, O'Fallon WM, Weyand CM. Value of Immunological Markers in Predicting Responsiveness to Influenza Vaccination in Elderly Individuals. *J Virol* (2001) 75(24):12182–7. doi: 10.1128/JVI.75.24.12182-12187.2001
3. Almanzar G, Schwaiger S, Jenewein B, Keller M, Herndler-Brandstetter D, Wurzner R, et al. Long-Term Cytomegalovirus Infection Leads to Significant Changes in the Composition of the CD8+ T-Cell Repertoire, Which may be the Basis for an Imbalance in the Cytokine Production Profile in Elderly Persons. *J Virol* (2005) 79(6):3675–83. doi: 10.1128/JVI.79.6.3675-3683.2005
4. Chidrawar S, Khan N, Wei W, McLarnon A, Smith N, Nayak L, et al. Cytomegalovirus-Seropositivity has a Profound Influence on the Magnitude of Major Lymphoid Subsets Within Healthy Individuals. *Clin Exp Immunol* (2009) 155(3):423–32. doi: 10.1111/j.1365-2249.2008.03785.x
5. Hadrup SR, Strindhall J, Kollgaard T, Seremet T, Johansson B, Pawelec G, et al. Longitudinal Studies of Clonally Expanded Cd8 T Cells Reveal a Repertoire Shrinkage Predicting Mortality and an Increased Number of Dysfunctional Cytomegalovirus-Specific T Cells in the Very Elderly. *J Immunol* (2006) 176(4):2645–53. doi: 10.4049/jimmunol.176.4.2645
6. Nikolich-Zugich J. The Twilight of Immunity: Emerging Concepts in Aging of the Immune System. *Nat Immunol* (2018) 19(1):10–9. doi: 10.1038/s41590-017-0006-x
7. Olsson J, Wikby A, Johansson B, Lofgren S, Nilsson B, Ferguson FG. Age-Related Change in Peripheral Blood T-lymphocyte Subpopulations and Cytomegalovirus Infection in the Very Old: The Swedish Longitudinal OCTO Immune Study. *Mech Ageing Dev* (2000) 121:187–201. doi: 10.1016/S0047-6374(00)00210-4
8. Koch S, Larbi A, Ozcelik D, Solana R, Gouttefangeas C, Attig S, et al. Cytomegalovirus Infection: A Driving Force in Human T Cell Immunosenescence. *Ann N Y Acad Sci* (2007) 1114:23–35. doi: 10.1196/annals.1396.043
9. Looney RJ, Falsey A, Campbell D, Torres A, Kolassa J, Brower C, et al. Role of Cytomegalovirus in the T Cell Changes Seen in Elderly Individuals. *Clin Immunol* (1999) 90(2):213–9. doi: 10.1006/clim.1998.4638
10. Derhovanessian E, Larbi A, Pawelec G. Biomarkers of Human Immunosenescence: Impact of Cytomegalovirus Infection. *Curr Opin Immunol* (2009) 21(4):440–5. doi: 10.1016/j.coi.2009.05.012
11. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJ, Furman D, et al. Variation in the Human Immune System is Largely Driven by non-Heritable Influences. *Cell* (2015) 160(1-2):37–47. doi: 10.1016/j.cell.2014.12.020
12. Aiello AE, Chiu YL, Frasca D. How Does Cytomegalovirus Factor Into Diseases of Aging and Vaccine Responses, and by What Mechanisms? *Geroscience* (2017) 39(3):261–71. doi: 10.1007/s11357-017-9983-9
13. Tu W, Rao S. Mechanisms Underlying T Cell Immunosenescence: Aging and Cytomegalovirus Infection. *Front Microbiol* (2016) 7:2111. doi: 10.3389/fmicb.2016.02111
14. Redeker A, Remmerswaal EBM, van der Gracht ETI, Welten SPM, Hollt T, Koning F, et al. The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose. *Front Immunol* (2017) 8:1953. doi: 10.3389/fimmu.2017.01953
15. Cicin-Sain L, Brien JD, Uhrlaub JL, Drabig A, Marandu TF, Nikolich-Zugich J. Cytomegalovirus Infection Impairs Immune Responses and Accentuates T-cell Pool Changes Observed in Mice With Aging. *PloS Pathog* (2012) 8(8):e1002849. doi: 10.1371/journal.ppat.1002849
16. Mekker A, Tchang VS, Haeberli L, Oxenius A, Trkola A, Karrer U. Immune Senescence: Relative Contributions of Age and Cytomegalovirus Infection. *PloS Pathog* (2012) 8(8):e1002850. doi: 10.1371/journal.ppat.1002850
17. McElhaney JE, Kuchel GA, Zhou X, Swain SL, Haynes L. T-Cell Immunity to Influenza in Older Adults: A Pathophysiological Framework for Development of More Effective Vaccines. *Front Immunol* (2016) 7:41. doi: 10.3389/fimmu.2016.00041
18. Reber A.J.C., T., Kim JH, Cao W, Biber R, Shay DK, Sambhara S. Immunosenescence and Challenges of Vaccination Against Influenza in the Aging Population. *Aging Dis* (2011) 3:68–90.
19. Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. Cytomegalovirus (CMV) Seropositivity Decreases B Cell Responses to the Influenza Vaccine. *Vaccine* (2015) 33(12):1433–9. doi: 10.1016/j.vaccine.2015.01.071
20. Merani S, Kuchel GA, Kleppinger A, McElhaney JE. Influenza Vaccine-Mediated Protection in Older Adults: Impact of Influenza Infection, Cytomegalovirus Serostatus and Vaccine Dosage. *Exp Gerontol* (2018) 107:116–25. doi: 10.1016/j.exger.2017.09.015

21. Wald A, Selke S, Magaret A, Boeckh M. Impact of Human Cytomegalovirus (CMV) Infection on Immune Response to Pandemic 2009 H1N1 Influenza Vaccine in Healthy Adults. *J Med Virol* (2013) 85(9):1557–60. doi: 10.1002/jmv.23642
22. den Elzen WP, Vossen AC, Cools HJ, Westendorp RG, Kroes AC, Gussekloo J. Cytomegalovirus Infection and Responsiveness to Influenza Vaccination in Elderly Residents of Long-Term Care Facilities. *Vaccine* (2011) 29(29-30):4869–74. doi: 10.1016/j.vaccine.2011.03.086
23. van den Berg SPH, Wong A, Hendriks M, Jacobi RHJ, van Baarle D, van Beek J. Negative Effect of Age, But Not of Latent Cytomegalovirus Infection on the Antibody Response to a Novel Influenza Vaccine Strain in Healthy Adults. *Front Immunol* (2018) 9:82. doi: 10.3389/fimmu.2018.00082
24. van den Berg SPH, Warmink K, Borghans JAM, Knol MJ, van Baarle D. Effect of Latent Cytomegalovirus Infection on the Antibody Response to Influenza Vaccination: A Systematic Review and Meta-Analysis. *Med Microbiol Immunol* (2019) 208(3-4):305–21. doi: 10.1007/s00430-019-00602-z
25. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, et al. Aging and Cytomegalovirus Infection Differentially and Jointly Affect Distinct Circulating T Cell Subsets in Humans. *J Immunol* (2014) 192(5):2143–55. doi: 10.4049/jimmunol.1301721
26. Weltevrede M, Eilers R, de Melker HE, van Baarle D. Cytomegalovirus Persistence and T-cell Immunosenescence in People Aged Fifty and Older: A Systematic Review. *Exp Gerontol* (2016) 77:87–95. doi: 10.1016/j.exger.2016.02.005
27. van der Heiden M, van Zelm MC, Bartol SJW, de Rond LGH, Berbers GAM, Boots AMH, et al. Differential Effects of Cytomegalovirus Carriage on the Immune Phenotype of Middle-Aged Males and Females. *Sci Rep* (2016) 6:26892. doi: 10.1038/srep26892
28. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, et al. Preexisting Influenza-Specific CD4+ T Cells Correlate With Disease Protection Against Influenza Challenge in Humans. *Nat Med* (2012) 18 (2):274–80. doi: 10.1038/nm.2612
29. Kreijtz JH, Fouchier RA, Rimmelzwaan GF. Immune Responses to Influenza Virus Infection. *Virus Res* (2011) 162(1-2):19–30. doi: 10.1016/j.virusres.2011.09.022
30. Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, et al. Cellular Immune Correlates of Protection Against Symptomatic Pandemic Influenza. *Nat Med* (2013) 19(10):1305–12. doi: 10.1038/nm.3350
31. Wang Z, Wan Y, Qiu C, Quinones-Parra S, Zhu Z, Loh L, et al. Recovery From Severe H7N9 Disease is Associated With Diverse Response Mechanisms Dominated by CD8(+) T Cells. *Nat Commun* (2015) 6:6833. doi: 10.1038/ncomms7833
32. Grant EJ, Quinones-Parra SM, Clemens EB, Kedzierska K. Human Influenza Viruses and CD8(+) T Cell Responses. *Curr Opin Virol* (2016) 16:132–42. doi: 10.1016/j.coviro.2016.01.016
33. Koutsakos M, Illing PT, Nguyen THO, Mifsud NA, Crawford JC, Rizzetto S, et al. Human Cd8(+) T Cell Cross-Reactivity Across Influenza a, B and C Viruses. *Nat Immunol* (2019) 20(5):613–25. doi: 10.1038/s41590-019-0320-6
34. Wang Z, Zhu L, Nguyen THO, Wan Y, Sant S, Quinones-Parra SM, et al. Clonally Diverse CD38(+)HLA-DR(+)CD8(+) T Cells Persist During Fatal H7N9 Disease. *Nat Commun* (2018) 9(1):824. doi: 10.1038/s41467-018-03243-7
35. Jansen JM, Gerlach T, Elbahesh H, Rimmelzwaan GF, Saletti G. Influenza Virus-Specific CD4+ and CD8+ T Cell-Mediated Immunity Induced by Infection and Vaccination. *J Clin Virol* (2019) 119:44–52. doi: 10.1016/j.jcv.2019.08.009
36. Khan N, Shariff N, Cobbold M, Bruton R, Ainsworth JA, Sinclair AJ, et al. Cytomegalovirus Seropositivity Drives the CD8 T Cell Repertoire Toward Greater Clonality in Healthy Elderly Individuals. *J Immunol* (2002) 169 (4):1984–92. doi: 10.4049/jimmunol.169.4.1984
37. Khan N, Hislop A, Gudgeon N, Cobbold M, Khanna R, Nayak L, et al. Herpesvirus-Specific CD8 T Cell Immunity in Old Age: Cytomegalovirus Impairs the Response to a Coresident EBV Infection. *J Immunol* (2004) 173 (12):7481–9. doi: 10.4049/jimmunol.173.12.7481
38. van de Berg PJ, van Stijn A, Ten Berge IJ, van Lier RA. A Fingerprint Left by Cytomegalovirus Infection in the Human T Cell Compartment. *J Clin Virol* (2008) 41(3):213–7. doi: 10.1016/j.jcv.2007.10.016
39. Pawelec G, Akbar A, Caruso C, Grubeck-Loebenstein B, Solana R, Wikby A. Human Immunosenescence: Is it Infectious? *Immunol Rev* (2005) 205:257–68. doi: 10.1111/j.0105-2896.2005.00271.x
40. Franceschi C, Campisi J. Chronic Inflammation (Inflammaging) and its Potential Contribution to Age-Associated Diseases. *J Gerontol A Biol Sci Med Sci* (2014) 69(Suppl 1):S4–9. doi: 10.1093/gerona/glu057
41. Jenkins C, Garcia W, Godwin MJ, Spencer JV, Stern JL, Abendroth A, et al. Immunomodulatory Properties of a Viral Homolog of Human interleukin-10 Expressed by Human Cytomegalovirus During the Latent Phase of Infection. *J Virol* (2008) 82(7):3736–50. doi: 10.1128/JVI.02173-07
42. Pawelec G, Goldeck D, Derhovanessian E. Inflammation, Ageing and Chronic Disease. *Curr Opin Immunol* (2014) 29:23–8. doi: 10.1016/j.coi.2014.03.007
43. Trzonkowski P, Myśliwska J, Szmit E, Wickiewicz J, Łukaszuk K, Brydak LB, et al. Association Between Cytomegalovirus Infection, Enhanced Proinflammatory Response and Low Level of Anti-Hemagglutinins During the Anti-Influenza Vaccination—an Impact of Immunosenescence. *Vaccine* (2003) 21(25-26):3826–36. doi: 10.1016/S0264-410X(03)00309-8
44. Prosch SS, Stein K, Liebenthal J, Stamminger C, Volk T, Kurger H. Stimulation of the Human Cytomegalovirus IE Enhancer/Promotor in HL-60 Cells by TNFa is Mediated Via Induction of NF-Kb. *Virology* (1995) 208:197–206. doi: 10.1006/viro.1995.1143
45. McElhaney JE, Ewen C, Zhou X, Kane KP, Xie D, Hager WD, et al. Granzyme B: Correlates With Protection and Enhanced CTL Response to Influenza Vaccination in Older Adults. *Vaccine* (2009) 27(18):2418–25. doi: 10.1016/j.vaccine.2009.01.136
46. McElhaney JE, Xie D, Hager WD, Barry MB, Wang Y, Kleppinger A, et al. T Cell Responses are Better Correlates of Vaccine Protection in the Elderly. *J Immunol* (2006) 176(10):6333–9. doi: 10.4049/jimmunol.176.10.6333
47. McElhaney JE, Zhou X, Talbot HK, Soethout E, Bleackley RC, Granville DJ, et al. The Unmet Need in the Elderly: How Immunosenescence, CMV Infection, Co-Morbidities and Frailty are a Challenge for the Development of More Effective Influenza Vaccines. *Vaccine* (2012) 30(12):2060–7. doi: 10.1016/j.vaccine.2012.01.015
48. Rosendahl Huber SK, Hendriks M, Jacobi RHJ, van de Kassteele J, Mandersloot-Oskam JC, van Boxtel RAJ, et al. Immunogenicity of Influenza Vaccines: Evidence for Differential Effect of Secondary Vaccination on Humoral and Cellular Immunity. *Front Immunol* (2018) 9:3103. doi: 10.3389/fimmu.2018.03103
49. van Beek J, Veenhoven RH, Bruin JP, van Boxtel RAJ, de Lange MMA, Meijer A, et al. Influenza-Like Illness Incidence Is Not Reduced by Influenza Vaccination in a Cohort of Older Adults, Despite Effectively Reducing Laboratory-Confirmed Influenza Virus Infections. *J Infect Dis* (2017) 216 (4):415–24. doi: 10.1093/infdis/jix268
50. Pel JZS. Proefonderzoek Naar De Frequentie En De Aetiologie Van Griepachtige Ziekten in De Winter 1963-1964. *Huisarts en Wetenschap* (1965) 86:321.
51. Tcherniaeva I, den Hartog G, Berbers G, van der Klis F. The Development of a Bead-Based Multiplex Immunoassay for the Detection of IgG Antibodies to CMV and EBV. *J Immunol Methods* (2018) 462:1–8. doi: 10.1016/j.jim.2018.07.003
52. Samson LD, Boots AMH, Ferreira JA, Picavet HSJ, de Rond LGH, de Zeeuw-Brouwer ML, et al. In-depth Immune Cellular Profiling Reveals Sex-Specific Associations With Frailty. *Immun Ageing* (2020) 17:20. doi: 10.1186/s12979-020-00191-z
53. Kotecha N, Krutzik PO, Irish JM. Web-Based Analysis and Publication of Flow Cytometry Experiments. *Curr Protoc Cytom* (2010) Chapter 10:Unit10 17. doi: 10.1002/0471142956.cy1017s53
54. Mamedov IZ, Britanova OV, Zvyagin IV, Turchaninova MA, Bolotin DA, Putintseva EV, et al. Preparing Unbiased T-cell Receptor and Antibody cDNA Libraries for the Deep Next Generation Sequencing Profiling. *Front Immunol* (2013) 4:456. doi: 10.3389/fimmu.2013.00456
55. Gerritsen B, Pandit A, Andeweg AC, de Boer RJ. RTCR: A Pipeline for Complete and Accurate Recovery of T Cell Repertoires From High Throughput Sequencing Data. *Bioinformatics* (2016) 32(20):3098–106. doi: 10.1093/bioinformatics/btw339
56. Derhovanessian E, Maier AB, Hahnel K, Beck R, de Craen AJM, Slagboom EP, et al. Infection With Cytomegalovirus But Not Herpes Simplex Virus Induces

the Accumulation of Late-Differentiated CD4+ and CD8+ T-Cells in Humans. *J Gen Virol* (2011) 92(Pt 12):2746–56. doi: 10.1099/vir.0.036004-0
57. Skowronski DM, Hottes TS, McElhaney JE, Janjua NZ, Sabaiduc S, Chan T, et al. Immuno-Epidemiologic Correlates of Pandemic H1N1 Surveillance Observations: Higher Antibody and Lower Cell-Mediated Immune Responses With Advanced Age. *J Infect Dis* (2011) 203(2):158–67. doi: 10.1093/infdis/jiq039
58. Deng Y, Jing Y, Campbell AE, Gravenstein S. Age-Related Impaired Type 1 T Cell Responses to Influenza: Reduced Activation Ex Vivo, Decreased Expansion in CTL Culture In Vitro, and Blunted Response to Influenza Vaccination In Vivo in the Elderly. *J Immunol* (2004) 172(6):3437–46. doi: 10.4049/jimmunol.172.6.3437
59. Nguyen THO, Sant S, Bird NL, Grant EJ, Clemens EB, Koutsakos M, et al. Perturbed Cd8+ T Cell Immunity Across Universal Influenza Epitopes in the Elderly. *J Leukoc Biol* (2017) 103:321–39. doi: 10.1189/jlb.5MA0517-207R
60. Murasko DMB, Gardner ED, Gross EM, Munk P, Dran G, Abrutyn S. Role of Humoral and Cell-Mediated Immunity in Protection From in¯Uenza Disease After Immunization of Healthy Elderly. *Exp Gerontol* (2002) 37:427–39. doi: 10.1016/S0531-5565(01)00210-8
61. Marandu TF, Oduro JD, Borkner L, Dekhtiarenko I, Uhrlaub JL, Drabig A, et al. Immune Protection Against Virus Challenge in Aging Mice Is Not Affected by Latent Herpesviral Infections. *J Virol* (2015) 89(22):11715–7. doi: 10.1128/JVI.01989-15
62. Derhovanessian E, Maier AB, Hahnel K, McElhaney JE, Slagboom EP, Pawelec G. Latent Infection With Cytomegalovirus is Associated With Poor Memory CD4 Responses to Influenza A Core Proteins in the Elderly. *J Immunol* (2014) 193(7):3624–31. doi: 10.4049/jimmunol.1303361
63. Merani S, Pawelec G, Kuchel GA, McElhaney JE. Impact of Aging and Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection. *Front Immunol* (2017) 8:784. doi: 10.3389/fimmu.2017.00784
64. Theeten H, Mathei C, Peeters K, Ogunjimi B, Goossens H, Ieven M, et al. Cellular Interferon Gamma and Granzyme B Responses to Cytomegalovirus-pp65 and Influenza N1 Are Positively Associated in Elderly. *Viral Immunol* (2016) 29(3):169–75. doi: 10.1089/vim.2015.0071
65. Smithey MJ, Venturi V, Davenport MP, Buntzman AS, Vincent BG, Frelinger JA, et al. Lifelong CMV Infection Improves Immune Defense in Old Mice by Broadening the Mobilized TCR Repertoire Against Third-Party Infection. *Proc Natl Acad Sci U.S.A.* (2018) 115(29):E6817–25. doi: 10.1073/pnas.1719451115
66. Chen G, Yang X, Ko A, Sun X, Gao M, Zhang Y, et al. Sequence and Structural Analyses Reveal Distinct and Highly Diverse Human Cd8+ TCR Repertoires to Immunodominant Viral Antigens. *Cell Rep* (2017) 19(3):569–83. doi: 10.1016/j.celrep.2017.03.072
67. Tavenier J, Margolick JB, Leng SX. T-Cell Immunity Against Cytomegalovirus in HIV Infection and Aging: Relationships With Inflammation, Immune Activation, and Frailty. *Med Microbiol Immunol* (2019) 208(3-4):289–94. doi: 10.1007/s00430-019-00591-z

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Memory CD8 T Cells Generated by Cytomegalovirus Vaccine Vector Expressing NKG2D Ligand Have Effector-Like Phenotype and Distinct Functional Features

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Viral vectors have emerged as a promising alternative to classical vaccines due to their great potential for induction of a potent cellular and humoral immunity. Cytomegalovirus (CMV) is an attractive vaccine vector due to its large genome with many non-essential immunoregulatory genes that can be easily manipulated to modify the immune response. CMV generates a strong antigen-specific CD8 T cell response with a gradual accumulation of these cells in the process called memory inflation. In our previous work, we have constructed a mouse CMV vector expressing NKG2D ligand RAE-1γ in place of its viral inhibitor m152 (RAE-1γMCMV), which proved to be highly attenuated *in vivo*. Despite attenuation, RAE-1γMCMV induced a substantially stronger CD8 T cell response to vectored antigen than the control vector and provided superior protection against bacterial and tumor challenge. In the present study, we confirmed the enhanced protective capacity of RAE-1γMCMV as a tumor vaccine vector and determined the phenotypical and functional characteristics of memory CD8 T cells induced by the RAE-1γ expressing MCMV. RNAseq data revealed higher transcription of numerous genes associated with effector-like CD8 T cell phenotype in RAE-1γMCMV immunized mice. CD8 T cells primed with RAE-1γMCMV were enriched in TCF1 negative population, with higher expression of KLRG1 and lower expression of CD127, CD27, and Eomes. These phenotypical differences were associated with distinct functional features as cells primed with RAE-1γMCMV showed inferior cytokine-producing abilities but comparable cytotoxic potential. After adoptive transfer into naive hosts, OT-1 cells induced with both RAE-1γMCMV and the control vector were equally efficient in rejecting established tumors, suggesting the context of latent infection and cell numbers as important determinants of enhanced anti-tumor response following RAE-1γMCMV vaccination. Overall, our results

shed new light on the phenotypical and functional distinctness of memory CD8 T cells induced with CMV vector expressing cellular ligand for the NKG2D receptor.

Keywords: memory T cells, CD8 T lymphocytes, cytomegalovirus, vaccine vector, tumor vaccine, Klrg1, TCF1

INTRODUCTION

In recent decades, various attempts have been made to harness the body's immune system in the fight against neoplastic cells by modulating various stages of the "cancer-immunity cycle" (1). Live replicating viral vectors, genetically engineered to express tumor epitopes, have a great potential in inducing potent and long-lasting cellular immunity against malignant cells. In this respect, cytomegalovirus (CMV) represents a particularly attractive viral vector candidate due to its life-long persistence and strong capacity to induce antigen-specific CD8 T cells, which gradually accumulate in the host (2). Moreover, CMV possesses many immunomodulatory genes that can be manipulated to modulate the immune response against viral or vectored epitopes (3).

RAE-1γ is a ligand of NKG2D, an activating receptor expressed on various immune cells, including NK and CD8 T cells (4). In our previous research, we have constructed a mouse CMV vector expressing RAE-1γ in place of its viral inhibitor m152 (RAE-1γMCMV). This vector proved to be highly attenuated *in vivo* in both BALB/c and C57BL/6J mice (5). Furthermore, co-expression of foreign CD8 T-cell epitope with RAE-1γ in CMV vector induced an augmented CD8 T cell response (6). When tested in the murine melanoma model, the MCMV vector expressing RAE-1γ and tumor antigen proved to be superior in delaying melanoma growth compared to the control vector (7). However, the mechanisms conferring this increased protection remained unclear.

The memory population of CD8 T cells consists of three major subsets: central memory (Tcm), effector memory cells (Tem), and tissue-resident memory cells (Trm). Tcm express CD62L and CCR7, transcription factors Eomes and TCF1 and are thought to have enhanced proliferative capabilities with low cytotoxic potential. On the other hand, Tem lack CD62L and CCR7 expression, express T-bet and Blimp 1 transcription factors, and are associated with lower proliferative capabilities but are considered to be highly cytotoxic (8–10). Further work identified subpopulation in the Tem compartment of effector-like cells expressing KLRG1 and conferring greater protective capabilities in certain models of infection (11, 12). However, this division based on a handful of molecules has come under intense scrutiny, as the advances of single-cell sequencing technologies and mass cytometry showed memory CD8 T cells to be more heterogeneous than previously thought (13).

This study confirmed the superiority of MCMV vector expressing RAE-1γ in conferring protection against subcutaneous tumor challenge in both prophylactic and therapeutic settings. RAE-1γMCMV induced substantially higher numbers of epitope-specific memory CD8 T cells, which had a highly differentiated, effector-like transcriptional profile. The majority of these cells lacked the expression of the TCF1 transcription factor, produced lower amounts of cytokines but exhibited similar cytotoxic capabilities compared to OT-1 cells primed with the control vector. Overall, our study revealed that the insertion of RAE-1γ into the CMV vector leads to gross differences in transcriptomic, phenotypical, and functional profiles of memory CD8 T cells.

METHODS

Mice, Viruses, Tumor Cell Lines and *In Vivo* Depletion

C57BL/6J, OT-1 (3831), $CD4^{cre}$ and $Klrk1^{fl/fl}$ mice were housed and bred under specific pathogen-free conditions at the Central Animal Facility, Faculty of Medicine, University of Rijeka, under the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. $CD4^{cre}$ mice were kindly provided by D. Littman. $Klrk1^{fl/fl}$ mice were generated as described previously (14). Adult female mice (6-12 weeks old) were strictly age-matched for use in experiments. The Ethics Committee at the Faculty of Medicine, Rijeka and Ethics Committee of the Veterinary Department of the Ministry of Agriculture, Croatia approved all experiments.

MCMV-SIINFEKL and RAE-1γMCMV-SIINFEKL were constructed as described previously (6, 15). Virus stocks were prepared as previously described (16). Mice were immunized with 2×10^5 PFU in the final volume of 50 µL DMEM *via* footpad (f.p.) route of injection.

E.G7-OVA cell line was kindly provided by V. Sexl (Vetmeduni, Vienna). Cells were cultured in RPMI 1640 (10% FCS) supplemented with G418 (Geneticin, Invivogen). B16-OVA cell line was kindly provided by T. Sparwasser (TWINCORE, Hannover) and cells were cultured in DMEM (10% FCS) also supplemented with G418. 10^6 E.G7-OVA and 10^5 B16-OVA cells were inoculated subcutaneously into an animal's right flank in 100 µL PBS. Tumor growth was measured using digital caliper two-three times a week, and mice were sacrificed when tumors reached approximately 1000^3 mm for ethical reasons.

In vivo CD8 T cell depletion was performed by i.p. injection of 150 µg of anti-CD8 antibody (YTS 169.4). Antibodies were administered once a week, over a period of 8 weeks.

Flow Cytometry

Flow cytometry was performed according to the *Guidelines for the use of flow cytometry and cell sorting in immunological studies* (17). Splenocytes were isolated using a standard protocol. Briefly, mice were sacrificed, spleens harvested, and homogenized,

followed by erythrocyte lysis. Blood samples were collected from a saphenous vein, followed by erythrocyte lysis. After leukocyte isolation, Fc receptors were blocked using a 2.4G2 antibody. For surface staining following antibodies were used: CD8α PerCP-Cy5.5 or SB780 (clone: 53-6.7; 1:400 or 1:200), CD45.1 e450 (clone: A20; 1:400), KLRG1 PE-e610 (clone:2F1, 1:100), CD127 APC (clone: SB/199, 1:100), CD44 A700 (clone: IM7, 1:100), PD1 PerCP-e710 (clone: RMP1-30; 1:200), TIM3 Pe-Cy7 (clone: RMT3-23; 1:100), CD27 PE-Cy7 (clone: LG.7F9; 1:200) and CD107a eF660 (clone: 1D4B; 1:400). Fixable Viability Dye (eBioscience) was used to exclude dead cells. For intracellular staining Intracellular fixation and permeabilization buffer set (eBioscience) was used along with following intracellular antibodies: IFNγ FITC (clone: XMG1.2; 1:100), TNFα PE-e610 (clone: MP6-XT22; 1:100), IL-2 PE-Cy7 (clone: JES6-5H4; 1:100), Granzyme B PE (clone: NGZB; 1:100). For transcription factor staining eBioscience Foxp3/Transcription Staining Buffer Set was used with: TCF1 A488 (clone:C63D9; 1:400), TOX PE (clone: TXRX10; 1:100), T-bet PerCP-Cy5.5 (clone:4B10; 1:400) and EOMES PE (clone:Dan11mag; 1:100). Antibody against TCF1 was produced by Cell Signaling Technology, and Invitrogen produced all other antibodies. Flow cytometry was performed on FACS Aria II, and data were analyzed using FlowJo v10 (Tree Star) software.

Biotinylated pMHC-I multimers were conjugated with streptavidin-PE, and splenocytes were stained as described previously (18).

In Vitro Stimulation and Killer Assay

Mice harboring memory OT-1 cells, primed with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL, were sacrificed and splenocytes isolated using a standard protocol. $2x10^6$ cells were then incubated for 6h with different concentrations of SIINFEKL peptide (JPT PeptideTechnologies GmbH) in RPMI 1640 (PAN-Biotech) supplemented with 10%FCS (PAN-Biotech), Brefeldin A (Invitrogen), Monensin (Invitrogen), and CD107 (Invitrogen) at 37°C.

For the *in vitro* killer assay, mice harboring memory OT-1 cells (CD45.1) were sacrificed and splenocytes isolated using a standard protocol. Splenocytes were pooled from 4-5 mice/group. CD8 T cells were purified by negative selection using magnetic beads (Miltenyi Biotec), and OT-1 (CD45.1) cells were stained with CD45.1 antibody and sorted using FACSAria II (BD) using high-speed sorting into RPMI supplemented with 20% FCS. Sorted OT-1 (CD45.1) cells were co-incubated with E.G7-OVA (CD45.2) cells for 4h in 2:1, 1:1, and 0.5:1 effector to target ratios at 37℃. After co-incubations, target cells were identified as CD45.1 negative, and viability was determined using Fixable Viability Dye (eBioscience). OT-1 cytotoxicity was calculated using following formula: [(% $FVD^+CD45.1^-$ cell-specific lysis – % $FVD^+CD45.1^-$ cell spontaneous lysis)/(100 – % $FVD^+CD45.1^-$ cell spontaneous lysis)] × 100 as described in (19).

Adoptive Transfer Experiments

Naïve OT-1 cells were purified by negative selection using magnetic beads (Miltenyi Biotec). 10^4 OT-1 (CD45.1) cells were adoptively transferred into naïve C57BL6/J (CD45.2) animals in 500 uL DMEM i.v. For adoptive transfer experiments, mice harboring memory OT-1 cells primed with viral vectors were sacrificed, splenocytes were isolated using a standard protocol, and CD8 T cells were purified using magnetic beads (Miltenyi Biotec). Following magnetic separation, OT-1 (CD45.1) cells were stained with CD45.1 (Invitrogen) antibody and sorted using FACSAria II (BD) using high-speed sorting into RPMI supplemented with 20% FCS. The purity and viability of sorted cells were checked immediately after sorting with PI staining and in all of the experiments exceeded 97%. Cells from 5-10 mice from each group were pooled, and $3x10^4$ cells were transferred in 500 uL DMEM i.v. into mice harboring tumors.

RNAseq Sample Preparation and Sequencing

Splenocytes were isolated from mice immunized with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL at day 36 post-immunization. CD8 T cells were purified using magnetic beads (Miltenyi Biotec). After magnetic beads separation, OT-1 (CD45.1) cells were stained with CD45.1 antibody (Invitrogen), high-speed sorted on FACSAria II (BD) directly into the RLT lysis buffer (QIAGEN), and their total RNA isolated using RNeasy Micro Kit (QIAGEN), according to manufacturers' protocol. Agilent Bioanalyzer 2100 and Agilent RNA 6000 Nano Kit were used to estimate sample quality and determine the quantity of isolated RNA. Before library generation, RNA was subjected to DNAse I digestion (Thermo Fisher Scientific) followed by RNeasy MinElute column clean up (Qiagen). RNAseq libraries were generated using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech Laboratories) following the manufacturer's recommendations. From cDNA, final libraries were generated utilizing the Nextera XT DNA Library Preparation Kit (Illumina). Concentrations of the final libraries were measured with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific), and fragment lengths distribution was analyzed with the DNA High Sensitivity Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). All samples were normalized to 2nM and pooled at equimolar concentrations. The library pool was sequenced on the NextSeq500 (Illumina) in a single 1x84bp run, producing 22.5 M to 29.7 M reads per sample from a total of six mRNAseq libraries. Adapter sequences were hard-clipped from raw sequencing reads as part of the bcl2fastq pipeline (version 2.20.0.422). The overall quality of the trimmed sequences was assessed by FastQC v0.11.9. Where applicable, quality data from individual analyses were aggregated using MultiQC v1.9.

RNAseq Data Processing and Analysis

RNASeq data processing and analysis were performed as described previously (20), with minor modifications specific to this study's experimental system. Briefly, following quality control using FastQC v0.11.9, sequencing libraries were searched for potentially contaminating sequences against an in-house database of common contaminants using FastQ Screen v0.14.0 (21) and Bowtie 2 v2.3.5.1 (22). Quality-checked

sequencing reads were then mapped to the mouse GRCm38.p6 (release M25) primary reference genome assembly (23) with STAR v2.7.6a (24–26), alignment files indexed using samtools v1.11 (27), and reads mapping to individual genes counted using featureCounts (28). Obtained uniquely mapped read counts were used for differential expression analysis, which was performed with the DESeq2 package (29), applying $p_{adj} < 0.05$ as a cutoff for statistical significance. Gene ontology overrepresentation analysis was performed using the clusterProfiler package v3.14.1 (30), and heatmaps were generated using R package pheatmap v.1.0.12 (https://CRAN.R-project.org/package=pheatmap).

Statistical Analysis

Unpaired t-test, ANOVA (followed by LSD post test), and log-rank Mantel-Coy test were performed using Prism software (GraphPad Software Inc., La Jolla, CA). P<0.05 was considered statistically significant.

RESULTS

RAE-1γMCMV Confers Robust Long-Term Protection Against Subcutaneous Tumor Challenges

To expand on our previous work (6) and investigate the robustness of protection conferred to mice immunized with RAE-1γMCMV against tumor challenge, we utilized viral vectors expressing H2Kb restricted SIINFEKL epitope (6, 15). C57BL6/J mice were immunized with MCMV-SIINFEKL, RAE-1γMCMV-SIINFEKL, or left unimmunized. Two months following the immunization, mice were challenged with E.G7-OVA lymphoma expressing SIINFEKL epitope, and tumor appearance and growth were followed over time (**Figure 1A**). Unimmunized mice rapidly developed large tumors while MCMV-SIINFEKL immunized mice developed smaller tumors that subsequently retracted (**Figure 1B**). Conversely, all of RAE-1γMCMV-SIINFEKL immunized mice failed to develop observable tumors during the initial three weeks after tumor inoculation, and the overall percentage of mice that developed tumors was much smaller than MCMV-SIINFEKL immunized animals (**Figure 1C**). Furthermore, immunization with either of these two vectors substantially increased the overall survival of mice after the tumor challenge. Although the survival rate was higher in mice immunized with RAE-1γMCMV-SIINFEKL than in MCMV-SIINFEKL immunized mice (80% vs. 64% survival), the difference was not statistically significant (**Figure 1D**). Next, two months after primary challenge, survivors of E.G7-OVA tumor were inoculated with a different, more malignant neoplasm expressing the same SIINFEKL epitope, B16-OVA melanoma (**Figure 1E**). Again, RAE-1γMCMV-SIINFEKL immunization led to an increase in the survival rate as 91% of mice survived the challenge in this group compared to 75% in the MCMV-SIINFEKL immunized group (**Figure 1F**). To confirm that the protection against secondary tumor challenge is mediated *via* CD8 T cells, half of the mice in each group were depleted of this lymphocyte population starting one day prior to the B16-OVA challenge. Mice lacking cytotoxic T cells quickly succumbed to tumor challenge, proving that this population mediates the protection.

We went on to investigate the therapeutic potential of the RAE-1γMCMV-SIINFEKL vector in the E.G7-OVA challenge. Mice were inoculated with E.G7-OVA subcutaneously and 6 days later immunized with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL, whereas one group of mice was left unimmunized (**Figure 1G**). RAE-1γMCMV-SIINFEKL vaccination was superior to MCMV-SIINFEKL vaccination in tumor size reduction (**Figure 1H**) and substantially increased the survival of mice inoculated with E.G7-OVA lymphoma cells (**Figure 1I**). Overall, the MCMV vector expressing RAE-1γ in place of its viral inhibitor proved more efficient than the wild-type MCMV vector in protection against subcutaneous tumor challenge in both prophylactic and therapeutic settings.

Memory CD8 T Cells Primed With RAE-1γMCMV Exhibit a Distinct Transcriptional Profile

We have previously shown that RAE1γMCMV-SIINFEKL has enhanced priming capacity compared to the wild-type MCMV vector (6, 7). However, differences in phenotype and function of memory populations of SIINFEKL specific CD8 T cells induced with these viral vectors remained poorly characterized. To screen for distinct characteristics in memory populations induced with indicated vectors, we compared their transcriptional profiles at the memory timepoint. 10^4 congenic OT-1 T cells (expressing H-2Kb-SIINFEKL restricted TCR) were transferred to naïve animals that were subsequently vaccinated with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL (**Figure 2A**). The transcriptional profile of OT-1 cells was determined on day 36 post-vaccination. Overall, 249 genes were differentially expressed between OT-1 cells primed with MCMV-SIINFEKL and RAE-1γMCMV-SIINFEKL (**Table S1**). Gene ontology overrepresentation analysis revealed that differentially expressed genes between these two groups participate in biological processes related to proliferation, cellular division, and cellular activation involved in immune response (**Figure 2B**). Interestingly, both *Sell* (CD62L) and *Ccr7*, coding for prototypical markers of central memory phenotype (31), as well as the antiapoptotic molecule *Bcl2*, were more strongly transcribed in the MCMV-SIINFEKL immunized group. Simultaneously, RAE-1γMCMV-SIINFEKL primed cells showed higher expression of transcripts associated with an effector-like phenotype, such as *Cx3Cr1* (32), *Gzma, Gzmb, Adam8, Cd244a, Lgals3*, and *Lgals1* (33, 34) (**Figure 2C**). These differences prompted us to analyze other genes associated with central memory and effector-like phenotype below the chosen significance threshold. In the MCMV-SIINFEKL group, we observed a clear trend in the expression of other transcripts associated with Tcm such as *Tcf7* (35), *Id3, Eomes, Il7r* (36), and *Cd27* (12) which were also elevated. At the same time, effector marker *Klrg1* (36) was more highly

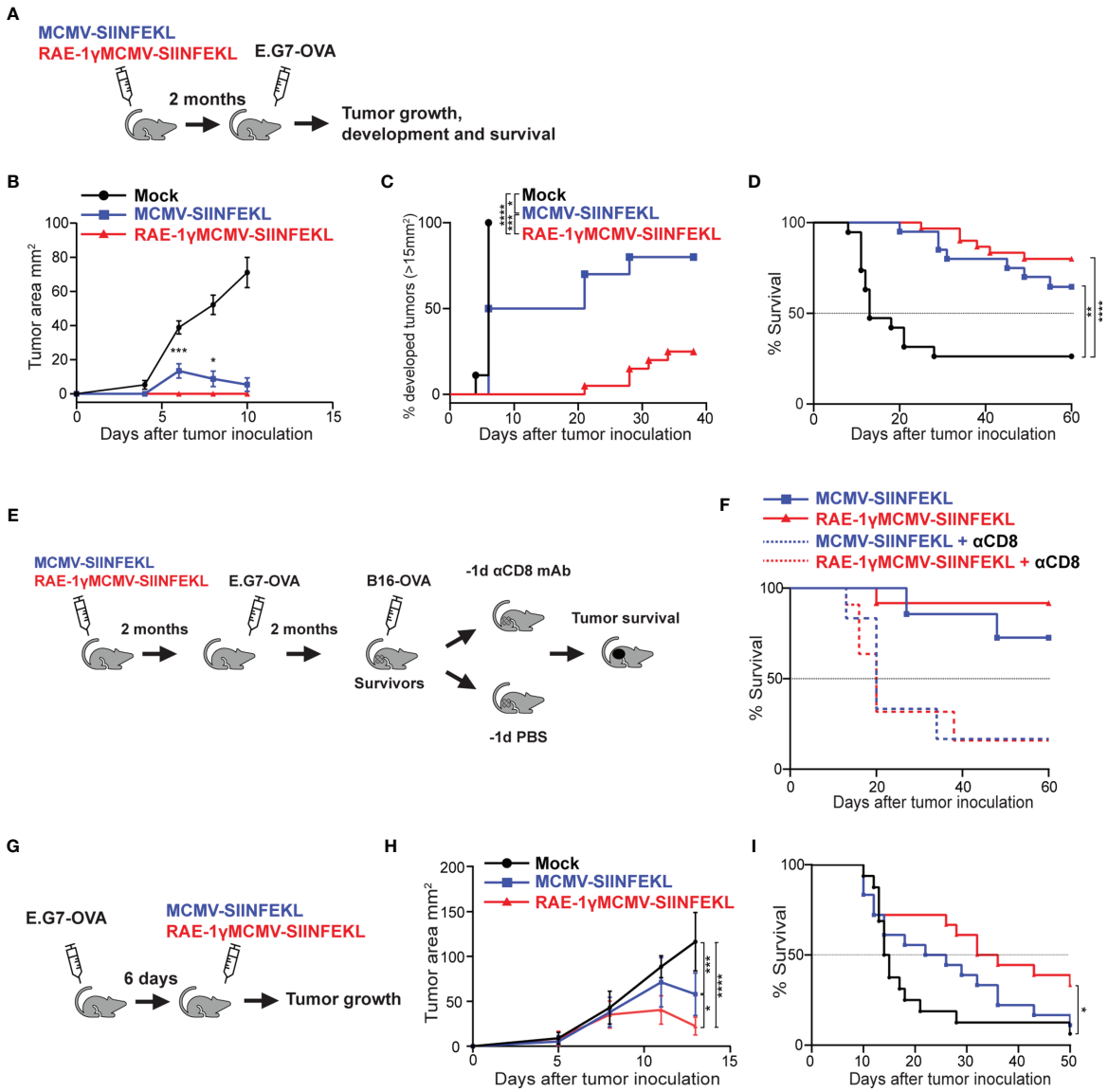

FIGURE 1 | RAE-1γMCMV-SIINFEKL confers robust long-term protection against subcutaneous tumor challenges. **(A)** Mice were immunized with either MCMV-SIINFEKL, RAE-1γMCMV-SIINFEKL, or left unimmunized (n=10-20). 2 months after immunization, mice were inoculated with E.G7-OVA tumor subcutaneously. **(B)** Tumor growth was followed over time. **(C)** Percent of mice that developed tumors (>15 mm^2). **(D)** Overall survival after E.G7-OVA challenge. **(E)** Mice that survived the initial E.G7-OVA challenge were further challenged with B16-OVA subcutaneously. Half of the survivors were depleted for CD8 T cells prior to the secondary challenge. **(F)** Overall survival of mice after secondary challenge. **(G)** Mice were inoculated with E.G7-OVA subcutaneously. 6 days later, mice were immunized with indicated vectors or left unimmunized (n=10). **(H)** Tumor growth and **(I)** overall survival were followed over time. Prophylactic data are from a single experiment representative of two independent experiments **(B, C)**. Survival data are pooled from two independent experiments **(D, F)**. Therapeutic data are from a single experiment representative of two independent experiments **(H)**, and survival data are pooled from two independent experiments **(I)**. Data are represented as mean ± SEM **(B, H)** and statistical significance is expressed as *p < 0.05, **p < 0.01, ***p < 0,001, ****p<0,0001. Statistical significance was tested using one-way ANOVA followed by LSD post test **(B, H)** or log-rank Mantel-Cox test for Kaplan-Maier curves **(C, D, F, I)**.

transcribed in RAE-1γMCMV-SIINFEKL immunized mice (**Figure 2D**). Furthermore, many genes linked with progression through the cell cycle were more highly transcribed in cells primed with RAE-1γMCMV-SIINFEKL, suggesting their higher proliferation rate (**Figure 2E**). Together, RNAseq data strongly associated OT-1 cells primed with RAE-1γMCMV-SIINFEKL and MCMV-SIINFEKL with effector-like and central memory phenotype, respectively.

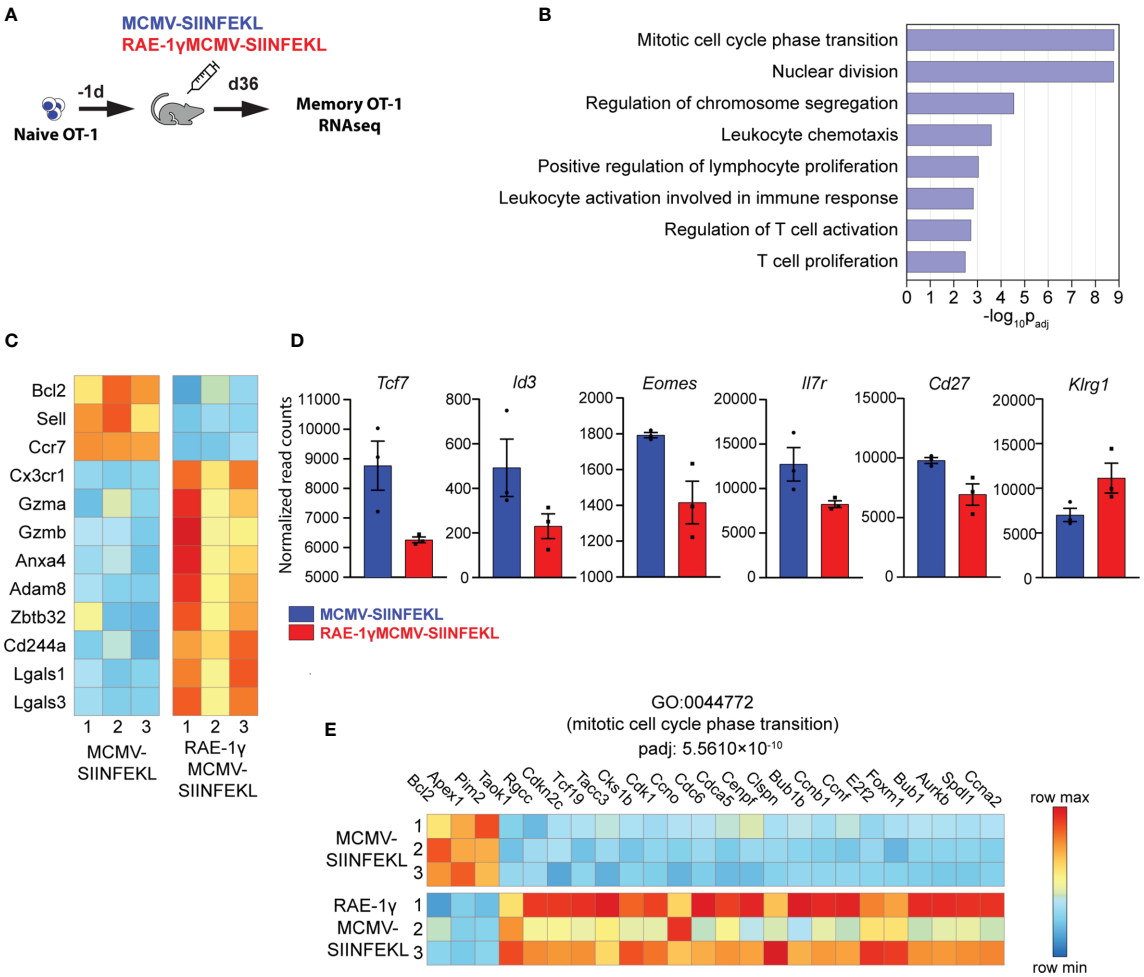

FIGURE 2 | Memory CD8 T cells primed with RAE-1γMCMV exhibit a distinct transcriptional profile. **(A)** 10^4 naive OT-1 cells were transferred to C57BL/6J mice one day prior to immunization with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL (n=3). **(B)** Gene ontology overrepresentation analysis (GO-ORA) of identified differentially expressed genes. Bar chart showing selected, statistically significant GO terms in the Biological process category, ranked by adjusted p-values. **(C)** Heat map showing the expression of indicated genes. **(D)** Transcription of indicated genes below chosen significance threshold. **(E)** Heat map showing the expression of genes belonging to the GO term "Mitotic cell cycle phase transition". Data are from a single experiment. Genes and GO terms with padj<0.05 were considered statistically significant.

RAE-1γMCMV-SIINFEKL Has Superior Priming Capacity and Generates Phenotypically Distinct Memory CD8 T Cells

We validated gene expression data and analyzed the kinetics and phenotype of OT-1 cells primed with viral vectors using flow cytometry. To that aim, 10^4 congenic OT-1 T cells were transferred to naïve animals that were subsequently vaccinated with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL (**Figure 3A**). The frequency of OT-1 T cells was followed over time. The initial expansion of OT-1 cells was substantially increased in RAE-1γMCMV-SIINFEKL immunized mice compared to MCMV-SIINFEKL immunized animals, and OT-1 T cells were maintained at significantly higher levels in the RAE1γMCMV-SIINFEKL group compared to MCMV-SIINFEKL group (**Figures 3B, C**). Importantly, OT-1 cells in RAE1γMCMV-SIINFEKL showed signs of memory inflation, as the frequency of these cells increased in later time points. Confirming our RNAseq data, we observed major phenotypical differences between OT-1 cells primed with different vectors. Notably, the frequency of cells expressing the TCF1 transcription factor was significantly lower in the RAE1γMCMV-SIINFEKL immunized group than in the MCMV-SIINFEKL immunized mice (**Figure 3D**, left). RAE-1γMCMV-SIINFEKL primed OT-1 cells showed a higher frequency of $KLRG1^+$ cells in accordance with RNAseq data (**Figure 3D**, right). Interestingly, these differences were not present at initial time points during acute CD8 T cell response but manifested themselves at memory time points, around day 30. A detailed phenotypical analysis of OT-1 cells at day 37 corroborated our RNAseq findings, as RAE-

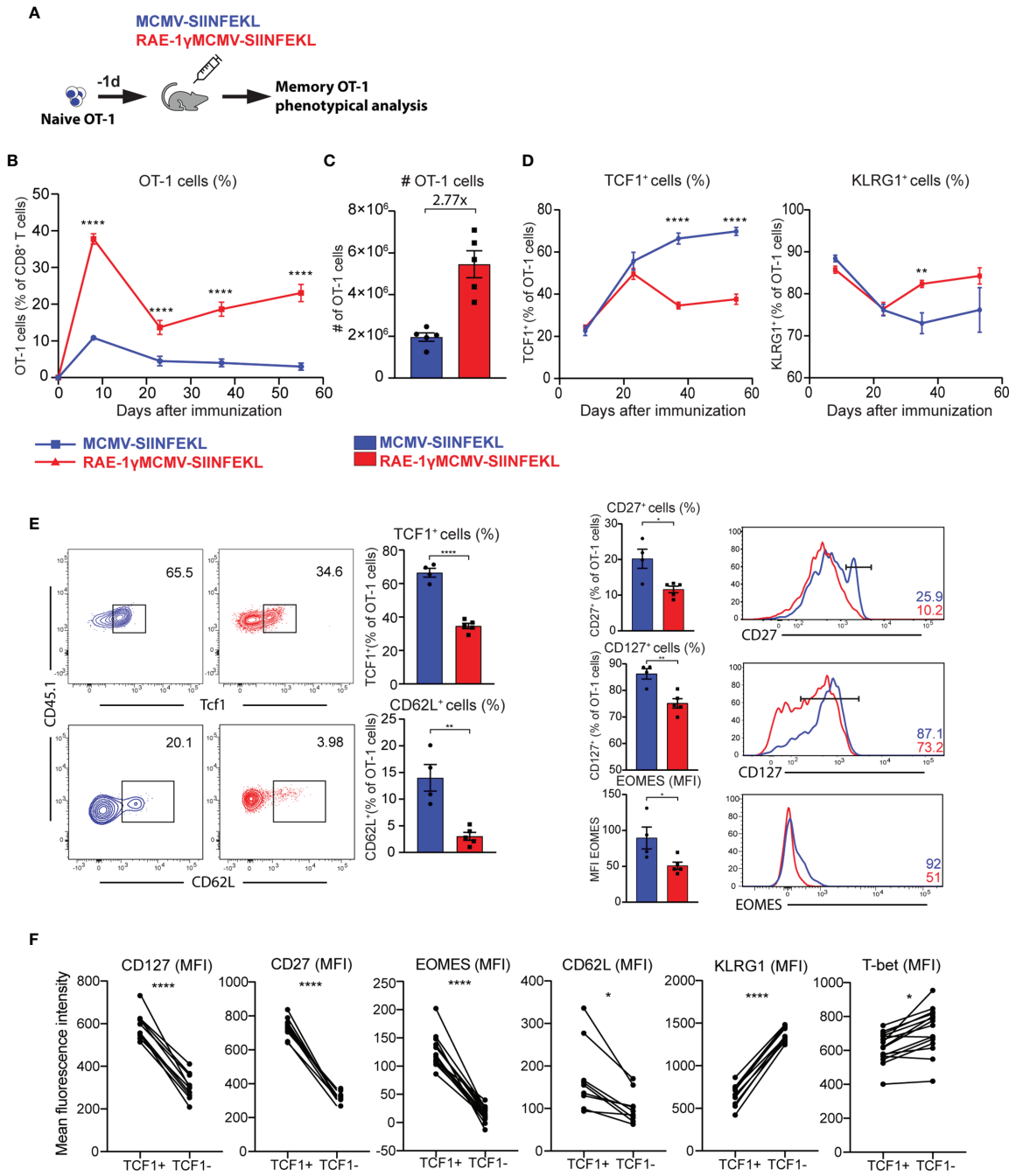

FIGURE 3 | RAE-1γMCMV-SIINFEKL has superior priming capacity and generates phenotypically distinct memory CD8 T cells. **(A)** 10^4 naive OT-1 cells were transferred to C57BL/6J mice one day prior to immunization with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL (n=4-5). **(B)** OT-1 frequency (blood) at indicated time points post-immunization. **(C)** Absolute numbers of OT-1 cells at day 7 post-immunization in the spleen. **(D)** Kinetics of $TCF1^+$ and $KLRG1^+$ populations were followed in blood over time. **(E)** The phenotype of memory OT-1 cells primed with indicated viruses at day 37 post-immunization shown as the percentage of $TCF1^+$, $CD62L^+$, $CD27^+$, $CD127^+$ OT-1 cells, and MFI of Eomes on OT-1 cells. **(F)** Expression of indicated molecules on $TCF1^+$ and $TCF1^-$ cells. Data are from a single experiment representative of two independent experiments. Data are represented as mean ± SEM and statistical significance as *p < 0.05, **p < 0.01, ****p<0,0001. Statistical significance was determined using unpaired Student t-test.

1γMCMV-SIINFEKL primed OT-1 cells expressed lower levels of CD62L, CD127, and CD27 as well as TCF1 and Eomes transcription factors (**Figure 3E**). To confirm that TCF1 expression successfully delineates two distinct populations with different phenotypical features, we compared the expression of several molecules on $TCF1^+$ and $TCF1^-$ cells. CD127, CD27, Eomes, and CD62L expression were all significantly elevated in $TCF1^+$ cells, while KLRG1 and Tbet showed a significant increase in $TCF1^-$ population (**Figure 3F**). These data convincingly demonstrate that RAE-1γMCMV-SIINFEKL induces memory CD8 T cells with distinct, effector-like phenotypical features that grossly differ from memory CD8 T cells primed with the vector lacking RAE-1γ expression.

CD8 T Cells Primed With RAE-1γMCMV Show Lower Cytokine-Producing Capabilities Than Cells Primed With the Virus Lacking RAE-1γ but Similar Cytotoxic Potential

Distinct phenotypical features are associated with different functionality of memory CD8 T cells (31, 36). To compare the cytokine-producing capabilities of cells primed with RAE1γMCMV-SIINFEKL or MCMV-SIINFEKL on a per cell basis, OT-1 cells were transferred to naïve animals that were immunized with indicated viruses the following day. On day 50, mice were sacrificed, splenocytes were isolated and stimulated with different SIINFEKL peptide concentrations (**Figure 4A**). OT-1 cells primed with the virus lacking RAE-1γ showed higher cytokine-producing capabilities as measured by frequency of IFNγ, TNFα and IL-2 positive cells (**Figures 4B, C**). Furthermore, MCMV-SIINFEKL induced memory OT-1 cells also showed enhanced degranulation ability as measured by mobilization of LAMP-1 (CD107a) molecule (**Figure 4D**). Despite the difference in CD107a mobilization, RAE-1γMCMV-SIINFEKL primed OT-1 cells showed equal or higher Granzyme B content (**Figure 4E**), suggesting the equal or higher cytotoxic potential of these cells when compared to OT-1 cells primed with wild-type virus. We sorted OT-1 cells from immunized mice at memory time point to compare the cytotoxicity of cells primed with these two vectors and incubated them in different ratios with E.G7-OVA cells. OT-1 cells primed with RAE-1γMCMV-SIINFEKL showed enhanced but not significantly different cytotoxic potential against tumor cells (**Figure 4F**), confirming previous studies that revealed that degranulation and cytotoxic potential do not necessarily correlate (37). $KLRG1^+$ population was enriched in OT-1 cells primed with RAE-1γ expressing vector, and we wondered whether there was any difference in the functional capacity of this population compared to cells lacking KLRG1 expression. $KLRG1^+$ cells had a lower frequency of IFNγ and IL-2 producing cells, showed lower degranulation capacity, but simultaneously had higher per cell content of cytotoxic Granzyme B molecule (**Figure 4G**). Therefore, RAE-1γMCMV-SIINFEKL primed OT-1 cells at the memory time point showed lower cytokine-producing capabilities and a lower percentage of cells mobilizing CD107a during *in vitro* stimulation. On the other hand, these cells had higher per cell Granzyme B content and similar or slightly elevated cytotoxic potential against tumor cells expressing their cognate antigen.

Adoptively Transferred CD8 T Cells Primed With RAE-1γMCMV Vector Show Protective Potential Similar to Cells Primed With the MCMV Lacking RAE-1γ

Prophylactic and therapeutic vaccination protocol established that immunization with RAE-1γMCMV-SIINFEKL confers greater protection against tumor challenge than immunization with a vector lacking RAE-1γ. To assess the protective capabilities of OT-1 cells generated with indicated vectors when an equal number of cells are transferred into naïve hosts, we performed an adoptive transfer experiment. OT-1 (CD45.1) cells were primed with indicated viruses, and at a memory time point, equal numbers of OT-1 cells were sorted and transferred into mice that were inoculated with E.G7-OVA tumors 5 days prior to transfer (**Figure 5A**). Both populations of transferred OT-1 cells were successful in tumor control (**Figure 5B**), and the survival rate was similar in both groups, 65% and 68% for MCMV-SIINFEKL and RAE-1γMCMV-SIINFEKL primed OT-1 cells, respectively (**Figure 5C**). Several studies associated $TCF1^+$ memory cells with increased proliferative capabilities (35, 38, 39), and accordingly, the initial response was somewhat augmented in OT-1 cells primed with the wild-type MCMV vector. However, by day 15 post-transfer, the difference was lost (**Figure 5D**). In line with previous findings (40), OT-1 cells in both groups became $TCF1^{low}$ during recall response, but RAE-1γMCMV primed cells still maintained elevated expression of KLRG1 (**Figure 5E**). We also analyzed the expression of CD8 T cell exhaustion markers PD-1 and Tim-3 on OT-1 cells, as well as Tox, a transcription factor crucially associated with T cell exhaustion (41). We found no difference in the expression of these molecules, suggesting the absence of CD8 T cell exhaustion in both groups (**Figure 5F**). Overall, OT-1 cells primed with both vectors showed comparable protective capabilities against subcutaneous tumor challenge after adoptive transfer into naive hosts.

DISCUSSION

In our previous work, we have demonstrated the potential of MCMV expressing NKG2D ligand RAE-1γ as a viral vaccine vector in the generation of CD8 T cell response to an inserted foreign epitope in the context of bacterial (6) and tumor challenge (7). We expanded our findings regarding the robust, long-term protection conferred by RAE-1γMCMV immunization against tumor challenge. First, we confirmed that this vector can induce substantially higher numbers of CD8 T cells specific for inserted foreign epitope than the vector lacking NKG2D ligand expression. Next, the transcriptomic analysis revealed ~250 differentially expressed genes between OT-1 cells derived from mice immunized with RAE-1γMCMV and WT MCMV vectors and uncovered

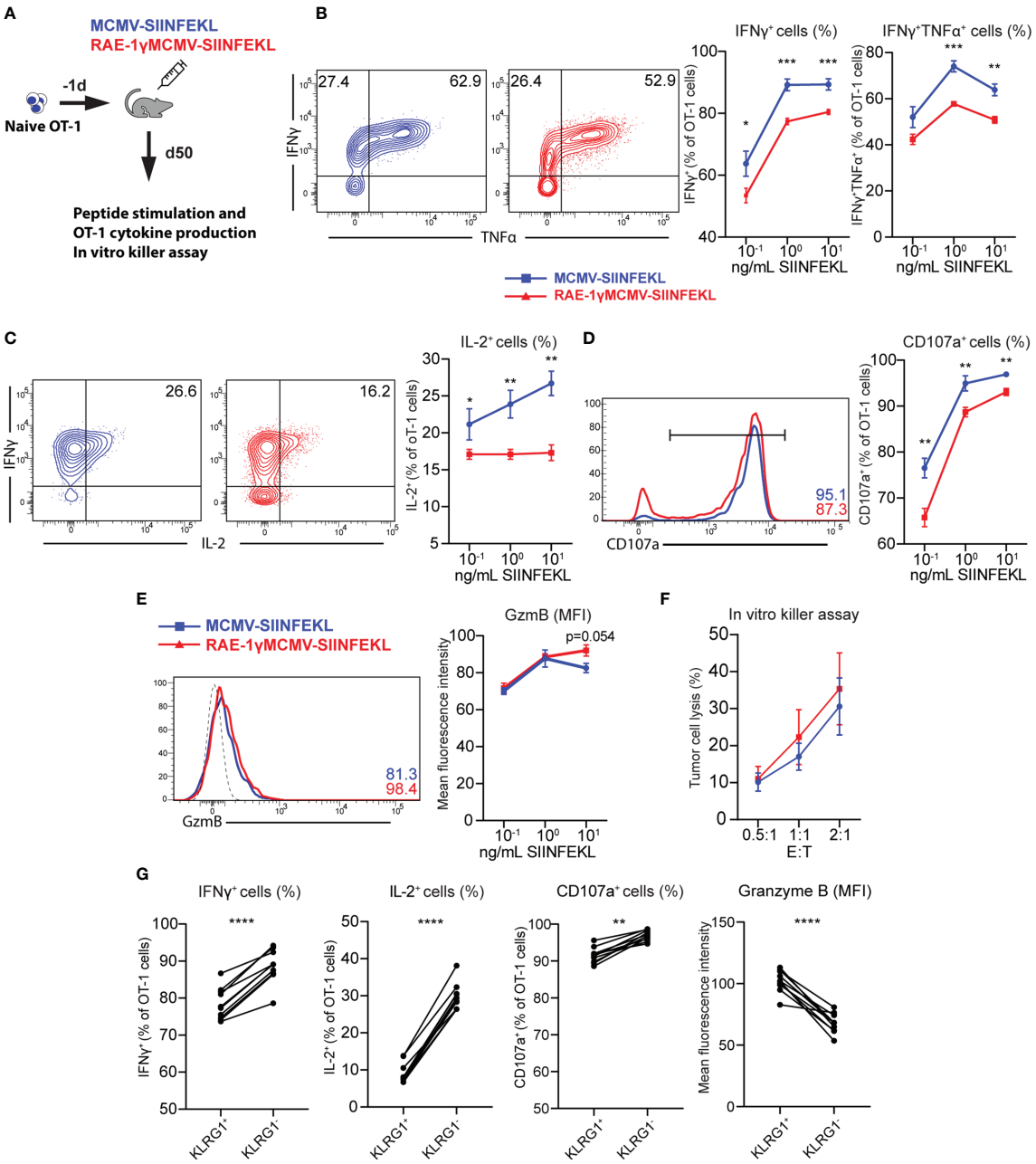

FIGURE 4 | CD8 T cells primed with RAE-1γMCMV show lower cytokine-producing capabilities than cells primed with the virus lacking RAE-1γ but similar cytotoxic potential. **(A)** Naive OT-1 (CD45.1) cells were transferred to CD57BL/6J mice (CD45.2). One day after the transfer, mice were immunized with indicated viruses (n=5-6). On day 50, mice were sacrificed, and splenocytes were stimulated with different concentrations of SIINFEKL peptide. Production of IFN-γ and TNF-α **(B)**, IL-2 **(C)**, CD107a **(D)**, Granzyme B **(E)** was analyzed using flow cytometry. **(F)** Memory time point OT-1 cells were incubated with E.G7-OVA cells in indicated effector:target ratios. **(G)** Percentage or MFI of indicated molecules in KLRG1$^+$ and KLRG1$^-$ cells. Data are from a single experiment representative of two independent experiments. Data is represented as mean ± SEM and statistical significance *p < 0.05, **p < 0.01, ***p < 0,001, ****p<0,0001. Statistical significance was determined using unpaired Student t-test.

that genes associated with effector-like phenotype and cellular proliferation are more strongly expressed in RAE-1γ MCMV primed cells. Using flow cytometry, we validated these findings on protein levels for several genes. Remarkably, CD8 T cells induced by RAE-1γ expressing MCMV vector were predominantly TCF1 negative and showed effector-like phenotype (KLRG1$^+$ CD127$^-$ CD27$^-$ CD62L$^-$). Finally, we showed that this phenotypical difference is associated with

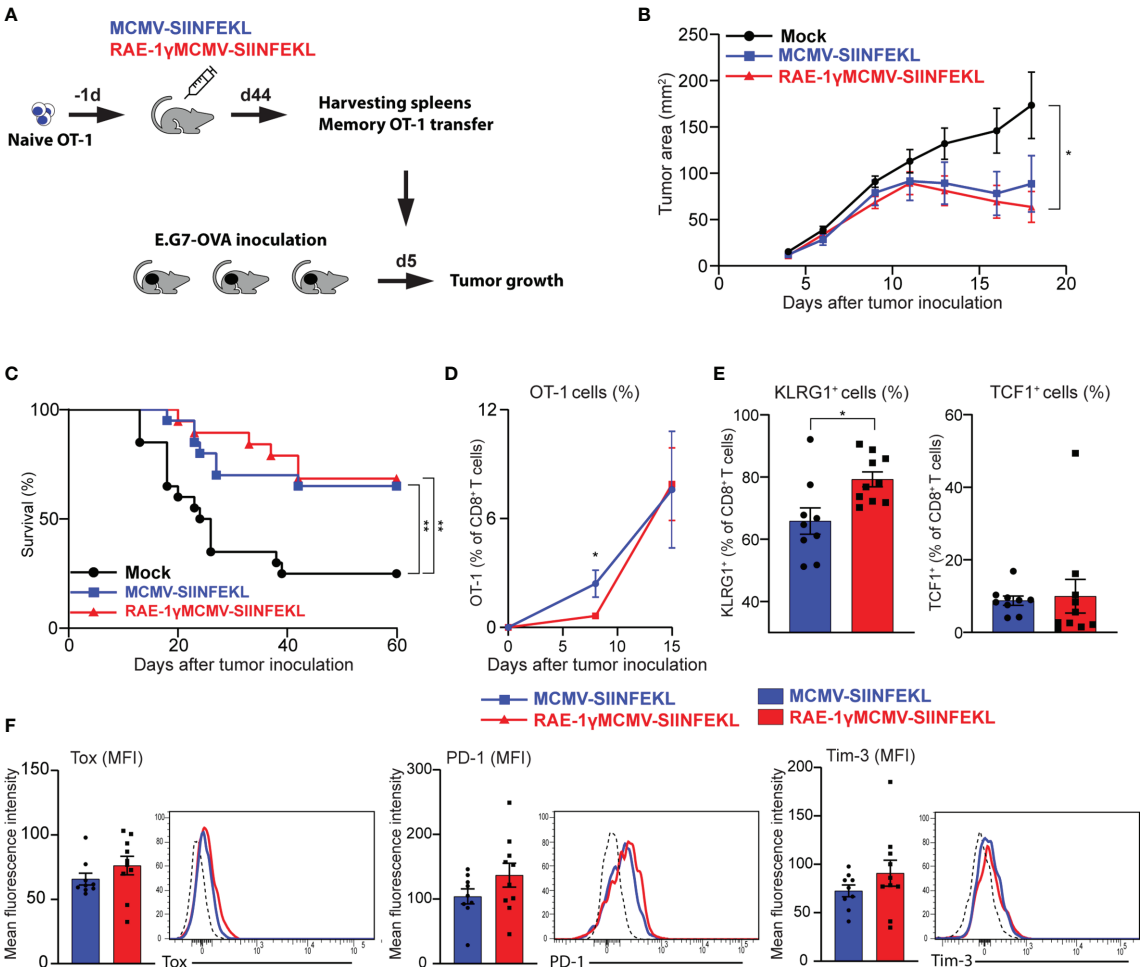

FIGURE 5 | Adoptively transferred CD8 T cells primed with RAE-1γMCMV vector show protective potential similar to cells primed with the MCMV lacking RAE-1γ. **(A)** Naïve OT-1 cells were transferred into C57BL/6 mice (n=8-10) one day prior to immunization with indicated viruses. On day 44 after immunization, $3x10^4$ memory OT-1 cells were sorted and transferred to mice (n=11) that were inoculated with E.G7-OVA tumors 5 days prior to OT-1 transfer. Tumor growth **(B)** and overall survival **(C)** were followed over time. **(D)** The frequency of OT-1 cells was determined in blood at indicated time points. **(E, F)** The phenotype of OT-1 cells was analyzed on day 15 following adoptive transfer in blood. Data are from a single experiment representative of two independent experiments **(B, D–F)**. Survival data are pooled from two independent experiments **(C)**. Data are represented as mean ± SEM and statistical significance *$p < 0.05$; **$p < 0.01$. Statistical significance was determined using one-way ANOVA followed by LSD post test **(B)**, log-rank Mantel-Cox test **(C)**, or unpaired Student t-test **(D–F)**.

distinct functional capabilities of cells primed with indicated vectors in a series of functional assays. RAE-1γMCMV primed OT-1 cells produced lower amounts of IFNγ, TNFα and IL-2, showed decreased degranulation potential, while simultaneously exhibiting similar cytotoxicity against tumor cells expressing their cognate antigen. However, when we transferred equal numbers of memory OT-1 cells primed with MCMV-SIINFEKL or RAE-1γMCMV-SIINFEKL into mice harboring subcutaneous tumors, OT-1 cells induced with both vectors were comparably successful in rejecting established tumors.

Memory CD8 T cells are traditionally divided by CD62L and/or CCR7 expression on central memory (Tcm) and effector memory (Tem) populations. These phenotypical properties were thought to entail functional distinction, as original reports identified the $CCR7^-$ $CD62L^-$ effector memory population as superior in cytokine production, whereas the central memory population was shown to exhibit enhanced proliferative capabilities (31). Further reports quickly led to a more complicated picture of functional division. For instance, Wherry et al. showed no substantial difference in cytokine-producing abilities (except IL-2 production) or cytotoxicity on per cell basis between Tcm and Tem in LCMV infection. At the same time, several other studies in different models obtained contrasting results, as Tem cells proved more protective and demonstrated higher cytotoxic capabilities (42, 43). In a recent study (11), the Tem population was further subdivided into effector-like $CD127^{low}$ $CD62L^{low}$ (terminal-Tem) and $CD127^{high}$ $CD62L^{low}$ (Tem). The effector-like terminal-Tem population (notably also expressing high levels of KLRG1) conferred the greatest protection against *Listeria monocytogenes* infection but

minimal protection against tumor challenge, indicating that protective capacity is highly dependent on the disease and therapeutic context. Our transcriptomic analysis revealed several effector genes, such as *Cx3Cr1, Gzma, Gzmb, Adam8, Cd244a*, and *Lgals3*, were upregulated in CD8 T cells primed with RAE-1γMCMV, suggesting their greater cytotoxic capabilities. *Cx3cr1* is a particularly interesting gene, as one study proposed memory CD8 T cell subset classification based on the expression of this marker (32). In that study, $CX3CR1^{high}$ cells were $CD27^-$, $CD127^-$ and $KLRG1^+$ and produced smaller amounts of cytokines but displayed greater cytotoxic potential, therefore closely corresponding to OT-1 cells primed with RAE-1γMCMV. Interestingly, despite somewhat enhanced cytotoxicity, we observed decreased degranulation of RAE-1γMCMV primed OT-1 cells as measured by LAMP1 (CD107a) mobilization. Previous reports also demonstrated decoupling of degranulation and cytotoxicity in memory CD8 T cells specific for viral antigens, confirming that our observation is not an isolated finding (37). Furthermore, both RNAseq and flow cytometry data demonstrated elevated levels of Granzyme B in RAE-1γMCMV primed OT-1 cells indicating granule content as a better predictor of cytotoxicity than degranulation per se. This is also illustrated by the fact that $KLRG1^+$ cells consistently showed lower levels of LAMP1 mobilization and substantially higher per cell content of Granzyme B.

Two aspects of CD8 T cell response are crucial for cellular immunity induced by a vaccine to be successful: cell numbers and their functionality. RAE-1γMCMV-SIINFEKL induced much higher numbers of OT-1 cells by day 7, which remained elevated throughout the experiment and showed distinct phenotypical and functional features. MCMV-SIINFEKL primed OT-1 cells, enriched in the $TCF1^+$ population, appeared to have slightly augmented recall response and cytokine-producing capabilities. However, when we transferred equal numbers of OT-1 cells primed with RAE-1γ expressing vector or wild-type viral vector, both of these populations successfully rejected established tumors. Therefore, superior cytokine production and recall response of MCMV-SIINFEKL induced OT-1 cells was, perhaps, compensated by slightly elevated cytotoxicity or different tumor-infiltrating potential of RAE-1γMCMV primed OT-1 cells, leading to a similar anti-tumor response *in vivo* by adoptively transferred cells. These results indicate that the enhanced anti-tumor potential demonstrated in **Figure 1**. might depend on the immunological milieu provided by chronic/latent infection by MCMV vector expressing RAE-1γ and the superior numbers of SIINFEKL specific CD8 T cells.

The mechanistic explanation for superior priming and maintenance of CD8 T cells in RAE-1γMCMV immunized mice remains unanswered. Increased frequency of CD8 T cells might be due to the costimulatory nature of NKG2D signaling on these cells. However, several studies have shown no substantial alterations in frequency or absolute numbers of CD8 T cells that lacked NKG2D signaling during priming (44–46), and we observed no diminishment in the frequency of CD8 T cells in RAE-1γMCMV immunized NKG2D deficient mice (6), nor in conditional knock-out mice in which NKG2D receptor is specifically lacking in T lymphocytes (**Figure S1**). It is also possible that RAE-1γ mediates its function through a yet unknown interaction partner, which would explain the persistence of this phenotype even in $Klrk1^{-/-}$ animals. On the other hand, in RAE-1γ expressing vector, the gene is inserted in place of its viral inhibitor *m152*, which has several functions, including the retention of MHC-I molecules in ERGIC-cis Golgi compartment (47). This suggests that CD8 T cells primed with RAE-1γMCMV have stronger TCR signalling, which is known to directly correlate with the magnitude of T cell response (48, 49) and thus provides a feasible hypothesis for superior initial expansion of epitope specific CD8 T cells in RAE-1γMCMV immunized mice. However, this would only apply to CD8 T cells primed *via* direct presentation by infected dendritic cells and not during cross-presentation which was shown to have a far more important role in T cell response to MCMV epitopes (50, 51). Finally, m152 protein delays STING protein trafficking to Golgy compartment and, hence abrogates type I IFN response (52). Type I interferons are potent modulators of T cell proliferation and differentiation (53) and this increased interferon signalling could have an impact on T cell expansion in mice immunized with RAE-1γ expressing vector. Our preliminary results suggest that this is not the case, as we failed to observe any difference in T cell response to RAE-1γMCMV-SIINFEKL in STING deficient animals compared to control animals (data not shown).

Tcm phenotype is crucially connected with TCF1 expression (35, 38). Major phenotypical differences between OT-1 cells primed with RAE-1γMCMV and wild type vector regarding the expression of this transcription factor became apparent only at memory time points, around day 30. Therefore, either $TCF1^+$ cells in the RAE-1γMCMV group died off or were converted to $TCF1^-$ population. This conversion might be the result of an antigen encounter. Welten et al. (54) showed that $TCF1^+$ cells give rise to $TCF1^-$ only in the presence of latent antigenic load. Although RAE-1γMCMV establishes a lower level of latent viral load (5), immunization with this vector might lead to more frequent reactivation events or higher antigen expression on latently infected cells and higher T cell stimulation. This would not only explain the difference in phenotype between T cells primed with indicated vectors, but also signs of memory inflation in RAE-1γMCMV immunized mice and the fact that some of the most abundantly transcribed genes in this group were associated with cellular proliferation and TCR stimulation (*Lgals3* and *Zbtb32*). Another explanation for the phenotypical and functional differences might be due to different priming conditions imprinting the long-term fate of these cells on the epigenetic level. Pace et al. identified histone methyltransferases Suv39h1 as a potent epigenetic silencer of genes related to stemness/memory potential such as *Il7r, Eomes, and Ccr7* (33). Epigenetic mechanisms play a crucial role in poising the cells towards particular phenotype and functionality (55), and it would be interesting to investigate whether RAE-1γMCMV confers distinct epigenetic states to cells primed with this vector.

Overall, our study confirms great potential of highly attenuated CMV viral vectors expressing NKG2D ligand in

prophylactic and therapeutic vaccine settings. The translational possibilities of CMV vectors expressing ligands for activating immune receptors are further supported by potent cellular immune response induced by human CMV vector expressing NKG2D ligand ULBP2 (56). More fundamentally, our study also indicates that small genetical changes of viral vectors can lead to gross differences in CD8 T cell expansion, phenotype, and function.

DATA AVAILABILITY STATEMENT

The RNAseq data has been uploaded to the European nucleotide archive, with accession number: PRJEB44407.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Veterinary Department of the Ministry of Agriculture.

AUTHOR CONTRIBUTIONS

MŠ, IB, AK, and SJ designed the study. MŠ, MC, JM, CR, VL, and LH performed the experiments. MŠ and BL analyzed the data. DI performed RNA sequencing. DB provided reagents. MŠ wrote the manuscript. SJ, AK, IB, and BL critically read and revised the manuscript. SJ and AK supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.681380/full#supplementary-material

REFERENCES

1. Chen DS, Mellman I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* (2013) 39(1):1–10. doi: 10.1016/j.immuni.2013.07.012
2. Klenerman P, Oxenius A. T Cell Responses to Cytomegalovirus. *Nat Rev Immunol* (2016) 16(6):367–77. doi: 10.1038/nri.2016.38
3. Berry R, Watson GM, Jonjic S, Degli-Esposti MA, Rossjohn J. Modulation of Innate and Adaptive Immunity by Cytomegaloviruses. *Nat Rev Immunol* (2020) Feb20(2):113–27. doi: 10.1038/s41577-019-0225-5
4. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of Ligands for the NKG2D Activating Receptor. *Annu Rev Immunol* (2013) 31:413–41. doi: 10.1146/annurev-immunol-032712-095951
5. Slavuljica I, Busche A, Babić M, Mitrović M, Gašparović I, Cekinović Đ, et al. Recombinant Mouse Cytomegalovirus Expressing a Ligand for the NKG2D Receptor is Attenuated and has Improved Vaccine Properties. *J Clin Invest* (2010) 120(12):4532–45. doi: 10.1172/JCI43961
6. Trsan T, Busche A, Abram M, Wensveen FM, Lemmermann NA, Arapovic M, et al. Superior Induction and Maintenance of Protective CD8 T Cells in Mice Infected With Mouse Cytomegalovirus Vector Expressing RAE-1. *Proc Natl Acad Sci* (2013) 110(41):16550–5. doi: 10.1073/pnas.1310215110
7. Tršan T, Vuković K, Filipović P, Brizić AL, Lemmermann NAW, Schober K, et al. Cytomegalovirus Vector Expressing RAE-1γ Induces Enhanced Anti-Tumor Capacity of Murine CD8+ T Cells. *Eur J Immunol* (2017) 47(8):1354–67. doi: 10.1002/eji.201746964
8. Herndler-Brandstetter D, Ishigame H, Shinnakasu R, Plajer V, Stecher C, Zhao J, et al. KLRG1+ Effector CD8+ T Cells Lose KLRG1, Differentiate Into All Memory T Cell Lineages, and Convey Enhanced Protective Immunity. *Immunity* (2018) 48(4):716–29.e8. doi: 10.1016/j.immuni.2018.03.015
9. Kaech SM, Cui W. Transcriptional Control of Effector and Memory CD8+ T Cell Differentiation. *Nat Rev Immunol* (2012) 12(11):749–61. doi: 10.1038/nri3307
10. Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, et al. Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of CD8+ Central Memory T Cells. *Immunity* (2014) 41(1):116–26. doi: 10.1016/j.immuni.2014.05.018
11. Milner JJ, Nguyen H, Omilusik K, Reina-Campos M, Tsai M, Toma C, et al. Delineation of a Molecularly Distinct Terminally Differentiated Memory CD8 T Cell Population. *Proc Natl Acad Sci USA* (2020) 117(41):25667–78. doi: 10.1073/pnas.2008571117
12. Olson JA, McDonald-Hyman C, Jameson SC, Hamilton SE. Effector-Like $CD8^+$ T Cells in the Memory Population Mediate Potent Protective Immunity. *Immunity* (2013) 38(6):1250–60. doi: 10.1016/j.immuni.2013.05.009
13. Jameson SC, Masopust D. Understanding Subset Diversity in T Cell Memory. *Immunity* (2018) 48(2):214–26. doi: 10.1016/j.immuni.2018.02.010
14. Lenartić M, Jelenčić V, Zafirova B, Ožanić M, Marečić V, Jurković S, et al. NKG2D Promotes B1a Cell Development and Protection Against Bacterial Infection. *J Immunol* (2017) 198(4):1531–42. doi: 10.4049/jimmunol.1600461
15. Lemmermann NAW, Gergely K, Böhm V, Deegen P, Däubner T, Reddehase MJ. Immune Evasion Proteins of Murine Cytomegalovirus Preferentially Affect Cell Surface Display of Recently Generated Peptide Presentation Complexes. *J Virol* (2010) 84(3):1221–36. doi: 10.1128/JVI.02087-09
16. Brizić I, Lisnić B, Brune W, Hengel H, Jonjić S. Cytomegalovirus Infection: Mouse Model. *Curr Protoc Immunol* (2018) 122(1):e51. doi: 10.1002/cpim.51
17. Cossarizza A, Chang H-D, Radbruch A, Acs A, Adam D, Adam-Klages S, et al. Guidelines for the Use of Flow Cytometry and Cell Sorting in Immunological Studies (Second Edition). *Eur J Immunol* (2019) 49(10):1457–973. doi: 10.1002/eji.201970107

18. Schober K, Voit F, Grassmann S, Müller TR, Eggert J, Jarosch S, et al. Reverse TCR Repertoire Evolution Toward Dominant Low-Affinity Clones During Chronic CMV Infection. *Nat Immunol* (2020) 21(4):434–41. doi: 10.1038/s41590-020-0628-2
19. Babić M, Pyzik M, Zafirova B, Mitrović M, Butorac V, Lanier LL, et al. Cytomegalovirus Immunoevasin Reveals the Physiological Role of "Missing Self" Recognition in Natural Killer Cell Dependent Virus Control In Vivo. *J Exp Med* (2010) 207(12):2663–73. doi: 10.1084/jem.20100921
20. Kveštak D, Juranić Lisnić V, Lisnić B, Tomac J, Golemac M, Brizić I, et al. NK/ILC1 Cells Mediate Neuroinflammation and Brain Pathology Following Congenital CMV Infection. *J Exp Med* (2021) 218(5):e20201503. doi: 10.1084/jem.20201503
21. Wingett SW, Andrews S. Fastq Screen: A Tool for Multi-Genome Mapping and Quality Control [Version 1; Referees: 3 Approved, 1 Approved With Reservations]. *F1000Research* (2018) 7(0):1–13. doi: 10.12688/f1000research.15931.2
22. Langmead B, Salzberg SL. Fast Gapped-Read Alignment With Bowtie 2. *Nat Methods* (2012) 9(4):357–9. doi: 10.1038/nmeth.1923
23. Frankish A, Diekhans M, Ferreira A-M, Johnson R, Jungreis I, Loveland J, et al. GENCODE Reference Annotation for the Human and Mouse Genomes. *Nucleic Acids Res* (2019) 47(D1):D766–73. doi: 10.1093/nar/gky955
24. Dobin A, Gingeras TR. Optimizing RNA-Seq Mapping With STAR. *Methods Mol Biol* (2016) 1415:245–62. doi: 10.1007/978-1-4939-3572-7_13
25. Dobin A, Gingeras TR. Mapping RNA-seq Reads With STAR. *Curr Protoc Bioinformatics* (2015) 51:11.14.1–11.14.19. doi: 10.1002/0471250953.bi1114s51
26. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: Ultrafast Universal RNA-seq Aligner. *Bioinformatics* (2013) 29(1):15–21. doi: 10.1093/bioinformatics/bts635
27. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map Format and SAMtools. *Bioinformatics* (2009) 25(16):2078–9. doi: 10.1093/bioinformatics/btp352
28. Liao Y, Smyth GK, Shi W. featureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features. *Bioinformatics* (2014) 30(7):923–30. doi: 10.1093/bioinformatics/btt656
29. Love MI, Huber W, Anders S. Moderated Estimation of Fold Change and Dispersion for RNA-seq Data With DESeq2. *Genome Biol* (2014) 15(12):550. doi: 10.1186/s13059-014-0550-8
30. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: An R Package for Comparing Biological Themes Among Gene Clusters. *OMICS* (2012) 16(5):284–7. doi: 10.1089/omi.2011.0118
31. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two Subsets of Memory T Lymphocytes With Distinct Homing Potentials and Effector Functions. *Nature* (1999) 401(6754):708–12. doi: 10.1038/44385
32. Gerlach C, Moseman EA, Loughhead SM, Alvarez D, Zwijnenburg AJ, Waanders L, et al. The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets With Distinct Roles in Immune Surveillance and Homeostasis. *Immunity* (2016) 45(6):1270–84. doi: 10.1016/j.immuni.2016.10.018
33. Pace L, Goudot C, Zueva E, Gueguen P, Burgdorf N, Waterfall JJ, et al. The Epigenetic Control of Stemness in CD8(+) T Cell Fate Commitment. *Science* (2018) 359(6372):177–86. doi: 10.1126/science.aah6499
34. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. Functional and Genomic Profiling of Effector CD8 T Cell Subsets With Distinct Memory Fates. *J Exp Med* (2008) 205(3):625–40. doi: 10.1084/jem.20071641
35. Zhou X, Yu S, Zhao DM, Harty JT, Badovinac VP, Xue HH. Differentiation and Persistence of Memory CD8+ T Cells Depend on T Cell Factor 1. *Immunity* (2010) 33(2):229–40. doi: 10.1016/j.immuni.2010.08.002
36. Martin MD, Badovinac VP. Defining Memory CD8 T Cell. *Front Immunol* (2018) 9(NOV):1–10. doi: 10.3389/fimmu.2018.02692
37. Wolint P, Betts MR, Koup RA, Oxenius A. Immediate Cytotoxicity But Not Degranulation Distinguishes Effector and Memory Subsets of CD8+ T Cells. *J Exp Med* (2004) 199(7):925–36. doi: 10.1084/jem.20031799
38. Jeannet G, Boudousquié C, Gardiol N, Kang J, Huelsken J, Held W. Essential Role of the Wnt Pathway Effector Tcf-1 for the Establishment of Functional CD8 T Cell Memory. *Proc Natl Acad Sci USA* (2010) 107(21):9777–82. doi: 10.1073/pnas.0914127107
39. Pais Ferreira D, Silva JG, Wyss T, Fuertes Marraco SA, Scarpellino L, Charmoy M, et al. Central Memory CD8+ T Cells Derive From Stem-Like Tcf7hi Effector Cells in the Absence of Cytotoxic Differentiation. *Immunity* (2020) 53(5):985–1000.e11. doi: 10.1016/j.immuni.2020.09.005
40. Lin WHW, Nish SA, Yen B, Chen YH, Adams WC, Kratchmarov R, et al. CD8+ T Lymphocyte Self-Renewal During Effector Cell Determination. *Cell Rep* (2016) 17(7):1773–82. doi: 10.1016/j.celrep.2016.10.032
41. Alfei F, Kanev K, Hofmann M, Wu M, Ghoneim HE, Roelli P, et al. TOX Reinforces the Phenotype and Longevity of Exhausted T Cells in Chronic Viral Infection. *Nature* (2019) 571(7764):265–9. doi: 10.1038/s41586-019-1326-9
42. Huster KM, Koffler M, Stemberger C, Schiemann M, Wagner H, Busch DH. Unidirectional Development of CD8+ Central Memory T Cells Into Protective Listeria-specific Effector Memory T Cells. *Eur J Immunol* (2006) 36(6):1453–64. doi: 10.1002/eji.200635874
43. Bachmann MF, Wolint P, Schwarz K, Jäger P, Oxenius A. Functional Properties and Lineage Relationship of CD8+ T Cell Subsets Identified by Expression of IL-7 Receptor Alpha and CD62L. *J Immunol* (2005) 175(7):4686–96. doi: 10.4049/jimmunol.175.7.4686
44. Kavazović I, Lenartić M, Jelenčić V, Jurković S, Lemmermann NAW, Jonjić S, et al. NKG2D Stimulation of CD8+ T Cells During Priming Promotes Their Capacity to Produce Cytokines in Response to Viral Infection in Mice. *Eur J Immunol* (2017) 47(7):1123–35. doi: 10.1002/eji.201646805
45. Wensveen FM, Lenartić M, Jelenčić V, Lemmermann NAW, ten Brinke A, Jonjić S, et al. NKG2D Induces Mcl-1 Expression and Mediates Survival of CD8 Memory T Cell Precursors Via Phosphatidylinositol 3-Kinase. *J Immunol* (2013) 191(3):1307–15. doi: 10.4049/jimmunol.1300670
46. Zloza A, Kohlhapp FJ, Lyons GE, Schenkel JM, Moore TV, Lacek AT, et al. NKG2D Signaling on CD8 + T Cells Represses T-bet and Rescues CD4-unhelped CD8 + T Cell Memory Recall But Not Effector Responses. *Nat Med* (2012) 18(3):422–8. doi: 10.1038/nm.2683
47. Ziegler H, Thale R, Lucin P, Muranyi W, Flohr T, Hengel H, et al. A Mouse Cytomegalovirus Glycoprotein Retains MHC Class I Complexes in the ERGIC/cis-Golgi Compartments. *Immunity* (1997) 6(1):57–66. doi: 10.1016/S1074-7613(00)80242-3
48. Wherry EJ, Puorro KA, Porgador A, Eisenlohr LC. The Induction of Virus-Specific CTL as a Function of Increasing Epitope Expression: Responses Rise Steadily Until Excessively High Levels of Epitope are Attained. *J Immunol* (1999) 163(7):3735–45.
49. Wherry EJ, McElhaugh MJ, Eisenlohr LC. Generation of CD8 + T Cell Memory in Response to Low, High, and Excessive Levels of Epitope. *J Immunol* (2002) 168(9):4455–61. doi: 10.4049/jimmunol.168.9.4455
50. Torti N, Walton SM, Murphy KM, Oxenius A. Batf3 Transcription Factor-Dependent DC Subsets in Murine CMV Infection: Differential Impact on T-cell Priming and Memory Inflation. *Eur J Immunol* (2011) 41(9):2612–8. doi: 10.1002/eji.201041075
51. Busche A, Jirmo AC, Welten SPM, Zischke J, Noack J, Constabel H, et al. Priming of CD8 + T Cells Against Cytomegalovirus-Encoded Antigens Is Dominated by Cross-Presentation. *J Immunol* (2013) 190(6):2767–77. doi: 10.4049/jimmunol.1200966
52. Stempel M, Chan B, Juranić Lisnić V, Krmpotić A, Hartung J, Paludan SR, et al. The Herpesviral Antagonist m152 Reveals Differential Activation of STING -Dependent IRF and NF -κB Signaling and STING 'S Dual Role During MCMV Infection. *EMBO J* (2019) 38(5):1–22. doi: 10.15252/embj.2018100983
53. Crouse J, Kalinke U, Oxenius A. Regulation of Antiviral T Cell Responses by Type I Interferons. *Nat Rev Immunol* (2015) 15(4):231–42. doi: 10.1038/nri3806
54. Welten SPM, Yermanos A, Baumann NS, Wagen F, Oetiker N, Sandu I, et al. Tcf1+ Cells are Required to Maintain the Inflationary T Cell Pool Upon MCMV Infection. *Nat Commun* (2020) 11(1):1–14. doi: 10.1038/s41467-020-16219-3
55. Henning AN, Roychoudhuri R, Restifo NP. Epigenetic Control of CD8+ T'cell Differentiation. *Nat Rev Immunol* (2018) 18(5):340–56. doi: 10.1038/nri.2017.146
56. Tomić A, Varanasi PR, Golemac M, Malić S, Riese P, Borst EM, et al. Activation of Innate and Adaptive Immunity by a Recombinant Human

Cytomegalovirus Strain Expressing an NKG2D Ligand. *PloS Pathog* (2016) 12 (12):1–27. doi: 10.1371/journal.ppat.1006015

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CMV Infection Is Directly Related to the Inflammatory Status in Chronic Heart Failure Patients

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High levels of inflammation play an important role in chronic heart failure (CHF). Patients with CHF have elevated levels of pro-inflammatory cytokines circulating systemically, mainly TNF and IL-6. However, there are almost no studies that relate these levels to the functional status of patients in CHF, much less to their CMV serostatus. In this study, patients with CHF (n=40; age=54.9 ± 6.3; New York Heart Association functional classification (NYHA, I-III) and healthy controls (n=40; age=53.5 ± 7.1) were analyzed. The serum concentrations of nine pro- and anti-inflammatory cytokines were measured by Luminex® xMap Technology and the basal level of mRNA expression of some immune molecules was quantified by TaqMan™ Array in CD4+ T-lymphocytes. The concentration of these cytokines in culture supernatants in response to anti-CD3 and LPS was also measured. The percentage of CD28null T-cells was determined, as well as the antibody titer against CMV. We found a higher concentration of all cytokines studied in CHF serum compared to healthy controls, as well as a direct correlation between functional status in CHF patients and levels of inflammatory cytokines. Moreover, the highest cytokine concentrations were found in patients with higher concentrations of lymphocytes lacking CD28 molecule. The cytokine production was much higher in CMV+ patients, and the production of these cytokines was found mainly in the T-lymphocytes of CMV+ patients in response to anti-CD3. Anti-CMV antibody levels were positively correlated with cytokine levels. The baseline expression of specific mRNA of the main molecules involved in the Th1 response, as well as molecules related to the CD4+ CD28 null subset was higher in CMV+ patients. The cytokine concentrations are higher in CHF CMV+ patients and these concentrations are related to the production of antibodies against CMV. These high levels of cytokines are also associated with the more differentiated CD28null lymphocyte populations. All this, together with the dynamics of the pathology itself, makes CMV+ patients present a worse functional status and possibly a worse evolution of the pathology.

Keywords: CHF, CMV, inflammation, T-lymphocyte, immunosenescence, TNF, IL-6

INTRODUCTION

The process known as immunosenescence may affect both the elderly and individuals of all ages with chronic inflammatory or infectious diseases. The changes produced by immunosenescence are therefore found in patients with chronic heart failure (CHF). The immunosenescence found in CHF patients is not only associated with the pathology itself but also with a worse functional status (1). The aging of the immune system, mainly adaptive, has been associated with the presence of chronic and persistent antigens, as well as a low-level inflammatory state, maintained for a considerable period of time. All these processes lead to a dysregulation of the immune system, compromising immune responses, producing an increase in the frequency of highly differentiated T-lymphocytes, mainly with the loss of the CD28 molecule (2, 3).

The inflammation found in patients with CHF may be a consequence of the increase in the pro-inflammatory cytokines expression as mediators of the protective effect on cardiac cells, as a rapid adaptation to the stress suffered by these cells (4). On the other hand, this increase in pro-inflammatory cytokines leads to an advance in cardiac pathology due to the harmful effect that these cytokine present on the heart-cells and on the systemic circulation (5, 6). IFN-γ production, mainly by CD4+ Th1 lymphocytes, is directly related to these deleterious effects produced by circulating pro-inflammatory cytokines (7–9). Accordingly, the differentiation of T-lymphocytes and the increase in the concentration of pro-inflammatory cytokines in physiological aging, and in certain chronic diseases, are events that occur at the same time. Because of this it is not at all clear what produces what. Differentiated T-lymphocytes are related to the production of inflammatory cytokines, while a high concentration of circulating cytokines has been related to the differentiation of T-lymphocytes.

Not much is known about the antigens involved in the differentiation of T-lymphocytes in the immunosenescence process in the context of CHF. T-lymphocytes could be activated repeatedly and continuously over time by antigens from chronic infections, and this continuous activation could be the cause of increased inflammatory degree and probable tissue damage. To date, the main known inducer of T-cell differentiation is CMV. This virus has been related to the immunosenescence process, even the antibody titer against CMV has been related to immunocompetence, and the degree of lymphocyte differentiation in the elderly (10–14). Recent studies in our laboratory have also found this association in patients with CHF (15).

The objective of this study was to investigate the implication of CMV infection in the production of pro-inflammatory cytokine, and its relationship with functional status in CHF patients.

MATERIALS AND METHODS

Study Population

Forty healthy volunteers and 40 chronic heart failure (CHF) patients were recruited for the study. Individuals in the study were divided into two groups: healthy control (n=40) and CHF patients (n=40). In turn, each of these groups was divided according to their CMV-seropositivity (**Table 1**). All volunteers were defined as individuals younger than 65 years old to reduce the effect of aging on the study. The control group was recruited from the Centro de Transfusiones del Principado de Asturias (Oviedo, Spain). CHF patients were classified according to the New York Heart Association functional classification (NYHA) and recruited from the Heart Failure Unit at Hospital Universitario Central de Asturias with symptomatic HF (NYHA class I to III). All subjects underwent a physical examination and answered a standardized questionnaire to assess their medical history, current illnesses, and any medication they were taking. Exclusion criteria included all conditions that might influence the immune system, such as a recent or current infection, autoimmune disease or tumor, malnutrition, abnormal laboratory data (hemoglobin < 12 g/dL, leukopenia < 3500 cells/μL, neutropenia < 1500 cells/μL, leukocytosis > 15000 cells/μL and platelets < 105 cells/μL), and pharmacological interference. Informed consent was obtained from all volunteers before participation in the study. The study was approved by the ethics committee of the Hospital Central de Asturias (Oviedo, Spain) with the number 82/17. Peripheral blood samples were drawn from all subjects for hematological and immunological analyses.

Hematological Analysis and Immunological Phenotyping

The hematological parameters were determined using a Sysmex XT-2000i (Sysmex, Hamburg-Norderstedt, Germany), and the biochemistry values using a Cobas c711 analyzer series (Roche Diagnostics, Indianapolis, USA). For flow cytometry analysis, peripheral blood cells were surface-stained with anti-CD4 (PerCP), anti-CD8 (PE), anti-CD3 (FITC) and anti-CD28 (APC) (Biolegend, San Diego, CA, USA). One hundred microliters of whole blood from volunteers were stained with the labeled monoclonal antibodies for 20 min at room temperature. Samples were red-blood lysed with FACS Lysing Solution (BD Biosciences), washed in PBS, and analyzed using Kaluza software in a Gallios cytometer (Beckman-Coulter, Brea, CA, USA). Appropriate isotype control mAbs were used for marker settings.

Cytomegalovirus Serology

Serum presence of CMV-specific antibodies was determined by an enzyme-linked immunosorbent assay, Vir-ELISA Anti-CMV-IgG (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany), according to the manufacturer's specifications. CMV-serostatus was interpreted by means of the calculation of the ratio: Cut-off Index = optical density (OD) value of sample / cut-off value, whereby a ratio of 1.0 is equivalent to the cut-off value. Cut-off indexes >1.1 were considered positive. Quantification of anti-CMV antibody titers was performed through a semi-quantitative titer calculation.

Isolation of PBMC and Cell Cultures

Peripheral blood mononuclear cells were isolated from peripheral blood that had been anticoagulated with EDTA by

TABLE 1 | Participant characteristics in relation to CMV serostatus.

	Chronic Heart Failure Patients (CHF) (n=40)		Healthy Controls (HYC) (n=40)		ANOVA		
	CMV- (n=13)	CMV+ (n=27)	CMV- (n=19)	CMV+ (n=21)	$P_{pathology}$	p_{CMV}	$p_{interaction}$
Demographic data							
Age ± SD	53.1 ± 8.9	56.7 ± 5.6	52.1 ± 7.5	54.9 ± 6.6	NS	NS	NS
Male (%)	10 (76.9)	22 (81.7)	16 (84.2)	13 (61.9)	NS	NS	NS
BMI ± SD (kg/m^2)	23.9 ± 3.6	24.4 ± 2.8	23.8 ± 3.6	25.0 ± 2.9	NS	NS	NS
Smoking status, current (%)	4 (30.8)	6 (22.2)	4 (21.1)	5 (23.8)	NS	NS	NS
Hypertension (%)	2 (15.4)	5 (18.5)	0 (0)	0 (0)		NA	
% LVEF ± SD	37.2 ± 15.4	36.1 ± 10.6	>60%	>60%		NA	
Diabetes mellitus (%)	3 (23.1)	6 (22.2)	0 (0)	0 (0)		NA	
Cholesterol ± SD (mg/dL)	173.1 ± 38.1	152.3 ± 25.5	ND	ND		NA	
NT-proBNP, pg/mL (IQR)	4985 (4759)	5304 (4172)	ND	ND		NA	
CRP, mg/dL (IQR)	1.6 (1.65)	1.6 (2.75)	ND	ND		NA	
CHF etiology							
Coronary artery disease (%)	5 (38.5)	12 (44.4)	NA	NA		NA	
Idiopathic dilated cardiomyopathy (%)	5 (38.5)	9 (33.3)	NA	NA		NA	
Others (%)	3 (23.1)	6 (22.2)	NA	NA		NA	
Hematological variables (mean and SD)							
WBCs (10^3/µl)	7.7 ± 1.9	8.2 ± 1.9	6.4 ± 1.8	6.9 ± 1.8	0.007	NS	NS
Neutrophils (10^3/µl)	5.0 ± 1.7	5.4 ± 1.8	3.9 ± 1.0	4.3 ± 1.0	0.025	NS	NS
Neutrophils (%)	64.1 ± 8.9	64.1 ± 8.1	54.2 ± 8.7	56.4 ± 6.6	0.001	NS	NS
Monocytes (10^3/µl)	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.3	0.4 ± 0.2	0.002	NS	NS
Monocytes (%)	8.9 ± 2.6	8.2 ± 2.5	7.0 ± 4.4	5.7 ± 2.3	0.009	NS	NS
Lymphocytes (10^3/µl)	1.7 ± 0.7	1.9 ± 0.6	2.6 ± 0.7	3.2 ± 0.7	<0.001	NS	NS
Lymphocytes (%)	23.3 ± 8.8	24.3 ± 7.2	36.2 ± 3.8	41.6 ± 5.0	<0.001	NS	NS

LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal protype B natriuretic peptide; BMI, body mass index; CRP, C-reactive protein; WBCs, white blood cells; SD, standard deviation; IQR, interquartile range; NA, not applicable; ND, not done; NS, not significant.

centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). Cultures were performed in RPMI 1640 medium containing 2x10-3 M L-glutamine and Hepes (BioWhitaker, Verviers, Belgium) and supplemented with 10% FCS (ICN Flow; Costa Mesa, CA, USA) and antibiotics. Cells were incubated at 37°C and 5% carbon dioxide.

Cytokine Quantification in Patient Serum and Supernatants

The sera of the individuals under study were collected and stored at -80°C until the cytokine quantification and analysis. Meanwhile, response to anti-CD3 (1 µg/mL) (eBioscience, San Diego, CA, USA) and to LPS (1 µg/mL) was analyzed in PBMCs (2x10^6 cells/ml) from CHF patients. PBMCs were cultivated alone or stimulated with anti-CD3 and LPS in 48-well plaques in a humidified 37°C incubator for three days. Finally, cell-free supernatants were collected and stored at −80°C for multiplexed cytokine analyses.

The production of 9 different cytokines (IFN-γ, IL-10, IL-12, IL-17, IL-1β, IL-2, IL-4, IL-6 and TNF) was quantified in sera and supernatants using the ProcartaPlex™ Mix & Match Panel (Affymetrix eBioscience, San Diego, USA) and the Luminex® xMap Technology (Luminex Corporation, Austin, USA) equipment following manufacturer's settings.

Cytokine Expression Array

To isolate CD4+ T-cells, PBMCs from 5 CMV-positive and 5 CMV-negative CHF patients were isolated by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway) after 20 min of incubation with the RosetteSep Human CD4+ T-cell Enrichment Cocktail (StemCell Technologies, Grenoble, France). In all cases, purity of isolated CD4+ T-lymphocytes, tested by flow cytometry was higher than 95%. mRNA was extracted using a Total RNA Isolation (Macherey-Nagel GmbH & CoKG, Düren, Germany) according to the manufacturer's instructions. Reverse transcription of mRNA isolated from each sample was carried out in a 20 µL final volume with the iScript cDNA Synthesis Kit (Bio-Rad, Life Science Research Group, Hercules, CA, USA) following manufacturer's instructions. The mixture was incubated at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min and stored at -80°C until required for the array. Equal quantities of cDNA were mixed to generate two pools, one with samples from CMV-seronegative patients and another one with samples from CMV-seropositive patients. Cytokine gene expression was examined through TaqMan™ Array Human Immune Response Real-Time PCR (Applied Biosystems, Foster City, CA, USA) using predesigned human gene-specific primers and probes based on published cytokine sequences and following manufacturer's instructions.

Statistical Analysis

Results are expressed as the median and interquartile range (IR) or the mean and standard deviation. Quantitative variables were compared using the analysis of variance (ANOVA) to study the effect of CMV (CMV- or CMV+), pathology (CHF+ or CHF-) or

NYHA (Class I+II or Class III) and adjusting for sex. If significant interactions were observed in any of these analyses, comparisons with a Bonferroni-correlated post-hoc test were performed. In order to perform these analyses, non-parametric variables were normalized by logarithmic transformation. Groups were compared using the non-parametric Mann-Whitney U test (for non-normally distributed data) or Student's t-test (for normally distributed data). To compare the results obtained in the expression arrays, we used the comparative ddCT method (16) to calculate relative quantitation of gene expression after outlier removal and data normalization based on the endogenous control genes expression (18S rRNA, GAPDH, HPRT1 and GUSB) using DataAssist software (Thermo Fisher Scientific). The list of analyzed genes and their assay IDs is presented in the **Supplementary File** (**Supplementary Table 1**). The outlier and the extreme values were calculated by adding 1.5 and 3 times the interquartile range (IR) to the 75th percentile, respectively. Correlations between variables were assessed using the non-parametric Spearman test (ρ). Analyses were performed using the PASW Statics 17.0 statistical software package (IBM SPSS, NY, USA) and p-values of 0.05 or less were considered significant.

RESULTS

Characteristics of Studied Groups Related to CMV-Serostatus and Cytokine Levels

In **Table 1** we can see the characteristics of the two studied groups, CHF patients and healthy control group (HC). All the participants in the study belong to the Caucasian ethnic group. Levels of the antibodies against CMV were measured in all participants. All study volunteers had a blood test and immunophenotype. When we made statistical comparisons with the ANOVA test, we found significantly higher levels in total white blood cells (WBCs), monocytes, and neutrophils and significantly decreased levels in lymphocytes (**Table 1**). We did not find any other difference in relation to CMV serostatus in any leukocyte subpopulation or in any other measured variable.

After measuring the cytokine concentrations of pro-inflammatory cytokines (IFN-γ, IL-12, IL-17, IL-1β, IL-2, TNF), anti-inflammatory (IL-10 and IL-4), and the pro- and anti-inflammatory cytokine IL -6, in the CHF group and controls, we found that the levels of cytokines were always significantly higher in the CHF group (Mann-Whitney Test, $p < 0.001$ in all cases except IL-17, p=0.015, and IL-1β, p=0.001) (**Figure 1**). In addition to the increased levels of the cytokines studied in CHF patients, all of them were positively correlated with each other (**Supplementary Table 2**). Therefore, in some of the figures we will only display the most representative cytokines, the rest of the figures of the cytokines can be consulted in the **Supplementary Material**.

Despite CHF patients showing higher CMV infection rate, total WBCs and leukocyte populations are related only to pathology and not to CMV serostatus in the groups studied. The levels of the cytokines studied are clearly increased in patients with CHF and all of them are correlated with each other.

Association Between CMV-Serostatus and Level of Cytokines in CHF

To evaluate the association of CMV infection with cytokine production we divided our CHF patients according to their CMV serostatus, 27 out of 40 were CMV-seropositive. Moreover, we classified the patients according to the extent of CHF by functional criteria (NYHA). As we only had three NYHA class I patients, we decided to combine the patients from groups I and II. We observed that the highest concentrations of cytokines were found in CMV-positive individuals from NYHA class III. We found that the cytokine concentrations were increased in the CMV+ patients in the case of IL-12, IL-17 and IL-6. These concentrations were also increased in CMV+ patients and belonging to NYHA class III in the case of IL-1β and TNF.

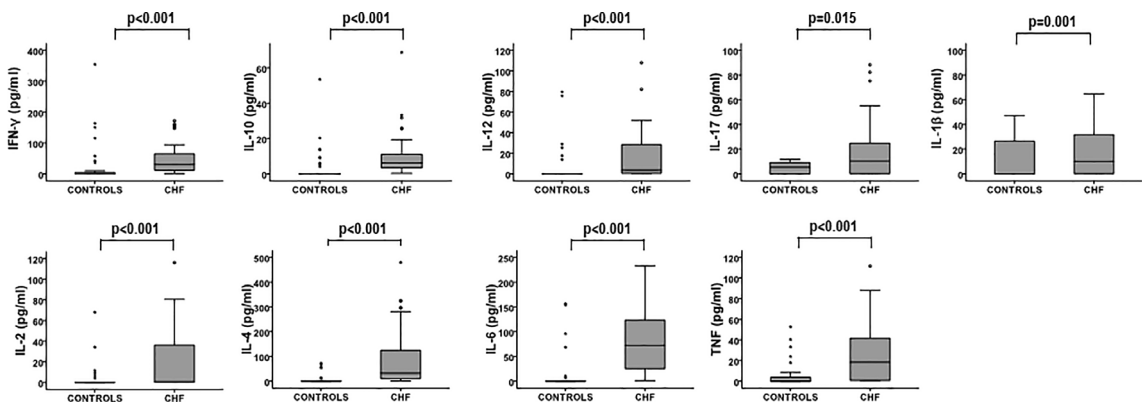

FIGURE 1 | Cytokine levels in the two studied groups. Cytokine concentrations were measured using Luminex multiplex technology. Levels of cytokines in CHF (n = 40) and controls (n = 40) are illustrated in the box plots. Differences between groups in the levels of cytokines were compared using the Mann-Whitney Test, p-values are depicted in the panels. Outlier values are represented by circles and extreme values by stars, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively.

When we analyzed IFN-γ and IL-10, we saw that their increase was only related to NYHA class III, but not to their CMV serostatus. In the case of IL-2 and IL-4, we did not find significant differences in patients with respect to their CMV serostatus or their functional class (ANOVA test, $p<0.05$) (**Figure 2** and **Supplementary Figure 1**).

Next, we wanted to find out if there was any type of correlation between the antibody titer against CMV and the level of the cytokines analyzed. Levels of anti-CMV antibodies in CHF individuals were measured and they showed a median concentration of 2,336 VU/ml (IR, 1,350 VI/ml). We found a clear correlation between the levels of the cytokines IL-17, IL-1β, IL-6 and TNF and the antibody titer against CMV (Spearman Rho test; $p<0.05$) (**Figure 3**), the antibody titer is positively correlated with the levels of these pro-inflammatory cytokines. In the case of the rest of cytokines, we did not find a significant relationship, but a clear trend (**Supplementary Figure 2**).

In summary, we can confirm that the increase in cytokine concentrations in CHF patients is related both to the worsening of the functional status of the patients and to their serostatus against CMV, and these levels of cytokines correlate with the levels of antibodies to CMV.

Relationship Between Cytokine Level and T-Lymphocyte Differentiation in CHF Patients

It is well known that the degree of differentiation in T-lymphocytes in the elderly is related to, among other things, an increase of a low basal inflammation or "inflammaging". We observed more differentiated phenotypes in T-cells in CHF patients related to the concentrations of IL-6 (1). T-lymphocytes can be separated into less differentiated subsets that express the CD28+ marker and the most differentiated subsets with loss of expression of CD28. We wanted to verify the association between the levels of the studied cytokines and the T-cell differentiation in the CHF patients. For this, we face the percentage of CD4 +CD28null T-cells and the level of cytokines in the CHF patients and we found a significant positive correlation between all the studied cytokines (Spearman Rho test; $p<0.05$), less in the case of IL-10, IL-2 and IL-4 where we did not find a significant correlation but a very marked trend in the case of IL-2 and IL-4 (**Figure 4A** and **Supplementary Figure 3A**). After analyzing the CD8+ T-lymphocyte populations, we also observed a strong correlation between all the studied cytokines (Spearman Rho test; $p<0.05$), but not in the case of IL-10. In patients with a larger CD8+CD28null population we found significant higher concentrations of all cytokines except for IL-10 (**Figure 4B** and **Supplementary Figure 3B**).

To test the implication of T-lymphocytes in pro-inflammatory cytokine production in CHF patients, we cultivated PBL's alone, in the presence of anti-CD3, and in the presence of LPS. We only found a significantly high production of cytokines compared to the culture without stimulation in the case of anti-CD3 (Student's t-test for paired data, $p<0.001$ in all cases except, curiously, IL-2). This could be indicating that most of these cytokines are being produced by T-lymphocytes. When we divide these results by CMV seropositivity, a significantly high production of cytokines is observed in all cases by CMV+

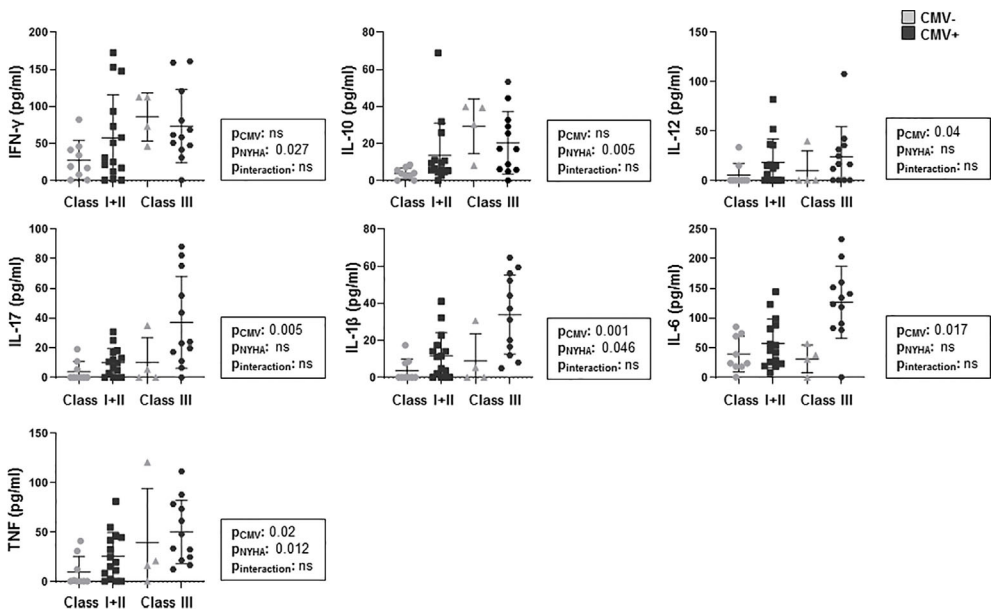

FIGURE 2 | Levels of cytokines analyzed in CHF with respect to the different degrees of HF classified according NYHA (New York Heart Association) in CMV- and CMV+ patients. Outlier values are represented by circles and extreme values by stars, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The ANOVA test was used to examine differences between the groups; p-values are depicted in the boxed text; the interaction is between NYHA and CMV serostatus. If significant interactions were observed, comparisons with a Bonferroni correlated *post hoc* test were performed and p-values are represented in the panels.

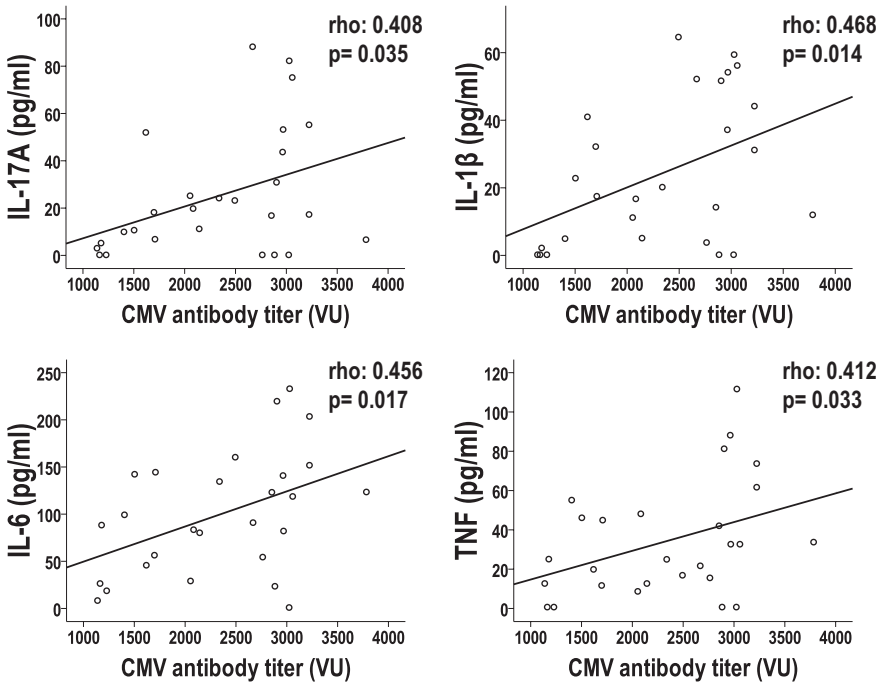

FIGURE 3 | Relationship between anti-CMV antibody titer with cytokine levels in CHF patients. The correlation of anti-CMV antibody titers and cytokine levels in CHF patients is represented in the dot plots. Spearman's test was applied to calculate the correlations; the p-value and coefficient of correlation are listed on the panels.

patients, except with anti-inflammatory cytokine IL- 10, where the highest production is observed in CMV- patients (Student's t-test for paired data, $p < 0.05$ in all cases except IFN-γ and IL-2) (**Figure 5**).

In summary, CD4+ and CD8+ phenotype is associated with the level of the cytokines; more differentiated CD4+ and CD8+ T-lymphocyte subsets are increased in CHF patients with higher pro-inflammatory cytokine levels. Furthermore, the production of these cytokines comes primarily from the T-lymphocyte population, with increased production in CMV+ patients.

Differential mRNA Expression in CMV- and CMV+ Patients

As it is already well known, the production of IFN-γ by the Th1 CD4+ T-lymphocytes are major contributors to heart failure (9). For this, we wanted to verify their influence in the high cytokine levels found in CHF patients. To this, we analyzed CD4+ baseline level of gene expression related to inflammation. We used TaqMan™ Array Human Immune Response plates where we measured separately the level of mRNA expression in a pool of 5 CMV- seronegative and 5 CMV-seropositive patients (**Figure 6**). We separated CD4+ lymphocytes, extracted their mRNA and quantified it in expression plate arrays.

After analyzing the results, we found that only two genes were under-expressed in the CHF CMV+ patients, IL-8 and CCL2, both involved in pro-inflammatory and chemotactic processes. A large number of genes were found to be overexpressed in CMV+ patients, and these genes can be categorized in some groups. We found IL-10 overexpressed, possibly as a reaction to the higher concentration of circulating pro-inflammatory cytokines that CMV+ patients present. We also found two of the main genes related to the Th1 cell response overexpressed, in this case TBX21 (T-bet) and IFN-γ. CD4+CD28null T-cells are associated with high cell differentiation and are found in a much higher proportion in CHF CMV+ patients (15). In addition, they present a cytotoxicity similar to CD8+ T-cells or NK cells and we found that the molecules related to this cytotoxicity are overexpressed, in this case, perforin, granzyme B and granulysin. We also observed overexpressed genes for molecules that are increased in activated CD4+ T-lymphocytes such as CD38, CTLA4, CD40LG, ICOS, CXCR3 and CCR5. Finally, the antiapoptotic genes BCL2 and BCL2L1 are also overexpressed in the CD4+ T-lymphocytes in CMV+ patients. Two other overexpressed genes were the SMAD3 genes, involved in the TGF-β production cascade, and the IKBKB gene, an inhibitor of the NF-κβ pathway.

In summary, we can say that CHF CMV+ patients present overexpressed genes of the Th1 pathway, molecules involved in the cytotoxicity of CD4+CD28null cells, molecules related to the activation of CD4+ T-cells and antiapoptotic molecules.

DISCUSSION

The present study exhibits that CHF patients show a higher level of cytokines than age-matched healthy controls, and this high level of cytokines is even higher in CMV+ CHF patients and in

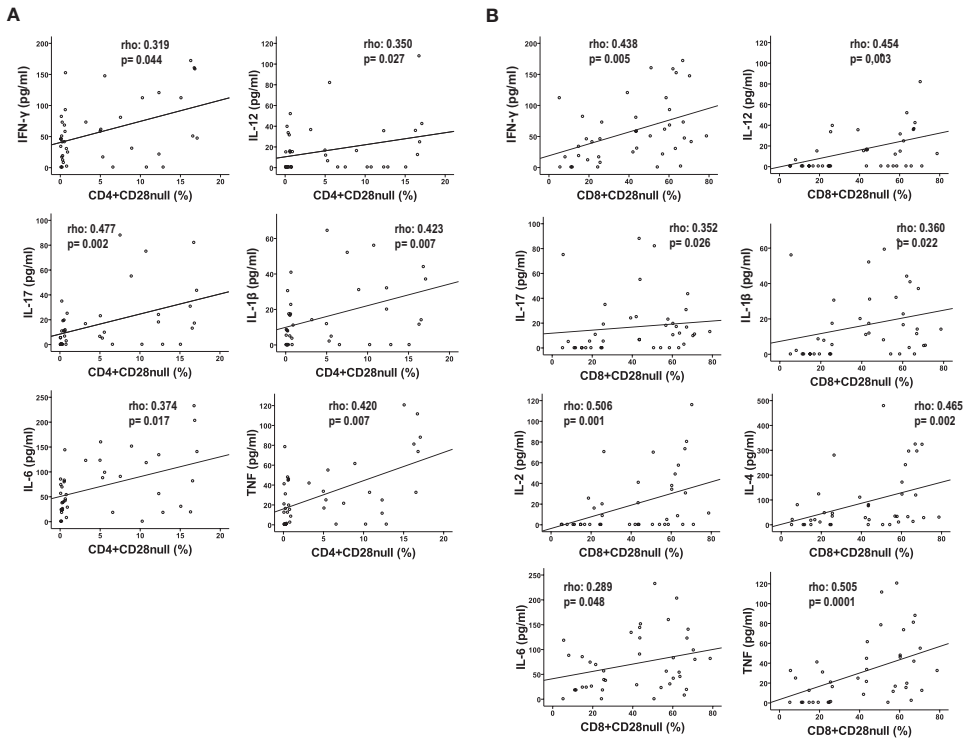

FIGURE 4 | Levels of cytokines correlated to CD28null T-lymphocyte populations in CHF patients. Levels of cytokines (pg/mL) in CD4+ **(A)** and CD8+ T-lymphocytes **(B)**. Spearman's test was applied to calculate the correlations; the p-value and coefficient of correlation are listed on the panels.

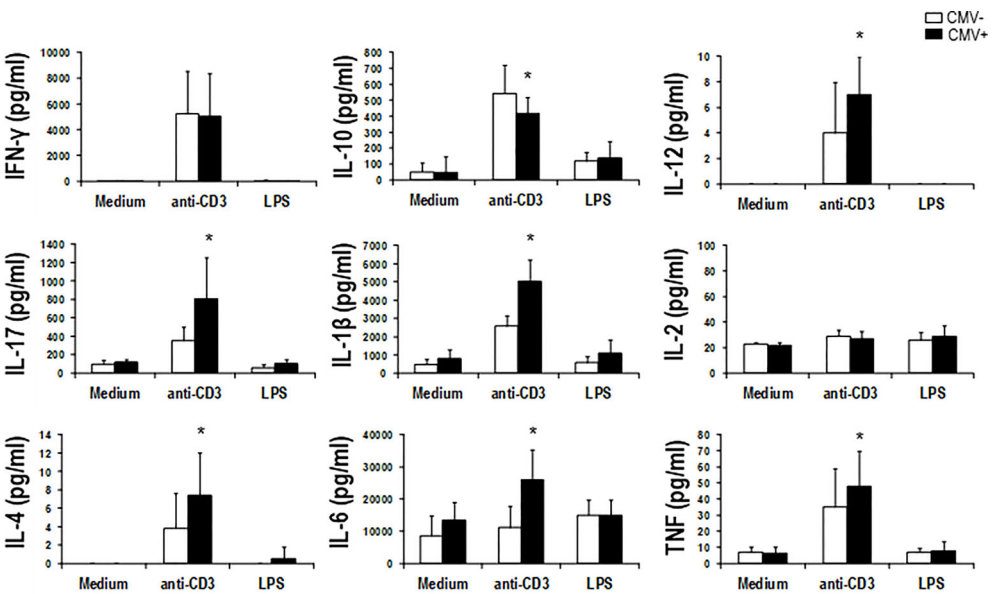

FIGURE 5 | Cytokine levels produced in response to different culture conditions in CHF patients according to their CMV-serostatus. PBMCs from CHF patients (CMV-, n = 7; CMV+, n = 9) were cultured for 5 days in medium alone, in medium containing anti-CD3 (1 μg/ml), or in medium containing LPS (1 μg/ml). In the supernatant of the different cultures, the concentrations of the different cytokines were measured using Luminex multiplex technology and analyzed according their CMV-serostatus. Bar graphs summarize the concentrations of the cytokines in the supernatants of the cultures with the different conditions studied. (means ± SEM) from the studied subjects. Paired t-test was used to compare paired means, and p-values are depicted in the panels. *indicates a significant difference ($p < 0.05$) compared to the other groups.

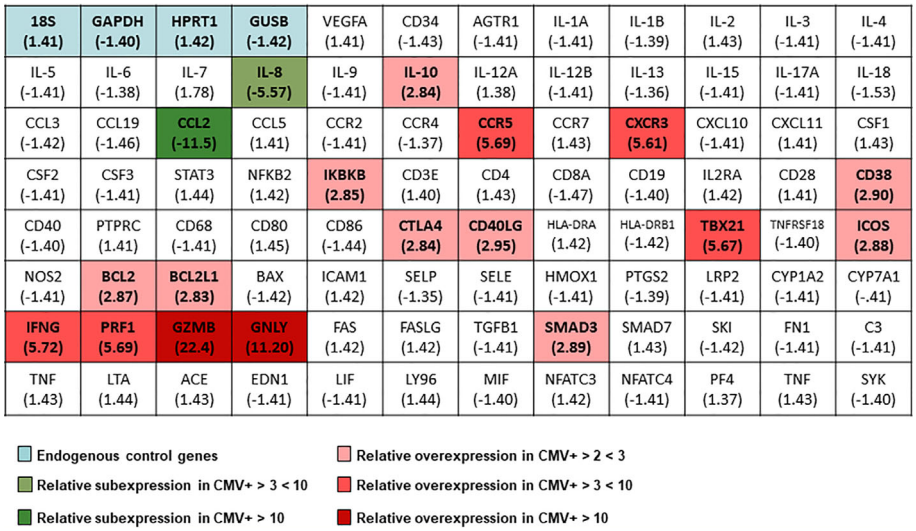

FIGURE 6 | Changes in the gene expression profile in CHF patients divided according to their seropositivity to CMV measured by TaqMan™ Array Human Immune Response. Heat map showing differential expression in patients with CHF CMV+ compared to CMV- patients. Green colors indicate under-expression in CMV+ patients and red tones overexpression in these patients. The blue color is indicating the genes used as endogenous control genes in 5 CHF CMV- and 5 CHF CMV+ patients. We used the comparative ddCT method for calculating relative quantitation of gene expression after outlier removal and data normalization based on the endogenous control genes expression using DataAssist software (Thermo Fisher Scientific).

those with worse functional status. Moreover, not only seropositivity but also serum titers of anti-CMV antibodies are related to a higher level of proinflammatory cytokines such as IL-17, TNF, IL-1β and IL-6. CHF patients may display immunocompromised responses, resulting in an inability in controlling viral reactivations where CMV may be exacerbating T-cell differentiation, being these populations, the main producers of the cytokines analyzed in this study.

CHF is a disease with high morbidity and mortality, despite the treatments that have emerged in recent years, which seems to indicate that the pathogenic mechanisms are not fully controlled by these treatments. Permanent inflammation may be one of these underlying mechanisms unaltered by current treatments (17, 18). After the finding of elevated levels of TNF in sera from patients with CHF, it was seen that other pro-inflammatory cytokines are also elevated (19). Since then, numerous evidences have pointed to the activation of inflammatory pathways as an important pathological event in the onset and progression of the syndrome (20–22). The increased concentration of pro-inflammatory cytokines in the serum of patients with CHF compared to healthy controls is a circumstance that can be caused by some relevant processes in the context of CHF, among others: global aging, metabolic syndrome, chronic obstructive pulmonary disease (COPD), chronic kidney disease (CKD), atrial fibrillation and neurohormonal hypothesis of renin-angiotensin-aldosterone system (23–26). Our results have shown that chronic CMV infection is a main factor related to this inflammatory status.

The relationship between poorer functional status, (measured as NYHA) and the level of pro-inflammatory cytokines, mainly with TNF and IFN-γ, had already been demonstrated in other studies (27–29). This relationship has even been correlated with increased mortality (30) but it had never been demonstrated in relation to CMV infection in CHF, although the role of CMV as a marker of disease severity in acute heart failure had been described and its possible implication in the development and worsening of other cardiac pathologies has been seen (31–33). What has never been proven is the relationship that we have shown between poorer functional status, levels of inflammatory cytokines, and CMV seropositivity. Seropositive patients have a higher concentration of pro-inflammatory cytokines, and the levels of antibodies against CMV are directly correlated with the level of inflammatory cytokines. In view of these results, lowering the levels of pro-inflammatory cytokines in CHF could improve quality of life. In this way, administration of methotrexate, with its anti-inflammatory effects, has demonstrated improving NYHA (34). In view of our results, the possible vaccination against CMV at an early age or once the disease is diagnosed, could also be a strategy to improve functional status in CHF patients (35, 36).

Like the concentration of pro-inflammatory cytokines, we also found elevated concentrations of the two anti-inflammatory cytokines studied, IL-10 and IL-4, contrary to what was found in another study from the 2000s (37). This elevation could be justified in the context of an environment with high concentrations of pro-inflammatory cytokines, in which IL-10 and IL-4 would act as feed-back, trying to reduce these levels of pro-inflammatory cytokines, although with little success, possibly because the mechanisms that are producing these high levels of inflammatory molecules are very powerful and difficult to counteract. IL-10 is one of the most powerful anti-inflammatory cytokines and is involved in various regulatory

actions of the immune and inflammatory systems (38). Some *in vitro* and *in vivo* studies have suggested that IL-10 could be used as a helpful therapeutic agent in the treatment of chronic and acute inflammatory processes, both systemic and localized (39). This cytokine has important suppressive properties in macrophages, T-cells and B cells (40). Both IL-10 and IL-4 have an important regulatory role in the cytokine network, acting as anti-inflammatory regulators in immune reactions in patients with CHF (37, 41). Curiously, we found a discrepancy between the levels detected in serum and the basal expression of mRNA, this could be due to an increased consumption of the cytokine in more inflammatory environments, or even to its blockage with the soluble form of its receptor (38).

The relationship between CMV and the host's immune system is very intimate and produces multiple changes in the lymphocyte compartment. This virus takes advantage of the host's inflammatory response to perpetuate itself and avoid being eliminated (42). In immunocompetent people, CMV is an asymptomatic, latent infection with periodic reactivations, whereas in immunosuppressed patients it usually causes acute pathology (43). The host's inflammatory response is essential for the reactivation of CMV and is very important in stimulating the gene expression of the virus (44). In turn, some of the virus gene products positively regulate the production by the host of a wide variety of pro-inflammatory mediators (IL-1β, IL-6, TNF) (45). Moreover, highly differentiated T-lymphocytes could be being activated by agents implicated in chronic infections, and this activation would lead to increased cytokine production and possible tissue damage. CMV reactivations could be producing this continuous activation of highly differentiated T-lymphocytes, exacerbating cardiac pathology and the defective response of these activated lymphocytes. Immunosuppressed individuals, as is well known, can suffer dire consequences in the context of a CMV reactivation. As is already known in the elderly, CMV reactivations in CHF patients may not give any kind of symptoms and may go completely unnoticed, despite being quite frequent (46). The greater lymphocyte differentiation found in patients with CHF may partly explain the higher production and concentration of pro-inflammatory cytokines in these patients, since the more differentiated lymphocytes are producers of large amounts of inflammatory products (12, 47, 48).

On the other hand, CMV seropositivity has recently been associated with gut damage and microbial translocation, markers of intestinal damage have been associated with IgG levels against CMV in elderly patients and this has been associated with increased inflammation (49). The translocation of microbial products into circulation further contributes to systemic immune activation. Microbial translocation was first described by quantifying levels of the bacterial lipopolysaccharide (LPS) in blood circulation. It has been demonstrated that plasma levels of a fungal cell wall component are also elevated and related to inflammation (50). It would be of great interest to be able to study these parameters in our patients and see if there is any relationship between gut damage or microbial translocation and inflammation and worse functional status.

It has been known for years that the immune response in patients with CHF is shifted towards the Th1 pathway (9) and in our study we have shown that this response is even more exacerbated in CMV+ patients, possibly as a control to the possible reactivations of the virus, with an increased expression of TBX21 and IFN-γ (51). The increased expression of the pro-inflammatory molecules IL-8 and CCL2 can be explained in the context of chronic inflammatory disease, these molecules producing an attraction for monocytes, neutrophils and lymphocytes, involved in the inflammatory state of the pathology. This infection control could also be involved in the increased expression of genes associated with the activation of CD4+ T-lymphocytes, since both CMV surveillance and the continuous presence of pro-inflammatory cytokines in the environment may be continuously activating these lymphocytes. CMV reactivations leads to an increase in CD4+CD28null T-cells (52, 53). One of the main characteristics of this lymphocyte population is the production of cytotoxic molecules, such as perforin and granzyme B (47). In fact, we have found genes involved in cytotoxicity, such as perforin, granzyme B, and granulysin, overexpressed in CD4+ T-lymphocytes. Another characteristic of this CD4+CD28null population is its resistance to apoptosis (54, 55) this is corroborated by the increased expression of the BCL2 and BCL2L1 genes, antiapoptotic molecules, in CHF CMV+ patients. As a whole, we can affirm that CHF CMV+ patients compared with CMV- patients present an expression profile in CD4+ T-lymphocytes Th1 type, more activated and with a highly differentiated and highly reactive CD4+CD28null T population, that is possibly the result of the own fight against CMV infection and the environment, even more pro-inflammatory than in CMV- patients.

In summary, the high levels of pro-inflammatory cytokines found in CHF patients are due, as was already known, to the processes present in the context of CHF but are also, and in a very important way, related to dynamics of CMV-infection, since these high levels of cytokines are related to anti-CMV antibody titers and not only to CMV-infection. The inflammation found and the consequent immunosuppression are probably the main causes of the re-emergences of CMV, and the great lymphocyte differentiation demonstrated in CHF patients. Both characteristics are enhanced in patients with worse functional status, probably due to the negative effects of the chronic inflammation present in these patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Hospital Central de Asturias. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows–RA-A and MM-G: designed the study. AG-T, EB-G, RL-M, BR-B, and CQ: prepared protocols, collected and processed all the samples, performed or oversaw the experimental protocols, and analyzed data. AG-T and RA-A: wrote the manuscript. SA-A, BD-M and JLL: selected, recruited and followed up volunteers. MM-G and RA-A reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.687582/full#supplementary-material

REFERENCES

1. Moro-García MA, Echeverría A, Galán-Artímez MC, Suárez-García FM, Solano-Jaurrieta JJ, Avanzas-Fernández P, et al. Immunosenescence and Inflammation Characterize Chronic Heart Failure Patients With More Advanced Disease. *Int J Cardiol* (2014) 174:590–9. doi: 10.1016/j.ijcard.2014.04.128
2. Hadrup SR, Strindhall J, Køllgaard T, Seremet T, Johansson B, Pawelec G, et al. Longitudinal Studies of Clonally Expanded CD8 T Cells Reveal a Repertoire Shrinkage Predicting Mortality and an Increased Number of Dysfunctional Cytomegalovirus-Specific T Cells in the Very Elderly. *J Immunol* (2006) 176(4):2645–53. doi: 10.4049/jimmunol.176.4.2645
3. Moro-García MA, Alonso-Arias R, López-Larrea C. When Aging Reaches CD4+ T-Cells: Phenotypic and Functional Changes. *Front Immunol* (2013) 4:107. doi: 10.3389/fimmu.2013.00107
4. Mann DL. Stress-Activated Cytokines and The Heart: From Adaptation to Maladaptation. *Annu Rev Physiol* (2003) 65:81–101. doi: 10.1146/annurev.physiol.65.092101.142249
5. Seta Y, Shan K, Bozkurt B, Oral H, Mann DL. Basic Mechanisms in Heart Failure: The Cytokine Hypothesis. *J Card Fail* (1996) 2(3):243–9. doi: 10.1016/S1071-9164(96)80047-9
6. Kallikourdis M, Martini E, Carullo P, Sardi C, Roselli G, Greco CM, et al. T Cell Costimulation Blockade Blunts Pressure Overload-Induced Heart Failure. *Nat Commun* (2017) 8:14680. doi: 10.1038/ncomms14680
7. Markó L, Kvakan H, Park JK, Qadri F, Spallek B, Binger KJ, et al. Interferon-γ Signaling Inhibition Ameliorates Angiotensin II-Induced Cardiac Damage. *Hypertension* (2012) 60(6):1430–6. doi: 10.1161/HYPERTENSIONAHA.112.199265
8. Laroumanie F, Douin-Echinard V, Pozzo J, Lairez O, Tortosa F, Vinel C, et al. CD4+ T Cells Promote the Transition From Hypertrophy to Heart Failure During Chronic Pressure Overload. *Circulation* (2014) 129(21):2111–24. doi: 10.1161/CIRCULATIONAHA.113.007101
9. Nevers T, Salvador AM, Velazquez F, Ngwenyama N, Carrillo-Salinas FJ, Aronovitz M, et al. Th1 Effector T Cells Selectively Orchestrate Cardiac Fibrosis in Nonischemic Heart Failure. *J Exp Med* (2017) 214(11):3311–29. doi: 10.1084/jem.20161791
10. Effros RB, Dagarag M, Spaulding C, Man J. The Role of CD8+ T-Cell Replicative Senescence in Human Aging. *Immunol Rev* (2005) 5(27):293–7. doi: 10.1111/j.0105-2896.2005.00259.x
11. Pawelec G, Akbar A, Caruso C, Solana R. Human Immunosenescence: Is it Infectious? *Immunol Rev* (2005) 205:257–68. doi: 10.1111/j.0105-2896.2005.00271.x
12. Alonso Arias R, Moro-Garcia MA, Echeverria A, Solano-Jaurrieta JJ, Suarez-Garcia FM, Lopez-Larrea C. Intensity of the Humoral Response to Cytomegalovirus Is Associated With the Phenotypic and Functional Status of the Immune System. *J Virol* (2013) 87:4486–95. doi: 10.1128/jvi.02425-12
13. Moro-García MA, Alonso-Arias R, López-Vázquez A, Suárez-García FM, Solano-Jaurrieta JJ, Baltar J, et al. Relationship Between Functional Ability in Older People, Immune System Status, and Intensity of Response to CMV. *Age (Omaha)* (2012) 34:479–95. doi: 10.1007/s11357-011-9240-6
14. Ramosa GC, Van Den Berg A, Nunes-Silva V, Weirather J, Peters L, Burkard M, et al. Myocardial Aging as a T-Cell-Mediated Phenomenon. *Proc Natl Acad Sci USA* (2017) 114(12):E2420–E2429. doi: 10.1073/pnas.1621047114
15. Moro-García MA, López-Iglesias F, Marcos-Fernández R, Bueno-García E, Díaz-Molina B, Lambert JL, et al. More Intensive CMV-Infection in Chronic Heart Failure Patients Contributes to Higher T-Lymphocyte Differentiation Degree. *Clin Immunol* (2018) 192:20–9. doi: 10.1016/j.clim.2018.03.015
16. Schmittgen TD, Livak KJ. Analyzing Real-Time PCR Data by the Comparative CT Method. *Nat Protoc* (2008) 3(6):1101–8. doi: 10.1038/nprot.2008.73
17. Aukrust P, Ueland T, Müller F, Andreassen AK, Nordøy I, Aas H, et al. Elevated Circulating Levels of C-C Chemokines in Patients With Congestive Heart Failure. *Circulation* (1998) 97(12):1136–43. doi: 10.1161/01.CIR.97.12.1136
18. Aukrust P, Ueland T, Lien E, Bendtzen K, Müller F, Andreassen AK, et al. Cytokine Network in Congestive Heart Failure Secondary to Ischemic or Idiopathic Dilated Cardiomyopathy. *Am J Cardiol* (1999) 83(3):376–82. doi: 10.1016/S0002-9149(98)00872-8
19. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated Circulating Levels of Tumor Necrosis Factor in Severe Chronic Heart Failure. *N Engl J Med* (1990) 323(4):236–41. doi: 10.1056/nejm199007263230405
20. Nishida K, Otsu K. Sterile Inflammation and Degradation Systems in Heart Failure. *Circ J* (2017) 81(5):622–628. doi: 10.1253/circj.CJ-17-0261
21. Ueland T, Gullestad L, Nymo SH, Yndestad A, Aukrust P, Askevold ET. Inflammatory Cytokines as Biomarkers in Heart Failure. *Clin Chim Acta* (2015) 443:71–7. doi: 10.1016/j.cca.2014.09.001
22. Hartupee J, Mann DL. Positioning of Inflammatory Biomarkers in the Heart Failure Landscape. *J Cardiovasc Transl Res* (2013) 6(4):485–92. doi: 10.1007/s12265-013-9467-y
23. Mocan M, Hognogi LDM, Anton FP, Chiorescu RM, Goidescu CM, Stoia MA, et al. Biomarkers of Inflammation in Left Ventricular Diastolic Dysfunction. *Dis Markers* (2019) 2019:7583690. doi: 10.1155/2019/7583690
24. Wu CK, Yang CY, Lin JW, Hsieh HJ, Chiu FC, Chen JJ, et al. The Relationship Among Central Obesity, Systemic Inflammation, and Left Ventricular Diastolic Dysfunction as Determined by Structural Equation Modeling. *Obesity* (2012) 20(4):730–7. doi: 10.1038/oby.2011.30
25. ter Maaten JM, Damman K, Verhaar MC, Paulus WJ, Duncker DJ, Cheng C, et al. Connecting Heart Failure With Preserved Ejection Fraction and Renal Dysfunction: The Role of Endothelial Dysfunction and Inflammation. *Eur J Heart Fail* (2016) 18(6):588–98. doi: 10.1002/ejhf.497
26. Sartipy U, Savarese G, Dahlström U, Fu M, Lund LH. Association of Heart Rate With Mortality in Sinus Rhythm and Atrial Fibrillation in Heart Failure With Preserved Ejection Fraction. *Eur J Heart Fail* (2019) 21(4):471–9. doi: 10.1002/ejhf.1389
27. Liu W, Ru L, Su C, Qi S, Qi X. Serum Levels of Inflammatory Cytokines and Expression of BCL2 and BAX mRNA in Peripheral Blood Mononuclear Cells

and in Patients With Chronic Heart Failure. *Med Sci Monit* (2019) 25:2633–9. doi: 10.12659/MSM.912457
28. Raymond RJ, Dehmer GJ, Theoharides TC, Deliargyris EN. Elevated Interleukin-6 Levels in Patients With Asymptomatic Left Ventricular Systolic Dysfunction. *Am Heart J* (2001) 141(3):435–8. doi: 10.1067/mhj.2001.113078
29. Eskandari V, Amirzargar AA, Mahmoudi MJ, Rahnemoon Z, Rahmani F, Sadati S, et al. Gene Expression and Levels of IL-6 and Tnfα in PBMCs Correlate With Severity and Functional Class in Patients With Chronic Heart Failure. *Ir J Med Sci* (2018) 187(2):359–68. doi: 10.1007/s11845-017-1680-2
30. Rauchhaus M, Doehner W, Francis DP, Davos C, Kemp M, Liebenthal C, et al. Plasma Cytokine Parameters and Mortality in Patients With Chronic Heart Failure. *Circulation* (2000) 102(25):3060–7. doi: 10.1161/01.CIR.102.25.3060
31. Núñez J, Chilet M, Sanchis J, Bodí V, Núñez E, Miñana G, et al. Prevalence and Prognostic Implications of Active Cytomegalovirus Infection in Patients With Acute Heart Failure. *Clin Sci* (2010) 119(10):443–52. doi: 10.1042/CS20100162
32. Vasilieva E, Gianella S, Freeman ML. Novel Strategies to Combat CMV-Related Cardiovascular Disease. *Pathog Immun* (2020) 5(1):240–74. doi: 10.20411/pai.v5i1.382
33. Li D, Li B, Yang L, Wang J. Human Cytomegalovirus Infection is Correlated With Atherosclerotic Plaque Vulnerability in Carotid Artery. *J Gene Med* (2020) 22(10):e3236. doi: 10.1002/jgm.3236
34. Gong K, Zhang Z, Sun X, Zhang X, Li A, Yan J, et al. The Nonspecific Anti-Inflammatory Therapy With Methotrexate for Patients With Chronic Heart Failure. *Am Heart J* (2006) 151(5):e5. doi: 10.1016/j.ahj.2005.02.040
35. Sommerer C, Schmitt A, Hückelhoven-Krauss A, Giese T, Bruckner T, Wang L, et al. Peptide Vaccination Against Cytomegalovirus Induces Specific T Cell Response in Responses in CMV Seronegative End-Stage Renal Disease Patients. *Vaccines* (2021) 9(2):133. doi: 10.3390/vaccines9020133
36. Gerna G, Lilleri D. Human Cytomegalovirus (HCMV) Infection/Re-Infection: Development of a Protective HCMV Vaccine. *New Microbiol* (2019) 42(1):1–20.
37. Stumpf C, Lehner C, Yilmaz A, Daniel WG, Garlichs CD. Decrease of Serum Levels of the Anti-Inflammatory Cytokine Interleukin-10 in Patients With Advanced Chronic Heart Failure. *Clin Sci* (2003) 105(1):45–50. doi: 10.1042/CS20020359
38. Moore KW, De Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the Interleukin-10 Receptor. *Annu Rev Immunol* (2001) 19:683–765. doi: 10.1146/annurev.immunol.19.1.683
39. Schulze-Tanzil G, Zreiqat H, Sabat R, Kohl B, Halder A, Muller R, et al. Interleukin-10 and Articular Cartilage: Experimental Therapeutical Approaches in Cartilage Disorders. *Curr Gene Ther* (2009) 9(4):306–15. doi: 10.2174/156652309788921044
40. Moore KW, O'Garra A, De Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol* (1993) 11:165–90. doi: 10.1146/annurev.iy.11.040193.001121
41. Yamaoka M, Yamaguchi S, Okuyama M, Tomoike H. Anti-Inflammatory Cytokine Profile in Human Heart Failure: Behavior of Interleukin-10 in Association With Tumor Necrosis Factor-Alpha. *Jpn Circ J* (1999) 63(12):951–6. doi: 10.1253/jcj.63.951
42. Stern-Ginossar N, Weisburd B, Michalski A, Le VTK, Hein MY, Huang SX, et al. Decoding Human Cytomegalovirus. *Science* (2012) 338(6110):1088–93. doi: 10.1126/science.1227919
43. Goodrum F. Human Cytomegalovirus Latency: Approaching the Gordian Knot. *Annu Rev Virol* (2016) 3(1):333–57. doi: 10.1146/annurev-virology-110615-042422
44. Söderberg-Nauclér C, Nelson JA. Human Cytomegalovirus Latency and Reactivation - A Delicate Balance Between the Virus and its Host's Immune System. *Intervirology* (1999) 42(5–6):314–21. doi: 10.1159/000053966
45. Mogensen TH, Paludan SR. Molecular Pathways in Virus-Induced Cytokine Production. *Microbiol Mol Biol Rev* (2001) 65(1):131–50. doi: 10.1128/mmbr.65.1.131-150.2001
46. Stowe RP, Kozlova EV, Yetman DL, Walling DM, Goodwin JS, Glaser R. Chronic Herpesvirus Reactivation Occurs in Aging. *Exp Gerontol* (2007) 42(6):563–70. doi: 10.1016/j.exger.2007.01.005
47. Alonso-Arias R, Moro-García MA, Vidal-Castiñeira JR, Solano-Jaurrieta JJ, Suárez-García FM, Coto E, et al. IL-15 Preferentially Enhances Functional Properties and Antigen-Specific Responses of CD4+CD28 Null Compared to CD4+CD28+ T Cells. *Aging Cell* (2011) 10:844–52. doi: 10.1111/j.1474-9726.2011.00725.x
48. Echeverría A, Moro-García MA, Asensi V, Cartón JA, López-Larrea C, Alonso-Arias R. CD4 + CD28 Null T Lymphocytes Resemble CD8 + CD28 Null T Lymphocytes in Their Responses to IL-15 and IL-21 in HIV-Infected Patients. *J Leukoc Biol* (2015) 98:373–84. doi: 10.1189/jlb.1a0514-276rr
49. Ramendra R, Isnard S, Lin J, Fombuena B, Ouyang J, Mehraj V, et al. Cytomegalovirus Seropositivity Is Associated With Increased Microbial Translocation in People Living With Human Immunodeficiency Virus and Uninfected Controls. *Clin Infect Dis* (2020) 71(6):1438–46. doi: 10.1093/cid/ciz1001
50. Isnard S, Fombuena B, Sadouni M, Lin J, Richard C, Routy B, et al. Circulating β-D-Glucan as a Marker of Subclinical Coronary Plaque in ART-Treated People Living With HIV. *Open Forum Infect Dis* (2021) 8(6):ofab109. doi: 10.1093/ofid/ofab109
51. Van De Berg PJ, Heutinck KM, Raabe R, Minnee RC, Young S, Van Donselaar-Van Der Pant KA, et al. Human Cytomegalovirus Induces Systemic Immune Activation Characterized by a Type 1 Cytokine Signature. *J Infect Dis* (2010) 202(5):690–9. doi: 10.1086/655472
52. Broadley I, Pera A, Morrow G, Davies KA, Kern F. Expansions of Cytotoxic CD4+CD28- T Cells Drive Excess Cardiovascular Mortality in Rheumatoid Arthritis and Other Chronic Inflammatory Conditions and Are Triggered by CMV Infection. *Front Immunol* (2017) 8:195. doi: 10.3389/fimmu.2017.00195
53. Slot MC, Kroon AA, Damoiseaux JGMC, Theunissen R, Houben AJHM, de Leeuw PW, et al. CD4+CD28null T Cells Are Related to Previous Cytomegalovirus Infection But Not to Accelerated Atherosclerosis in ANCA-Associated Vasculitis. *Rheumatol Int* (2017) 37(5):791–8. doi: 10.1007/s00296-016-3643-8
54. Kovalcsik E, Antunes RF, Baruah P, Kaski JC, Dumitriu IE. Proteasome-Mediated Reduction in Proapoptotic Molecule Bim Renders CD4+CD28null T Cells Resistant to Apoptosis in Acute Coronary Syndrome. *Circulation* (2015) 131(8):709–20. doi: 10.1161/CIRCULATIONAHA.114.013710
55. Vallejo AN, Schirmer M, Weyand CM, Goronzy JJ. Clonality and Longevity of CD4 + CD28 Null T Cells Are Associated With Defects in Apoptotic Pathways. *J Immunol* (2000) 165(11):6301–7. doi: 10.4049/jimmunol.165.11.6301

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In-Depth Profiling of T-Cell Responsiveness to Commonly Recognized CMV Antigens in Older People Reveals Important Sex Differences

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The impact of biological sex on T-cell immunity to Cytomegalovirus (CMV) has not been investigated in detail with only one published study comparing CMV-specific T-cell responses in men and women. Many studies, however, have shown an association between CMV infection and immunosenescence, with broad effects on peripheral blood lymphocyte subsets as well as the T and B-cell repertoires. Here, we provide a detailed analysis of CMV-specific T-cell responses in (n=94) CMV+ older people, including 47 women and 47 men aged between 60 and 93 years. We explore sex differences with respect to 16 different CMV proteins arranged in 14 peptide pools (overlapping peptides). Following ex vivo stimulation, CD4 and CD8 T-cells producing IFN-γ, TNF, and IL-2 were enumerated by flow-cytometry (intracellular cytokine staining). T-cell responses were evaluated in terms of each cytokine separately or in terms of cytokines produced simultaneously (polyfunctionality). Surface memory phenotype and CD3 downmodulation were assessed in parallel. The polyfunctionality index and a memory subset differentiation score were used to identify associations between response size, cytokine production, polyfunctionality, and memory subset distribution. While no significant sex differences were found with respect to overall CMV target protein selection, the T-cell response in men appeared more focused and accompanied by a more prominent accumulation of CMV-specific memory CD4 and CD8 T-cells. T-cell polyfunctionality and differentiation were similar in the sexes, however, CMV-specific T-cells in men produced more pro-inflammatory cytokines. Particularly, TNF production by CD4 T-cells was stronger in men than in women. Also, compared with women, men had larger responses to CMV proteins with immediate-early/early kinetics than women, which might have been driven by CMV reactivation. In conclusion, the CMV-specific T-cell response in men was larger and more

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pro-inflammatory than in women. Our findings may help explain sex differences in CMV-associated pathologies.

Keywords: aging, T cell, Cytomegalovirus (CMV), immunosenescence, biological sex

INTRODUCTION

Cytomegalovirus (CMV) infection has a major impact on the immune system, in particular on the distribution of lymphocyte subsets (1, 2). CMV prevalence increases with age, which also has a profound effect on the immune system by reducing its ability to respond to infection and cancer (immunosenescence). This CMV prevalence results in increased morbidity and mortality in older people. Biological sex appears to modify both the effect of CMV infection and aging (3–6). It is important to note that immunosenescence also occurs in the absence of CMV infection, however, changes resulting from CMV infection may accelerate the effects of immunosenescence.

A role of CMV infection in immunosenescence has been supported by numerous publications since the early 2000s. Loss of the costimulatory molecule CD28 on CD4 T-cells (generating 'CD28^{null} CD4 T-cells') has been recognized as a hallmark of immune ageing since 1999 (7) and for just as long, it has been clear that CMV is associated with its occurrence (8). This culminated in the hypothesis that immunosenescence may be 'infectious' (9). Our own recent work (10) has clarified that CD28^{null} CD4 T-cells do not increase with age among CMV-seronegative (CMV-) people showing that accumulation of these cells is not related to aging *per se*. So, aging and immune aging are not the same thing, they just coincide in older people. CD28^{null} CD4 T-cells clearly have features of 'aged' immune cells (e.g., replicative senescence) and the immune system of people with expanded CD28^{null} CD4 T-cells would, therefore, appear more aged. Since older people tend to be more frequently CMV-seropositive (CMV+) than younger individuals, the effects of aging and CMV cannot be easily differentiated in the elderly. CMV prevalence can be up to 90% or more in many parts of the world (11).

CMV has been implicated in memory T-cell inflation, in particular in mouse models (12), and in a number of conditions associated with changes to the peripheral blood T-cell compartment, including autoimmune disease, atherosclerosis, and cardiovascular disease (CVD). CVD is not only more common in older ages, but also shows considerable sex bias (13, 14). Our own recent work demonstrated an association of CMV infection with increased aortic stiffness in healthy, White British older males (15). Although confirmation of these findings in populations of different genetic backgrounds and social circumstances is pending, our findings suggest a potentially very important effect of biological sex on CMV-induced immunopathology. However, despite some inroads, detailed knowledge of sex-related differences in CMV-associated immunity and immunopathology is lacking. A number of studies have established that CMV infection biases the differentiation of the overall T-cell repertoire (2, 3, 6), however, it is still unclear to what extent CMV-specific T-cells may account for this effect. The Berlin Aging Study II (BASE-II) showed that both age and CMV infection drive T-cell differentiation, in both the CD4 and CD8 compartments, with men accumulating higher frequencies of terminally differentiated CD57+ CD8 T-cells. These cells are strongly associated with CMV infection and have high cytotoxic potential (3). Overall, older people had significantly higher proportions of late-differentiated TEMRA cells (T effector memory cells re-expressing CD45RA) among both men and women, but, interestingly, these were exclusively accounted for by CMV+ subjects. Our own recent study on vascular stiffness also suggested a stronger effect of CMV on memory T-cell differentiation in men than in women, which could be one possible explanation for increased vascular stiffness in CMV+ older men (15).

To our knowledge, the only published study on sex differences with respect to CMV-antigen specific T-cell responses dates back to 2004 (16). It showed that the *in vitro* CD4 T-cell response to a CMV virus lysate was dominated by IFN-γ and that women exhibited higher levels of IL-2, IL-2-secreting cells, and proliferation than men, suggesting that women react more strongly to CMV than men. Since T-cells are the mainstay of CMV-specific immunity, it is surprising that such differences have not been explored in more detail since. In this study, we use overlapping peptide pools for T-cell stimulation rather than a virus lysate. This has a number of advantages, not least the fact that both CD4 and CD8 T-cells can be stimulated efficiently (17, 18). A landmark study on CMV-specific T-cell immunity published in 2005 showed that this approach is very powerful (19). In that study CD4 and CD8 T-cell responses to 213 different CMV proteins (ORFs) were tested in 33 adults of different ages and biogeographical ancestries. This historic dataset was later reanalyzed to gain insight into the variability of CMV-specific T-cell immunity in different individuals (20), but the cohort was too small to robustly address sex differences. Our current dataset, however, is ideally suited for this purpose; the population of n=94 individuals of at least 60 years of age is evenly split into women and men, which ensures not only that the effects of CMV on the immune system are visible in most participants, but also that comparisons between the sexes are statistically robust. Our work focuses on the most important CMV target proteins in order to highlight patterns of responsiveness, including response size, differentiation, (poly-)functionality, and efficiency of activation (CD3 downmodulation). This study is the first ever to show a range of significant sex differences in CMV-specific T-cell immunity that will help explain different immunopathologies in women and men following CMV infection.

MATERIALS AND METHODS

Ethics Statement

Study approved by the UK National Research Ethics Service (NRES) London Centre (Reference 13/LO/1270). Written informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki.

Participants and Samples

Generally healthy (n=94), CMV+ older volunteers (60-94 years) were recruited through general practices (GP) in Southern England with help of the primary care research network (PCRN). Individuals with previous vascular events, such as TIA, stroke or CV complications were included if generally well with normal physical activity. However, individuals with advanced CV morbidity were excluded.

Inclusion criteria were: White British ethnicity and age 60 years or older; exclusion criteria were: known immunodeficiency (including HIV infection), organ transplantation, use of immunosuppressive or immune-modulating drugs within the last year (excluding acetylsalicylic acid ≤ 100mg/day), cancer or treatment for cancer within the previous 5 years, insulin dependent diabetes, moderate or advanced renal failure, liver disease, endocrine disorders (except corrected thyroid dysfunction), manifest autoimmune disease, dementia/mental incompetence, known alcoholism or other drug abuse, acute infection or illness in the last 4 weeks, raised body temperature (>37.5°C), moderate or severe heart failure (NYHA III or IV), inability to lie flat. Individuals with typical, age-related cardiovascular morbidity were not excluded.

Participant Data and Sample Collection

Peripheral blood from each subject was collected by venipuncture in sodium Heparin-containing tubes. PBMCs were isolated after collection using Ficoll-Hypaque density gradient centrifugation (Sigma Aldrich, Steinheim, Germany).

CMV Serology

CMV immunoglobulin G (IgG) serology (Architect CMV IgG, Abbot, Maidenhead, UK) was performed at the Brighton and Sussex University Hospital Trust (BSUHT) virology laboratory.

CMV Peptides

Overlapping peptide pools representing 16 CMV proteins arranged in 14 pools (**Table 1**) were used in T-cell stimulation assays [for details see **Supplementary Material** and (15)].

CMV Reactivity of T-Cells

PBMCs were stimulated overnight (16h) with CMV peptide pools. The following morning cells, were stained and acquired by flow-cytometry. Phenotype markers included CD3, CD4, CD8, CD45RA and CCR7 (surface staining). Activation markers included IL-2, TNF, and IFN-γ (ICS). The gating strategy is shown in **Figure S1**. Responses were considered positive when a clustered population of activated cells was identified (visual inspection) above 0.01% of CD4 or CD8 T-cells after background subtraction from the unstimulated sample. For more details, see **Supplementary Material** and (15).

T-Cell Counts

T-cell counts per nanoliter (nL) of blood were determined using a dual platform approach. White blood counts were obtained from the routine clinical laboratory using a Sysmex counter (Sysmex, UK). Using fresh whole blood, CD45+ cells (white blood cells) were gated as well as CD4 and CD8 T-cells (using CD3, CD4, and CD8). CD4 and CD8 T-cells per nL of blood were determined in two steps. First, the proportion of CD4 and CD8 T-cells among WBC was determined by dividing the CD4 and CD8 T-cell counts in the CD45+ gate (absolute event count) by the CD45+ gate count (absolute event count). This proportion was then multiplied with the WBC. Absolute CD4 and CD8 T-cell counts were available in 47 men (100%) and 42 women (89.4%).

TABLE 1 | CMV protein-covering peptide-pools used for T-cell stimulation[a].

Pool	CMV Protein(s)	Kinetic class[c] (21)	No. of Peptides
1	none	n.a.	n.a.
2	UL55	L	224
3	UL83 ('pp65')	L	138
4	UL86	E-L	340
5	UL122 ('IE2')	IE (L)	120
6	UL123 ('IE1')	IE	143
7	UL153	L	67
8	UL32	L	260
9	UL28	L	92
10	UL48A[b]	L	281
11	UL48B[b]	L	281
12	US3	IE	44
13	UL151& UL82	unclassified & L	219 (82 &137)
14	UL94 & US29	L&E-L	197 (84 &113)
15	US24 & UL36	E&E(IE)	240 (123 &117)
16	SEB (positive control)		

[a]A panel of 19 CMV protein-spanning peptide pools was previously shown to correlate highly with the CD4 and CD8 T-cell response against 203 tested CMV proteins (19). The original panel contained UL99, UL103, and US32 in addition, but were left out here since responses were absent in >100 White British people. [b]UL48 was divided into two pools (UL48A and UL48B), however, results were combined with respect to determining T-cell reactivity. [c]IE, immediate early; E, early; L, late; n.a., not applicable.

Data Processing

The raw data obtained from Flow Cytometry (FlowJo v9.6) was processed by a php script running PHP Version 7.1.33. The data processing involves the computation of percentage counts for each patient and tube, background subtraction, sums of subset counts for each patient (and type of subset), and the production of FunkyCells compatible data files, containing percentages for each T-cell memory subset.

HLA Typing

HLA-typing was performed at the Institute for Transfusion Medicine, Hannover Medical School, Hannover/Germany [see (10)].

Polyfunctionality Index and Differentiation Score

Polyfunctionality analysis was performed using FunkyCells software (www.Funkycells.com). The polyfunctionality index was described previously (22). Of note, here we performed the analysis on antigen-specific T-cells by gating on cells secreting at least one cytokine.

$$\text{Polyfunctionality Index } = \Sigma_{i=0}^{k} F_i * \frac{i}{k},$$

where k is the total number of functions studied, F_i is the frequency (%) of cells performing i functions.

The differentiation profiles of T-cells were based on CD45RA and CCR7 expression. The four differentiation states were identified as Naive (i=0, CD45RA+CCR7+), Central Memory (i=1, CD45RA-CCR7+), Effector Memory (i=2, CD45RA-CCR7+) and Revertant (i=3, CD45RA+CCR7-).

$$\text{Differentiation Score } = \Sigma_{i=0}^{3} \% \, Subset * \frac{i}{3}$$

Statistical Analysis

Sidak's (biological sex) and Tukey's (CMV proteins) multiple comparison correction were applied for multiple end-point correction with respect to gender and protein-specific T-cell reactivity, and gender and protein-specific CD3 downregulation in 2-way ANOVA analyses.

Charts

The 3D bar charts were created by a Python program running Python version 3.3.7. The following python libraries were used: numpy, pandas, matplotlib, math, and scipy.stats. Scatter pie charts were generated using R (version 3.6.2) (23) and the packages ggplot2 (24) and scatterpie (25).

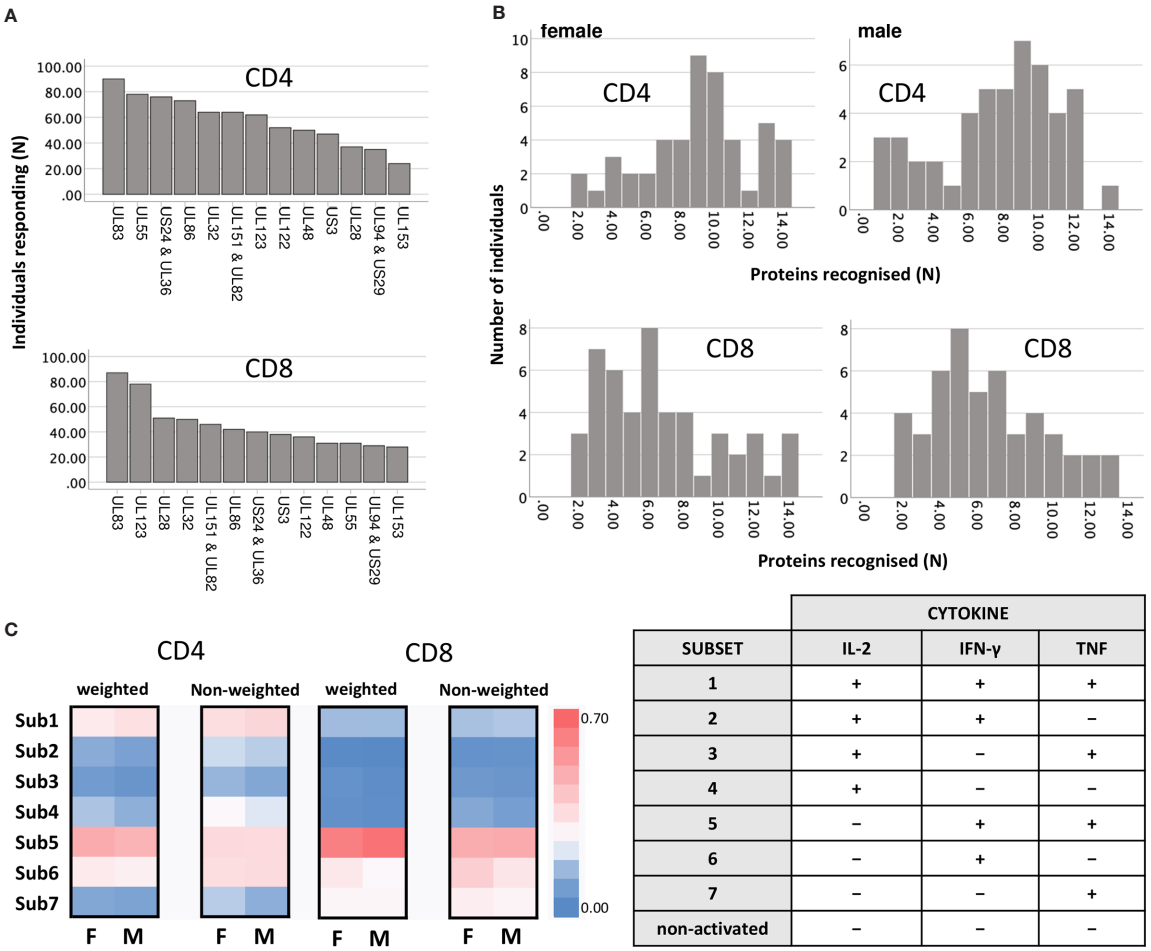
RESULTS

Global Distribution of CMV Protein Reactivity Does Not Differ Between Males and Females

We recently recruited a cohort of 108 CMV seropositive and 101 CMV seronegative individuals between 60 and 93 years of age (10, 15). The present analysis comprises 94 CMV+ participants, in whom T-cell responses to CMV were measured including 47 women and 47 men of very similar age distribution (70.7 ± 6.5 years and 71.1 ± 8.2 years, respectively, mean ± SD). For testing T-cell reactivity to CMV, we used overlapping peptide pools covering 16 CMV proteins arranged in 14 stimulation pools (**Table 1**). The 16 proteins were selected from a group of 19 proteins previously identified to correlate highly with the T-cell response to all CMV proteins (19), but three proteins previously not inducing a single response in >100 White British individuals were omitted (26). Note that the selected proteins cover different kinetic classes (**Table 1**), which are being considered as part of the analysis. The division of proteins by kinetic classes accounts for the fact that different CMV proteins occur at different times after CMV reactivation. More precisely, those detectable at the earliest times are referred to as 'immediate early' (IE) and those that appear last are part of the virion at late (L) times. Some proteins appear in between, i.e., at early (E) and early-late (E-L) times (21, 27).

For the representation of protein-specific reactivity, the responses to two pools including UL48 peptides (UL48A and UL48B) were combined (**Figure 1**). We first analyzed CD4 and CD8 T-cell responses to each pool with respect to the three cytokines measured (IL-2, TNF, IFN-γ). This was done cytokine by cytokine and in combination. (**Figure S1**, gating strategy). Logical combination of two possible states for each cytokine (i.e., IL-2+ or IL-2-, TNF+ or TNF-, IFN-γ+ or IFN-γ-) resulted in seven non-overlapping (Boolean) subsets of cells with one or more positive markers (**Figure 1C**, 1-7 hereafter), and one non-activated all-negative subset (IL-2-/TNF-/IFN-γ-). Individuals were considered 'reactive' to a pool if the percentage of activated events was equal to or larger than 0.01% (1/10,000) within CD4 and CD8 T-cells. After ranking the responses according to the number of individuals reacting to each CMV protein pool, the top five CD4 T-cell targets (UL83, UL55, US24 & UL36, UL86 and UL32) and top two CD8 T-cell targets (UL83 and UL123) were the same as in our previous study (28), but the order deviated slightly for the less dominant proteins (**Figure 1A**). The number and frequency distribution of protein target-specific T-cell responses were very similar in the two sexes (**Figure 1B**). For CD4 T-cells the median number of recognized proteins was 9 (IQR 7-11) in women and 8 (IQR 6-10) in men, the difference being borderline significant (p=0.082), for CD8 T-cells it was 6 (IQR 4-9) in both groups.

Theoretically, CMV protein recognition by T-cells should be affected by HLA-type. Peptides arising from a given CMV target protein will bind to some, but not all HLA-alleles. Individuals possessing the right alleles are, therefore, more likely to respond to these peptides than other individuals. All the participants of this study were White British and no significant difference between men and women in terms of target protein recognition was observed (**Figure 1B**). Regarding protein-specific response size, we observed a statistically significant difference between men and women in the size of the CD8 T-cell response to UL123 (IE-1) and UL153 proteins ($p \leq 0.05/14$, i.e., $p \leq 0.0036$, Bonferroni correction for 14 endpoints), which could have been caused by differences in the



SUBSET	CYTOKINE		
	IL-2	IFN-γ	TNF
1	+	+	+
2	+	+	–
3	+	–	+
4	+	–	–
5	–	+	+
6	–	+	–
7	–	–	+
non-activated	–	–	–

FIGURE 1 | Reactivity of CD4 and CD8 T-cells against dominant CMV proteins. **(A)** The indicated proteins (overlapping peptide pools) were used for PBMC stimulation. Responses (IL-2, TNF, or IFN-γ) equal to or higher than 0.01% (1/10,000) of the reference population (CD4 or CD8) were included. The graph shows the number of individuals recognising the indicated CMV protein pools within the entire cohort (n=94). **(B)** The graph shows the number of recognised proteins per individual (x-axis) in females (right panels, n=47) and males (left panels, n=47), respectively. **(C)** Heatmaps show the frequency distribution of functional Subsets 1 to 7 (respectively: IL-2+/TNF+/IFN-γ+; IL-2+/TNF+/IFN-γ-; IL-2+/TNF-/IFN-γ+; IL-2+/TNF-/IFN-γ-; IL-2-/TNF+/IFN-γ+; IL-2-/TNF+/IFN-γ-; and IL-2-/TNF-/IFN-γ+, right table) within CD4 and CD8 T-cells, in females and in males. The heatmaps on the left show the contribution of each subset after weighing this relatively to the overall response size, while the heatmaps on the right show the unweighted contribution (not normalised for the response size).

frequencies of one or more class-I HLA-alleles. HLA-typing (2-field-code) was available for only 28 women and 25 men. The difference with respect to these protein-specific responses, however, was not significant among the HLA-typed individuals, so that a possible effect of HLA-type on these responses could not be explored. As would be expected in a group of this size (given the vast polymorphism of the HLA-locus), HLA-alleles were not distributed completely evenly between women and men. Among the 53 HLA-typed participants, a total of 109 class-I and class-II HLA-alleles were present. Using the CHI^2 test (Fisher's exact test), we identified one single (class-II) HLA allele that was distributed significantly differently between the sexes at the $p \leq 0.05$ level (HLA-DQB1*03:01, p=0.02, CHI^2 test). Multiple end-point correction, however, would reduce the significance level to $p \leq 0.05/109$, i.e., $p \leq 0.00046$. No allele difference between the sexes was significant at that level. On the whole, the number of HLA-typed individuals was too small to allow a rigorous analysis of the effect of HLA-type on protein-specific T-cell response sizes in women and men.

Next, the distribution of functional subsets 1 to 7 across all CMV proteins by size (**Figure 1C**) was determined either as weighted (contribution is proportional to protein-specific response sizes) or unweighted (all responses make the same contribution). No significant sex differences in functional subset distribution were detected at this level.

Among Men, Response Profiles Are More Focused on Fewer Dominant Responses Than in Women

To explore potential sex differences, we conducted an in-depth analysis of T-cell responsiveness to a range of known and dominant target proteins (**Table 1**) using *ex vivo* stimulation

followed by intracellular cytokine detection. First, we investigated whether the overall CMV-specific T-cell response size differed between the sexes. This was achieved by summing up all CMV protein-specific responses (i) with respect to each cytokine separately (IL-2, TNF and IFN-γ) and (ii) all cytokines in combination. Although men generally showed higher (median) responses, no statistically significant sex differences were detected with respect to the overall CD4 T-cell response. However, CD8 T-cell responses measured in percent of CD8 T-cells were significantly bigger in men than in women with respect to TNF, IFN-γ, or 'any' activation marker. This was also true (and included IL-2) when considering T-cell counts per volume of blood (**Table 2**). Note that for the purpose of analyzing T-cell response size, we mostly rely on subset percentages in terms of CD4 and CD8 T-cells, which agrees with our own previous work and most reports in the literature. The percentage reflects the degree of commitment of the overall resource (CD4 and CD8 T-cells) to certain phenotypic/functional subsets, making them amenable to comparison. We have, however, also performed analyses in terms of cell counts per volume of blood. Overall, the two measures show a very high correlation (**Figure S2** and **Table S1**).

We then investigated the question whether the size of the summated CMV-specific T-cell response correlated with the number of recognized CMV proteins in each individual. There was a weak, but statistically significant correlation among CD4 (R=0.258, p=0.012), but not CD8 T-cells (**Table S2**). The lack of a stronger correlation between these parameters reflected that the contribution of individual CMV protein-specific responses to overall response size was clearly highly variable (**Figures S3**, **S4**). The UL83 and UL55-specific responses among CD4 T-cells were significantly bigger than other responses. IL-2+ CD4 T-cell responses were dominated by responses to the late (L) structural target proteins, UL55, UL83, and UL86 (**Figure S3**). However, in regards to TNF and IFN-γ responses, the immediate early (IE) protein-specific responses also played a major role. US3 was slightly less frequently recognized than UL123 (IE-1) and UL122 (IE-2) (**Figure 1A**), yet clearly induced bigger CD4 T-cell responses (**Figure S3**). As previously reported (26, 28), the CD8 T-cell response was dominated by UL83 and UL123 (**Figure 1A** and **Figure S3**). Of note, we found that CD8 T-cell responses to UL28 were very large in some individuals, but more variable than responses to UL83 and UL123. Essentially, the same patterns were revealed when using cell count per volume of blood (cells/nL) instead of percentages of CD4 or CD8 T-cells (**Figure S4**).

We subsequently explored which proteins showed the biggest sex differences in response magnitude and if there were sex differences in regards to protein immunodominance (**Figure 2**). Overall, there was a clearly visible trend towards higher TNF and IFN-γ responses in men (**Figure 2**, heatmaps) both among CD4 and CD8 T-cells. However, using stringent multiple endpoint correction, only the difference in the size of the IFN-γ CD4 T-cell response to the UL86 protein was statistically significant. US3-specific CD4 T-cell responses were four to five times bigger in men than in women, but the difference did not reach statistical significance (**Figure 2**).

Interestingly, the CD4 T-cell response to UL55 differed significantly in size from responses to multiple other CMV proteins in men (blue) but not women (orange), underscoring the dominance of UL55 as CD4 T-cell target, particularly in men (**Figure 2**). In particular, with respect to TNF+ and IFN-γ+ CD4 T-cells, UL55-specific responses were significantly higher than responses to nine other proteins, but only in men. Also, UL123 CD8 T-cell responses were significantly higher than three other responses in men, but not in women. Together, these data suggest that protein recognition is more focused on dominant proteins in men than in women, with UL55, UL86 and US3-specific CD4 T-cell responses and UL123-specific CD8 T-cell responses contributing most to the overall larger summated response sizes.

We subsequently sought to investigate cytokine dominance in CMV-specific T-cell responses in the two sexes. In the entire cohort and with respect to each peptide pool, TNF and IFN-γ CD4 T-cell responses were on average higher than IL-2 responses (p<0.0001 for each comparison), and TNF responses were bigger than IFN-γ responses (Wilcoxon test, related samples, p<0.001). For CD8 T-cells, both TNF and IFN-γ responses were bigger

TABLE 2 | Overall CMV-specific response size in men and women.

Parameter	All Median (IQR)	Women Median (IQR)	Men Median (IQR)	Women *vs* Men p^a
CD4 IL-2 (%)	0.65 (1.34)	0.60 (0.97)	1.21 (1.73)	0.069
CD4 TNF (%)	2.42 (3.61)	2.28 (2.34)	3.08 (5.86)	0.192
CD4 IFN-γ (%)	2.17 (2.78)	2.06 (2.27)	2.23 (5.39)	0.268
CD4 (any) (%)	2.84 (3.81)	2.59 (2.53)	3.27 (6.43)	0.143
CD8 IL-2 (%)	0.29 (0.52)	0.20 (0.56)	0.35 (0.63)	0.128
CD8 TNF (%)	5.55 (6.64)	3.82 (5.90)	7.26 (7.91)	**0.008**
CD8 IFN-γ (%)	5.63 (7.26)	3.68 (6.56)	7.29 (7.22)	**0.004**
CD8 (any) (%)	6.53 (8.61)	4.10 (7.55)	8.61 (9.21)	**0.008**
CD4 IL-2 (cells/nl)	0.50 (0.98)	0.45 (0.59)	0.68 (1.11)	0.324
CD4 TNF (cells/nL)	1.74 (2.54)	1.83 (1.85)	1.70 (3.27)	0.593
CD4 IFN-γ (cells/nL)	1.29 (2.04)	1.53 (1.46)	1.18 (3.13)	0.721
CD4 (any) (cells/nL)	1.95 (2.69)	2.02 (1.75)	1.92 (3.59)	0.593
CD8 IL-2 (cells/nL)	0.09 (0.25)	0.08 (0.10)	0.14 (0.29)	**0.030**
CD8 TNF (cells/nL)	2.00 (3.25)	1.32 (3.00)	2.91 (3.26)	**0.006**
CD8 IFN-γ (cells/nL)	1.78 (3.43)	1.09 (2.88)	3.13 (3.40)	**0.005**
CD8 (any) (cells/nL)	2.39 (4.27)	1.56 (3.74)	3.61 (3.62)	**0.009**

[a]*Mann-Whitney test, not corrected for multiple endpoints. Significant values (p≤0.05) are shown in bold.*

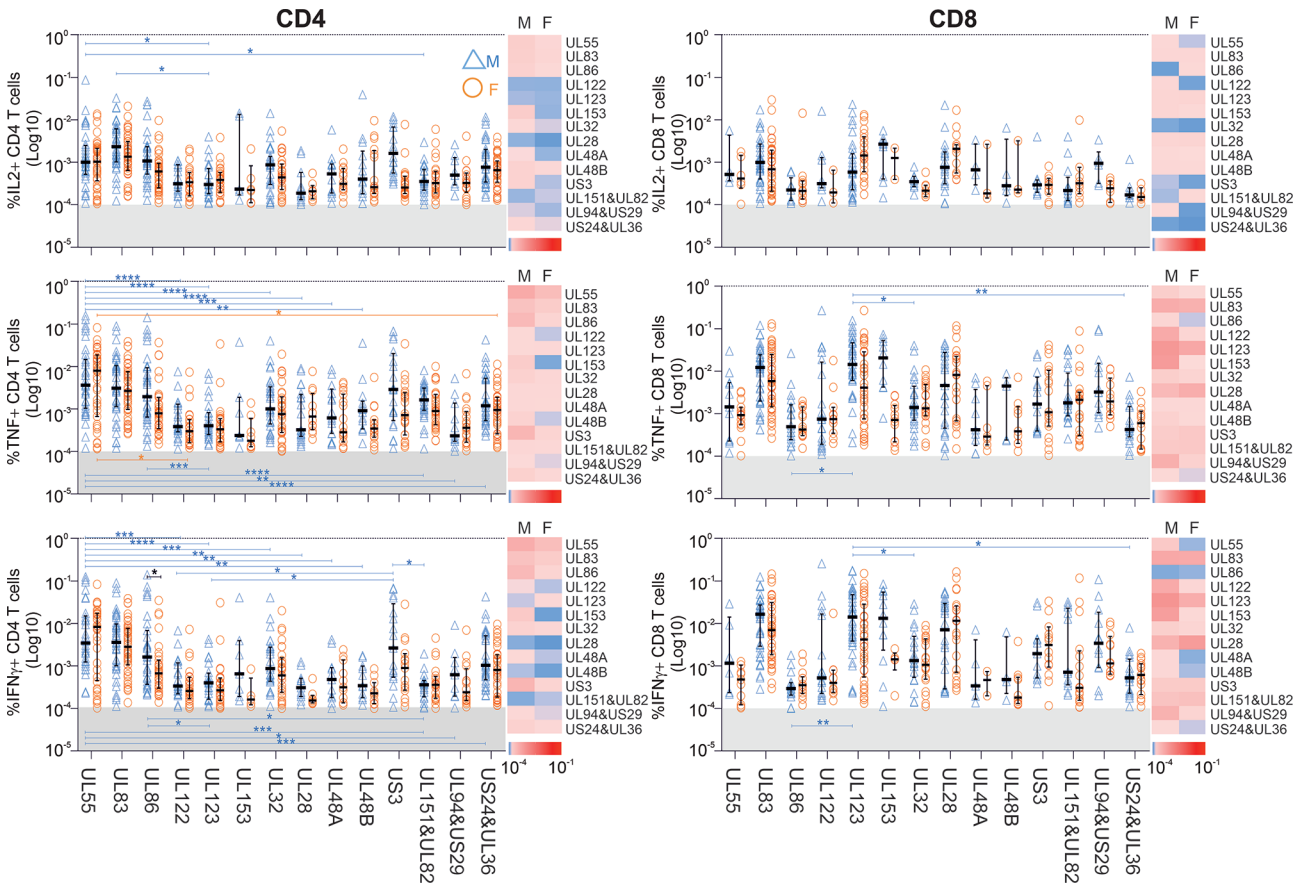


FIGURE 2 | Impact of biological sex on the CD4 and CD8 T-cell reactivity against 14 different CMV peptide pools. PBMCs from males (n=47) and females (n=47) were stimulated with CMV peptide pools; responses below the 1/10,000 threshold were excluded (grey shaded area of each plot). Heatmaps represent response size in males (M) and females (F). As indicated in the scale, darker shades of red represent large responses, while darker shades of blue correspond to low response frequencies. Scatter plots show the frequencies of CD4 and CD8 T-cells responding to the indicated CMV proteins in males (blue empty triangles, M) and females (orange empty circles, F). Error bars show median and interquartile range. Statistical differences in response sizes with respect to different CMV protein were assessed in 2-way ANOVA (addressing both the effect of 'sex' and 'CMV protein' on the size of the cytokine response, **Table S3**) followed by multiple end-point correction. Differences between proteins within males and females are shown by blue and orange lines, respectively, and differences between males and females by black lines. Significance levels are: *$p \leq 0.05$; **$p \leq 0.005$; ***$p \leq 0.0005$; ****$p \leq 0.0001$.

than IL-2 responses ($p<0.0001$ for each comparison), however, the differences between TNF and IFN-γ responses were not statistically significant. All of the above differences persisted when men and women were analyzed separately.

Sex Differences Are Reflected by CMV Target Protein-Associated CD3 Downregulation Patterns

With respect to most tested CMV-proteins, T-cell responses tended to be larger in men than in women (**Figure 2**). Consequently, we investigated if such differences were reflected by different degrees of activation-induced CD3 downmodulation, which is often used as a measure of stimulation efficiency. In each individual, CD3 downmodulation was assessed relative to the non-activated (i.e., cytokine-negative) portion of CD4 and CD8 T-cells. Because differences in cytokine production may be associated with different propensities to respond to antigenic challenge, we analyzed relative CD3 downmodulation in each of the seven functional subsets defined by IL-2, TNF, and IFN-γ production (**Figure 1C**). CD3 downmodulation was calculated as:

$$(\textit{MFI of the non-responsive T-cell population} - \textit{MFI of the activated T-cell population})/\textit{MFI of the non-responsive T-cell population}$$

This typically resulted in values between 0 and 1 ('1' indicating 100% downmodulation). However, in a few cases, this ratio was negative (if the activated events had a higher MFI than the non-responsive population) or >1 (if the MFI of the activated T-cell population was below baseline, i.e., recorded with a negative value).

Interestingly, CD3 downregulation varied significantly between the seven functional subsets in regards to each protein, but also the responses to different proteins. It also varied between women and men (**Figures 3** and **S5, S6**). Functional subset 5 (IL-2-/TNF +/IFN-γ+, 'Sub5') showed the biggest CD3 downmodulation of all subsets both in CD4 and CD8 T-cells (**Figure S5**). CD3 downmodulation in Sub5 was frequently close to 100% in CD4 T-cells but somewhat less often in CD8 T-cells. The heatmaps in **Figure S5** show a tendency towards lesser CD3 downmodulation in Sub5 in men than in women (dominance of red over green, **Figure S5**). CD3 downmodulation of Sub5 among UL28 and UL48A-specific CD4 T-cells was weaker than among CD4 T-cells specific to other proteins. With respect to CD8 T-cells, UL83 showing the highest levels of downregulation in both sexes, followed by UL55 and US3. No significant differences were elicited but, surprisingly, CD3 downmodulation overall was weaker in the CD8 T-cells (**Figure S5**).

Next, in order to establish if there was a hierarchy of CD3 downmodulation across the functional subsets, we standardized CD3 downmodulation across all subsets to the degree of CD3 downmodulation found in Sub5, which was the most downmodulated subset (i.e., the level of CD3 downmodulation of each subset was divided by that of Sub5) (**Figures 3** and **S6**). Relative CD3 downmodulation for each subset was hence between 0 and 1 with a very small number of exceptions in individuals where a subset other than Sub5 showed the strongest CD3 downmodulation (**Figures 3** and **S6**).

With respect to CD4 T-cells (**Figure 3**), Sub1 showed a degree of CD3 downmodulation similar to Sub5 (values close to 1, in general). In regards to the most immunodominant proteins, UL55, UL83 and UL86 (**Figure 2**), CD3 downmodulation was high in virtually all functional CD4 subsets in both men and women (**Figure 3** and **S6**). In CD8 T-cells, interestingly, CD3 downregulation across the functional subsets was less consistent

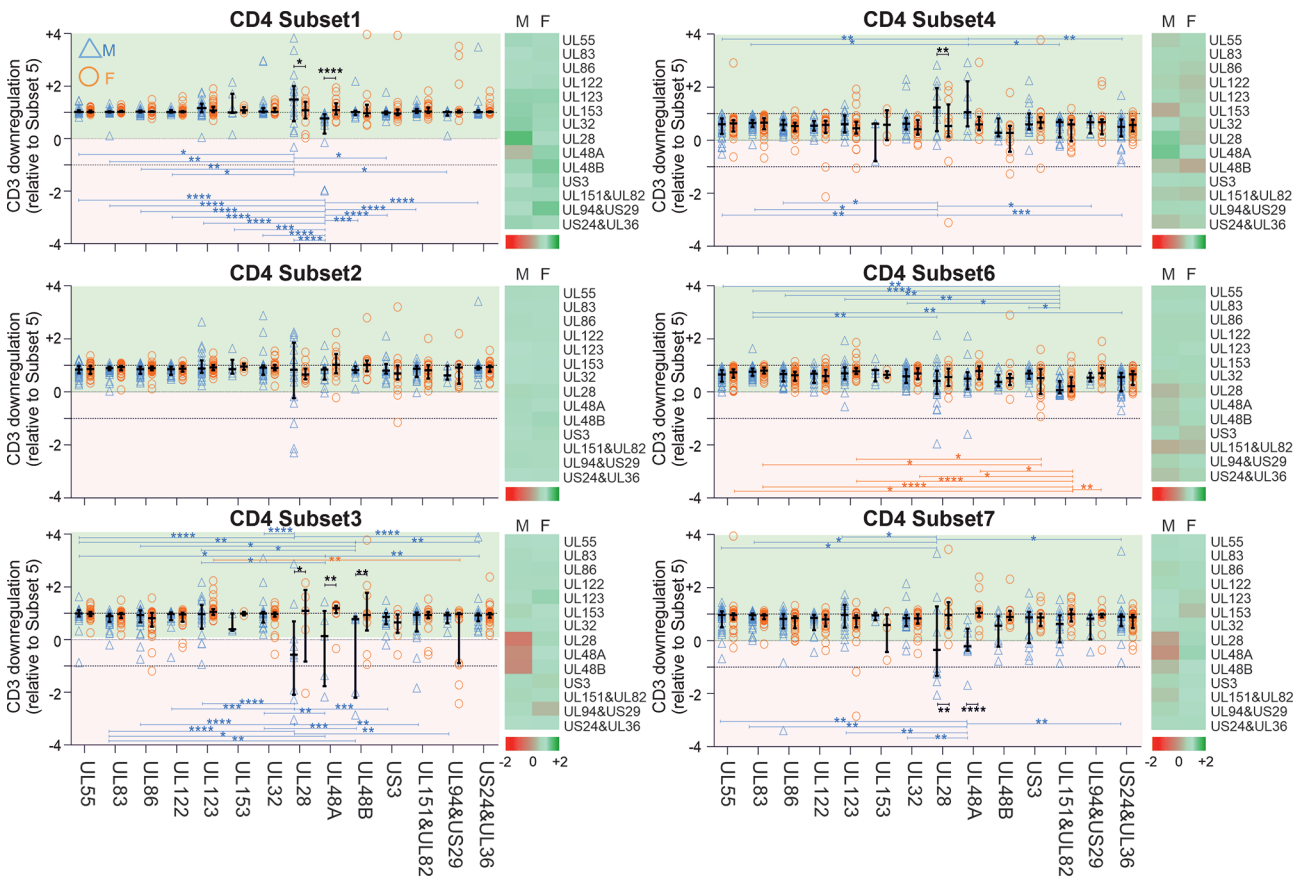


FIGURE 3 | CD3 downregulation takes place to different degrees in different functional CD4 T-cell subsets and reveals sex differences in regards to select proteins. PBMCs from men (n=47) and females (n=47) were stimulated with specific CMV-protein peptide mixes and processed for intracellular cytokine staining, excluding responses below the 1/10,000 threshold. Levels of CD3 downregulation in functional subsets (Sub1 through Sub7) were standardized relative to the downregulation in Sub5. Heatmaps show CD3 downmodulation in males (M) compared with females (F). Darker shades of red represent low and darker shades of green high CD3 downmodulation. Scatter plots show CD3 downregulation in males (blue triangles) and females (orange circles). Error bars show median and interquartile range. Statistically significant differences (i) between sexes are indicated by black lines and (ii) within sexes between proteins by blue and orange lines for men and women, respectively. Statistical significance was determined by 2-way ANOVA followed by multiple end-point correction. Significance levels are $^{*}P \leq 0.05$; $^{**}P \leq 0.005$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

and showed more heterogeneity across different target proteins (**Figures S5** and **S6**). **Table S4** shows *p*-values for differences in CD3 downmodulation between the functional subsets as well as between the sexes. These results agree with the sex differences reported in the previous sections. Generally, sex differences were more visible in CD4 than in CD8 T-cells. In men the CMV-specific immune response appeared more polarized towards dominant targets than in women.

Men Accumulate More CMV-Specific CD4 and CD8 Memory T-Cells Than Women

We wondered whether the biological sex differences detected above in cytokine responses were mirrored in the distribution of canonical peripheral blood T-cell subsets. The surface markers C-C chemokine receptor 7 (CCR7) and CD45RA were used to identify canonical T-cell 'memory subsets' including naïve/naïve-like T-cells (CCR7+ CD45RA+, Na), central memory (CCR7+CD45RA-, CM), effector memory (CCR7-CD45RA-, EM), and revertant memory T-cell subsets (CCR7-CD45RA+, Rv) (**Figure S1**) (15). Of note, the 'naïve' phenotype will include a variable and generally small number of T-cells that are antigen experienced, but exhibit a phenotype similar to that of naïve cells, referred to as naïve-like cells (Na). The distribution of these four subsets among CMV-reactive T-cells was analyzed in women and men with respect to each protein/protein-combination pair (**Figure S7**) and the overall distribution (**Figure 4**).

We initially examined differences in the proportions of all memory subsets among CMV-specific T-cells, which may identify changes in response composition between the sexes (**Figure 4A**).

Importantly, to generate these diagrams, the percentages of each respective subset in each individual were first added up across all CMV proteins (top), immediate-early/early proteins (IE/E, middle), and early-late/late proteins (E-L/L, bottom) and then the sums were normalized to 100% for representation in the pie charts. Larger responses to some proteins, therefore, have a stronger effect on these pie charts than smaller responses and hence have more weight; we refer to these charts as 'weighted' (averaging the proportions across the different proteins in the first step, by contrast, would give each protein the same weight). Regarding all CMV-proteins together, the memory subset composition of CD4

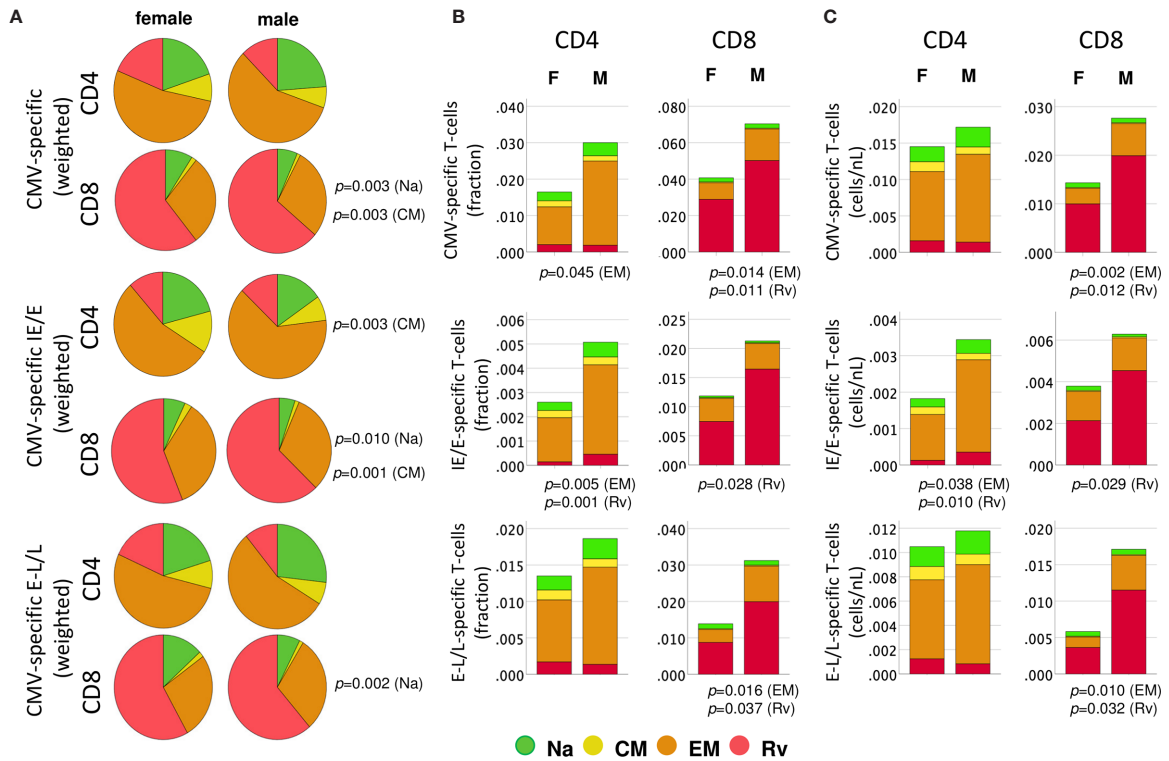


FIGURE 4 | Distribution of protein-specific T-cells across canonical blood T-cell subsets by protein kinetic class. CMV-specific T-cells (any activation marker) were analyzed in regards to memory subset composition including naïve-like/stem-cell memory (Na, CCR7+CD45RA+, green), central memory, (CM, CCR7+CD45RA-, yellow), effector memory (EM, CCR7-CD45RA-, orange) and revertant memory (Rv, CCR7-CD45RA+, red). **(A)** Pie charts show weighted average percentages of memory T-cell subsets (composition). Top, middle, and bottom panels show all CMV-protein reactive T-cells, IE/E-protein-and E-L/L-protein-specific T-cells, respectively. Weighted percentages were obtained by adding up responder frequency for all selected proteins, and then the sums for each subset were normalized to 100%. **(B)** Stacked bar charts show non-normalized CD4 or CD8 T-cell subset percentages added up across all CMV proteins, IE/E and E-L/L proteins, visualizing overall subset size differences between men and women. **(C)** Stacked bar charts show non-normalized CD4 or CD8 T-cell counts/nL of blood added up across all CMV proteins, IE/E and E-L/L proteins, providing an alternative visualization of subset size differences between men and women. *p*-values indicate significant ($p \leq 0.05$) differences between females and males.

T-cell responses did not significantly differ between women and men. With respect to CD8 T-cells, however, the Na and CM compartments were significantly larger in women than in men (**Figure 4A**, top). Significant sex differences were also found in regards to IE/E-specific CM CD4 T-cells, Na and CM CD8 T-cells, and E-L/L-specific Na CD8 T-cells (**Figure 4A**, middle and bottom). These results suggest that the proportions of the Na and/or CM CD8 T-cell (less differentiated) compartments in men were generally smaller. **Figure 4B** shows memory subset frequencies (summed percentages) in terms of CD4 and CD8 T-cells rather than response composition. With respect to the overall frequencies of CMV-specific peripheral T-cells (top), EM CD4 T-cells (p=0.045) as well as EM (p=0.014) and Rv (p=0.011) CD8 T-cells were significantly larger in men than women. In regards to IE/E protein-specific T-cells (middle), men showed significantly more EM (p=0.005) and Rv (p=0.001) CD4 T-cells and, in addition, Rv (p=0.028) CD8 T-cells. Regarding E-L/L proteins (bottom), men had higher EM (p=0.016) and Rv (p=0.037) CD8 T-cells. **Figure 4C** confirms the identified sex differences showing, however, median counts per volume of blood (cells/nL), instead of percentages. The Rv CD8 T-cell subset in particular was clearly bigger in men than in women, for example, with respect to E-L/L proteins it was 3.7 times bigger in men (**Table 3**). On the whole, when considering response size both in terms of percentages and counts per volume of blood, significant sex differences became apparent, particularly regarding the sizes of the EM and/or Rv compartments among CD4 and CD8 T-cells. EM and Rv CD4 T-cell responses to IE/E as well as EM and Rv CD8 T-cell responses to E-L/L proteins were between two- and threefold higher in men than in women (**Table 3**).

The Amount of Cytokine Produced by CMV-Specific T-Cells Is Higher in Men Than in Women

In addition to response size, we also examined the overall IL-2, TNF, IFN-γ secretion 'potential' of CMV-specific CD4 and CD8 T-cells. This was done in order to explore if certain proteins had more intrinsic inflammatory potential than others and to elicit additional potential differences between men and women. Note that, at the single cell level, the mean fluorescence intensity (MFI) of the cytokine detection antibody directly correlates with the amount of intracellular cytokine. However, at the population level, the average MFI of CMV protein-specific responses does not provide a good measure of cytokine production unless the number of cells producing the cytokine is taken into account. By multiplying the

TABLE 3 | Biological sex differences in memory subset size and distribution.

	Women Median (IQR)	Men Median (IQR)	Women/Men Ratio	*P*
Na CD4 IE/E (%)	0.03 (0.06)	0.06 (0.09)	0.57	n.s.
CM CD4 IE/E (%)	0.03 (0.05)	0.03 (0.04)	0.92	n.s.
EM CD4 IE/E (%)	0.18 (0.28)	0.37 (1.16)	**0.49**	**0.005**
Rv CD4 IE/E (%)	0.01 (0.03)	0.05 (0.15)	**0.32**	**0.001**
Na CD8 IE/E (%)	0.03 (0.07)	0.03 (0.05)	0.90	n.s.
CM CD8 IE/E (%)	0.01 (0.04)	0.01 (0.03)	0.99	n.s.
EM CD8 IE/E (%)	0.32 (0.62)	0.37 (0.9)	0.88	n.s.
Rv CD8 IE/E (%)	0.68 (1.09)	1.24 (2.48)	**0.54**	**0.028**
Na CD4 E-L/L (%)	0.19 (0.35)	0.28 (0.61)	0.70	n.s.
CM CD4 E-L/L (%)	0.14 (0.16)	0.11 (0.22)	1.19	n.s.
EM CD4 E-L/L (%)	0.85 (1.73)	1.34 (2.67)	0.64	n.s.
Rv CD4 E-L/L (%)	0.17 (0.34)	0.14 (0.65)	1.25	n.s.
Na CD8 E-L/L (%)	0.13 (0.39)	0.13 (0.3)	1.01	n.s.
CM CD8 E-L/L (%)	0.02 (0.03)	0.03 (0.04)	0.75	n.s.
EM CD8 E-L/L (%)	0.34 (1.01)	0.89 (1.42)	**0.39**	**0.016**
Rv CD8 E-L/L (%)	0.79 (3.85)	1.88 (4.15)	**0.42**	**0.037**
Na CD4 IE/E (cells/nL)	0.02 (0.04)	0.04 (0.06)	0.61	n.s.
CM CD4 IE/E (cells/nL)	0.02 (0.04)	0.02 (0.03)	1.18	n.s.
EM CD4 IE/E (cells/nL)	0.13 (0.19)	0.25 (0.56)	**0.50**	**0.038**
Rv CD4 IE/E (cells/nL)	0.01 (0.02)	0.04 (0.08)	**0.36**	**0.010**
Na CD8 IE/E (cells/nL)	0.02 (0.02)	0.01 (0.03)	1.62	n.s.
CM CD8 IE/E (cells/nL)	0 (0.02)	0.01 (0.01)	0.82	n.s.
EM CD8 IE/E (cells/nL)	0.13 (0.25)	0.15 (0.44)	0.87	n.s.
Rv CD8 IE/E (cells/nL)	0.21 (0.69)	0.4 (1.14)	**0.52**	**0.029**
Na CD4 E-L/L (cells/nL)	0.16 (0.26)	0.19 (0.28)	0.86	n.s.
CM CD4 E-L/L (cells/nL)	0.11 (0.14)	0.09 (0.13)	1.26	n.s.
EM CD4 E-L/L (cells/nL)	0.65 (1.32)	0.82 (1.97)	0.80	n.s.
Na CD4 E-L/L (cells/nL)	0.12 (0.28)	0.08 (0.26)	1.50	n.s.
Rv CD8 E-L/L (cells/nL)	0.06 (0.1)	0.07 (0.13)	0.88	n.s.
CM CD8 E-L/L (cells/nL)	0.01 (0.01)	0.01 (0.02)	0.75	n.s.
EM CD8 E-L/L (cells/nL)	0.14 (0.25)	0.45 (0.69)	**0.31**	**0.010**
Rv CD8 E-L/L (cells/nL)	0.28 (1.62)	1.02 (2.06)	**0.27**	**0.032**

Statistical significances ($p \leq 0.05$) are shown in bold.
n.s., non-significant.

cell number (percentage of reference population) and MFI, a surrogate for the amount of produced cytokine can be calculated that is sometimes referred to as 'integrated MFI' (iMFI) (29). We, therefore, used the iMFI to compare responses between women and men in addition to response size alone. Also note that with respect to cytokine production, the MFI of different protein-specific responses cannot reasonably be added up in order to summarize the amount of cytokine produced overall. Because the iMFI is a surrogate for cytokine amount, however, it is permissible (and makes sense, mathematically) to add iMFIs across responses. This allows for the computation of cytokine production in response to several proteins. **Table 4** shows that, with respect to CD4 and CD8 T-cells and responses to all CMV proteins, the iMFI for TNF and IFN-γ tends to be higher in men than in women. These differences were statistically significant for CD8 T-cells only.

CMV Reactivation Responses Are Bigger in Men and Associate With Bigger E-L/L Responses, But Not Vice Versa

CMV reactivation is a frequent event in CMV-infected people and thought to be probably the main driver of CMV-specific T-cell expansions (30). As a result, CMV reactivation is also likely to drive differences in CMV-specific T-cell responses between women and men. Accordingly, we examined differences between men and women regarding proteins whose expression is related to different times after activation. We hypothesized that proteins produced at immediate early and early times may have a bigger impact of shaping T-cell immunity than those produced later and that they may have different impact in women and men. We, therefore, calculated the median response iMFI (IL-2, TNF and IFN-γ) for all pools containing IE/E proteins (UL122, UL123, US3, US24&UL36) as well as E-L/L proteins (UL55, UL83, UL32, UL28, UL48, UL151&UL83, UL94&US29) and compared these between men and women (**Figure 5A**). Additionally, summated response sizes with respect to the same proteins (median) were compared between men and women (**Figure 5B**). The lower panel of **Figure 5B** shows counts per volume of blood, essentially confirming the results obtained with percentages. With respect to CD4 T-cells (**Figure 5A**, left), the median iMFI of the IL-2, TNF, and IFN-γ responses to IE/E proteins (top) was significantly higher in men than in women, as well as the iMFI of the E-L/L protein-specific IL-2 response (bottom). Regarding CD8 T-cells (**Figure 5A**, right), the median iMFI of the TNF response to IE/E proteins (top) as well as the median iMFI of IL-2, TNF, and IFN-γ responses to E-L/L proteins (bottom) were all significantly higher in men than in women. At the level of response sizes, CD4 T-cells response to IE/E and E-L/L proteins and CD8 T-cells response to E-L/L proteins were higher in men compared with women (**Figure 5B**). Interestingly, there was a moderate association between the size of the CD4 T-cell response to IE/E proteins and that to E-L/L proteins (any cytokine, R=0.325, p=0.003).

We also investigated if there was an effect of the frequently reactivated responses to IE/E proteins on the response to any other proteins, potentially indicating that even incomplete reactivation would push all CMV-specific responses to a more differentiated phenotype. We, therefore, examined if the cytokine iMFIs of responses to IE/E (compared with E-L/L) proteins were associated with IE/E and E-L/L overall response sizes. For CD4 and CD8 T-cells we ran bivariate correlations between iMFI of IL-2, TNF, and IFN-γ responses to any CMV protein (14 pools) and the size of the responses directed against IE/E and E-L/L antigens (**Figure 6**). Note that correlations between the iMFI of IE/E protein-specific response with the overall size of the response to IE/E proteins would be expected as the iMFI as such incorporates the size of the response. However, a correlation of the iMFI of IE/E protein-specific responses and the size or iMFI of E-L/L protein specific responses would not be expected, unless reactivation (mostly marked by responses to IE/E protein-antigens) were to somehow enhance E-L/L-driven responses. Weak-to-moderate, yet significant, positive associations between the iMFIs (IL2, TNF and IFN-γ) of IE/E protein-specific CD4 T-cell responses and the size of the E-L/L protein specific CD4 T-cell response were found (**Figure 6**; see inset tables). This effect was not identified in CD8 T-cells, indicating that reactivation might have a stronger overall effect on the size of the CD4 T-cell response.

Associations of Polyfunctionality With T-Cell Differentiation Do Not Differ Between Women and Men

In order to analyze associations of T-cell differentiation and polyfunctionality, we used the previously described polyfunctionality index (PI) (22), which allows one to summarize the composition of each T-cell response in terms of non-overlapping, functional Boolean subsets as a single number. The advantage of using this index is that polyfunctionality becomes a continuous variable that can be correlated with other parameters, for example, response size. We previously reported that, contrary to common belief, polyfunctionality is increased in large CMV-specific responses (26, 31). Here we sought to confirm this relationship and, in addition, explore differences between men and women in regard to protein-specific polyfunctionality, MFI/iMFI, memory differentiation and

TABLE 4 | iMFI for TNF and IFN-γ between biological sex groups.

Parameter	Women		Men		p
	Median	(IQR)	Median	(IQR)	
CD4 TNF	468.37	(715.33)	644.04	(1941.48)	0.228
CD4 IFN-γ	70.42	(64.24)	86.26	(156.47)	0.189
CD8 TNF	546.55	(1055.63)	1299.14	(1405.64)	**0.006**
CD8 IFN-γ	171.42	(382.69)	364.62	(414.93)	**0.004**

Statistical significances ($p \leq 0.05$) are shown in bold

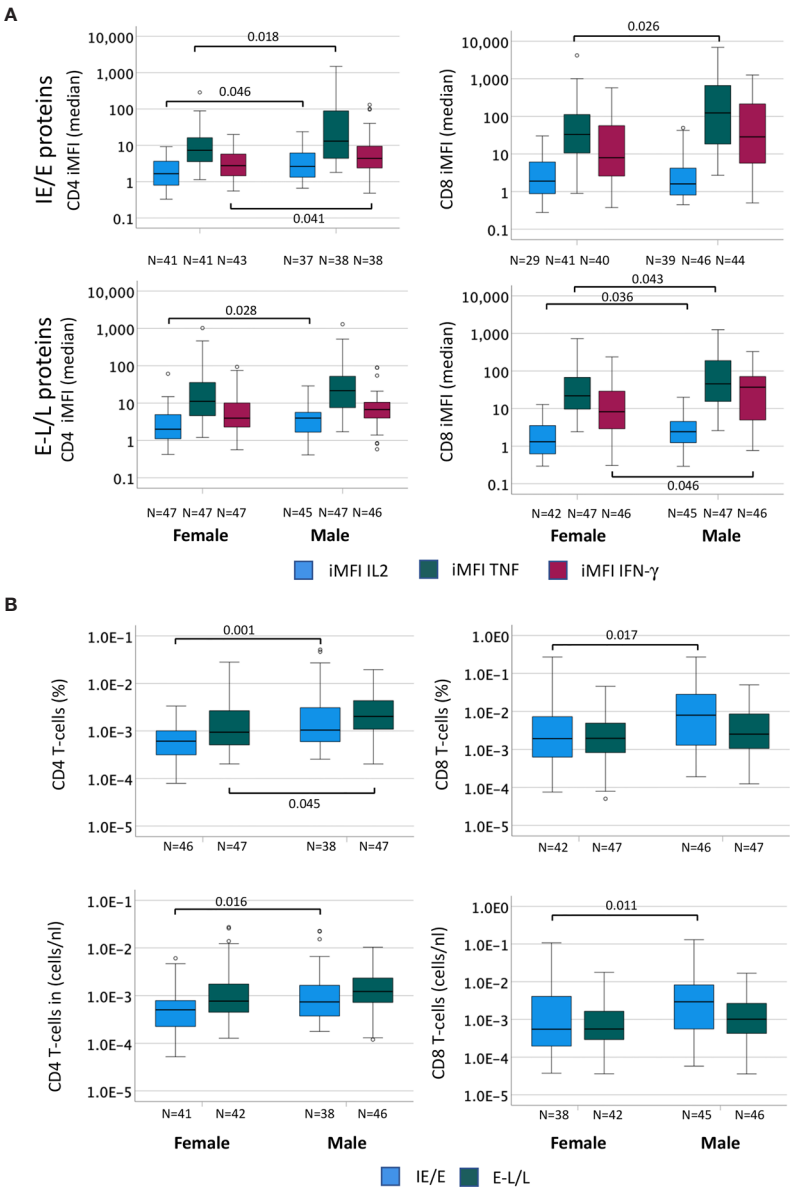
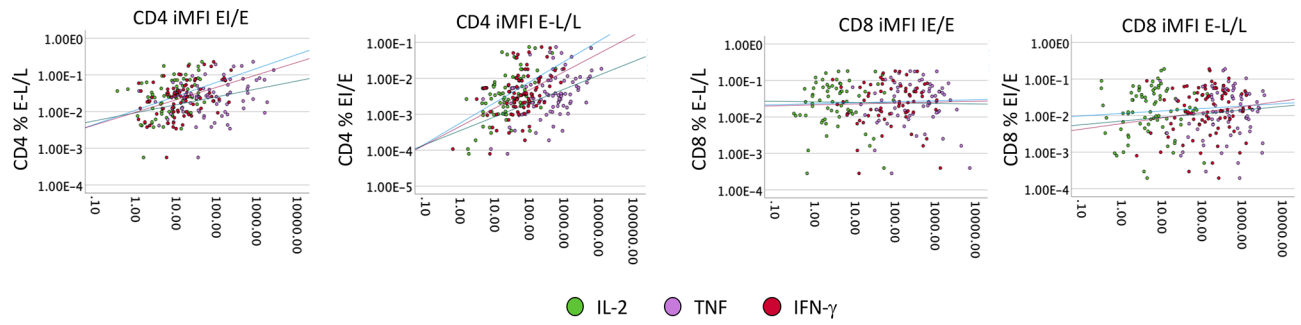


FIGURE 5 | The cytokine content of CMV-specific T-cell responses is higher in men than in women. In order to compare the cytokine amounts produced by CMV-specific T-cells in men and women the percentage (in terms of CD4 or CD8 T-cells) of each cytokine response was multiplied by the MFI to generate the 'integrated MFI' (iMFI), for CD4 and CD8 T-cells. Responses were grouped by adding up the iMFI of responses to IE/E proteins (UL122, UL123, US3, US24&UL36), more likely to reflect CMV reactivation, and those to E-L/L proteins (UL55, UL83, UL32, UL28, UL48, UL151&UL83, UL94&US29). **(A)** Box plots show the median iMFIs for each cytokine response to IE/E (top) or E-L/L proteins (bottom) by CD4 (left) and CD8 (right) T-cells. **(B)** Box plots show the median of the summed frequencies or counts (cells/nL) of CD4 (left) and CD8 (right) T-cells responding to IE/E and E-L/L proteins. Box plots show median and interquartile range, outlier limits (whiskers, LQ-1.5*IQR, UQ+1.5*IQR), outliers (o). Within each graph, left and right boxes show responses in females and males, respectively.

response size. For the purpose of representing memory differentiation in correlations, we generated a differentiation score (DSc). This score captures the percentages of CD4 and CD8 T-cells in each of the non-naïve compartments and assigns progressive weight to each compartment (see *Polyfunctionality Index and Differentiation Score* for a formal definition). The polyfunctionality of responses to CMV is more homogeneous (showing a tighter cluster of pies) for some CMV proteins (US3 and the four late proteins: UL28, UL48A, UL48B, UL151 & IL82) than others, in both CD4 and CD8 T-cells (**Figure 7**). Compared with CD4 T-cells, fewer CD8 T-cells produce IL-2, resulting in lower PI values for CD8 (compare **Figures 7A, B**). Also, the DSc is higher in CD8 than CD4 T-cells (with a DSc hardly exceeding 0.67) because CD4 T-cells rarely reacquire CD45RA expression (revertant cells) (**Figure 4**). Nevertheless, the correlation between polyfunctionality and differentiation score is less evident in CD8 than in CD4 T-cells, where there is a clear increase of



	CD4 iMFI IL2 IE/E	CD4 iMFI TNF IE/E	CD4 iMFI IFN-γ IE/E	CD4 iMFI IL2 E-L/L	CD4 iMFI TNF E-L/L	CD4 iMFI IFN-g E-L/L
CD4% IE/E	.558**	.929**	.922**	.345**	.238*	.264*
P	0.000	0.000	0.000	0.001	0.030	0.015
N	78	79	81	84	84	84
CD4% E-L/L	.316**	.315**	.351**	.666**	.914**	.914**
P	0.005	0.005	0.001	0.000	0.000	0.000
N	78	79	81	92	94	93

	CD8 iMFI IL2 IE/E	CD8 iMFI TNF IE/E	CD8 iMFI IFN-γ IE/E	CD8 iMFI IL2 E-L/L	CD8 iMFI TNF E-L/L	CD8 iMFI IFN-g E-L/L
CD8% IE/E	.588**	.959**	.874**	-0.053	-0.129	-0.114
P	0.000	0.000	0.000	0.633	0.229	0.295
N	68	87	84	82	88	86
CD8% E-L/L	0.033	-0.151	-0.094	.411**	.911**	.876**
P	0.786	0.162	0.395	0.000	0.000	0.000
N	68	87	84	87	94	92

FIGURE 6 | IE/E and E-L/L CD4 and CD8 T-cell response correlation. PBMCs from males (n=47) and females (n=47) were stimulated and processed for intracellular cytokine staining. Thereafter, the iMFI for each cytokine was calculated. Scatter plots show the association of IE/E protein specific with E-L/L protein-specific CD4 and CD8 T-cell responses. The inset tables show the Pearson R correlation coefficient for each association comparison with the level of significance. *$p \leq 0.05$; **$p \leq 0.005$.

polyfunctionality with higher differentiation (**Figure 7A**). Of note, the PI tends to rise along with the DSc until a certain point, after which it decreases, as most responses predominantly originate from revertant cells (Rv, red) (e.g., UL86, UL24&UL36, UL153 and UL48A/B in CD4 T-cells and UL55, UL28 and UL48A in CD8 T-cells, **Figure 7**). This agrees with our own previously published data comparing MFIs in different memory T-cell compartments (previously defined by CD27 and CD45RA) (26). While no significant sex differences were observed for CD4 and CD8 T-cell polyfunctionality to CMV, some non-significant trends towards lower polyfunctionality were apparent from UL153 in women (**Figure S8**).

A Population View of CMV-Target Protein-Specific T-Cell Responses Reveals TNF-Dominated CD4 T-Cell Responses in Men That Are Absent in Women

In order to visualize T-cell responses as a whole in the entire participant population, we plotted donor (x-axis) against protein pool (y-axis), response sizes in percentages of CD4 or CD8 T-cells (z-axis) as well as the MFI for TNF or IFN-γ (color gradient of the columns). This four-dimensional view shows a stark difference between women and men in regards to CD4 T-cells, in particular those producing TNF (**Figures 8**, **9**). It illustrates an excess of T-cell reactivity in terms of the measured cytokines in men and in particular the presence of response outliers. Sex differences in regards to IFN-γ producing CD4 T-cells were observed only in terms of response size. In CD8 T-cells sex differences were not nearly as obvious as in CD4 T-cells (**Figure 9**). The same analysis was performed using cells counts (cell/nL) obtaining similar results (**Figures S9**, **S10**).

In CD4 T-cells, response size measured in percentage (and cell counts) of TNF-producing cells was hence positively correlated with the MFI of TNF (i.e., the bigger response, the higher the MFI of TNF). However, response size measured in percentage (and cell counts) of IFN-γ-producing T-cells was negatively associated with the MFI of IFN-γ (the larger the response, the lower the MFI of IFN-γ). **Table 5** shows that in CD4 T-cells these positive and negative associations between response size and cytokine MFI were statistically significant for all dominant target proteins. Among CD8 T-cells the pattern was less clear overall, although clearly the MFIs of TNF and IFN-γ were significantly positively correlated with the respective response size in regards to several dominant responses but not the (most dominant) response to UL83. The pattern appeared less clear than in CD4 T-cells.

DISCUSSION

Previous studies exploring the effect of biological sex on CMV-specific immunity had shown that CMV infection affects T-cell differentiation differently in women and men (3, 15). However, these studies did not explore the actual CMV-specific T-cell

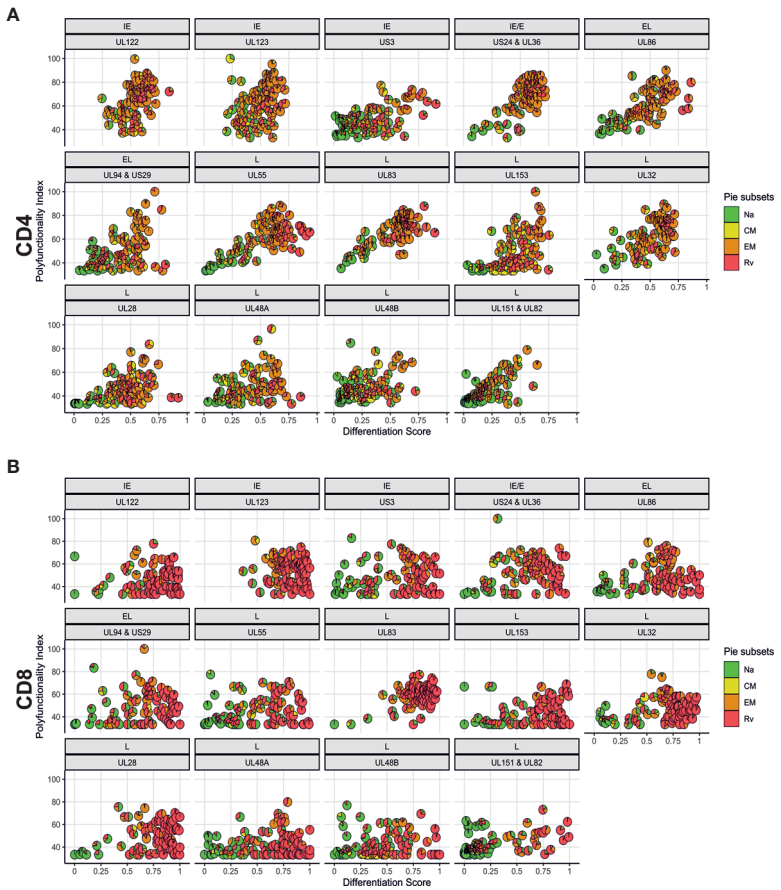


FIGURE 7 | Polyfunctionality plotted *versus* differentiation score reveals distinct, protein-associated patterns. **(A)** CD4 and **(B)** CD8 T-cell polyfunctionality (Polyfunctionality Index) in relation to differentiation score for each CMV protein pool stratified according to the kinetics of the antigens. CMV protein designation and kinetic class are indicated at the top of each graph (in panels **A, B**). Pie charts show memory subset distributions including naive-like/stem-cell memory (Na, green), (CM, yellow), effector memory (EM, orange), and revertant memory (Rv, red).

response in much detail, let alone the response to individual CMV proteins representing different phases of reactivation. The present study is the first to provide a detailed analysis of sex differences in the T-cell response to a range of dominant CMV T-cell target proteins.

Based on a selection of CMV proteins known to represent frequent and dominant T-cell response targets (19), we initially explored protein recognition (i.e., presence or absence of a response in regards to each protein in each individual) in a recently recruited cohort of 94 CMV-seropositive individuals. As expected, hierarchies in terms of protein recognition frequency differed between CD4 and CD8 T-cells. However, our results confirmed the previously identified hierarchy in an equally White British cohort with respect to the most dominant target proteins (26, 28). Minor differences detected in target protein response hierarchy between the historic cohort (19) and the present one may be related to differences in HLA-allele frequencies, since the historic cohort was ethnically more diverse.

Interestingly, the numbers of protein targets recognized by CD4 T-cells and CD8 T-cells in each individual were significantly correlated, despite the fact that CD4 and CD8 T-cells recognize peptides in the context of class-II and class-I MHC, respectively. This might be explained by an accumulation of HLA-alleles favoring CMV-peptide recognition during evolution. The distribution of T-cell responses in terms of recognized target proteins was very similar in women and men.

In agreement with the literature and our own previous reports (19, 20, 26, 28, 31), both the CD4 and CD8 T-cell compartments make a significant contribution to the CMV-specific T-cell response. CD4 T-cells may respond to antigens presented in the context of class-II MHC on APCs, but also endothelial cells. In addition, infected endothelial cells (and possibly other tissues) produce non-infectious exosomes containing CMV proteins. These are taken up by APCs and presented to CD4 T-cells (32). CD8 T-cells however seem to be mainly primed by cross presentation as CMV induces downmodulation of MHC-I

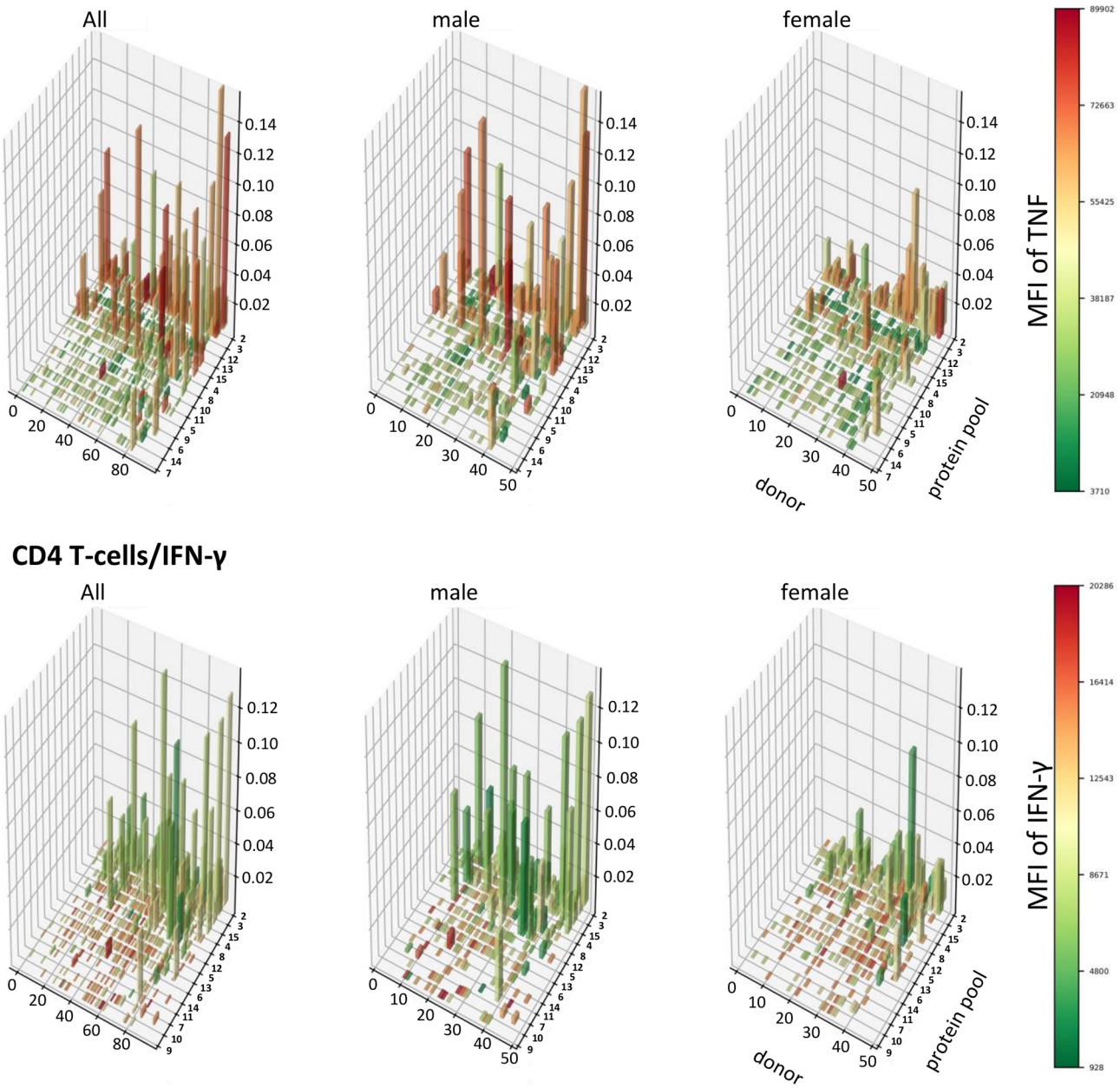


FIGURE 8 | Large CD4 T-cell responses producing high levels of TNF are found preferentially in men. 3D bar charts show all individual CD4 T-cell responses to each CMV peptide pool in the entire population (left), men alone (middle) and women alone (right). Participants are ordered by increasing median T-cell response size from left to right with respect to the entire population. Peptide pools are ordered by increasing median response size induced from front to back, also with respect to the entire population. Bar height corresponds to the percentage of cytokine producing CD4 T-cells, i.e., response size. Bar color corresponds to the MFI of the measured cytokine according to the displayed scale.

molecules in infected cells (33). It was previously reported that immunogenic ORFs were represented across all kinetic classes of HCMV proteins, and it was also shown that the frequency of T-cells specific to a given protein was proportional to the representation of the corresponding ORF in the entire CMV proteome (19).

In this study, T-cell activation was identified by intracellular IL-2, TNF, and IFN-γ. This may be a limited panel, but these cytokines are critical for T-cell proliferation and effector function. TNF was the cytokine most frequently produced by activated CD4 T-cells, whereas among CD8 T-cells TNF and IFN-γ were equally dominant. We previously reported that the largest age-associated

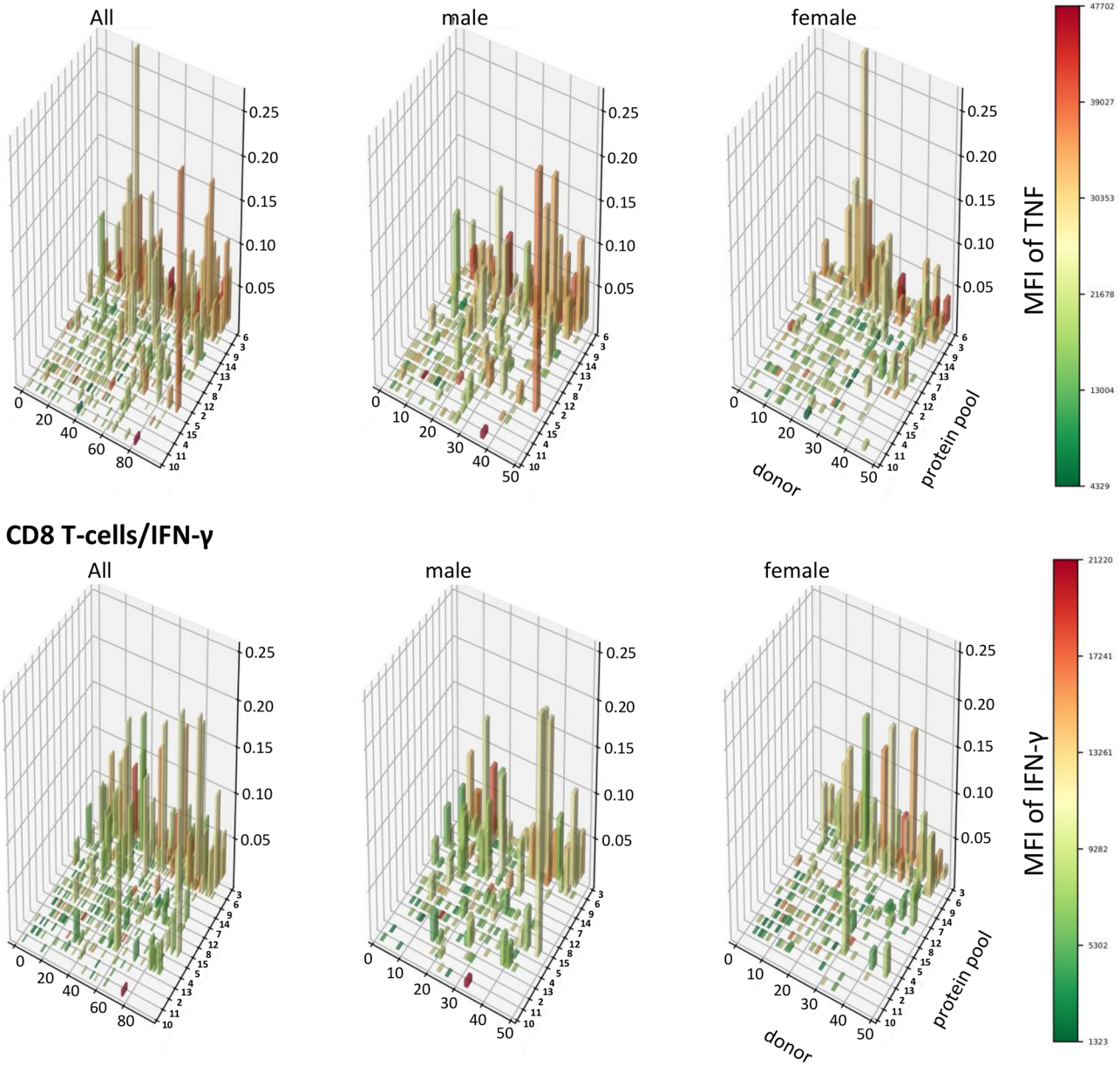


FIGURE 9 | Patterns of TNF and IFN-γ producing CD8 T-cells appear similar in terms of size and distribution among men and women. 3D bar charts show all individual CD8 T-cell responses to each CMV peptide pool in the entire population (left), men alone (middle) and women alone (right). Participants are ordered by increasing median T-cell response size from left to right with respect to the entire population. Peptide pools are ordered by increasing median response size induced from front to back, also with respect to the entire population. Bar height corresponds to the percentage of cytokine producing CD8 T-cells, i.e., response size. Bar color corresponds to the MFI of the measured cytokine according to the displayed scale.

increase in CMV-specific T-cell responses occurred in regards to TNF-producing CD4 T-cells (28), which is of interest in this context, since the participants of this study were 60 years old or older. Our previous study, however, did not explore sex differences, but our current work clearly shows that men have overall larger T-cell responses to CMV proteins than women with a more pro-inflammatory CD4 T-cell component.

The combination of the three cytokines further allowed the division of activated T-cells into seven non-overlapping (Boolean) functional subsets. The relative contribution of these subsets to the overall response, as expected, differed a lot between CD4 and CD8 T-cells, and this was clearly the effect of CD8 T-cells not producing as much IL-2 as CD4 T-cells. The most 'polyfunctional' subset simultaneously producing IL-2, TNF, and

TABLE 5 | Association of T-cell response size with cytokine MFI.

Response size in % of reference T-cell population	CD4 T-cells % IL-2 with MFI IL-2	CD4 T-cells % TNF with MFI TNF	CD4 T-cells % IFN-γ with MFI IFN-γ	CD8 T-cells % IL-2 with MFI IL-2	CD8 T-cells % TNF with MFI TNF	CD8 T-cells % IFN-γ with MFI IFN-γ
UL55[5]	0.028	0.358**	-0.310*	0.834**	-0.007	-0.220
UL83 ('pp65')	0.085	0.483***	-0.285**	-0.168	0.111	0.161
UL86	0.006	0.577***	-0.335**	0.350	-0.035	0.283
UL122	0.029	0.763***	-0.297*	-0.089	0.572**	0.409*
UL123 ('IE-1')	-0.096	0.366**	-0.168	0.009	0.353**	0.250*
UL153	-0.273	0.393	-0.269	-0.059	0.363	0.218
UL32	-0.076	0.194	-0.152	0.59*2	0.227	0.278
UL28	0.307	-0.218	-0.320	0.137	0.374**	0.360*
UL48A[a]	0.242	0.183	0.014	0.062	0.520*	0.977***
UL48B[a]	0.424*	0.007	-0.228	0.846**	0.432	0.253
US3	-0.153	0.533***	-0.357*	-0.311	0.036	-0.268
UL151& UL82	-0.049	-0.072	-0.199	0.456*	0.516***	0.061
UL94 & US29	-0.131	0.075	0.172	-0.151	-0.025	0.159
US24 & UL36	0.006	0.482***	-0.227	-0.068	0.519**	0.075
Response size in cells/nL of reference T-cell population	**CD4 T-cells cells/ nL IL-2 with MFI IL-2**	**CD4 T-cells cells/ nL TNF with MFI TNF**	**CD4 T-cells cells/ nL IFN-γ with MFI IFN- γ**	**CD8 T-cells cells/ nL IL-2 with MFI IL-2**	**CD8 T-cells cells/ nL TNF with MFI TNF**	**CD8 T-cells cells/nL IFN- γ with MFI IFN- γ**
UL55[5]	0.048	0.264*	-0.337**	0.912**	0.007	-0.214
UL83 ('pp65')	0.175	0.501***	-0.245*	-0.100	0.213	0.190
UL86	0.055	0.592***	-0.331*	0.346	0.029	0.029
UL122	0.101	0.714***	-0.249	-0.173	0.635***	0.443*
UL123 ('IE-1')	-0.045	0.298*	-0.145	0.037	0.266	0.114
UL153	-0.316	0.394	-0.271	-0.167	0.287	0.187
UL32	-0.074	0.113	-0.102	0.038	0.284	0.211
UL28	0.447	-0.123	-0.258	0.098	0.303*	0.261
UL48A[a]	0.052	0.214	0.037	0.099	0.248	0.841*
UL48B[a]	0.312	0.010	-0.089	0.918**	0.560	0.351
US3	-0.106	0.636***	-0.362	-0.288	0.069	-0.216
UL151& UL82	0.037	0.060	-0.042	0.024	0.600***	0.034
UL94 & US29	-0.115	0.004	0.452*	-0.244	-0.086	0.121
US24 & UL36	0.024	0.387**	-0.197	-0.022	0.568**	0.059

[a]*A panel of 19 CMV protein-spanning peptide pools was previously shown to correlate highly with the CD4 and CD8 T-cell response against 203 tested CMV proteins (19). The original panel contained UL99, UL103, and US32 in addition, but were left out here since responses were absent in >100 White British people. UL48 was divided into two pools (UL48A and UL48B), however, results were combined with respect to determining T-cell reactivity. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.*

Statistical significances (p-values ≤0.05) are shown in bold.

IFN-γ was, therefore, much smaller in the CD8 T-cell compartment. In both T-cell compartments, however, the largest subset was generally subset 5 producing TNF and IFN-γ, but not IL-2. We found no sex difference in regards to the distribution of these subsets.

Another way of subdividing activated T-cell subsets is by their expression of markers defining 'canonical' memory T-cell subsets (i.e., naïve/naive-like stem cell memory, central memory, effector memory and revertant). This allows some conclusions as to the role that each protein plays in terms of driving T-cell memory. We analyzed the distribution of these subsets in regards to CD4 and CD8 responses to every single protein and also in terms of kinetic classes. The division of proteins by their assigned kinetic classes accounts for the fact that different CMV proteins may have more or less influence on T-cell responsiveness, depending on their presence or absence during the (frequent) reactivation of latent CMV infection (21, 27). IE protein expression will dominate immediately after reactivation and, in particular, if reactivation is abrogated and remains incomplete. The major immediate early promoter (MIEP) regulates IE protein expression and appears to be regulated itself by cellular chromatin activity, an increase of which is the common endpoint of a host of signaling pathways, particularly in inflammation (34). Memory subset composition was hence analyzed across all CMV proteins but also grouped by kinetic class, combining IE and E protein-specific responses in one group and E-L/L protein specific responses in the other. Responses to individual proteins varied significantly in terms of memory subset distribution both among CD4 and CD8 T-cells. Whereas the (normalized) composition of responses in terms of memory subset contribution indicated that there were smaller naïve and/or CM contributions to the CD4 and CD8 T-cell response in men, comparing the summed responses to these subsets showed that, not only the entire response (all memory subsets together) was bigger in men, but that in particular the EM and/or revertant subset contributions to responses against all CMV proteins and IE/E proteins were significantly bigger in men than in women. So, not only did men have bigger responses than women, but also the EM and Rv component of these responses appeared to contribute most to that difference.

It is a limitation of our work that viral load was not determined in our cohort. Previous work has shown that in otherwise healthy older people viral load is generally negative, however, digital droplet PCR may detect viral load in healthy older people over 70 years of age (35). We are unable to link T-cell responsiveness to viral loads since performing this very costly

assay in all participants was outside the scope of our work. However, it is a possibility that a higher frequency of (potentially incomplete) CMV reactivation in men might contribute to a dominance of T-cell responsiveness to IE/E proteins. This would need to be addressed in future studies. The possible role of latency-associated proteins was not explored. Responses to these proteins appear to have very different functional characteristics than responses to lytic phase proteins (36), interestingly, however, changes to chromatin structure induced by histone deacetylases (HDACs) may lead to the transient expression of IE proteins without leading to full viral reactivation. This can be induced pharmacologically (37), but may also occur as a result of chromatin activity during cellular activation (34). It might be speculated that such an effect is more prominent in men.

Recent work suggests that persistent stimulation of CMV-specific T-cells may additionally result from a continuous low-level gene expression during latency with increasing evidence coming from both human and mouse models (38–40). This low-level gene expression may be involved in regulating the switch between latency and reactivation, and in the murine infection model appears to have random (stochastic) characteristics with a skewing to IE transcripts at about 8 months after latent infection of lungs (40). We speculate that increased proinflammatory cytokine secretion in response to immune stimulation in men (41) may increase low-level latent CMV gene expression and so contribute to the gender difference we have observed in the present study.

T-cell polyfunctionality is thought to be related to T-cell response efficacy after vaccination (42, 43). We used the polyfunctionality index (22) to gauge T-cell polyfunctionality as a compound measure allowing us to correlate it to other variables. Our data confirms that the most differentiated subsets (Rv) have reduced polyfunctionality, but the bulk of expanded and highly differentiated (EM and Rv) CD4 T-cells appears to retain two or more functions. This is particularly visible in the diagrams showing the differentiation score *versus* the polyfunctionality index. It is also in agreement with our previous report that expanded T-cells in older ages retain polyfunctionality (26).

The combination of polyfunctionality index and differentiation score observed in T-cells responding to different CMV proteins might reflect the role of these proteins in driving T-cell responsiveness. Responses to some proteins showed a wider spread along the axes than others. CD4 and CD8 T-cells responding to the UL123 protein, for example, generally had a high differentiation score and appeared more homogeneous in that sense than the response to US3, which included many cells at an early stage of memory differentiation. The T-cell response to UL83, one of the most dominant T-cell target proteins for both CD4 and CD8 T-cells, also included predominantly cells in an EM/Rv differentiation stage.

More inflammation, therefore, means more CMV reactivation. US3 is an IE protein and appears to be the earliest protein expressed that contributes to immune evasion. It does so by reducing the number of mature MHC complexes in the surface of infected cells (44, 45). Strong T-cell immunity against US3 might curtail this mechanism of immune evasion. US3 was recognized in about 50% of individuals with respect to CD4 T-cells (23 women and 22 men) and in about 40% with respect to CD8 T-cells (21 men and 15 women). The response to US3 was four to five times bigger in men than in women, but this difference reached statistical significance only in regards to IL-2+ CD4 T-cells. Differences with respect to the UL122 (IE-2) and UL123 (IE-1), two other dominant IE proteins, were not statistically significant, however there was a trend to larger TNF and IFN-γ UL123-specific CD8 T-cell responses in males. Overall, men appeared to have bigger responses to IE and E proteins than women. Responses to these immediate-early/early gene products may be of particular importance in driving the size of the T-cell immune response to CMV (44).

We also analyzed sex differences regarding the pro-inflammatory cytokine content of CMV-specific T-cell responses. Since we had not directly measured cytokine production in the supernatant of stimulated cells, we used the integrated MFI (iMFI) as a surrogate marker for the amount of cytokine produced (29, 46). With respect to CD4 and CD8 T-cells and all CMV proteins, the summated iMFI for TNF and IFN-γ was found higher in men than in women. The biggest sex differences in the CD4 T-cell compartment were related to IE/E proteins and in the CD8 compartment to E-L/L proteins. A limitation of these results might be that IE proteins are clearly overrepresented in our selection. This selection, however, was not random, but based on an analysis of overall protein immunodominance (19).

CD3/TCR downregulation occurs following T-cell recognition of MHC-peptide complexes (47) and, generally, correlates with T-cell activation and, more specifically, the strength of the initial activation (48, 49). The extent of CD3 downmodulation varied across the functional subsets defined by the presence or absence of IL-2, TNF, and IFN-γ. Sub1 (IL-2+/TNF+/IFN-γ+) and Sub5 (IL-2-/TNF+/IFN-γ+) showed the highest relative CD3 downregulation in CD4 T cells and Sub5 in CD8 T-cells. One benefit of CD3 downmodulation might be that, as a result of stronger downmodulation, high avidity, highly pro-inflammatory T-cell clones may compete less with lower avidity clones and so avoid excessive inflammatory responses (50). This would fit, for example, with the lesser CD3 downmodulation in men in regards to CD4 T-cell responses to a portion of the very abundant late protein UL48 (UL48A peptide pool) and would be commensurate with the equally observed trend to higher TNF-production by CD4 T-cells in response to the same peptide pool in men. However, our understanding of the biological significance of CD4 and CD8 T-cell responses to individual CMV proteins remains limited and we are unable to explain the majority of patterns of cytokine production *versus* CD3 downmodulation that we have observed. One might also speculate that effector T-cells that readily downregulate CD3/TCR may not progress towards terminal differentiation. This would favor the retention of IL-2 and TNF production, possibly alongside IFN-γ, and promote the emergence of long-lived polyfunctional T-cells with high avidity. The exact mechanisms of CD3/TCR downmodulation are still being debated. Beyond the TCR signal, they are involved in costimulation/immunoregulatory pathways [e.g., PD-1:PDL-1 (51)].

CONCLUSIONS

Our work shows a number of statistically significant sex differences with respect to CMV-specific T-cell immunity (besides a number of

statistically not significant, yet obvious trends). While CMV infection has been reported to cause broad changes to the peripheral blood B- and T-cell compartments with significant differences between women and men, sex differences regarding CMV protein-specific T-cells have not been widely discussed. The trends we observed all point in the same direction, suggesting that older men have a stronger and more inflammatory response to CMV than older women. This may be driven by more frequent CMV reactivation (or partial CMV reactivation) throughout life, which is suggested by higher T-cell responses to IE/E proteins in men. These findings are particularly relevant as CMV infection has been increasingly associated with CVD, which also has a higher incidence in men. Many of the differences we observed as trends would have reached statistical significance, had we not applied stringent multiple end-point corrections. While these remain a useful statistical safeguard against falling for random observations, future studies are warranted revisiting these observations. We are hoping that our work described here will have a seminal effect and make others examine gender differences in the adaptive immune system as we move towards a more personalized and stratified medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UK National Research Ethics Service (NRES) London Centre (Reference 13/LO/1270). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, FK. Methodology, GM and AB, and AP. Formal analysis, FK, BR, SC, ML, and AP. HLA typing and analysis, MH. Donor selection, CR. Writing—original draft preparation, FK, AP, and SC. Writing—review and editing, AB, GM, BR, SC, ML, FK, and AP. Project administration, AP and FK. Funding acquisition, CR and FK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.707830/full#supplementary-material

REFERENCES

1. Solana R, Tarazona R, Aiello AE, Akbar AN, Appay V, Beswick M, et al. CMV and Immunosenescence: From Basics to Clinics. *Immun Ageing* (2012) 9 (1):23. doi: 10.1186/1742-4933-9-23
2. Pourgheysari B, Khan N, Best D, Bruton R, Nayak L, Moss PA. The Cytomegalovirus-Specific CD4+ T-Cell Response Expands With Age and Markedly Alters the CD4+ T-Cell Repertoire. *J Virol* (2007) 81(14):7759–65. doi: 10.1128/JVI.01262-06
3. Di Benedetto S, Derhovanessian E, Steinhagen-Thiessen E, Goldeck D, Muller L, Pawelec G. Impact of Age, Sex and CMV-Infection on Peripheral T Cell Phenotypes: Results From the Berlin BASE-II Study. *Biogerontology* (2015) 16 (5):631–43. doi: 10.1007/s10522-015-9563-2
4. Campos C, Pera A, Sanchez-Correa B, Alonso C, Lopez-Fernandez I, Morgado S, et al. Effect of Age and CMV on NK Cell Subpopulations. *Exp Gerontol* (2014) 54:130–7. doi: 10.1016/j.exger.2014.01.008
5. Reed RG, Al-Attar A, Presnell SR, Lutz CT, Segerstrom SC. A Longitudinal Study of the Stability, Variability, and Interdependencies Among Late-Differentiated T and NK Cell Subsets in Older Adults. *Exp Gerontol* (2019) 121:46–54. doi: 10.1016/j.exger.2019.03.006
6. van der Heiden M, van Zelm MC, Bartol SJW, de Rond LGH, Berbers GAM, Boots AMH, et al. Differential Effects of Cytomegalovirus Carriage on the Immune Phenotype of Middle-Aged Males and Females. *Sci Rep* (2016) 6:26892. doi: 10.1038/srep26892
7. Vallejo AN, Nestel AR, Schirmer M, Weyand CM, Goronzy JJ. Aging-Related Deficiency of CD28 Expression in CD4+ T Cells Is Associated With the Loss of Gene-Specific Nuclear Factor Binding Activity. *J Biol Chem* (1998) 273 (14):8119–29. doi: 10.1074/jbc.273.14.8119
8. Hooper M, Kallas EG, Coffin D, Campbell D, Evans TG, Looney RJ. Cytomegalovirus Seropositivity Is Associated With the Expansion of CD4+CD28- and CD8+CD28- T Cells in Rheumatoid Arthritis. *J Rheumatol* (1999) 26(7):1452–7.
9. Pawelec G, Koch S, Franceschi C, Wikby A. Human Immunosenescence: Does It Have an Infectious Component? *Ann NY Acad Sci* (2006) 1067:56–65. doi: 10.1196/annals.1354.009
10. Pera A, Caserta S, Albanese F, Blowers P, Morrow G, Terrazzini N, et al. $CD28^{null}$ Pro-Atherogenic CD4 T-Cells Explain the Link Between CMV Infection and an Increased Risk of Cardiovascular Death. *Theranostics* (2018) 8(16):4509–19. doi: 10.7150/thno.27428
11. Cannon MJ, Schmid DS, Hyde TB. Review of Cytomegalovirus Seroprevalence and Demographic Characteristics Associated With Infection. *Rev Med Virol* (2010) 20(4):202–13. doi: 10.1002/rmv.655
12. Klenerman P, Dunbar PR. CMV and the Art of Memory Maintenance. *Immunity* (2008) 29(4):520–2. doi: 10.1016/j.immuni.2008.09.008

13. Bots SH, Peters SAE, Woodward M. Sex Differences in Coronary Heart Disease and Stroke Mortality: A Global Assessment of the Effect of Ageing Between 1980 and 2010. *BMJ Glob Health* (2017) 2(2):e000298. doi: 10.1136/bmjgh-2017-000298
14. Vrijenhoek JE, Den Ruijter HM, De Borst GJ, de Kleijn DP, De Vries JP, Bots ML, et al. Sex Is Associated With the Presence of Atherosclerotic Plaque Hemorrhage and Modifies the Relation Between Plaque Hemorrhage and Cardiovascular Outcome. *Stroke* (2013) 44(12):3318–23. doi: 10.1161/STROKEAHA.113.002633
15. Kirkham F, Pera A, Simanek AM, Bano A, Morrow G, Reus B, et al. Cytomegalovirus Infection Is Associated With an Increase in Aortic Stiffness in Older Men Which May Be Mediated in Part by CD4 Memory T-Cells. *Theranostics* (2021) 11(12):5728–41. doi: 10.7150/thno.58356
16. Villacres MC, Longmate J, Auge C, Diamond DJ. Predominant Type 1 CMV-Specific Memory T-Helper Response in Humans: Evidence for Gender Differences in Cytokine Secretion. *Hum Immunol* (2004) 65(5):476–85. doi: 10.1016/j.humimm.2004.02.021
17. Kern F, Faulhaber N, Frommel C, Khatamzas E, Prosch S, Schonemann C, et al. Analysis of CD8 T Cell Reactivity to Cytomegalovirus Using Protein-Spanning Pools of Overlapping Pentadecapeptides. *Eur J Immunol* (2000) 30(6):1676–82. doi: 10.1002/1521-4141(200006)30:6<1676::AID-IMMU1676>3.0.CO;2-V
18. Maecker HT, Dunn HS, Suni MA, Khatamzas E, Pitcher CJ, Bunde T, et al. Use of Overlapping Peptide Mixtures as Antigens for Cytokine Flow Cytometry. *J Immunol Methods* (2001) 255(1-2):27–40. doi: 10.1016/s0022-1759(01)00416-1
19. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly Targeted Human Cytomegalovirus-Specific CD4+ and CD8+ T Cells Dominate the Memory Compartments of Exposed Subjects. *J Exp Med* (2005) 202(5):673–85. doi: 10.1084/jem.20050882
20. Sylwester A, Nambiar KZ, Caserta S, Klenerman P, Picker LJ, Kern F. A New Perspective of the Structural Complexity of HCMV-Specific T-Cell Responses. *Mech Ageing Dev* (2016) 158:14–22. doi: 10.1016/j.mad.2016.03.002
21. Wathen MW, Stinski MF. Temporal Patterns of Human Cytomegalovirus Transcription: Mapping the Viral RNAs Synthesized at Immediate Early, Early, and Late Times After Infection. *J Virol* (1982) 41(2):462–77. doi: 10.1128/JVI.41.2.462-477.1982
22. Larsen M, Sauce D, Arnaud L, Fastenackels S, Appay V, Gorochov G. Evaluating Cellular Polyfunctionality With a Novel Polyfunctionality Index. *PloS One* (2012) 7(7):e42403. doi: 10.1371/journal.pone.0042403
23. R-Core-Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing (2019). Available at: https://www.R-project.org/.
24. Wickham H. *Ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag (2016).
25. Yu G. *Scatterpie: Scatter Pie Plot. R Package Version 0.1.5*. (2020). Available at: https://CRAN.R-project.org/package=scatterpie.
26. Bajwa M, Vita S, Vescovini R, Larsen M, Sansoni P, Terrazzini N, et al. Functional Diversity of Cytomegalovirus-Specific T Cells Is Maintained in Older People and Significantly Associated With Protein Specificity and Response Size. *J Infect Dis* (2016) 214(9):1430–7. doi: 10.1093/infdis/jiw371
27. Chambers J, Angulo A, Amaratunga D, Guo H, Jiang Y, Wan JS, et al. DNA Microarrays of the Complex Human Cytomegalovirus Genome: Profiling Kinetic Class With Drug Sensitivity of Viral Gene Expression. *J Virol* (1999) 73(7):5757–66. doi: 10.1128/JVI.73.7.5757-5766.1999
28. Bajwa M, Vita S, Vescovini R, Larsen M, Sansoni P, Terrazzini N, et al. CMV-Specific T-Cell Responses at Older Ages: Broad Responses With a Large Central Memory Component May Be Key to Long-Term Survival. *J Infect Dis* (2017) 215(8):1212–20. doi: 10.1093/infdis/jix080
29. Shooshtari P, Fortuno ES 3rd, Blimkie D, Yu M, Gupta A, Kollmann TR, et al. Correlation Analysis of Intracellular and Secreted Cytokines via the Generalized Integrated Mean Fluorescence Intensity. *Cytometry A* (2010) 77 (9):873–80. doi: 10.1002/cyto.a.20943
30. van den Berg SPH, Pardieck IN, Lanfermeijer J, Sauce D, Klenerman P, van Baarle D, et al. The Hallmarks of CMV-Specific CD8 T-Cell Differentiation. *Med Microbiol Immunol* (2019) 208(3-4):365–73. doi: 10.1007/s00430-019-00608-7
31. Lachmann R, Bajwa M, Vita S, Smith H, Cheek E, Akbar A, et al. Polyfunctional T Cells Accumulate in Large Human Cytomegalovirus-Specific T Cell Responses. *J Virol* (2012) 86(2):1001–9. doi: 10.1128/jvi.00873-11
32. Walker JD, Maier CL, Pober JS. Cytomegalovirus-Infected Human Endothelial Cells Can Stimulate Allogeneic CD4+ Memory T Cells by Releasing Antigenic Exosomes. *J Immunol* (2009) 182(3):1548–59. doi: 10.4049/jimmunol.182.3.1548
33. Busche A, Jirmo AC, Welten SP, Zischke J, Noack J, Constabel H, et al. Priming of CD8+ T Cells Against Cytomegalovirus-Encoded Antigens Is Dominated by Cross-Presentation. *J Immunol* (2013) 190(6):2767–77. doi: 10.4049/jimmunol.1200966
34. Mason R, Groves IJ, Wills MR, Sinclair JH, Reeves MB. Human Cytomegalovirus Major Immediate Early Transcripts Arise Predominantly From the Canonical Major Immediate Early Promoter in Reactivating Progenitor-Derived Dendritic Cells. *J Gen Virol* (2020) 101(6):635–44. doi: 10.1099/jgv.0.001419
35. Parry HM, Zuo J, Frumento G, Mirajkar N, Inman C, Edwards E, et al. Cytomegalovirus Viral Load Within Blood Increases Markedly in Healthy People Over the Age of 70 Years. *Immun Ageing* (2016) 13:1. doi: 10.1186/s12979-015-0056-6
36. Mason GM, Jackson S, Okecha G, Poole E, Sissons JG, Sinclair J, et al. Human Cytomegalovirus Latency-Associated Proteins Elicit Immune-Suppressive IL-10 Producing CD4(+) T Cells. *PloS Pathog* (2013) 9(10):e1003635. doi: 10.1371/journal.ppat.1003635
37. Krishna BA, Lau B, Jackson SE, Wills MR, Sinclair JH, Poole E. Transient Activation of Human Cytomegalovirus Lytic Gene Expression During Latency Allows Cytotoxic T Cell Killing of Latently Infected Cells. *Sci Rep* (2016) 6:24674. doi: 10.1038/srep24674
38. Shnayder M, Nachshon A, Krishna B, Poole E, Boshkov A, Binyamin A, et al. Defining the Transcriptional Landscape During Cytomegalovirus Latency With Single-Cell RNA Sequencing. *mBio* (2018) 9(2):e00013–18. doi: 10.1128/mBio.00013-18
39. Cheng S, Caviness K, Buehler J, Smithey M, Nikolich-Zugich J, Goodrum F. Transcriptome-Wide Characterization of Human Cytomegalovirus in Natural Infection and Experimental Latency. *Proc Natl Acad Sci USA* (2017) 114(49): E10586–95. doi: 10.1073/pnas.1710522114
40. Griessl M, Renzaho A, Freitag K, Seckert CK, Reddehase MJ, Lemmermann NAW. Stochastic Episodes of Latent Cytomegalovirus Transcription Drive CD8 T-Cell "Memory Inflation" and Avoid Immune Evasion. *Front Immunol* (2021) 12:668885. doi: 10.3389/fimmu.2021.668885
41. Aulock SV, Deininger S, Draing C, Gueinzius K, Dehus O, Hermann C. Gender Difference in Cytokine Secretion on Immune Stimulation With LPS and LTA. *J Interferon Cytokine Res* (2006) 26(12):887–92. doi: 10.1089/jir.2006.26.887
42. Precopio ML, Betts MR, Parrino J, Price DA, Gostick E, Ambrozak DR, et al. Immunization With Vaccinia Virus Induces Polyfunctional and Phenotypically Distinctive CD8(+) T Cell Responses. *J Exp Med* (2007) 204 (6):1405–16. doi: 10.1084/jem.20062363
43. Boyd A, Almeida JR, Darrah PA, Sauce D, Seder RA, Appay V, et al. Pathogen-Specific T Cell Polyfunctionality Is a Correlate of T Cell Efficacy and Immune Protection. *PloS One* (2015) 10(6):e0128714. doi: 10.1371/journal.pone.0128714
44. Hegde NR, Tomazin RA, Wisner TW, Dunn C, Boname JM, Lewinsohn DM, et al. Inhibition of HLA-DR Assembly, Transport, and Loading by Human Cytomegalovirus Glycoprotein US3: A Novel Mechanism for Evading Major Histocompatibility Complex Class II Antigen Presentation. *J Virol* (2002) 76 (21):10929–41. doi: 10.1128/jvi.76.21.10929-10941.2002
45. Liu Z, Winkler M, Biegalke B. Human Cytomegalovirus: Host Immune Modulation by the Viral US3 Gene. *Int J Biochem Cell Biol* (2009) 41 (3):503–6. doi: 10.1016/j.biocel.2008.10.012
46. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 Cells Define a Correlate of Vaccine-Mediated Protection Against Leishmania Major. *Nat Med* (2007) 13(7):843–50. doi: 10.1038/nm1592
47. Valitutti S, Muller S, Dessing M, Lanzavecchia A. Different Responses are Elicited in Cytotoxic T Lymphocytes by Different Levels of T Cell Receptor Occupancy. *J Exp Med* (1996) 183(4):1917–21. doi: 10.1084/jem.183.4.1917
48. Bachmann MF, Oxenius A, Speiser DE, Mariathasan S, Hengartner H, Zinkernagel RM, et al. Peptide-Induced T Cell Receptor Down-Regulation on Naive T Cells Predicts Agonist/Partial Agonist Properties and Strictly Correlates With T Cell Activation. *Eur J Immunol* (1997) 27(9):2195–203. doi: 10.1002/eji.1830270912
49. Viola A, Lanzavecchia A. T Cell Activation Determined by T Cell Receptor Number and Tunable Thresholds. *Science* (1996) 273(5271):104–6. doi: 10.1126/science.273.5271.104

50. Gallegos AM, Xiong H, Leiner IM, Susac B, Glickman MS, Pamer EG, et al. Control of T Cell Antigen Reactivity *via* Programmed TCR Downregulation. *Nat Immunol* (2016) 17(4):379–86. doi: 10.1038/ni.3386
51. Escors D, Bricogne C, Arce F, Kochan G, Karwacz K. On the Mechanism of T Cell Receptor Down-Modulation and Its Physiological Significance. *J Biosci Med* (2011) 1(1):2011.5.

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Therapeutic Vaccination of Hematopoietic Cell Transplantation Recipients Improves Protective CD8 T-Cell Immunotherapy of Cytomegalovirus Infection

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Reactivation of latent cytomegalovirus (CMV) endangers the therapeutic success of hematopoietic cell transplantation (HCT) in tumor patients due to cytopathogenic virus spread that leads to organ manifestations of CMV disease, to interstitial pneumonia in particular. In cases of virus variants that are refractory to standard antiviral pharmacotherapy, immunotherapy by adoptive cell transfer (ACT) of virus-specific $CD8^+$ T cells is the last resort to bridge the "protection gap" between hematoablative conditioning for HCT and endogenous reconstitution of antiviral immunity. We have used the well-established mouse model of $CD8^+$ T-cell immunotherapy by ACT in a setting of experimental HCT and murine CMV (mCMV) infection to pursue the concept of improving the efficacy of ACT by therapeutic vaccination (TherVac) post-HCT. TherVac aims at restimulation and expansion of limited numbers of transferred antiviral $CD8^+$ T cells within the recipient. Syngeneic HCT was performed with C57BL/6 mice as donors and recipients. Recipients were infected with recombinant mCMV (mCMV-SIINFEKL) that expresses antigenic peptide SIINFEKL presented to $CD8^+$ T cells by the MHC class-I molecule K^b. ACT was performed with transgenic OT-I $CD8^+$ T cells expressing a T-cell receptor specific for SIINFEKL-K^b. Recombinant human CMV dense bodies (DB-SIINFEKL), engineered to contain SIINFEKL within tegument protein pUL83/pp65, served for vaccination. DBs were chosen as they represent non-infectious, enveloped, and thus fusion-competent subviral particles capable of activating dendritic cells and delivering antigens directly into the cytosol for processing and presentation in the MHC class-I pathway. One set of our experiments documents the power of vaccination with DBs in protecting the immunocompetent host against a challenge infection. A further set of experiments revealed a significant improvement of antiviral control in HCT recipients by combining ACT with TherVac. In both settings, the benefit from vaccination with DBs proved to be strictly epitope-specific. The capacity to protect was lost when DBs included

the peptide sequence SIINFEKA lacking immunogenicity and antigenicity due to C-terminal residue point mutation L8A, which prevents efficient proteasomal peptide processing and binding to K^b. Our preclinical research data thus provide an argument for using pre-emptive TherVac to enhance antiviral protection by ACT in HCT recipients with diagnosed CMV reactivation.

Keywords: adoptive cell transfer, antiviral protection, HCMV dense bodies, subviral particles, T cell receptor transgenic cells, T cell priming, $CD8^+$ T cells, vaccine

INTRODUCTION

Human cytomegalovirus (hCMV) is the prototype member of the beta-subfamily of herpesviruses (1). Primary infection is rarely diagnosed, because it passes without overt symptoms of disease in the immunologically mature, immunocompetent host. Resolution of acute, productive infection results in maintenance of the viral genome in a non-replicative state, referred to as latent infection or, briefly, "latency". Latency is defined by the presence of reactivation-competent viral genomes in certain cell types [for an overview, see (2)] in absence of infectious virus (3). The medical relevance of hCMV infection is based on birth defects caused by congenital infection of fetuses through diaplacental virus transmission, as well as on multiple organ disease in immunocompromised patients. Major groups at risk of lethal disease from primary infection or productive reactivation from latency are recipients of solid organ transplantation (SOT) and hematopoietic cell transplantation (HCT) [for clinical reviews, see (4–6)]. This report focuses on the further advancement of an established mouse model of experimental HCT and murine cytomegalovirus (mCMV) infection [for reviews on the model, see (7–9)] aiming at a preclinical proof-of-concept evaluation of therapeutic vaccination (TherVac) as a new option to improve immunotherapy by adoptive cell transfer (ACT) of virus-specific $CD8^+$ T cells.

HCT is the therapy of choice for aggressive forms of hematopoietic malignancies that resist standard chemotherapy. Tumor cells become wiped out by hematoablative treatment that, unavoidably, co-depletes bone marrow and the immune system. Transplanted hematopoietic (stem) cells (HC) repopulate the bone marrow stroma and differentiate into all hematopoietic cell lineages, eventually reconstituting a functional immune system. Transient immunodeficiency in the period between HCT and completed reconstitution poses a "window of risk" during which latent hCMV present in transplanted donor cells or in tissues of the recipient can reactivate to productive infection that causes histopathology resulting in organ failure. Interstitial pneumonia represents the most critical organ manifestation of reactivated infection, specifically in the context of HCT. The risk of progression to CMV disease in a latently infected recipient is primarily associated with latent viral genome load in the recipient's tissues, so that it cannot be avoided by selection of an hCMV-negative HC donor (2, 10). Close follow-up monitoring of HCT recipients for viral DNA in the blood by quantitative PCR has become clinical routine to initiate treatment with antiviral drugs upon earliest evidence of hCMV reactivation. This strategy, which is known as "pre-emptive antiviral therapy", aims at preventing ongoing virus replication, inter- and intra-tissue spread, and organ manifestations (6, 11, 12). Although pre-emptive antiviral therapy has significantly reduced the incidence of post-HCT CMV disease, adverse side effects of antivirals (13) and drug-refractory virus variants (14–17) have made it necessary to develop the alternative strategy of immunotherapy by ACT of virus-specific $CD8^+$ T cells as the last therapeutic option. ACT aims at bridging the "protection gap" between hematoablative conditioning for HCT and the completion of endogenous reconstitution of antiviral immunity (18–21).

ACT has been the validity check for the predictive value of the mouse model of CMV infection, disease, and $CD8^+$ T-cell immunotherapy in the immunocompromised host, specifically also in HCT recipients under conditions of transient immunodeficiency during ongoing hematopoietic reconstitution [reviewed in (7–9)]. Prevention of a lethal CMV organ infection by ACT of virus-specific $CD8^+$ T cells was originally demonstrated in the preclinical model of mice infected with mCMV after sublethal γ-irradiation (22–24), years before ACT with cell culture-propagated $CD8^+$ cytolytic T-cell lines (CTLL) specific for the hCMV tegument protein pUL83/pp65 was shown to control human infection (18, 19). The combination of experimental HCT and ACT (HCT-ACT) in the mouse model revealed that ACT not only prevents lethal organ infection and histopathology but also reduces the latent viral genome load in organs and the incidence of recurrent CMV infection (25). Showing this was possible by an experimental induction of virus reactivation, an approach that can be taken only in animal models. Addressing this question by clinical investigation would require viral genome load determinations in biopsies from HCT patients who recovered from CMV reactivation, and waiting for an unpredictable, incidental secondary immunosuppression.

More recently, ACT in the mouse model of experimental HCT and CMV infection has shown that antiviral $CD8^+$ T cells not only prevent viral histopathology in organs but also preclude graft failure (26) from CMV-associated inhibition of the hematopoietic repopulation of bone marrow stroma (27–29). Another valuable insight originally contributed by the mouse model is the loss of per-cell functional activity in CTLL compared to *ex vivo* isolated and directly transferred donor $CD8^+$ central memory T cells (TCM) specific for the same viral epitope, the IE1 peptide YPHFMPTNL in the specific example (30, 31). Subsequent to this, high protective antiviral activity in low-dose ACT was also reported for *ex vivo* sorted hCMV epitope-specific human TCM with stemness (32–36). Yet, a direct comparison of cohorts of ACT recipients receiving CTLL or TCM of identical epitope-specificity was, of course, not feasible in a controlled clinical trial. So, again, it was up to the mouse model to have provided proof-of-concept.

The source of virus-specific $CD8^+$ T cells used in clinical ACT is usually a CMV-experienced, latently infected donor, ideally, the HCT donor who is matched to the HCT recipient for avoiding a graft-*versus*-host (GvH) response against MHC (in humans, HLA) antigens. Thus, HCT-ACT donor and recipient usually share antigen-presenting MHC class-I molecules. Preferably, however, the HCT donor should be CMV-negative to avoid a contribution of transplanted latently infected hematopoietic cells to the risk of reactivation. Besides this, in clinical practice, optimized donor-recipient matching always has priority over donor CMV-status. In cases of a CMV-naïve HCT-ACT donor, $CD8^+$ cells can be transduced with an engineered T-cell receptor (TCR) specific for MHC class-I-presented antigenic viral peptide to generate CMV-TCR transgenic cells for ATC (37, 38). Again, the mouse model provided proof-of-concept by showing that ACT of hCMV-TCR transgenic human $CD8^+$ T cells protects HLA-transgenic mice infected with a recombinant mCMV engineered to express an antigenic peptide of hCMV (39, 40).

Although Odendahl et al. (35) reported on a clinical-scale cell isolation, the logistics for providing sufficient cell numbers, in particular of CMV-TCR transgenic $CD8^+$ T cells generated from CMV-negative donors, remains demanding, and has so far precluded CMV-specific ACT from becoming clinical routine. Here, we further developed the mouse model to pursue the concept of improving the efficacy of low-dose ACT in CMV-infected HCT recipients by further expanding limited numbers of transferred donor $CD8^+$ T cells within the HCT recipient by TherVac. We employed the highly versatile model of TCR-transgenic OT-I $CD8^+$ T cells, which are specific for the ovalbumin (OVA)-derived antigenic peptide SIINFEKL (41, 42), for investigating the potential of TherVac to enhance the efficacy of ACT. Specifically, recipients of HCT and OT-I ACT were infected with a recombinant mCMV expressing SIINFEKL (mCMV-SIINFEKL), and TherVac was performed with recombinant hCMV dense bodies (DB-SIINFEKL) containing SIINFEKL within tegument protein pUL83/pp65. DBs were chosen for TherVac, because they represent non-infectious, enveloped and thus fusion-competent subviral particles capable of activating dendritic cells (DC) and delivering antigens into the cytosol for processing and presentation in the MHC class-I pathway (43–46). Our data show that TherVac by DB-SIINFEKL drives the proliferation of transferred OT-I cells in lymphoid tissues of ACT recipients and that equivalence in the efficacy of antiviral control in HCT recipients is reached with a significantly lower number of OT-I cells when ACT and TherVac are combined.

MATERIALS AND METHODS

Cells and Mice

Murine embryonic fibroblasts (MEF) were prepared and cultivated in minimal essential medium (MEM, Thermo Fisher Scientific), supplemented with 10% fetal calf serum (FCS, Thermo Fisher Scientific) and Penicillin/Streptomycin (Thermo Fisher Scientific), by standard protocol (47). Primary human foreskin fibroblasts (HFF) were grown in MEM supplemented with 10% FCS, 2 mM L-glutamine, 50 ng gentamicin ml^{-1}, and 0.5 ng basic fibroblast growth factor ml^{-1} (bFGF, Thermo Fisher Scientific).

Female C57BL/6 (8-week-old) mice were purchased from Harlan Laboratories and were housed under specified pathogen-free (SPF) conditions in the Translational Animal Research Center (TARC) of the University Medical Center of the Johannes Gutenberg-University Mainz. TCR-transgenic OT-I mice (42) were bred and housed in the TARC under SPF conditions.

Generation of Recombinant mCMVs

Recombinant viruses mCMV-SIINFEKL and mCMV-SIINFEKA were generated by two-step replacement BAC mutagenesis in the mCMV BAC plasmid pSM3frΔm157luc (48), replacing a sequence that codes for an endogenous D^d-presented antigenic peptide in the non-essential gene *m164* with sequences coding for peptides SIINFEKL or SIINFEKA (49, 50). The recombinant mCMVs were reconstituted in MEF and were propagated for removal of BAC sequences and for amplification (51). Infectious virions were purified according to standard procedures (47). Reconstituted and purified virus derived from the parental BAC plasmid pSM3frΔm157luc served as a control virus, for the sake of brevity herein referred to as mCMV, despite features included for a multi-purpose usage not applying to this report.

Generation of Recombinant hCMV Dense Bodies (DBs)

Recombinant DBs HB5-pp65_SIINFEKL (briefly DB-SIINFEKL) and HB5-pp65_SIINFEKA (briefly DB-SIINFEKA) were generated by using the galK positive/negative selection procedure as described (45). In essence, the DNA sequence encoding peptides SIINFEKL or SIINFEKA was integrated into the hCMV open reading frame UL83, which is contained within BAC plasmid HB5 (52), to express fusion proteins in which SIINFEKL or SIINFEKA are integrated at amino acid position W175 of the tegument protein pUL83/pp65 (53). Unmodified hCMV DB (DB-Ø) were also HB5-derived (46). Viruses were reconstituted and stocks were prepared as described (54). DBs were purified from late-stage infected HFF by glycerol-tartrate gradient ultracentrifugation (43). In one experiment, purified ovalbumin (OVA) was used (catalog number 9006-59-1; Sigma-Aldrich Chemie, Steinheim, Germany). JPT Peptide Technologies (Berlin, Germany) synthesized OVA peptide SIINFEKL.

Adoptive Cell Transfer (ACT)

$CD8^+$ T cells were isolated from spleens of 10- to 20-week-old OT-I mice (42) by immune-magnetic cell sorting. This yields an almost pure population of Vα2Vβ5 TCR-transgenic OT-I cells specific for the peptide-MHC class-I (pMHC-I) complex SIINFEKL-K^b. For immunotherapy by ACT, these cells were infused intravenously into total-body γ-irradiated (7 Gy) C57BL/6 mice, followed by intraplantar (left hind footpad) infection of the recipients with 10^5 plaque-forming units (PFU) of recombinant mCMVs.

In Vivo Proliferation Assay

OT-I cells were fluorescence-labeled by incubation for 4 min at 37°C at a concentration of 1×10^7 cells/ml with 5 μM of 5(6)-carboxyfluorescein diacetate (CFDA; Merck Darmstadt) in phosphate-buffered saline (PBS). CFDA converts intracellularly into the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE). The reaction was stopped with FCS, and the cells were washed three times with PBS [(50) and references therein]. CFSE-labeled OT-I cells (10^7) were administered intravenously into immunocompetent C57BL/6 mice. Intraplantar infection or application of DBs was performed 24 h later. At the indicated times, OT-I cells that have homed to the spleen or the popliteal lymph node (PLN) were enriched by positive immune-magnetic sorting of $CD8^+$ T cells. To assess their proliferation, loss of CFSE fluorescence with every cell division was determined by cytofluorometric analysis.

Experimental HCT

Syngeneic HCT with 9-week-old female C57BL/6 mice as bone marrow cell (BMC) donors and recipients was performed as described in greater detail previously (29, 47). In brief, hematoablative conditioning was performed by sublethal total-body γ-irradiation with a single dose of 7 Gy. Femoral and tibial donor BMC were depleted of $CD8^+$ and $CD4^+$ T cells, present within bone marrow capillaries, by negative immune-magnetic cell sorting. Donor hematopoietic cells (5×10^6/mouse) were infused into the tail vein of the recipients at 6 h after irradiation, followed by intraplantar infection (see above).

T-Cell Depletion

In vivo depletion of $CD8^+$ T cells was performed by a single intravenous injection of purified monoclonal antibody (clone: YTS169.4; 1.3 mg/mouse) directed against the CD8 molecule.

Cytofluorometric Analyses

Single-cell suspensions were prepared from lymph nodes, spleen, and lungs, as described (47, 55). Unspecific staining was blocked with unconjugated anti-FcγRII/III antibody (anti-CD16/CD32, clone 2.4G2; BD Biosciences). Cells were stained with the following antibodies for multi-color cytofluorometric analyses: ECD-conjugated anti-CD8α (clone 53-6.7, Beckman Coulter), PE-conjugated anti-TCR Vβ5.1/5.2 (clone MR9-4, BD Biosciences), and APC-conjugated anti-TCR Vα2 (clone B20.1, BD Biosciences). Peptide/epitope-specific $CD8^+$ T cells were identified with APC-conjugated MHC-I dextramer H-2Kb/SIINFEKL (Immudex, Copenhagen, Denmark). For the analyses, a "live gate" was routinely set on leukocytes in the forward scatter (FSC) *versus* sideward scatter (SSC) plot. All cytofluorometric analyses were performed with flow cytometer FC500 and CXP analysis software (Beckman Coulter).

Quantitation of Tissue Infection and T-Cell Infiltration

Infectious virus in spleen, lungs, liver, and salivary glands was quantitated in the respective organ homogenates with high sensitivity by virus plaque assay performed under conditions of "centrifugal enhancement of infectivity" [(47, 56) and references therein]. Infected cells and tissue infiltration by $CD8^+$ T cells, which include OT-I cells, were detected and quantified in liver tissue sections by two-color immunohistochemistry (2C-IHC) specific for the intranuclear viral protein IE1 (red staining) and the CD8 molecule (black staining), using monoclonal antibodies Croma101 and anti-mouse CD8 (clone 4SM15, eBioscience), respectively (47, 51). The total numbers N of infected $IE1^+$ liver cells (mostly hepatocytes) and of liver-infiltrating or liver-localizing $CD8^+$ T cells were calculated according to the formula:

$$N = n \times V/V^{\star} \times d/(D + d)$$

n = number of stained cells counted in a 10-mm^2 tissue section area (mean of five independent areas);

V = volume of a mouse liver embedded in paraffin (mean of 10 livers = 420 mm^3);

$V^{\star}$ = volume of the count disc = 10 $mm^2 \times d$ = 0.02 mm^3;

d = thickness of the tissue section = 0.002 mm;

D = maximal diameter of the counted object;

D-$CD8^+$ T cell = 0.010 mm;

D-$IE1^+$ hepatocyte nucleus = 0.006 mm.

From this it follows that:

$$N - \mathrm{CD8^+cells} = n \times 3,500;$$

$$N - IE1^+\mathrm{hepatocytes} = n \times 5,250.$$

The correction term $d/(D+d)$ takes into account that an object can be cut more than once by tissue sections if $d < D$.

Quantitation and Avidity Distributions of Viral Epitope-Specific $CD8^+$ T Cells

An 18-h IFNγ-based enzyme-linked immunospot (ELISpot) assay was used to determine the frequency of epitope-specific $CD8^+$ T cells [(30, 57) and references therein]. Briefly, graded numbers of immune-magnetically purified $CD8^+$ T cells were sensitized in triplicate assay cultures by EL-4 ($H\text{-}2^b$) cells that were exogenously loaded with epitope-representing synthetic peptides at the indicated molar loading concentrations. Custom peptide synthesis with a purity of >80% was performed by JPT Peptide Technologies (Berlin, Germany). Spots, representing single cells stimulated to secrete IFNγ, were counted automatically, based on standardized criteria using ImmunoSpot S4 Pro Analyzer (Cellular Technology Limited, Cleveland, OH, USA). Avidity distributions were derived by calculation from frequencies of $CD8^+$ T cells responding to stimulation by graded target cell loading concentrations of synthetic peptides (cumulative avidity distribution), as explained in greater detail previously (55, 58).

Determination of Viral Doubling Times

Virus growth is quantitated from log-linear regression lines [$\log N(t) = at + \log N(0)$], where $N(t)$ is an infection parameter (ordinate), such as the number of PFU or of infected cells, measured at time t (abscissa) after infection, a is the slope of the regression line, and log $N(0)$ is its ordinate intercept. Linear regression was calculated with GraphPad Prism 6.07 (GraphPad Software). The viral doubling time (vDT) is calculated according to the formula: $vDT = \log 2/a$. The upper and lower 95%

confidence limit values of slope *a* (determined from the ellipsoidal parameter confidence region) define the 95% confidence intervals of *vDT* (51).

Statistics

Frequencies (most probable numbers) of cells responding in the ELISpot assay and the corresponding 95% confidence intervals were calculated by intercept-free linear regression analysis from the linear portions of regression lines, based on spot counts from triplicate assay cultures for each of the graded cell numbers seeded (30, 57). To evaluate statistical significance of differences between two independent sets of log-transformed, log-normally distributed data, the two-sided unpaired *t*-test with Welch's correction of unequal variances was used. In case of data sets that include data below the detection limit of the assay, which excludes log-transformation, the distribution-free Wilcoxon Mann Whitney test was applied. Differences were considered as statistically significant for *P*-values of <0.05 (*), <0.01 (**), and <0.001 (***). Calculations were performed with GraphPad Prism 6.07 (GraphPad Software).

RESULTS

Recombinant mCMV Expressing Peptide SIINFEKL Primes CD8$^+$ T Cells and Drives the Proliferation of Transferred OT-I Cells in an Epitope-Specific Manner

It was the aim of this study to develop an advanced preclinical model for enhancing the efficacy of ACT as pre-emptive immunotherapy of CMV infection in HCT patients by TherVac. To build this model, its modular components were first characterized and tested individually.

As module-1 of the mouse model, we generated recombinant viruses mCMV-SIINFEKL and mCMV-SIINFEKA expressing the antigenic model peptide SIINFEKL or its non-immunogenic analog SIINFEKA, respectively. We have previously shown that point mutation L8A of the C-terminal amino acid residue destroys immunogenicity by reducing proteasomal cleavage as well as binding to the presenting MHC class-I molecule K^b (49). With this strategy, one can generate an optimal control for epitope-specificity, as all other infection parameters remain conserved [for a review, see (59)]. To avoid interference with viral replicative fitness, SIINFEKL/A peptide-coding sequences replaced an endogenous antigenic sequence in a non-essential viral gene. Despite this rationale of virus design, independently generated recombinant viruses are never identical in terms of replicative fitness and should be tested before use in experiments for avoiding a misinterpretation of quantitative differences. To focus on non-immunological parameters, replicative fitness for all viruses is determined by growth kinetics and the associated viral doubling time (*vDT*) in various organs of immunocompromised mice (60–62). In the specific case here, *vDT* values differed between different organs, as it was expected from previous experience, but, within each organ, control virus mCMV as well as recombinant viruses mCMV-SIINFEKL and mCMV-SIINFEKA replicated almost equivalently, as indicated by overlapping 95% confidence intervals for the *vDT* values (**Supplementary Figure S1**).

Based on this verified comparability in replicative fitness, we primed immunocompetent C57BL/6 (haplotype *H-2^b*) mice by local, intraplantar infection [(57), for a discussion of priming route in vaccination, see (55)], and quantitated viral epitope-specific and functional IFNγ-secreting CD8$^+$ T cells present in the spleen (**Figure 1A**, protocol; **Figure 1B**, results). As one could expect, all three viruses primed cells specific for a panel of endogenous antiviral peptides of mCMV in the *H-2^b* haplotype (63), whereas only mCMV-SIINFEKL successfully primed SIINFEKL-specific CD8$^+$ T cells, thereby verifying the prevention of immunogenicity by point mutation L8A in mCMV-SIINFEKA.

As module-2 of the mouse model, we introduced TCR-transgenic OT-I cells for performing ACT. OT-I cells express a Vα2Vβ5 TCR specific for the pMHC-I complex SIINFEKL-K^b (41, 42). As revealed by cytofluorometric analysis, >90% of the CD8$^+$ T cells isolated from spleens of OT-I mice expressed the transgenic TCR (**Supplementary Figure S2A**).

Combining module-1 and module-2 first in immunocompetent C57BL/6 mice, we addressed the question if an infection set on the day after intravenous ACT and at a peripheral site, specifically the mouse footpad, would drive the proliferation of CFSE fluorescence-labeled OT-I cells in lymphoid tissues in an epitope-specific manner. We have recently used this approach to document the presentation of SIINFEKL in mice latently infected with mCMV-SIINFEKL (50). In a pilot experiment, we first showed that mCMV-SIINFEKL drives the proliferation of transferred OT-I cells in the ipsilateral PLN, which is the draining regional lymph

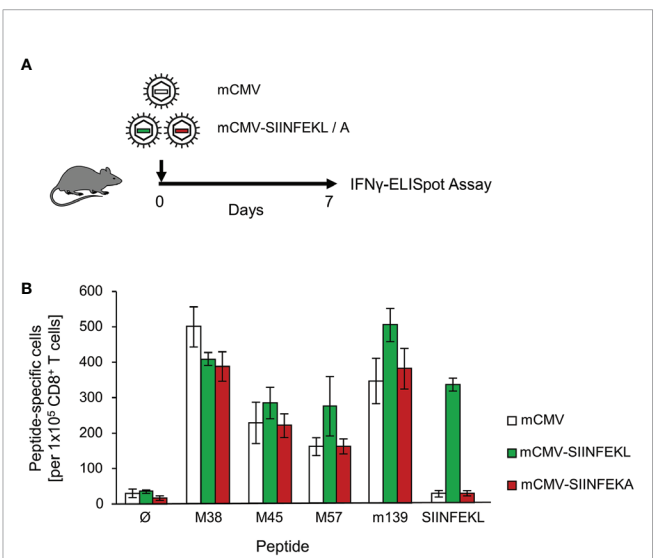


FIGURE 1 | Priming of viral epitope-specific CD8$^+$ T cells. **(A)** Experimental protocol. On day 7 after intraplantar infection of immunocompetent C57BL/6 mice with 10^5 PFU of the viruses indicated, frequencies of functional, epitope-specific CD8$^+$ T cells present in the spleen (from three mice pooled) were determined by an IFNγ-ELISpot assay. **(B)** Frequencies of primed cells specific for the epitopes indicated. For stimulation in the assay, EL-4 cells were exogenously loaded with the corresponding synthetic antigenic peptides at a saturating concentration of 10^{-7}M. (Ø), no peptide loaded. Bars represent numbers of responding cells. Error bars indicate the 95% confidence intervals.

node in the case of unilateral intraplantar infection, but not in the corresponding contralateral PLN (**Supplementary Figure S3**). This shows that intravenously administered OT-I cells home to PLN and that local infection drives their proliferation. In a following experiment, we studied OT-I proliferation in the spleen in the time course and verified the epitope-specificity (for the protocol, see **Figure 2A**). Whereas proliferation was absent in the basal control group consisting of uninfected C57BL/6 mice, OT-I cells underwent several cell divisions when the recipients were infected with mCMV-SIINFEKL expressing the cognate antigenic peptide. In contrast, only few cell divisions were observed after infection with epitope mutant virus mCMV-SIINFEKA (**Figure 2B**). These findings thus indicated some epitope-independent, but infection-related proliferation of transferred OT-I cells, most likely driven by virally induced cytokines, as well as a much stronger component of an epitope-specific proliferation.

ACT With OT-I Cells Protects Against Infection of Immunocompromised Recipients in a Cell Dose-Dependent and Epitope-Specific Manner

Continuing with combining module-1 and module-2, now in ACT recipients immunocompromised by sublethal total-body γ-irradiation, the antiviral potential of pre-emptive immunotherapy by ACT with OT-I cells was tested by transferring graded numbers of OT-I cells, followed by infection with mCMV-SIINFEKL or mCMV-SIINFEKA (**Figure 3A**). Virus epitope-specific $CD8^+$ T cells can only protect against infection when their functional avidity is high enough to detect pMHC-I complexes presented at the cell surface after endogenous antigen processing and presentation. In CMV infections, the demand for high avidity is tightened by the expression of immune evasion proteins that interfere with cell surface trafficking of pMHC-I complexes [for

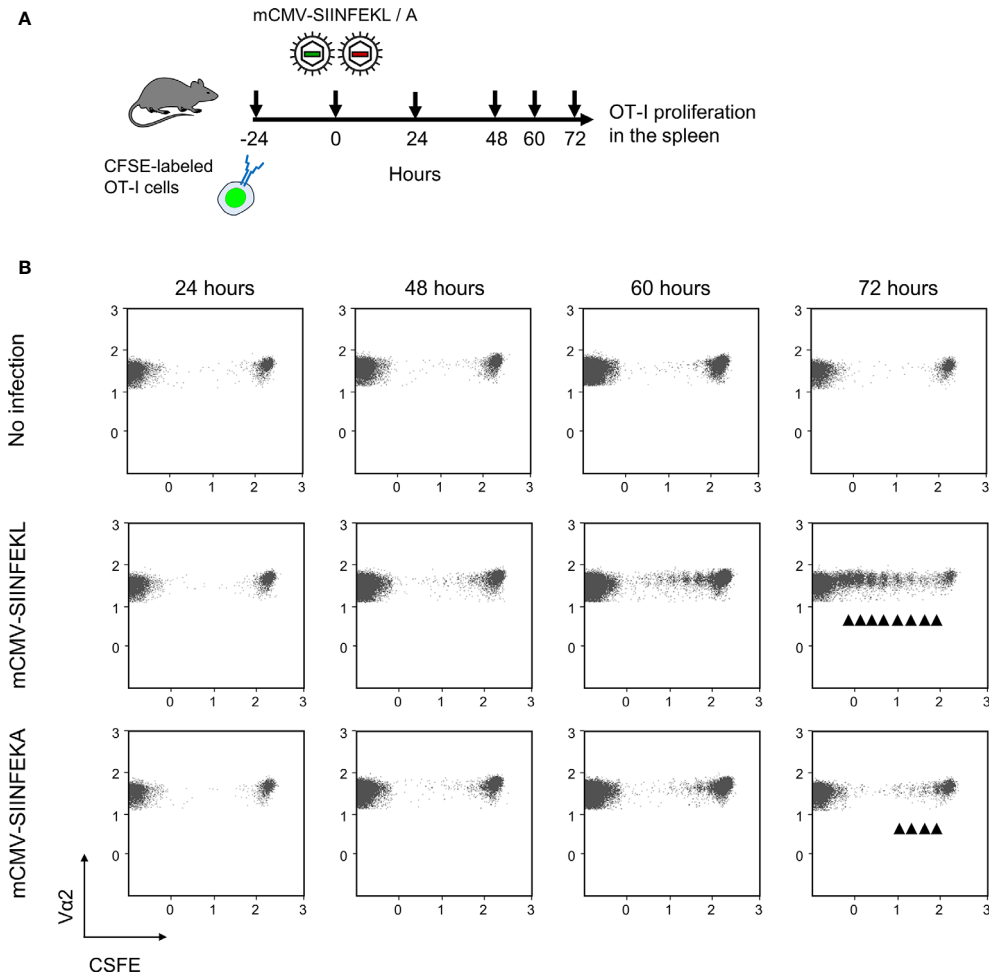


FIGURE 2 | Lymphoid-tissue homing and epitope-specific proliferation of transferred OT-I cells. **(A)** Experimental protocol. CFSE fluorescence-labeled OT-I cells were transferred intravenously into immunocompetent C57BL/6 mice. Proliferation of OT-I cells recovered from the spleen (from three mice pooled) was measured at the indicated times after intraplantar infection with 10^5 PFU of viruses mCMV-SIINFEKL or mCMV-SIINFEKA. **(B)** Cytofluorometric measurement of the loss of the fluorescence label due to the proliferation of OT-I cells over time. (Ordinate), fluorescence specific for the Vα2 TCR chain expressed by OT-I cells. (Abscissa), CFSE fluorescence. For groups of most interest, arrowheads mark cell divisions.

mCMV, see (49, 64, 65)]. As we have shown recently, recognition of infected cells requires a functional avidity that corresponds to the recognition of ≤10^{-9} M of exogenously loaded antigenic peptide (55). We have therefore determined the avidity distribution for OT-I cells and found that most functional cells in the OT-I population have an avidity corresponding to 10^{-10} M, and a significant proportion even to 10^{-11} M (**Supplementary Figure S2B**). Thus, OT-I cells fulfill a prerequisite for protecting against mCMV infection upon ACT despite the expression of viral immune evasion proteins in infected tissue cells of the recipients.

This prediction came true in ACT, demonstrating a cell dose-dependent protection against infection with mCMV-SIINFEKL in spleen, lungs, and liver (**Figure 3B**). Notably, low-dose ACT with just 100 OT-I cells significantly reduced the infection in all three organs tested, which is in good agreement with clinical data on low-dose ACT with streptamer-enriched hCMV-specific $CD8^+$ T cells into HCT recipients, as reported by Stemberger and colleagues (33). In contrast, as a specificity control that cannot be accomplished in a clinical trial, even a 10,000-fold higher number of transferred OT-I cells failed to reduce the infection with mCMV-SIINFEKA, in which the cognate epitope is selectively missing, while all other parameters associated with infection are maintained. These include cytokine network perturbation, innate immune responses, non-cognate antigen presentation, and a general immune system remodeling.

After infection with mCMV-SIINFEKL, 2C-IHC images of liver tissue sections (**Figure 4A**) show disseminated tissue infection with extended plaque-forming clusters of infected cells, mostly of hepatocytes (61, 66), when no OT-I cells were transferred. In contrast, after transfer of as few as 100 OT-I cells, liver tissue infection was largely controlled by tissue-infiltrating OT-I cells that confine and eventually resolve the infection by aggregating around few remaining infected cells, thereby forming nodular inflammatory foci (NIF). These are micro-anatomical structures that are indicative of epitope-specific protection (31, 39, 67, 68). In the absence of the epitope, that is, after infection with mCMV-SIINFEKA, few OT-I cells are seen being randomly distributed in highly infected liver tissue, failing to form NIF (**Supplementary Figure S4**).

It appears to be obvious that protection against mCMV-SIINFEKL in different organs cannot be exerted by the 100 OT-I cells transferred, but depends on clonal expansion in the recipients (33, 34). For a minimum estimate of OT-I cell divisions, we determined the absolute numbers of OT-I cells present in the

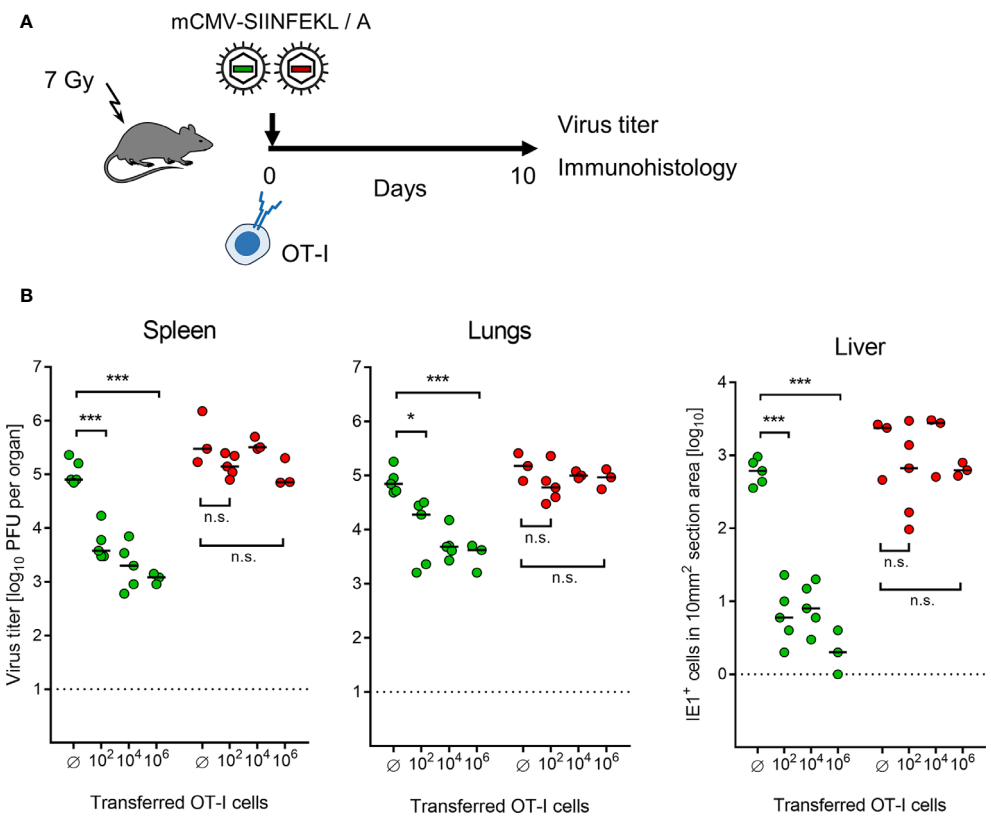


FIGURE 3 | Control of organ infection by ACT of OT-I cells. **(A)** Experimental protocol. OT-I cells were transferred intravenously into immunocompromised C57BL/6 mice on the day of intraplantar infection with 10^5 PFU of viruses mCMV-SIINFEKL or mCMV-SIINFEKA. **(B)** Virus titers in spleen and lungs, as well as numbers of infected $IE1^+$ liver tissue cells in representative 10-mm^2 section areas were determined on day 10 after infection and transfer of graded numbers of OT-I cells. (Ø) no OT-I cells transferred. Symbols (green: mCMV-SIINFEKL, red: mCMV-SIINFEKA) represent data from individually tested mice, with the median values marked. Significance levels (*) $P < 0.05$, (***) $P < 0.001$. (n.s.), not significant ($P > 0.05$).

liver on day 10 after transfer of an initial number of 100 OT-I cells, and paired these numbers with the absolute numbers of infected liver cells in 5 mice analyzed by 2C-IHC individually (**Figure 4B**). A control group not receiving OT-I cells served to quantitate residual liver-resident $CD8^+$ T cells for subtraction. Control of infection clearly correlated with tissue infiltration by OT-I cells. The mean number of OT-I cells per liver was 1.445×10^6, which corresponds to 13–14 cell divisions. This has to be interpreted as a minimum estimate, as OT-I cells infiltrate also other tissues.

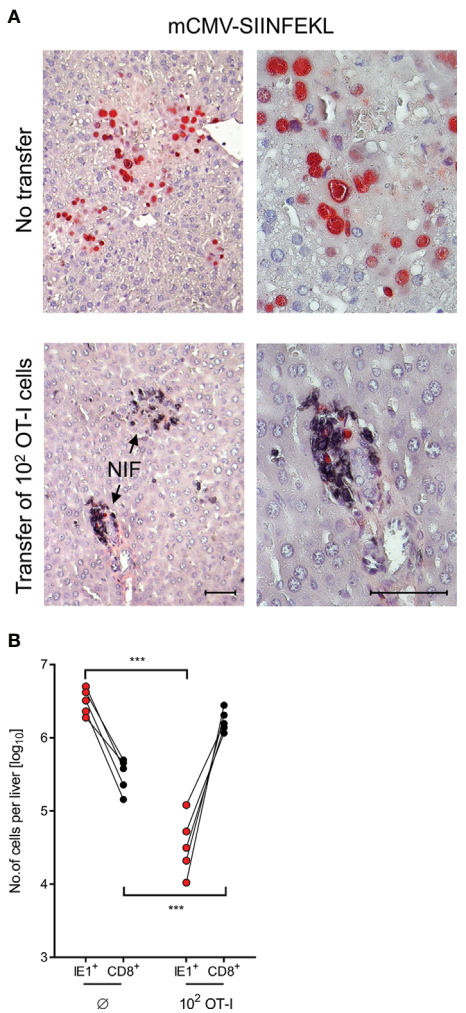


FIGURE 4 | Control of liver infection by tissue infiltrating OT-I cells. **(A)** 2C-IHC images of liver tissue sections, corresponding to groups with no cell transfer and with transfer of 100 OT-I cells in Figure 3. Liver cells, mostly hepatocytes, infected with mCMV-SIINFEKL are identified by expression of the intranuclear viral protein IE1 (red staining). Residual $CD8^+$ T cells and infiltrating OT-I cells are visualized by black staining of the CD8 molecule. (Left images), low-magnification overview. (Right images), resolution to greater detail by higher magnification. Bar markers, 50 µm. (NIF), nodular inflammatory foci that represent microanatomical sites where protective OT-I cells aggregate at infected liver cells to confine and resolve the infection. **(B)** Absolute numbers of infected liver cells (red symbols) and of residual $CD8^+$ T cells (Ø, no transfer control group) as well as tissue-infiltrating OT-I cells (both with black symbols) were determined by quantitative 2C-IHC for five mice individually. Paired data are connected by lines. (***) $P < 0.001$.

Recombinant Dense Bodies (DBs) Prime Epitope-Specific $CD8^+$ T Cells

As module-3 of the mouse model, we introduced hCMV DBs for future use in TherVac. Recombinant DBs were engineered to contain peptides SIINFEKL or SIINFEKA integrated within the protein pUL83/pp65, which is a tegument protein of hCMV virions and the major component of DBs (69). The potential of DB-SIINFEKL for priming of SIINFEKL-specific $CD8^+$ T cells in immunocompetent C57BL/6 mice was tested by intraplantar application of graded doses of purified DBs, and frequencies of functional, IFNγ-secreting $CD8^+$ T cells were determined on day 7 in the spleen (**Figure 5A**). The response increased steadily with increasing doses of DBs and was strictly epitope-specific. Only DB-SIINFEKL, but not DB-SIINFEKA, induced SIINFEKL-K^b specific $CD8^+$ T cells. An unrelated mCMV peptide, namely, peptide m139 that is also presented by K^b, was not recognized after application of either type of DB (**Figure 5B**).

Vaccination With Recombinant DBs Protects Immunocompetent Mice Against a High-Dose Challenge Infection

Combining module-1 and module-3, we tested the capacity of DBs to serve as a prophylactic intraplantar vaccine for protection against a subsequent high-dose intravenous challenge infection of immunocompetent C57BL/6 mice (**Figure 6A**). Compared to a control group with no vaccination, intraplantar priming (recall **Figure 5**) with DB-SIINFEKL significantly reduced the replication of challenge virus mCMV-SIINFEKL in all organs tested, but not of challenge virus mCMV-SIINFEKA lacking the cognate epitope. Accordingly, vaccination with the epitope-deletion variant DB-SIINFEKA failed to protect against challenge infection with either virus (**Figure 6B**).

To identify the protective cell type primed by DB-vaccination, we depleted $CD8^+$ T cells 6 days after priming with DB-SIINFEKL and one day before a high-dose intravenous challenge infection with mCMV-SIINFEKL (**Figure 7A**). Again, confirming the results of the experiment shown above (**Figure 6**), vaccination with DB-SIINFEKL significantly reduced virus replication in all organs tested when compared to the control group with no vaccination. This protection was abolished by depletion of $CD8^+$ T cells shortly before challenge infection (**Figure 7B**).

So far, SIINFEKL was included in the DBs as part of a fusion protein with pUL83/pp65, from which it is released after DB uptake through envelope-cell membrane fusion followed by proteasomal processing. One important feature of DBs is their capacity to stimulate maturation and activation of DCs (46), so that they are source of antigen and adjuvant both in one. We therefore wondered if these two roles can be separated. Indeed, whereas purified ovalbumin (OVA) alone failed to prime protective SIINFEKL-specific $CD8^+$ T cells, non-antigenic DB-Ø (**Figure 8A**) as well as non-antigenic DB-SIINFEKA (**Figure 8B**) mediated uptake and processing of OVA for priming a protective SIINFEKL-specific response. Finally, non-antigenic DB-

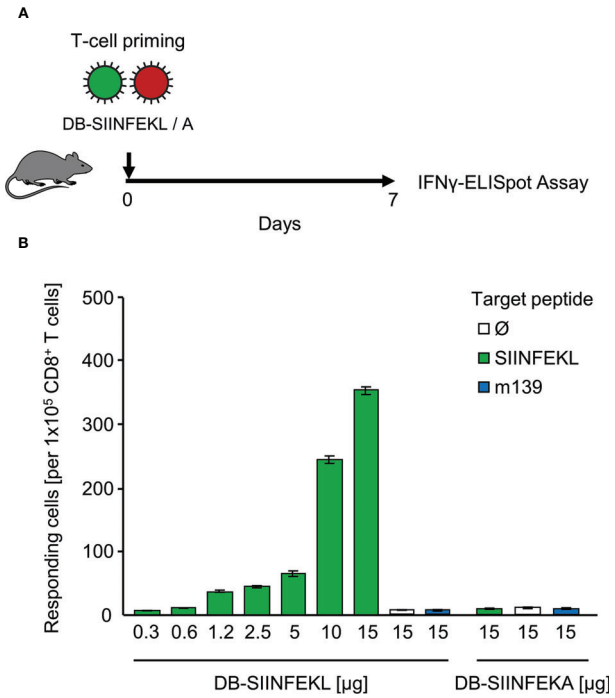


FIGURE 5 | Epitope-specific priming of $CD8^+$ T cells by recombinant dense bodies (DBs). **(A)** Experimental protocol. On day 7 after intraplantar application of graded µg-doses of DB-SIINFEKL or DB-SIINFEKA to immunocompetent C57BL/6 mice, frequencies of functional, viral epitope-specific $CD8^+$ T cells recovered from the spleen (from three mice pooled) were determined by an IFNγ-ELISpot assay. **(B)** Frequencies of primed cells specific for the viral epitopes indicated. For stimulation in the assay, EL-4 cells were exogenously loaded with the corresponding synthetic antigenic peptides at a saturating concentration of 10^{-7} M. (Ø), no peptide loaded. Bars represent numbers of responding cells. Error bars indicate the 95% confidence intervals.

SIINFEKA adjuvanted the priming by purified antigenic peptide SIINFEKL (**Figure 8C**). While significance of protection after priming with DB-SIINFEKL was always achieved and in both spleen and liver, DB-adjuvanted priming with OVA protein or SIINFEKL peptide was less efficient in the control of liver infection and did not always reach statistical significance. Thus, providing the antigen as fusion protein within DBs remains the strategy of choice.

In conclusion, this set of experiments has verified efficient priming of protective SIINFEKL-specific $CD8^+$ T cells by vaccination with DB-SIINFEKL.

Vaccination With Recombinant DBs Drives the Epitope-Specific Proliferation of Adoptively Transferred TCR-Transgenic T Cells in Immunocompetent ACT Recipients

We then combined module-2 and module-3 for testing the capacity of recombinant DBs to drive the proliferation of adoptively transferred OT-I cells in immunocompetent ACT recipients. A pilot experiment showed that sensitization by DB-SIINFEKL was as efficient as infection with mCMV-SIINFEKL in driving the proliferation of OT-I cells in the spleen (**Supplementary Figure S5**). In the main experiment, we looked for the proliferation of transferred CFSE-labeled OT-I cells in the PLN that drains the site of DB vaccine application (**Figure 9A**). Epitope-specific sensitization by DB-SIINFEKL induced more cell divisions and faster than did an epitope-independent sensitization by DB-SIINFEKA (**Figure 9B**). Some delayed activation of OT-I cells by DB-SIINFEKA may result from cytokines expressed in response to antigen-independent DC activation by DBs (43–46).

TherVac With Recombinant DBs Enhances the Efficacy of Low-Dose Antiviral Immunotherapy in Immunocompromised ACT Recipients

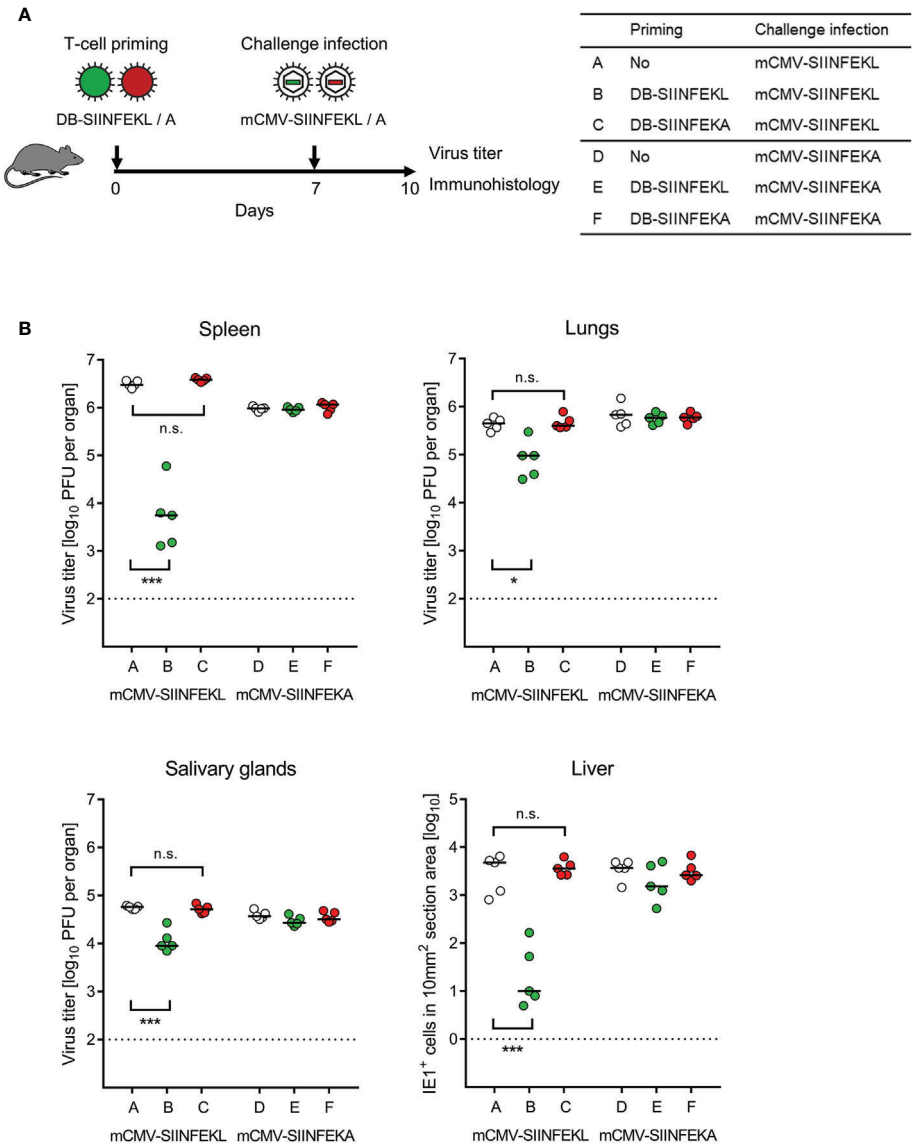
The three modules were combined to test if TherVac can enhance the antiviral efficacy of ACT in immunocompromised recipients. TherVac by intraplantar application of DB-SIINFEKL was carried out on the day after intravenous infusion of OT-I cells. A scenario of early virus reactivation was modeled by infection with mCMV-SIINFEKL at the site and time of TherVac (**Figure 10A**). The decisive question was if antiviral T cells in the circulation would migrate to a peripheral site of vaccine application and receive their signals from local antigen presentation.

We expected to see an enhancement of protection by TherVac especially under conditions when low-dose ACT alone is not sufficient. It is our long experience in this model that infection is generally more difficult to control in the lungs compared to spleen and liver [(22) and many publications to follow], and depends on mast cell-derived chemokine CCL5 for enhancing tissue infiltration by protective pulmonary $CD8^+$ T cells (70). In accordance with this, a benefit from TherVac after low-dose ACT with just 100 OT-I cells was most significant in the lungs (**Figure 10B**). This is of interest, because the lungs are the clinically most relevant organ site of CMV disease, a fact that reflects the inefficient immune control in the lungs. Thus, enhancing the pulmonary immune response by TherVac may be an option to, at least, reduce the severity of CMV-associated interstitial pneumonia.

TherVac With Recombinant DBs Enhances the Efficacy of Antiviral Immunotherapy by ACT in HCT Recipients

ACT without HCT has no clinical correlate. The clinical demand is to bridge the critical phase of transient immunodeficiency between hematoablative treatment and endogenous hematopoietic reconstitution by HCT in leukemia/lymphoma patients who are at risk of CMV disease from hCMV reactivation. For studying this situation, we added HCT as module-4 to our mouse model, combining HCT with ACT by OT-I cells, TherVac by recombinant DBs, and mCMV infection. HCT and ACT were performed combined by intravenous transfer of HC and OT-I cells in a mixture. TherVac and infection followed the next day, both by intraplantar application in a mixture (**Figure 11A**).

Endogenous reconstitution of antiviral $CD8^+$ T cells by syngeneic HCT eventually led to clearance of productive

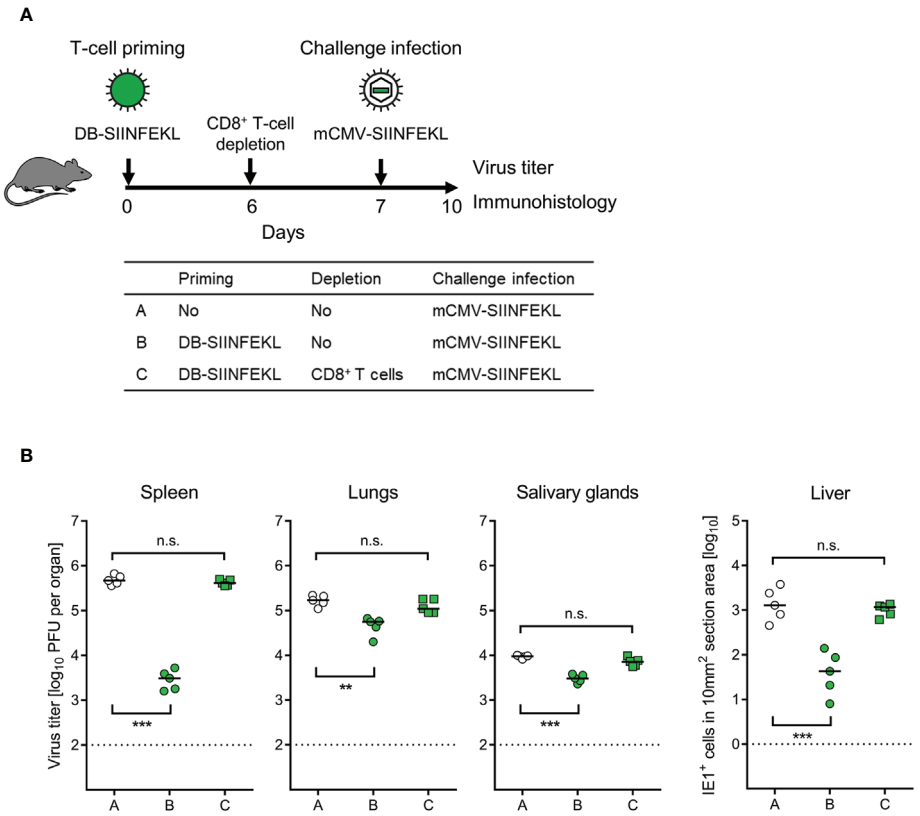


	Priming	Challenge infection
A	No	mCMV-SIINFEKL
B	DB-SIINFEKL	mCMV-SIINFEKL
C	DB-SIINFEKA	mCMV-SIINFEKL
D	No	mCMV-SIINFEKA
E	DB-SIINFEKL	mCMV-SIINFEKA
F	DB-SIINFEKA	mCMV-SIINFEKA

FIGURE 6 | Protection against challenge infection by vaccination with recombinant DBs. **(A)** Sketch of the protocol and table of experimental groups A–F. Immunocompetent C57BL/6 mice were primed (vaccinated) by intraplantar application of 20-µg doses of DB-SIINFEKL or DB-SIINFEKA. High-dose (10^6 PFU) intravenous challenge infections with mCMV-SIINFEKL or mCMV-SIINFEKA were performed on day 7 after priming (vaccination). Control of organ infection was assessed on day 10. **(B)** Virus titers in spleen, lungs, and salivary glands, as well as numbers of infected $IE1^+$ liver tissue cells in representative 10-mm^2 section areas. Symbols (open circles: no vaccination; green closed circles: vaccination with DB-SIINFEKL; red closed circles: vaccination with DB-SIINFEKA) represent data from individually tested mice, with the median values marked. Significance levels (*) $P < 0.05$, (***) $P < 0.001$. (n.s.), not significant ($P > 0.05$). (Dotted lines), detection limits of the assays.

infection by mCMV-SIINFEKL over time in all organs tested (**Figure 11B**), which is in accordance with previous experience [for reviews, see (7, 8)]. ACT by SIINFEKL-specific OT-I cells reduced virus spread early on and accelerated clearance of productive infection. Specifically, ACT by OT-I cells alone terminated liver infection after HCT by day 14, so that TherVac made no difference. In contrast, in all other organs, TherVac by DB-SIINFEKL, but not by DB-SIINFEKA, further reduced viral replication and accelerated the clearance of productive infection.

We finally performed ACT in HCT recipients with graded numbers of OT-I cells to determine the benefit from TherVac by DB-SIINFEKL in terms of ACT cell numbers required for equivalent protection (**Figure 12A**). Control of mCMV-SIINFEKL infection by 100 OT-I cells combined with TherVac by DB-SIINFEKL, but not by DB-SIINFEKA, was equivalent in all tested organs to infection control by 10,000 OT-I cells in absence of TherVac (**Figure 12B**). Thus, in this experiment, TherVac gave a 100-fold benefit in terms of ACT cell numbers saved.



	Priming	Depletion	Challenge infection
A	No	No	mCMV-SIINFEKL
B	DB-SIINFEKL	No	mCMV-SIINFEKL
C	DB-SIINFEKL	$CD8^+$ T cells	mCMV-SIINFEKL

FIGURE 7 | Identification of the protective T-cell subpopulation. **(A)** Sketch of the protocol and table of experimental groups A–C. Immunocompetent C57BL/6 mice were primed (vaccinated) by intraplantar application of 20-µg doses of DB-SIINFEKL. $CD8^+$ T cells were depleted on day 6, followed by high-dose (10^6 PFU) intravenous challenge infection with mCMV-SIINFEKL on day 7. Control of organ infection was assessed on day 10. **(B)** Virus titers in spleen, lungs, and salivary glands, as well as numbers of infected $IE1^+$ liver tissue cells in representative 10-mm^2 section areas. Symbols (open circles: no vaccination; green closed circles: vaccination with DB-SIINFEKL; green closed squares: vaccination with DB-SIINFEKL followed by depletion of $CD8^+$ T cells) represent data from individually tested mice, with the median values marked. Significance levels (**) $P < 0.01$, (***) $P < 0.001$, (n.s.), not significant ($P > 0.05$). Dotted lines, detection limits of the assays.

DISCUSSION

This study in the mouse model has provided proof-of-concept for enhancing the efficacy of low-dose ACT against CMV disease in an HCT setting by TherVac: no more, no less. This may encourage clinical investigation and trials, just as the first demonstration of CMV-specific ACT in the mouse model (22–24) has intellectually paved the way to clinical ACT as an immune-therapeutical approach to the prevention of disease from hCMV reactivation in HCT patients [for a review on medical translation of results from animal CMV models, see (9)].

In the weakness of any reductionistic approach in animal models to never be able to reproduce the clinical reality in all its complexity (71) lies also the strength of a less obstructed view on fundamental principles. The outcome of CMV infection in HCT patients is highly individual, as many variables, which are difficult to control, determine the individual fate. To begin with, an underestimated and sometimes ignored factor is a pre-existing defect from the primary disease, that is, a hematopoietic malignancy and associated chemotherapy. Leukemia relapse after therapy by HCT is a major concern. Thus, in histocompatibility antigen-mismatched "allogeneic" HCT, mature T cells are not depleted from the transplant to maintain a graft-*versus*-leukemia (GvL) response, taking the risk of a GvH response, which is associated with a higher risk of CMV reactivation (72). In addition, the CMV status of donor and recipient decides on whether virus reactivation occurs within the transplanted HC or within the recipient's own tissues, or both [reviewed in (2)], and the individual's genetic constitution and infection history defines the latent CMV genome load and incidence of reactivation [discussed in (73)]. The time of virus reactivation in an individual HCT recipient is not predictable, and viral loads after reactivation vary dramatically between individuals (10). To conclude this certainly not comprehensive list of variables, genetic and phenotypic differences between hCMV strains/variants, which are rarely typed in HCT clinical routine, can have a fundamental impact on cell-type tropism and thus on the pathogenicity of the reactivating virus (74, 75). With all this in mind, it becomes evident that no experimental animal model will ever perfectly suit human CMV disease in any individual HCT recipient.

Pre-emptive immunotherapy of hCMV reactivation by ACT with virus-specific $CD8^+$ T cells is an option to prevent CMV organ disease in HCT recipients who are latently infected

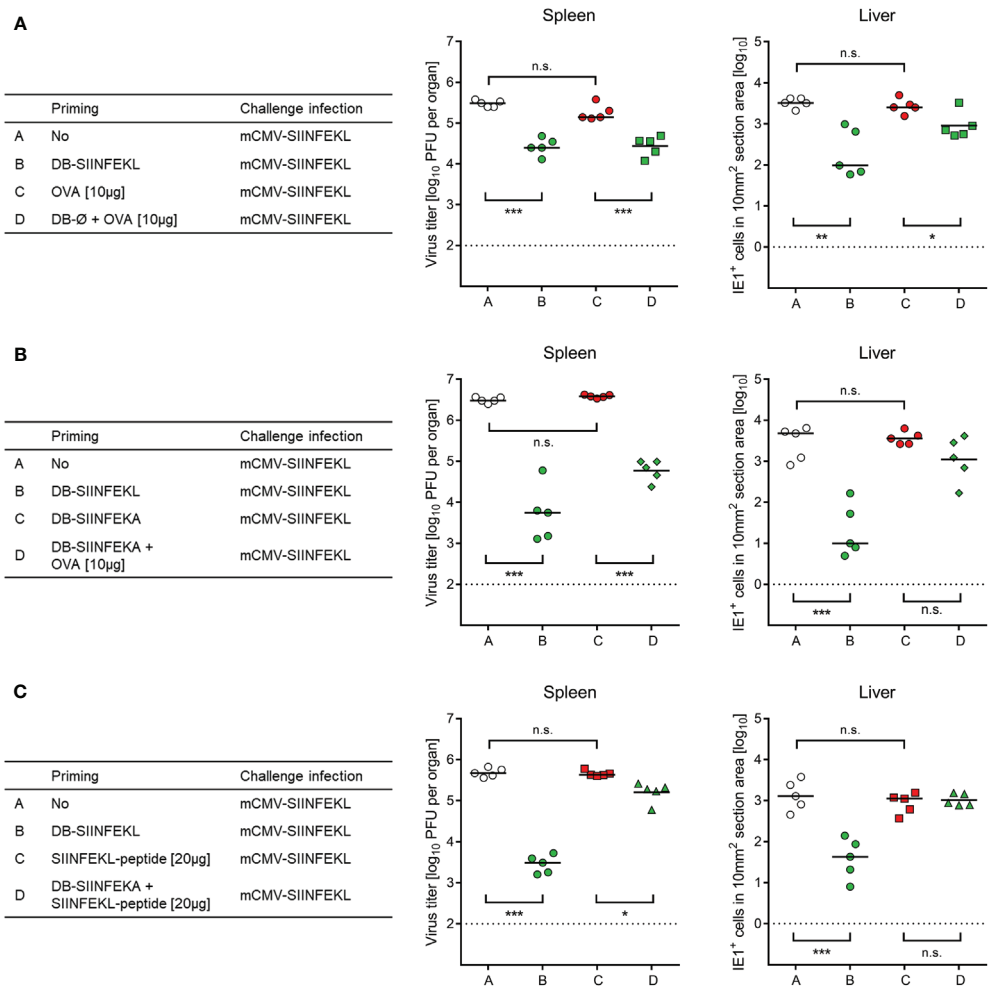


FIGURE 8 | Non-antigenic DBs facilitate priming of a protective response by OVA protein or SIINFEKL peptide. **(A)** Adjuvant role of unmodified DB (DB-Ø) for vaccination with OVA. **(B)** Adjuvant role of non-antigenic DB-SIINFEKA for vaccination with OVA. **(C)** Adjuvant role of non-antigenic DB-SIINFEKA for vaccination with peptide SIINFEKL. Symbols represent data from individually tested mice, with the median values marked. (Open circles), no vaccination. (Green symbols), SIINFEKL-specific priming accomplished. (Red symbols), no SIINFEKL-specific priming accomplished. Significance levels (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (n.s.), not significant ($P > 0.05$). Dotted lines, detection limits of the assays.

(constellation D^-R^+), who receive an HC transplant derived from a latently infected donor (constellation D^+R^-), or who combine both risk factors (constellation D^+R^+). In HCT, the risk of virus reactivation is highest in D^-R^+ patients who do not receive donor immunity with the transplant, as well as in D^+R^+ patients who receive a T cell-depleted transplant for avoiding GvH disease [for a review, see (2)]. ACT with purified virus-specific $CD8^+$ T-cell preparations avoids GvH disease while selectively targeting infected cells. It is a general alternative to pharmacotherapy with antiviral drugs for avoiding myelosuppressive or nephrotoxic side effects, and it is the last resort to fight infection by virus strains/variants that are refractory to antiviral medication (15–17, 21) from the outset or that develop resistance and become selected under longer-term treatment (see also the Introduction).

In case of the HCT constellations with D^+ (see above), the HCT donor is the first choice as a source of virus-specific $CD8^+$ T cells for ACT. HLA/MHC class-I restriction of $CD8^+$ T-cell function does here not seriously pose a limitation, because an unrelated HCT donor and the recipient need to be HLA type-matched to share HLA antigens as complete as possible for avoiding GvH disease. An expanding list of viral peptides identified to be presented by more common HLA class-I molecules allows purification of viral epitope-specific donor $CD8^+$ T cells for ACT by various cell sorting techniques [(32–35), for reviews, see (76, 77)]. In case of HCT constellations with an hCMV-negative donor (D^-), $CD8^+$ T cells can be derived from an unrelated, hCMV-experienced third-party donor sharing HLA molecules with the HCT/ACT recipient (77). As a more recent strategy of ACT, naïve or memory $CD8^+$ T cells can be equipped with an engineered, transgenic TCR specific for an HLA class-I presented antigenic peptide, as studied in viral and tumor models [(38–40), for reviews, see (78, 79)]. The first

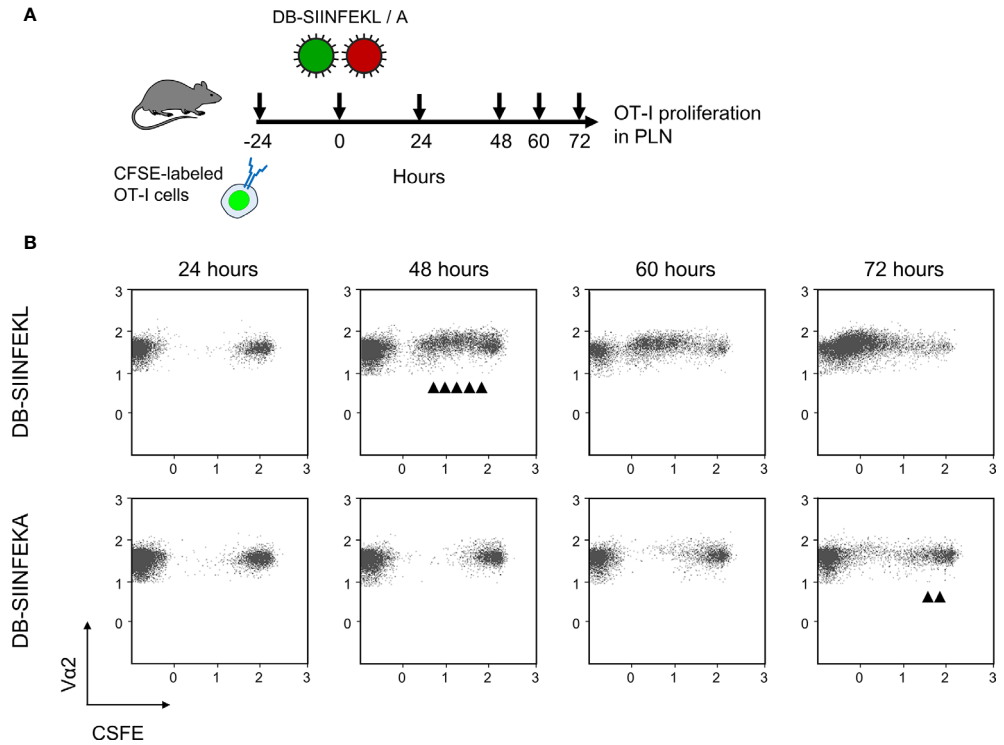


FIGURE 9 | DB-driven epitope-specific proliferation of transferred OT-I cells. **(A)** Experimental protocol. Fluorescence-labeled OT-I cells were transferred intravenously into immunocompetent C57BL/6 mice. Proliferation of OT-I cells recovered from the draining ipsilateral popliteal lymph node (from three mice pooled) was measured at the indicated times after intraplantar priming (vaccination) with 20-µg doses of DB-SIINFEKL or DB-SIINFEKA. **(B)** Cytofluorometric measurement of the loss of the fluorescence label due to the proliferation of OT-I cells over time. (Ordinate) fluorescence specific for the Vα2 TCR chain expressed by OT-I cells. (Abscissa) CFSE fluorescence. For groups of most interest, arrowheads mark cell divisions.

clinical approaches of ACT for preventing hCMV infection in HCT recipients were performed with cell culture-propagated, and thereby highly expanded, clonal $CD8^+$ CTLL (18, 19). By directly comparing ACTs with CTLL and *ex vivo* isolated $CD8^+$ T cells of the same specificity, studies in the mouse model revealed that the benefit from expanding antiviral cells to high cell numbers by recombinant IL2 is largely dashed by a loss of per-cell antiviral activity (30, 31). More recent studies indicated that autocrine IL2 induced by cultivation in the presence of IL7 and IL15, but also co-stimulatory signals, can improve persistence and proliferation potential of cell culture-propagated $CD8^+$ T cells upon ACT [(80), for a review, see (81)].

It is an underappreciated finding that combining protective epitope specificities failed to improve the antiviral efficacy of ACT (82). This can be explained by the fact that an infected cell cannot "die two deaths". This insight is of practical importance, as sorting of $CD8^+$ T cells specific for a single type of antigenic peptide presented in both the ACT donor and recipient, or a single type of engineered viral epitope-specific TCR, should suffice for the control of the infection upon ACT. It is also worth to note that protective efficacy upon ACT is not linked to the immunodominance of an antigenic peptide in the natural immune response against the virus (58, 83). It rather depends on the structural avidity of the TCR and the functional avidity with which $CD8^+$ T cells interact with pMHC-I complexes on the surface of infected cells (78, 84, 85). This parameter becomes most important when viral immune evasion proteins limit antigen presentation. Specifically, a functional avidity corresponding to the recognition of target cells exogenously loaded with $\leq 10^{-9}$ M of an antigenic peptide is required for the recognition of mCMV-infected cells when immune evasion molecules are expressed (55).

A clinical application of ACT in HCT recipients diagnosed to have latent hCMV reactivated has so far not become a routine therapy, mainly because the logistics for providing sufficiently high donor cell numbers is demanding. Already shortly after the first experimental ACT with $CD8^+$ T cells in the mouse model of CMV infection of the immunocompromised host (22), we pursued the idea to expand low numbers of transferred cells within the mCMV-infected recipients. Specifically, inspired by earlier work on IL2-enhanced ACT for cancer therapy (86–88), we showed that recombinant IL2 administered in 12-hour intervals resulted in enhanced antiviral protection equivalent to daily doublings of the effector cell population (23). However, besides enormous costs for recombinant cytokines, meanwhile including also IL7, -12, -15, and -21, adverse side effects by unspecific activation need to be considered (81).

Here we have pursued the alternative strategy of expanding adoptively transferred cells in CMV-infected, combined HCT

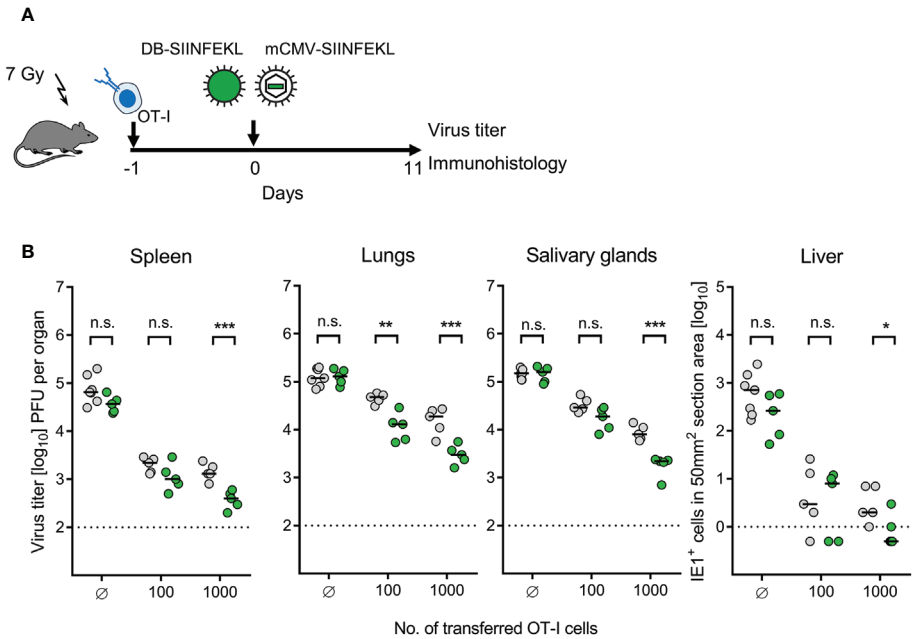
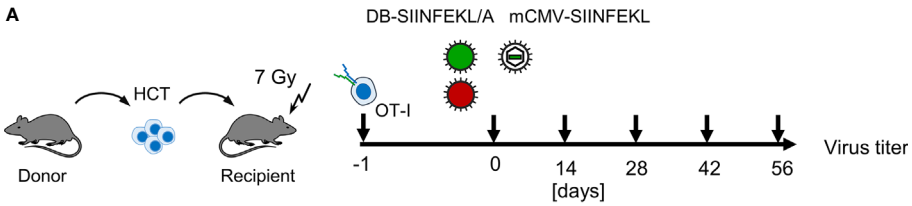


FIGURE 10 | Enhancement of protection by combining ACT with DB-based TherVac. **(A)** Sketch of the protocol. Immunocompromised C57BL/6 mice received either no ACT for control or ACT with 10^2 or 10^3 OT-I cells. On the following day, intraplantar TherVac with 20 µg of DB-SIINFEKL and infection with 10^5 PFU of mCMV-SIINFEKL were performed combined. Control of organ infection was assessed on day 11. **(B)** Virus titers in spleen, lungs, and salivary glands, as well as numbers of infected IE1$^+$ liver tissue cells in representative 10-mm^2 section areas. Symbols (grey-shaded closed circles: no vaccination; green closed circles: vaccination with DB-SIINFEKL) represent data from individually tested mice, with the median values marked. Significance levels (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. (n.s.), not significant ($P > 0.05$). Dotted lines, detection limits of the assays.

and ACT recipients by TherVac, a concept discussed also for tumor therapy [reviewed in (81)]. As a model for CD8$^+$ T cells with transgenic TCR, modeling ACT with cells from CMV-negative donors, we used OT-I cells specific for the pMHC-I complex SIINFEKL-K^b. Syngeneic HCT was performed with C57BL/6 mice (*H-2^b* haplotype) as donors and recipients in order not to complicate the model by consequences of mismatch in major or minor histocompatibility antigens. According to most recent findings, such mismatches, rather than causing a GvH reaction, inhibit the reconstitution of protective high-avidity CD8$^+$ T cells by inducing non-cognate transplantation tolerance (67, 68). Our data show an exquisite epitope-specific function of OT-I cells. ACT controlled virus mCMV-SIINFEKL, but not the epitope-loss mutant mCMV-SIINFEKA, in the tissues of immunocompromised recipients.

For TherVac, we chose hCMV DBs, which represent non-infectious, DNA-free subviral particles consisting of enveloped viral tegument proteins (43, 69). DBs can be modified to package recombinant tegument protein pp65/UL83 containing integrated immunogenic peptides of interest (45). As an advantage of the DB concept of vaccination, envelope glycoprotein complexes mediate the fusion of DBs with the cell membrane of target cells and deliver the tegument proteins directly into the cytosol for antigen presentation in the MHC-I pathway (43–45). Thus, also cells of non-hematopoietic cell lineages can present antigenic peptides to already primed CD8$^+$ T cells used for ACT. In addition, the DB entry process directly activates professional antigen-presenting cells (profAPCs), such as DC (46) for the priming of naïve CD8$^+$ T cells, so that a DB-based vaccine does not require adjuvantation (53). For use as a vaccine against hCMV, new generations of DBs (89, 90) have the additional advantage of priming an antibody response directed against the virion trimeric and pentameric entry complexes in order to cover hCMV strains that differ in cell tropism. Our data show an exquisite epitope-specific expansion of OT-I cells in that they proliferated and exerted an enhanced antiviral protection only after TherVac with DB-SIINFEKL but not with DB-SIINFEKA. Although we have here chosen recombinant DBs for TherVac, it is important to emphasize that the concept of TherVac is, of course, not limited to the use of a DB-based vaccine, but is open for alternative vaccination strategies.

A difference between vaccination of immunocompetent hosts and TherVac in immunocompromised recipients of combined HCT and ACT is the transient shortness of hematopoietic lineage-derived profAPCs at early times after HCT. As we have shown previously in a sex-chimeric mouse model of HCT with male *sry*$^+$ donors and female *sry*$^-$ recipients, CD11c$^+$ DC are preferentially lost in the recipients after hematoablative treatment and replaced with donor-derived cells only with delay (91, 92). So, immediate expansion of transferred antiviral CD8$^+$ T cells depends on direct antigen expression by non-hematopoietic cells targeted by the vaccine. It was therefore an open question if limited numbers of transferred cells would at all encounter host cells presenting the vaccine antigen for driving clonal expansion. Our data show that TherVac amplifies the



	Adoptive cell transfer	Vaccination	Infection
○	No	No	mCMV-SIINFEKL
●	10^2 OT-I cells	No	mCMV-SIINFEKL
●	10^2 OT-I cells	DB-SIINFEKL	mCMV-SIINFEKL
●	10^2 OT-I cells	DB-SIINFEKA	mCMV-SIINFEKL

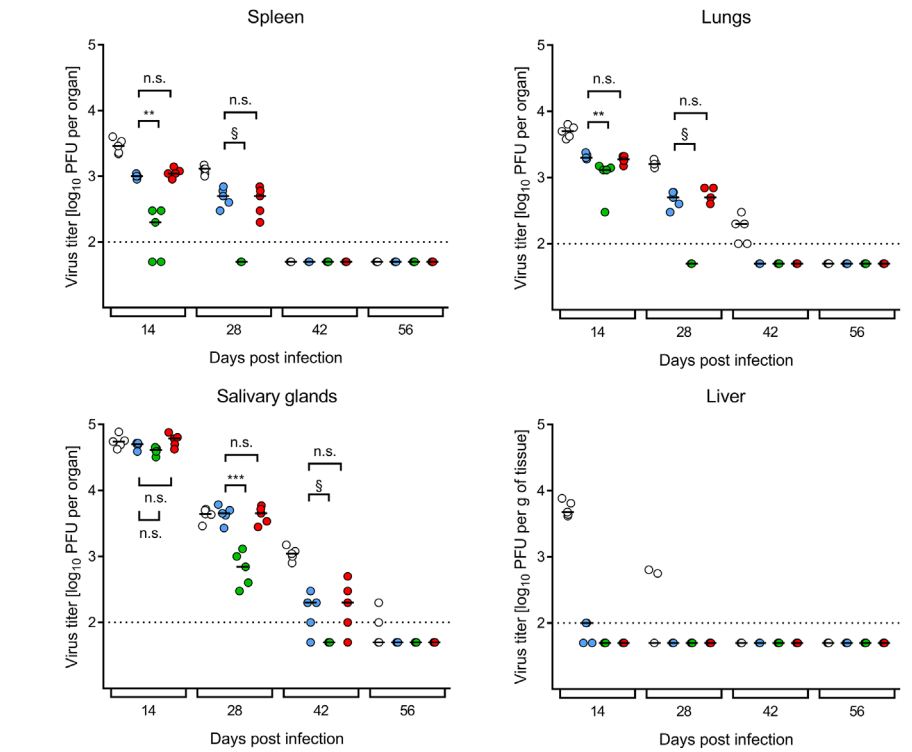


FIGURE 11 | Kinetics of epitope-specific enhancement of protection by combining ACT with DB-based TherVac in HCT recipients. **(A)** Sketch of the protocol and table of experimental groups. Transiently immunocompromised C57BL/6 recipients of syngeneic HCT received ACT with 10^2 OT-I cells or were left with no ACT for control. On the following day, intraplantar TherVac with 20 µg of DB-SIINFEKL or control TherVac with 20 µg DB-SIINFEKA was combined with infection by 10^5 PFU of mCMV-SIINFEKL. Control of organ infection was assessed at the indicated times after infection. **(B)** Virus titers in organs indicated. Symbols, color-coded as defined in (A, table), represent data from individually tested mice, with the median values marked. Significance levels (**) $P < 0.01$, (***) $P < 0.001$. (n.s.), not significant ($P > 0.05$). §, de facto significant difference, although calculation of P values is pointless when all data of one group in the comparison are below the detection limit of the assay (dotted line).

protective efficacy of limited numbers of OT-I cells even when ACT is performed intravenously and TherVac locally into a footpad. As we have discussed recently (55), intraplantar vaccine application in the mouse is a good model for subcutaneous or intramuscular vaccine application into the upper arm of vaccinees, the favored site for routine vaccination of humans. This finding increases the chances for a clinical translation to TherVac in human recipients of HCT-ACT.

Let us speculate on a translation of our findings to a clinical application. Clinical studies discussed already above (32–36)

A

	Adoptive cell transfer	Vaccination	Infection
A	No	No	mCMV-SIINFEKL
B	10^2 OT-I cells	No	mCMV-SIINFEKL
C	10^3 OT-I cells	No	mCMV-SIINFEKL
D	10^4 OT-I cells	No	mCMV-SIINFEKL
E	10^5 OT-I cells	No	mCMV-SIINFEKL
F	10^2 OT-I cells	DB-SIINFEKL	mCMV-SIINFEKL
G	10^2 OT-I cells	DB-SIINFEKA	mCMV-SIINFEKL

B

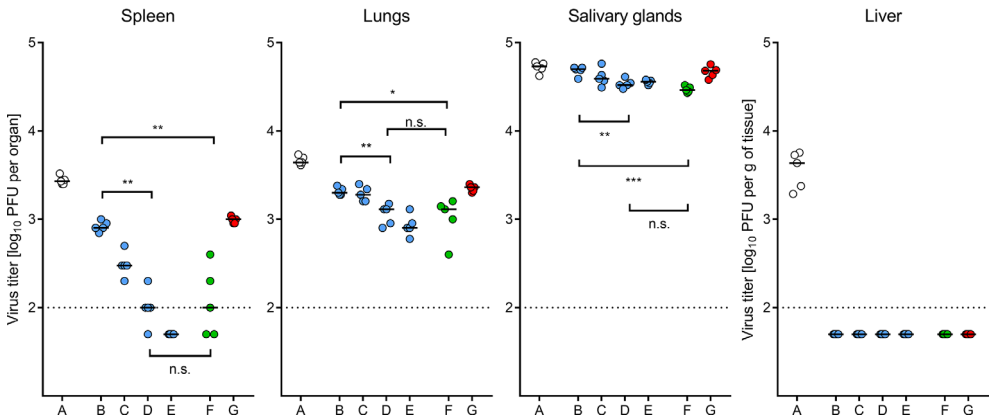


FIGURE 12 | ACT dose-benefit from TherVac in HCT recipients. **(A)** Table of experimental groups A–G. For the protocol, see the sketch in Figure 11A, with the modification that ACTs alone were performed with graded numbers of OT-I cells. Organ infection was assessed on day 14 after infection with mCMV-SIINFEKL. **(B)** Virus titers in organs indicated. Symbols (open circles: no ACT, blue closed circles: ACT alone, green closed circles: ACT and TherVac with DB-SIINFEKL, red closed circles: ACT and control TherVac with DB-SIINFEKA) represent data from individually tested mice, with the median values marked. Significance levels (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. (n.s.), not significant ($P > 0.05$). Dotted lines, detection limit of the assay.

have shown that low-dose ACT works, in principle, with no need for TherVac. This might be raised as an argument against TherVac. However, a further enhancement by TherVac may be beneficial to control infection also at "immune-privileged" sites at which infection is more difficult to control. Notably, these studies have also revealed that control of infection depends on massive clonal expansion of the initially few cells transferred, and this expansion depends on stimulation by cognate antigen. As we have shown here, OT-I cells expand in the infected recipients within 10 days from 100 cells to more than 10^6 cells (recall **Figure 4B**) when driven by the cognate epitope SIINFEKL. The dependence on antigen implies that the expansion, and thus the efficacy of ACT, is low when antigen presentation is limited, for instance, at a very early stage of virus reactivation, which is a stochastic event occurring in only few cells (93, 94). Likewise, an expansion will predictably be limited, when viral replication, and hence antigen presentation, is inhibited by antiviral drugs, so that combining antiviral pharmacotherapy with immunotherapy by ACT makes little sense. In such scenarios, specifically in a phase when antiviral medication is planned to taper off, TherVac-enhanced ACT may be an option to prevent a relapse of infection.

Altogether, the possibility to apply the vaccine locally and without adjuvant, combined with strict epitope-specificity of the enhancement of the protective effector function, makes TherVac superior to cytokine cocktails for the post-ACT clonal expansion of protective antiviral $CD8^+$ T cells and gives new options for the prevention of CMV disease in HCT recipients.

BIOSAFETY STATEMENT

The work was done according to German federal law GenTG and BioStoffV. The generation of recombinant mCMV and hCMV was approved by the 'Struktur- und Genehmigungsdirektion Süd' (SDG, Neustadt, Germany), permission numbers 24.1-886.3 and 25.2-886.3, respectively.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of the "Landesuntersuchungsamt Rheinland-Pfalz" according to German federal law §8 Abs. 1 TierSchG (animal protection law), permission numbers 177-07/G 10-1-52.

AUTHOR CONTRIBUTIONS

MR and NL are responsible for conception and design of the study, analysis, and interpretation of the data. RH and BP provided essential material and designed parts of the study. KG, JP, SB, KF, SK, NB, and NL conducted the work and analyzed the data. MR wrote the first draft of the manuscript. NL wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.694588/full#supplementary-material

Supplementary Figure 1 | Viral replicative fitness. Virus growth *in vivo* in absence of immune control was determined in the indicated organs of C57BL/6 mice that were immunocompromised by sublethal total-body γ-irradiation with a single dose of 7 Gy, followed by intraplantar infection with 1×10^5 PFU of the indicated viruses. At the indicated times after infection, virus growth parameters were measured, specifically burden of infectious virus (PFU in spleen, lungs, and salivary glands) or numbers of infected cells in representative 10-mm^2 areas of liver tissue sections. Symbols represent data from three to four mice tested individually per time of assay. Graphs show log-linear regression lines (based on data from all time points) and their corresponding 95% confidence regions bordered by dotted lines. Viral doubling times (*vDT*) were calculated based on the slopes of the regression lines according to the formula $vDT = \log 2/a$. The 95% confidence intervals of *vDT* are given in parentheses.

Supplementary Figure 2 | Phenotypic and functional characterization of TCR-transgenic OT-I cells. **(A)** Cytofluorometric quantitation of cells expressing SIINFEKL-specific Vα2Vβ5 TCR among $CD8^+$ T cells derived from the spleen of OT-I mice. **(B)** Determination of the functional avidity of OT-I cells in an IFNγ-ELISpot assay with EL-4 stimulator cells exogenously loaded with synthetic peptide SIINFEKL in the graded molar concentrations indicated. Bars represent numbers of OT-I cells responding with IFNγ secretion. Error bars represent 95% confidence intervals. (Top panel), cumulative avidity distribution revealing frequencies of OT-I cells responding to the indicated peptide concentration tested, which includes cells that would also respond to lower concentrations. (Bottom panel), Gaussian-like avidity distribution revealing frequencies of OT-I cells responding exactly to the peptide concentration indicated. This distribution is deduced from the cumulative avidity distribution values by plotting the difference to the respective lower-concentration frequency.

Supplementary Figure 3 | Lymphoid homing and OT-I proliferation driven by local infection. Proliferation of intravenously transferred, CFSE-labeled OT-I cells (transfer on day -1) in popliteal lymph nodes (PLN) of C57BL/6 mice, determined at 60 h after unilateral intraplantar infection with mCMV-SIINFEKL expressing the cognate epitope. (Top panel), lack of OT-I proliferation in the contralateral PLN not draining the site of infection. (Bottom panel), OT-I proliferation in the ipsilateral PLN draining the site of infection. FSC, forward scatter. CFSE, fluorescence intensity. Arrowheads mark cell divisions.

Supplementary Figure 4 | OT-I cells fail to control liver infection when the cognate epitope is not expressed. Corresponding to **Figure 4A**, 2C-IHC images of liver tissue sections show extensive virus spread and random distribution of liver-infiltrating OT-I cells after infection of immunocompromised C57BL/6 mice with mCMV-SIINFEKA not expressing the cognate epitope. (Left images), low magnification overviews. (Right images), resolved to greater detail by higher magnification. (Black staining), $CD8^+$ T cells. (Red staining), infected liver cells. Bar markers, 50 μm. For additional information, see the legend of **Figure 4**.

Supplementary Figure 5 | Equivalence of OT-I proliferation driven by mCMV-SIINFEKL and DB-SIINFEKL. Proliferation of intravenously transferred, CFSE-labeled OT-I cells (transfer on day -1) in the spleen of C57BL/6 mice, determined at 72 h after infection or vaccination. FSC, forward scatter. CFSE, fluorescence intensity. Arrowheads mark cell divisions.

REFERENCES

1. Davison AJ, Holton M, Dolan A, Dargan DJ, Gatherer D, Hayward GS. Comparative Genomics of Primate Cytomegaloviruses. In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. 1. Norfolk, UK: Caister Academic Press (2013). p. 1–22.
2. Reddehase MJ, Lemmermann NA. Cellular Reservoirs of Latent Cytomegaloviruses. *Med Microbiol Immunol* (2019) 208:391–403. doi: 10.1007/s00430-019-00592-y
3. Roizman B, Sears AE. An Inquiry Into the Mechanisms of Herpes Simplex Virus Latency. *Annu Rev Microbiol* (1987) 41:543–71. doi: 10.1146/annurev.mi.41.100187.002551
4. Ho M. The History of Cytomegalovirus and Its Diseases. *Med Microbiol Immunol* (2008) 197:65–73. doi: 10.1007/s00430-007-0066-x
5. Boppana SB, Britt WJ. Synopsis of Clinical Aspects of Human Cytomegalovirus Disease. In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*. Norfolk, UK: Caister Academic Press (2013) 2:1–25.
6. Seo S, Boeckh M. Clinical Cytomegalovirus Research: Haematopoietic Cell Transplantation. In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*. Norfolk, UK: Caister Academic Press (2013) 2:335–51.
7. Holtappels R, Ebert S, Podlech J, Fink A, Böhm V, Lemmermann NA, et al. Murine Model for Cytoimmunotherapy of CMV Disease After Haematopoietic Cell Transplantation. In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*. Norfolk, UK: Caister Academic Press (2013) 2:354–81.
8. Reddehase MJ. Mutual Interference Between Cytomegalovirus and Reconstitution of Protective Immunity After Hematopoietic Cell Transplantation. *Front Immunol* (2016) 7:294. doi: 10.3389/fimmu.2016.00294
9. Reddehase MJ, Lemmermann NA. Mouse Model of Cytomegalovirus Disease and Immunotherapy in the Immunocompromised Host: Predictions for Medical Translation That Survived the "Test of Time". *Viruses* (2018) 10:693. doi: 10.3390/v10120693
10. Emery VC. Relative Importance of Cytomegalovirus Load as a Risk Factor for Cytomegalovirus Disease in the Immunocompromised Host. *Monogr Virol* (1998) 21:288–301. doi: 10.1159/000061707
11. Hebart H, Einsele H. Clinical Aspects of CMV Infection After Stem Cell Transplantation. *Hum Immunol* (2004) 65:432–36. doi: 10.1016/j.humimm.2004.02.022
12. Stern L, Withers B, Avdic S, Gottlieb D, Abendroth A, Blyth E, et al. Human Cytomegalovirus Latency and Reactivation in Allogeneic Hematopoietic Stem

Cell Transplant Recipients. *Front Microbiol* (2019) 10:1186. doi: 10.3389/fmicb.2019.01186
13. Maffini E, Giaccone L, Festuccia M, Brunello L, Busca A, Bruno B. Treatment of CMV Infection After Allogeneic Hematopoietic Stem Cell Transplantation. *Expert Rev Hematol* (2016) 9:585–96. doi: 10.1080/17474086.2016.1174571
14. Michel D, Chevilotte M, Mertens T. Antiviral Therapy, Drug Resistance and Computed Resistance Profiling. In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*. Norfolk, UK: Caister Academic Press (2013) 2:402–23.
15. Campos AB, Ribeiro J, Boutolleau D, Sousa H. Human Cytomegalovirus Antiviral Drug Resistance in Hematopoietic Stem Cell Transplantation: Current State of the Art. *Rev Med Virol* (2016) 26:161–82. doi: 10.1002/rmv.1873
16. Pei XY, Zhao XY, Chang YJ, Liu J, Xu LP, Wang Y, et al. Cytomegalovirus-Specific T-Cell Transfer for Refractory Cytomegalovirus Infection After Haploidentical Stem Cell Transplantation: The Quantitative and Qualitative Immune Recovery for Cytomegalovirus. *J Infect Dis* (2017) 216:945–56. doi: 10.1093/infdis/jix357
17. Chemaly RF, Chou S, Einsele H, Griffiths P, Avery R, Razonable RR, et al. Definitions of Resistant and Refractory Cytomegalovirus Infection and Disease in Transplant Recipients for Use in Clinical Trials. *Clin Infect Dis* (2019) 68:1420–6. doi: 10.1093/cid/ciy696
18. Riddell SR, Watanabe KS, Goodrich M, Li CR, Agha ME, Greenberg PD. Restoration of Viral Immunity in Immunodeficient Humans by the Adoptive Transfer of T Cell Clones. *Science* (1992) 257:238–41. doi: 10.1126/science.1352912
19. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas D, et al. Reconstitution of Cellular Immunity Against Cytomegalovirus in Recipients of Allogeneic Bone Marrow by Transfer of T-Cell Clones From the Donor. *N Engl J Med* (1995) 333:1038–44. doi: 10.1056/NEJM199510193331603
20. Moss P, Rickinson A. Cellular Immunotherapy for Viral Infection After HSC Transplantation. *Nat Rev Immunol* (2005) 5:9–20. doi: 10.1038/nri1526
21. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive Transfer of pp65-Specific T Cells for the Treatment of Chemorefractory Cytomegalovirus Disease or Reactivation After Haploidentical and Matched Unrelated Stem Cell Transplantation. *Blood* (2010) 116:4360–7. doi: 10.1182/blood-2010-01-262089
22. Reddehase MJ, Weiland F, Münch K, Jonjic S, Lüske A, Koszinowski UH. Interstitial Murine Cytomegalovirus Pneumonia After Irradiation: Characterization of Cells That Limit Viral Replication During Established Infection of the Lungs. *J Virol* (1985) 55:264–73. doi: 10.1128/jvi.55.2.264-273.1985
23. Reddehase MJ, Mutter W, Koszinowski UH. *In Vivo* Application of Recombinant Interleukin 2 in the Immunotherapy of Established Cytomegalovirus Infection. *J Exp Med* (1987) 165:650–6. doi: 10.1084/jem.165.3.650
24. Reddehase MJ, Mutter W, Münch K, Bühring HJ, Koszinowski UH. CD8-Positive T Lymphocytes Specific for Murine Cytomegalovirus Immediate-Early Antigens Mediate Protective Immunity. *J Virol* (1987) 61:3102–8. doi: 10.1128/jvi.61.10.3102-3108.1987
25. Steffens HP, Kurz S, Holtappels R, Reddehase MJ. Preemptive CD8 T-Cell Immunotherapy of Acute Cytomegalovirus Infection Prevents Lethal Disease, Limits the Burden of Latent Viral Genomes, and Reduces the Risk of Virus Recurrence. *J Virol* (1998) 72:1797–804. doi: 10.1128/JVI.72.3.1797-1804.1998
26. Renzaho A, Podlech J, Kühnapfel B, Blaum F, Reddehase MJ, Lemmermann NA. Cytomegalovirus-Associated Inhibition of Hematopoiesis is Preventable by Cytoimmunotherapy With Antiviral CD8 T Cells. *Front Cell Infect Microbiol* (2020) 10:138. doi: 10.3389/fcimb.2020.00138
27. Mutter W, Reddehase MJ, Busch FW, Bühring HJ, Koszinowski UH. Failure in Generating Hemopoietic Stem Cells Is the Primary Cause of Death From Cytomegalovirus Disease in the Immunocompromised Host. *J Exp Med* (1988) 167:1645–58. doi: 10.1084/jem.167.5.1645
28. Mayer A, Podlech J, Kurz S, Steffens HP, Maiberger S, Thalmeier K, et al. Bone Marrow Failure by Cytomegalovirus Is Associated With an *In Vivo* Deficiency in the Expression of Essential Stromal Hemopoietin Genes. *J Virol* (1997) 71:4589–98. doi: 10.1128/jvi.71.6.4589-4598.1997
29. Steffens HP, Podlech J, Kurz S, Angele P, Dreis D, Reddehase MJ. Cytomegalovirus Inhibits the Engraftment of Donor Bone Marrow Cells by Downregulation of Hemopoietin Gene Expression in Recipient Stroma. *J Virol* (1998) 72:5006–15. doi: 10.1128/JVI.72.6.5006-5015.1998
30. Pahl-Seibert MF, Juelch M, Podlech J, Thomas D, Deegen P, Reddehase MJ, et al. Highly Protective *In Vivo* Function of Cytomegalovirus IE1 Epitope-Specific Memory CD8 T Cells Purified by T-Cell Receptor-Based Cell Sorting. *J Virol* (2005) 79:5400–13. doi: 10.1128/JVI.79.9.5400-5413.2005
31. Böhm V, Podlech J, Thomas D, Deegen P, Pahl-Seibert MF, Lemmermann NA, et al. Epitope-Specific *In Vivo* Protection Against Cytomegalovirus Disease by CD8 T Cells in the Murine Model of Preemptive Immunotherapy. *Med Microbiol Immunol* (2008) 197:135–44. doi: 10.1007/s00430-008-0092-33
32. Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H, et al. Adoptive Transfer of Cytomegalovirus-Specific CTL to Stem Cell Transplant Patients After Selection by HLA-Peptide Tetramers. *J Exp Med* (2005) 202:379–86. doi: 10.1084/jem.20040613
33. Stemberger C, Graef P, Odendahl M, Albrecht J, Dössinger G, Anderl F, et al. Lowest Numbers of Primary CD8(+) T Cells can Reconstitute Protective Immunity Upon Adoptive Immunotherapy. *Blood* (2014) 124:628–37. doi: 10.1182/blood-2013-12-547349
34. Graef P, Buchholz VR, Stemberger C, Flossdorf M, Henkel L, Schiemann M, et al. Serial Transfer of Single-Cell-Derived Immunocompetence Reveals Stemness of CD8(+) Central Memory T Cells. *Immunity* (2014) 41:116–26. doi: 10.1016/j.immuni.2014.05.018
35. Odendahl M, Grigoleit GU, Bönig H, Neuenhahn M, Albrecht J, Anderl F, et al. Clinical-Scale Isolation of 'Minimally Manipulated' Cytomegalovirus-Specific Donor Lymphocytes for the Treatment of Refractory Cytomegalovirus Disease. *Cytotherapy* (2014) 16:1245–56. doi: 10.1016/j.jcyt.2014.05.023
36. Neuenhahn M, Albrecht J, Odendahl M, Schlott F, Dössinger G, Schiemann M, et al. Transfer of Minimally Manipulated CMV-Specific T Cells From Stem Cell or Third-Party Donors to Treat CMV Infection After Allo-HSCT. *Leukemia* (2017) 31:2161–71. doi: 10.1038/leu.2017.16
37. Schub A, Schuster IG, Hammerschmidt W, Moosmann A. CMV-Specific TCR-Transgenic T Cells for Immunotherapy. *J Immunol* (2009) 183:6819–30. doi: 10.4049/jimmunol.0902233
38. Thomas S, Klobuch S, Besold K, Plachter B, Dörrie J, Schaft N, et al. Strong and Sustained Effector Function of Memory- *Versus* Naïve-Derived T Cells Upon T-Cell Receptor RNA Transfer: Implications for Cellular Therapy. *Eur J Immunol* (2012) 42:3442–53. doi: 10.1002/eji.201242666
39. Thomas S, Klobuch S, Podlech J, Plachter B, Hoffmann P, Renzaho A, et al. Evaluating Human T-Cell Therapy of Cytomegalovirus Organ Disease in HLA-Transgenic Mice. *PloS Pathog* (2015) 11:e1005049. doi: 10.1371/journal.ppat.1005049
40. Lemmermann NA, Reddehase MJ. Refining Human T-Cell Immunotherapy of Cytomegalovirus Disease: A Mouse Model With 'Humanized' Antigen Presentation as a New Preclinical Study Tool. *Med Microbiol Immunol* (2016) 205:549–61. doi: 10.1007/s00430-016-0471-0
41. Rötzschke O, Falk K, Stevanović S, Jung G, Walden P, Rammensee HG. Exact Prediction of a Natural T Cell Epitope. *Eur J Immunol* (1991) 21:2891–4. doi: 10.1002/eji.1830211136
42. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T Cell Receptor Antagonist Peptides Induce Positive Selection. *Cell* (1994) 76:17–27. doi: 10.1016/0092-8674(94)90169-4
43. Pepperl S, Münster J, Mach M, Harris JR, Plachter B. Dense Bodies of Human Cytomegalovirus Induce Both Humoral and Cellular Immune Responses in the Absence of Viral Gene Expression. *J Virol* (2000) 74:6132–46. doi: 10.1128/jvi.74.13.6132-6146.2000
44. Pepperl-Klindworth S, Frankenberg N, Riegler S, Plachter B. Protein Delivery by Subviral Particles of Human Cytomegalovirus. *Gene Ther* (2003) 10:278–84. doi: 10.1038/sj.gt.3301879
45. Mersseman V, Besold K, Reddehase MJ, Wolfrum U, Strand D, Plachter B, et al. Exogenous Introduction of an Immunodominant Peptide From the non-Structural IE1 Protein of Human Cytomegalovirus Into the MHC Class I Presentation Pathway by Recombinant Dense Bodies. *J Gen Virol* (2008) 89:369–79. doi: 10.1099/vir.0.83380-0
46. Sauer C, Klobuch S, Herr W, Thomas S, Plachter B. Subviral Dense Bodies of Human Cytomegalovirus Stimulate Maturation and Activation of Monocyte-Derived Immature Dendritic Cells. *J Virol* (2013) 87:11287–91. doi: 10.1128/JVI.01429-13
47. Podlech J, Holtappels R, Grzimek NK, Reddehase MJ. Animal Models: Murine Cytomegalovirus. In: SHE Kaufmann, D Kabelitz, editors. *Methods in*

Microbiology. Immunology of Infection. London, UK: Academic Press (2002). p. 493–525.
48. Klenovsek K, Weisel F, Schneider A, Appelt U, Jonjic S, Messerle M, et al. Protection From CMV Infection in Immunodeficient Hosts by Adoptive Transfer of Memory B Cells. *Blood* (2007) 110:3472–9. doi: 10.1182/blood-2007-06-095414
49. Lemmermann NA, Gergely K, Böhm V, Deegen P, Däubner T, Reddehase MJ. Immune Evasion Proteins of Murine Cytomegalovirus Preferentially Affect Cell Surface Display of Recently Generated Peptide Presentation Complexes. *J Virol* (2010) 84:1221–36. doi: 10.1128/JVI.02087-09
50. Lemmermann NAW, Reddehase MJ. Direct Evidence for Viral Antigen Presentation During Latent Cytomegalovirus Infection. *Pathogens* (2021) 10:731. doi: 10.3390/pathogens10060731
51. Lemmermann NA, Podlech J, Seckert CK, Kropp KA, Grzimek NK, Reddehase MJ, et al. CD8 T Cell Immunotherapy of Cytomegalovirus Disease in the Murine Model. In: D Kabelitz, SHE Kaufmann, editors. *Methods in Microbiology*, vol. 37 . London, UK: Academic Press (2010). p. 369–420. Immunology of infection.
52. Borst EM, Hahn G, Koszinowski UH, Messerle M. Cloning of the Human Cytomegalovirus (HCMV) Genome as an Infectious Bacterial Artificial Chromosome in Escherichia Coli: A New Approach for Construction of HCMV Mutants. *J Virol* (1999) 73:8320–9. doi: 10.1128/JVI.73.10.8320-8329.1999
53. Becke S, Aue S, Thomas D, Schader S, Podlech J, Bopp T, et al. Optimized Recombinant Dense Bodies of Human Cytomegalovirus Efficiently Prime Virus Specific Lymphocytes and Neutralizing Antibodies Without the Addition of Adjuvant. *Vaccine* (2010) 28:6191–8. doi: 10.1016/j.vaccine.2010.07.016
54. Besold K, Wills M, Plachter B. Immune Evasion Proteins gpUS2 and gpUS11 of Human Cytomegalovirus Incompletely Protect Infected Cells From CD8 T Cell Recognition. *Virology* (2009) 391:5–19. doi: 10.1016/j.virol.2009.06.004
55. Holtappels R, Freitag K, Renzaho A, Becker S, Lemmermann NA, Reddehase MJ. Revisiting CD8 T-Cell 'Memory Inflation': New Insights With Implications for Cytomegaloviruses as Vaccine Vectors. *Vaccines* (2020) 8:402. doi: 10.3390/vaccines8030402
56. Kurz S, Steffens HP, Mayer A, Harris JR, Reddehase MJ. Latency *Versus* Persistence or Intermittent Recurrences: Evidence for a Latent State of Murine Cytomegalovirus in the Lungs. *J Virol* (1997) 71:2980–7. doi: 10.1128/JVI.71.4.2980-2987.1997
57. Böhm V, Simon CO, Podlech J, Seckert CK, Gendig D, Deegen P, et al. The Immune Evasion Paradox: Immunoevasins of Murine Cytomegalovirus Enhance Priming of CD8 T Cells by Preventing Negative Feedback Regulation. *J Virol* (2008) 82:11637–50. doi: 10.1128/JVI.01510-08
58. Holtappels R, Simon CO, Munks MW, Thomas D, Deegen P, Kühnapfel B, et al. Subdominant CD8 T-Cell Epitopes Account for Protection Against Cytomegalovirus Independent of Immunodomination. *J Virol* (2008) 82:5781–96. doi: 10.1128/JVI.00155-08
59. Lemmermann NA, Kropp KA, Seckert CK, Grzimek NK, Reddehase MJ. Reverse Genetics Modification of Cytomegalovirus Antigenicity and Immunogenicity by CD8 T-Cell Epitope Deletion and Insertion. *J BioMed Biotechnol* (2011) 2011:812742. doi: 10.1155/2011/812742
60. Kropp KA, Simon CO, Fink A, Renzaho A, Kühnapfel B, Podlech J, et al. Synergism Between the Components of the Bipartite Major Immediate-Early Transcriptional Enhancer of Murine Cytomegalovirus Does Not Accelerate Virus Replication in Cell Culture and Host Tissues. *J Gen Virol* (2009) 90:2395–401. doi: 10.1099/vir.0.012245-0
61. Lemmermann NA, Krmpotic A, Podlech J, Brizic I, Prager A, Adler H, et al. Non-Redundant and Redundant Roles of Cytomegalovirus gH/gL Complexes in Host Organ Entry and Intra-Tissue Spread. *PloS Pathog* (2015) 11: e1004640. doi: 10.1371/journal.ppat.1004640
62. Podlech J, Reddehase MJ, Adler B, Lemmermann NA. Principles for Studying *In Vivo* Attenuation of Virus Mutants: Defining the Role of the Cytomegalovirus gH/gL/gO Complex as a Paradigm. *Med Microbiol Immunol* (2015) 204:295–305. doi: 10.1007/s00430-015-0405-2
63. Munks MW, Gold MC, Zajac AL, Doom CM, Morello CS, Spector DH, et al. Genome-Wide Analysis Reveals a Highly Diverse CD8 T Cell Response to Murine Cytomegalovirus. *J Immunol* (2006) 176:3760–6. doi: 10.4049/jimmunol.176.6.3760
64. Lemmermann NA, Fink A, Podlech J, Ebert S, Wilhelmi V, Böhm V, et al. Murine Cytomegalovirus Immune Evasion Proteins Operative in the MHC Class I Pathway of Antigen Processing and Presentation: State of Knowledge, Revisions, and Questions. *Med Microbiol Immunol* (2012) 201:497–512. doi: 10.1007/s00430-012-0257-y
65. Becker S, Fink A, Podlech J, Giese I, Schmiedeke JK, Bukur T, et al. Positive Role of the MHC Class-I Antigen Presentation Regulator m04/gp34 of Murine Cytomegalovirus in Antiviral Protection by CD8 T Cells. *Front Cell Infect Microbiol* (2020) 10:454. doi: 10.3389/fcimb.2020.00454
66. Sacher T, Podlech J, Mohr CA, Jordan S, Ruzsics Z, Reddehase MJ, et al. The Major Virus-Producing Cell Type During Murine Cytomegalovirus Infection, the Hepatocyte, Is Not the Source of Virus Dissemination in the Host. *Cell Host Microbe* (2008) 3:263–72. doi: 10.1016/j.chom.2008.02.014
67. Holtappels R, Schader SI, Oettel O, Podlech J, Seckert CK, Reddehase MJ, et al. Insufficient Antigen Presentation Due to Viral Immune Evasion Explains Lethal Cytomegalovirus Organ Disease After Allogeneic Hematopoietic Cell Transplantation. *Front Cell Infect Microbiol* (2020) 10:157. doi: 10.3389/fcimb.2020.00157
68. Gezinir E, Podlech J, Gergely KM, Becker S, Reddehase MJ, Lemmermann NAW. Enhancement of Antigen Presentation by Deletion of Viral Immune Evasion Genes Prevents Lethal Cytomegalovirus Disease in Minor Histocompatibility Antigen-Mismatched Hematopoietic Cell Transplantation. *Front Cell Infect Microbiol* (2020) 10:279. doi: 10.3389/fcimb.2020.00279
69. Büscher N, Paulus C, Nevels M, Tenzer S, Plachter B. The Proteome of Human Cytomegalovirus Virions and Dense Bodies Is Conserved Across Different Strains. *Med Microbiol Immunol* (2015) 204:285–93. doi: 10.1007/s00430-015-0397-y
70. Ebert S, Becker M, Lemmermann NA, Büttner JK, Michel A, Taube C, et al. Mast Cells Expedite Control of Pulmonary Murine Cytomegalovirus Infection by Enhancing the Recruitment of Protective CD8 T Cells to the Lungs. *PloS Pathog* (2014) 10:e1004100. doi: 10.1371/journal.ppat.1004100
71. Griffiths P, Reeves M. Pathogenesis of Human Cytomegalovirus in the Immunocompromised Host. *Nat Rev Microbiol* (2021). doi: 10.1038/s41579-021-00582-z
72. Söderberg-Nauclér C, Fish KN, Nelson JA. Reactivation of Latent Human Cytomegalovirus by Allogeneic Stimulation of Blood Cells From Healthy Donors. *Cell* (1997) 91:119–26. doi: 10.1016/s0092-8674(01)80014-3
73. Adler SP, Reddehase MJ. Pediatric Roots of Cytomegalovirus Recurrence and Memory Inflation in the Elderly. *Med Microbiol Immunol* (2019) 208:323–28. doi: 10.1007/s00430-019-00609-6
74. Adler B, Sinzger C. Cytomegalovirus Interstrain Variance in Cell Type Tropism. In: MJ Reddehase, editor. *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*, vol. 1 . Norfolk, UK: Caister Academic Press (2013). p. 297–321.
75. Wilkinson GW, Davison AJ, Tomasec P, Fielding CA, Aicheler R, Murrell I, et al. Human Cytomegalovirus: Taking the Strain. *Med Microbiol Immunol* (2015) 204:273–84. doi: 10.1007/s00430-015-0411-4
76. Kaeuferle T, Krauss R, Blaeschke F, Willier S, Feuchtinger T. Strategies of Adoptive T -Cell Transfer to Treat Refractory Viral Infections Post Allogeneic Stem Cell Transplantation. *J Hematol Oncol* (2019) 12:13. doi: 10.1186/s13045-019-0701-1
77. Ottaviano G, Chiesa R, Feuchtinger T, Vickers MA, Dickinson A, Gennery AR, et al. Adoptive T Cell Therapy Strategies for Viral Infections in Patients Receiving Haematopoietic Stem Cell Transplantation. *Cells* (2019) 8:47. doi: 10.3390/cells8010047
78. D'Ippolito E, Schober K, Nauerth M, Busch DH. T Cell Engineering for Adoptive T Cell Therapy: Safety and Receptor Avidity. *Cancer Immunol Immunother* (2019) 68:1701–12. doi: 10.1007/s00262-019-02395-9
79. Schober K, Müller TR, Busch DH. Orthotopic T-Cell Receptor Replacement-an "Enabler" for TCR-Based Therapies. *Cells* (2020) 9:1367. doi: 10.3390/cells9061367
80. Redeker A, Welten SP, Baert MR, Vloemans SA, Tiemessen MM, Staal FJ, et al. The Quantity of Autocrine IL-2 Governs the Expansion Potential of CD8+ T Cells. *J Immunol* (2015) 195:4792–801. doi: 10.4049/jimmunol.1501083
81. Redeker A, Arens R. Improving Adoptive T Cell Therapy: The Particular Role of T Cell Costimulation, Cytokines, and Post-Transfer Vaccination. *Front Immunol* (2016) 7:345. doi: 10.3389/fimmu.2016.00345
82. Holtappels R, Janda J, Thomas D, Schenk S, Reddehase MJ, Geginat G. Adoptive CD8 T Cell Control of Pathogens Cannot be Improved by

Combining Protective Epitope Specificities. *J Infect Dis* (2008) 197:622–29. doi: 10.1086/526791
83. Ebert S, Lemmermann NA, Thomas D, Renzaho A, Reddehase MJ, Holtappels R. Immune Control in the Absence of Immunodominant Epitopes: Implications for Immunotherapy of Cytomegalovirus Infection With Antiviral CD8 T Cells. *Med Microbiol Immunol* (2012) 201:541–50. doi: 10.1007/s00430-012-0268-8
84. Ebert S, Podlech J, Gillert-Marien D, Gergely KM, Büttner JK, Fink A, et al. Parameters Determining the Efficacy of Adoptive CD8 T-Cell Therapy of Cytomegalovirus Infection. *Med Microbiol Immunol* (2012) 201:527–39. doi: 10.1007/s00430-012-0258-x
85. Nauerth M, Weißbrich B, Knall R, Franz T, Dössinger G, Bet J, et al. TCR-Ligand Koff Rate Correlates With the Protective Capacity of Antigen-Specific CD8+ T Cells for Adoptive Transfer. *Sci Transl Med* (2013) 5:192ra87. doi: 10.1126/scitranslmed.3005958
86. Ettinghausen SE, Rosenberg SA. The Adoptive Immunotherapy of Cancer Using Lymphokine Activated Killer Cells and Recombinant Interleukin-2. *Springer Semin Immunopathol* (1986) 9:51–71. doi: 10.1007/BF00201905
87. Cheever MA, Thompson DB, Klarnet JP, Greenberg PD. Antigen-Driven Long Term-Cultured T Cells Proliferate *In Vivo*, Distribute Widely, Mediate Specific Tumor Therapy, and Persist Long-Term as Functional Memory T Cells. *J Exp Med* (1986) 163:1100–12. doi: 10.1084/jem.163.5.1100
88. Lotze MT, Rosenberg SA. Results of Clinical Trials With the Administration of Interleukin 2 and Adoptive Immunotherapy With Activated Cells in Patients With Cancer. *Immunobiology* (1986) 172:420–37. doi: 10.1016/S0171-2985(86)80122-X
89. Gogesch P, Penner I, Krauter S, Büscher N, Grode L, Aydin I, et al. Production Strategies for Pentamer-Positive Subviral Dense Bodies as a Safe Human Cytomegalovirus Vaccine. *Vaccines* (2019) 7:104. doi: 10.3390/vaccines7030104
90. Lehmann C, Falk JJ, Büscher N, Penner I, Zimmermann C, Gogesch P, et al. Dense Bodies of a gH/gL/UL128/UL130/UL131 Pentamer-Repaired Towne Strain of Human Cytomegalovirus Induce an Enhanced Neutralizing Antibody Response. *J Virol* (2019) 93:e00931–19. doi: 10.1128/JVI.00931-19
91. Seckert CK, Renzaho A, Tervo HM, Krause C, Deegen P, Kühnapfel B, et al. Liver Sinusoidal Endothelial Cells Are a Site of Murine Cytomegalovirus Latency and Reactivation. *J Virol* (2009) 83:8869–84. doi: 10.1128/JVI.00870-09
92. Seckert CK, Schader SI, Ebert S, Thomas D, Freitag K, Renzaho A, et al. Antigen-Presenting Cells of Haematopoietic Origin Prime Cytomegalovirus-Specific CD8 T-Cells But Are Not Sufficient for Driving Memory Inflation During Viral Latency. *J Gen Virol* (2011) 92:1994–2005. doi: 10.1099/vir.0.031815-0
93. Reddehase MJ, Balthesen M, Rapp M, Jonjić S, Pavić I, Koszinowski UH. The Conditions of Primary Infection Define the Load of Latent Viral Genome in Organs and the Risk of Recurrent Cytomegalovirus Disease. *J Exp Med* (1994) 179:185–93. doi: 10.1084/jem.179.1.185
94. Kurz SK, Reddehase MJ. Patchwork Pattern of Transcriptional Reactivation in the Lungs Indicates Sequential Checkpoints in the Transition From Murine Cytomegalovirus Latency to Recurrence. *J Virol* (1999) 73:8612–22. doi: 10.1128/JVI.73.10.8612-8622.1999

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REVIEW
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Immune Landscape of CMV Infection in Cancer Patients: From "Canonical" Diseases Toward Virus-Elicited Oncomodulation

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Human Cytomegalovirus (HCMV) is an immensely pervasive herpesvirus, persistently infecting high percentages of the world population. Despite the apparent robust host immune responses, HCMV is capable of replicating, evading host defenses, and establishing latency throughout life by developing multiple immune-modulatory strategies. HCMV has coexisted with humans mounting various mechanisms to evade immune cells and effectively win the HCMV-immune system battle mainly through maintaining its viral genome, impairing HLA Class I and II molecule expression, evading from natural killer (NK) cell-mediated cytotoxicity, interfering with cellular signaling, inhibiting apoptosis, escaping complement attack, and stimulating immunosuppressive cytokines (immune tolerance). HCMV expresses several gene products that modulate the host immune response and promote modifications in non-coding RNA and regulatory proteins. These changes are linked to several complications, such as immunosenescence and malignant phenotypes leading to immunosuppressive tumor microenvironment (TME) and oncomodulation. Hence, tumor survival is promoted by affecting cellular proliferation and survival, invasion, immune evasion, immunosuppression, and giving rise to angiogenic factors. Viewing HCMV-induced evasion mechanisms will play a principal role in developing novel adapted therapeutic approaches against HCMV, especially since immunotherapy has revolutionized cancer therapeutic strategies. Since tumors acquire immune evasion strategies, anti-tumor immunity could be prominently triggered by multimodal strategies to induce, on one side, immunogenic tumor apoptosis and to actively oppose the immune suppressive microenvironment, on the other side.

Keywords: HCMV, immunosuppression, immune evasion, immunosenescense, TME, oncomodulation, therapeutic approaches

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BACKGROUND

HCMV or human herpesvirus 5 (HHV-5) has co-evolved with mammalian hosts over millennia infecting almost 83% of the world's population, impending 100% in developing countries (1). After initial infection, HCMV can establish lifelong persistence within its corresponding host as well as possessing the reactivation potential; viral persistence depends on composite interactions among various

viral and host determinants. Such interactions mostly generate an equilibrium between the immunocompetent host and the virus itself. In the host, HCMV infrequently causes symptoms unless this balance is demolished by the minimized host immune proficiency (atypical settings) leading to substantial pathology (1, 2). Upon viewing several forms of viral-host interaction, the explicit HCMV reactivation in immunosuppressed patients (organ transplant recipients) and immunocompromised patients (septic patients, elderly, HIV-infected patients, etc.) is considered a well identifiable disease state (3, 4). Hence, in immunocompetent patients, HCMV is considered a multifaceted beta herpesvirus that is viewed as an asymptomatic and mildly pathogenic virus, but may nevertheless cause chronic infections along with acute and serious complications in immunocompromised individuals (5). HCMV persistence can also have a key influence on the host, even in healthy carriers, through the attenuation of innate and adaptive immune responses since HCMV starts to counteract several host immune response mechanisms required to control the infection (1, 2). HCMV potentially triggers the host immune responses starting by the mechanisms of innate immunity, including inflammatory cytokines resulting from virus/cell binding and NK cell induction which consequently drives adaptive immune responses, involving production of antibodies and the initiation of $CD4^+$ and $CD8^+$ T-cell responses. However, HCMV encodes various immune evasion mechanisms; hence, expressing several genes that influence both innate and adaptive immunity (5, 6).

HCMV, a leading viral cause of birth defects, has been linked to several mortality and morbidity conditions (7). The stage of HCMV acquisition may affect the range of associated clinical manifestations and the effectiveness of the immune responses exerted against HCMV (7, 8). Regarding congenital CMV infection, neurological defects might be experienced as mental retardation, cerebral palsy, and hearing impairment; newborns may experience either symptomatic or asymptomatic infections (7, 9, 10). Symptomatic infections might cause petechiae, low birth weight, jaundice, hepatosplenomegaly, seizures, and microcephaly; they appear to be more common in newborns infected during the first trimester of pregnancy (7). In case of premature birth, sepsis and respiratory distress can develop (11). Compared to adults, these observations indicate that controlling CMV replication is restricted during early stages of life and is associated with delayed immune responses and increased risk of symptomatic infection (7). Years later, persistent HCMV infection might be considered a potential risk factor exacerbating age-associated diseases and immunosenescence which is defined as the age-associated deterioration in overall immune condition (12–14) although some studies have indicated that the link between HCMV and immune aging is obscure (3, 15). Further, various stimuli can induce HCMV reactivation; it might be triggered in SARS-CoV-2 infected patients (16, 17) thus exacerbating the risk of coronavirus disease 2019 (COVID-19) (18, 19). Even if this interaction is still elusive and additional large scale studies are recommended (16), CMV testing and treatment should be taken into consideration in such critical conditions (18). CMV status must be taken into account for several vaccine responses, especially cancer despite the use of HCMV-based therapeutic cancer vaccines (20), since it has been suggested that with advanced age and due to CMV-associated altered immunity in both healthy and immunocompromised hosts, vaccine immunogenicity was modulated (21–23). Thus, recent studies are concerned about targeting HCMV to decrease the sensitivity to other infectious diseases and cancer, and to prevent poor responses to vaccination (21, 22).

The contribution of HCMV infection in late inflammatory complications highlights its potential association with chronic diseases, such as atherosclerosis, chronic rejection following solid-organ transplantation, and malignancies (24). Recent investigations have reported the prevalence of HCMV infection in tumoral tissues of malignancies such as malignant glioma, breast and colon cancer, negative Hodgkin's disease, Epstein-Barr virus (EBV), liver cancer, cervical cancer, and prostatic carcinoma (1, 25, 26). Despite the fact that HCMV is not yet included in the oncogenic viruses list, its possible contribution in carcinogenesis as initiator or promoter is significantly reported suggesting that HCMV and tumors express a symbiotic relationship (26–29). HCMV aids the tumor to escape immune surveillance by encoding viral proteins and inducing various cellular factors, in addition to the HCMV-induced immune tolerance which favors tumor growth. In return, HCMV harbors in the immunologically weak environment of the cancerous cells (6). This review accentuates the considerable influence of HCMV on the immune landscape and its oncomodulatory signals that might contribute to oncogenesis.

HOST IMMUNE RESPONSES AGAINST HCMV INFECTION

HCMV, a double-stranded DNA (dsDNA) genome beta-herpesvirus is considered the largest virus among the human herpesviruses (30). Upon HCMV infection and despite the counteracting host response, this virus powerfully adapts to the human immune system. HCMV is certainly not eradicated from the HCMV-positive immunocompetent patient, in whom the virus establishes latency (31). Thus, the human immune system is incompetent to clear the latent HCMV, however it mounts an immune defense targeting multiple viral proteins (8). Due to the existing coevolution between HCMV and the host immune system for millions of years, it's informative to study the immune defense strategies and pathogen counterstrategies (12). Innate immunity, in addition to adaptive humoral and cell-mediated immune responses, are induced by HCMV infection; such responses lead to the resolution of acute primary infection (5). Such immune responses differ during distinct life stages; throughout pregnancy, maternal anti-HCMV antibodies participate in preventing congenital fetal CMV infection (32). In addition, studies have shown that despite the detection of primary humoral and cellular immune responses in neonates, cell-mediated immune responses are delayed compared to adults which justifies the reason behind uncontrolled viremia and serious clinical harm in early life CMV infections (8, 32, 33). CMV-specific $CD8^+$ T-cell responses in congenitally infected newborns were characterized by lower IFN-γ levels and elevated

levels of IL-8 compared to adults (33). Finally, elderly people have increased sensitivity and susceptibility to serious infections and diseases most likely due to immunosenescence (14).

HCMV Entry

HCMV exhibits a wide host cell range, possessing the ability to infect several cell types for instance endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, leukocytes, and dendritic cells (DCs) (8, 34). In healthy persons, HCMV initiates its replication in the mucosal epithelium; thereafter, it disperses to monocytes and CD34+ cells, where it institutes a latent infection. Upon differentiation of HCMV-infected monocytes into macrophages, a viral infection could be initiated (35). Infection of both hematopoietic and endothelial cells systemically eases the viral spread within the host (36), unlike prevalent cell types infection including smooth muscle cells and fibroblasts which enhances efficient proliferation of the virus (35).

Innate Immunity

As HCMV enters the cells, virions are firstly recognized by the host thus activating multiple pathways and strategies of innate immunity which is known as the primary host defense against HCMV infection (8). These involve inflammatory cytokines, type I interferon (IFN), and upregulation of CD80 and CD86 (37) that are essential for limiting pathogen's spread and thereafter priming the adaptive immune response (5, 8). The stimulation of the NF-κB pathway and predominant inflammatory cytokines production for example interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (38) result from the interaction of viral envelope glycoproteins B (gB) and glycoprotein H (gH) with the immune-sensor molecules namely, toll-like receptors 2 (TLR2) (5, 12, 39, 40). Such inflammatory cytokines are capable of inducing and triggering phagocytic cells, such as dendritic cells, which have the ability to clear HCMV-infected cells (5, 8, 38). In the initial infection sites, NK cells are activated to eradicate HCMV-infected cells by the liberation of cytotoxic proteins (38). Furthermore, studies have shown NK cells' role in inhibiting HCMV transmission in fibroblasts, epithelial, and endothelial cells and this through inducing IFN-β in target infected cells (41) and secretion of IFN-γ (42). NK cells, crucial guards of the immune system, produce a cytokine environment that triggers the consequential maturation of adaptive immune responses particularly T-cells (5, 8, 43).

Adaptive Immunity

The adaptive immunity which contributes to the control of HCMV infection is among the strongest responses in which it fully engages humoral and cellular immune responses. Adaptive immunity is necessary to fundamentally manage HCMV primary infection, afterwards HCMV will enter into a latent state (44). The development of a sustained adaptive immune response is essential to preserve HCMV latency, avert acute viremia, and avert lytic infection which, in contrast, is frequent in patients on immunosuppressive therapies and immunocompromised individuals often leading to unrestricted replication and clinically severe HCMV morbidity and mortality (45).

Humoral Responses

Following a primary HCMV infection, the initiation of a robust immune response to control HCMV is essentially required. Many evidences supported the role of humoral immunity in limiting viral propagation and HCMV severity through antibody production targeting multiple CMV proteins, envelope glycoproteins, and genes (8, 32). The key target for antibody neutralization against HCMV is gB since it is related to cellular adhesion and invasion; besides, gH is considered the secondary target as it is involved in the fusion of the host cell membrane with the viral envelope (37). Other targets include the structural tegument proteins (pp65 and pp150) and non-structural proteins (IE1) (8, 32). A study shows that pregnant women, primarily infected with HCMV, having HCMV specific IgM antibodies and missing neutralizing IgG antibodies are at greater risk of transmitting HCMV to their fetus in contrast to seropositive mothers experiencing a recurrent infection (45). Thus, underlying the critical role of humoral immunity, especially HCMV IgG, in controlling HCMV infection and spread.

T-Cell Mediated Immune Responses

Due to the fact that the immune response stimulated by primary infection does not eliminate HCMV, HCMV-specific $CD4^+$ T-cells, $CD8^+$ T-cells, and gamma delta (γδ) T-cells have been considered as critical players in restricting viral replication in hosts acquiring persistent infections (38, 46, 47). With regard to $CD8^+$ T-cells, the $CD8^+$ HCMV-specific T-cell response is targeted toward HCMV proteins which are being expressed at different stages of viral replication (IE, early, early-late, and late) in addition to other proteins possessing various functions (capsid, tegument, glycoprotein, DNA-regulatory, and immune escape) (37). It is worth noting that the most immunodominant antigens to which HCMV-specific $CD8^+$ T-cells react are addressed toward IE1 (UL123), IE2 (UL122), and pp65 (UL83) (37, 48). Even though the major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell immune response role in targeting HCMV is evidently marked, there exists a significant indication that $CD4^+$ T-cells are as well fundamental in controlling HCMV infections (37). Further studies reveal the attainment of a cytolytic potential by pp65-specific $CD4^+$ T-cells and gB-specific $CD4^+$ CTL *in vivo* where $CD4^+$ T-cells released granzyme B in reaction to glia presenting endogenous gB (49). The recruitment of HCMV-specific T-cells into the memory compartment is stimulated by the relatively prolonged viral replication of HCMV since T-cells are mandatory to limit HCMV viral replication and impede certain diseases (50). HCMV-specific T-cell responses inflate throughout life leading to a significant fraction of memory T-cells in healthy seropositive individuals (50–52). Hence, HCMV-seropositive immunocompetent people maintain lifetime protection despite the insufficient or minimal HCMV-specific T-cell responses. Cellular responses from $CD4^+$ and $CD8^+$ T-cells vary among individuals (50). HCMV-positive serostatus has been associated with $CD8^+$ T-cell compartment expansion, reduced CD4:CD8 T-cell ratio as well as alterations in the expression of CD8+ T-cell senescence related markers (53, 54). These senescent cells are characterized by a progressive loss of CD28 and CD27,

upregulation of CD57 expression which is known as the classical immune senescence marker, replicative senescence, and shortened telomeres resulting in a limited cell proliferation capacity and finally the inability to eliminate the HCMV infection (14, 55). Other cellular responses include γδ T-cells and NK cells. Although the previously mentioned cells are not targeted specifically against HCMV, they can still successfully lyse HCMV-infected endothelial cells and fibroblasts in consequence of a cellular stress response that upregulates the endothelial protein C receptor (EPCR) in addition to CD54 (Intercellular adhesion molecule-1, *ICAM-1*) (56). γδ T-cells contribute to dual immune response, anti-infectious and antitumor. Activated γδ T-cells are essential immune effectors against HCMV in which they stimulate IFN-γ and TNF-α production that may synergize to inhibit HCMV replication (38).

HCMV PERSISTENCE DESPITE ANTIVIRAL IMMUNITY

In healthy individuals, the operative homeostatic equilibrium established between HCMV and the host prevents serious HCMV complications. Conversely, in an immunocompromised host, fetus, and neonates, HCMV infection can cause multiple forms of clinical harm (8). Disequilibrium in immunocompromised patients can result in unhindered viral replication followed by the reactivation of the latent virus, with subsequent morbidity and mortality (5). Despite a powerful immunity involving both arms of the immune system, HCMV establishes latency. In that context, HCMV-encoded determinants of tropism for endothelial cells, an imperative objective of the infection, were considered. It was stated that in endothelial cells the UL133-UL138 locus, encoded in the ULb′ region of the HCMV genome, is essential for the viral late-stage response (57). In infected cells, this locus was mandatory for preserving membrane organization and is required for the progeny viruses' maturation. However, it's not necessary for early/late gene expression or viral genome synthesis. Viruses missing the *UL133-UL138* region, produce progeny viruses that are deprived of tegument and envelopes, leading to deficient viral yields. *UL135* and *UL136* genes, encoded in the UL133-UL138 region, promote viral maturation. Additional recent data propose that this locus involves the main molecular switch among latency and reactivation, including the opposing roles of *UL135* and *UL138*. Moreover, a study reported that the outcome of antiviral immunity might be influenced by numerous viral determinants, including HCMV strain, virulence, MHC I downregulation, and other escape strategies elicited by HCMV during the early virus-host interaction (3).

HCMV ESCAPE MACHINERIES AND IMMUNOSUPPRESSION

HCMV has evolved manifold immune evasion strategies that modulate the host immune system and promotes more efficient infection and dissemination within the host. A chief evasion strategy depends on hindering the MHC class I-restricted antigen presentation (58). Throughout the immediate early HCMV infection phase, a cytotoxic T-lymphocyte (CTL) response counteract antigenic peptides resulting from the IE1 transcription factor (59, 60). The matrix protein, pp65, possessing a kinase activity, phosphorylates the IE1 protein and specifically inhibit the presentation of IE-derived antigenic peptides to escape immune recognition of the early produced viral proteins (59). Knowing that pp65 is delivered directly into the cells during the viral fusion phase, HCMV will instantly escape from immunological surveillance, till further immune evasion related proteins are secreted (61). HCMV-specific viral proteins and genes that are associated with the host interferon responses (pp65), inhibit NK cell detection or activation (37, 62), and inhibit the recognition of $CD4^+$ and $CD8^+$ T-cell by preventing MHC Class I and II antigen processing and appearance (1, 37, 61). HCMV infected cells produce viral IL-10 homolog which further suppresses $CD4^+$ and $C8^+$ T-cell responses (1, 61). The previously mentioned evasion mechanisms are summarized in **Figure 1**.

In the absence of MHC class expression, HCMV must be susceptible to NK cell-mediated lysis; however, HCMV donates a large proportion of its genome to down-regulate the NK cell activity (63). Consequently, the surface expression of HLA-E and HLA-G is stimulated by gpUL40 and CMV-IL10 respectively (64–66). In addition, the expression of UL16 supports HCMV to block natural killer group 2D (NKG2D)-mediated NK-cell activation and this is by adopting a blocking strategy that hinders the binding of NKG2D to UL16 binding proteins (ULBPs) namely, ULBP1 and ULBP2, and to the MHC class I chain-related gene B (MICB gene) (37, 61). HCMV US18 and US20 proteins stimulate the deterioration of a major stress protein namely, MHC class I polypeptide-related sequence A (MICA); hence, preventing the NK cell from recognizing infected cells' stress signals (67). Other machineries considered by HCMV to escape NK cell lysis involve the inhibition of NK cell-activating receptor (NKp30) by pp65 (37), UL122-encoded microRNA that represses *MICB* gene expression (68), and blocking of the expression of CD155 by HCMV-UL141 (37).

To counteract apoptosis and further evade the immune system, HCMV overexpresses anti-apoptotic proteins and inhibits pro-apoptotic molecules and death receptors. The former is achieved by upregulating B-cell lymphoma 2 (Bcl-2) in HCMV-infected cells (69) and expressing Fas-associated death-domain-like IL-1β-converting enzyme-inhibitory proteins (FLIP) by IE2 (70). On the other hand, pUL36 inhibits the induction of procaspase 8 to the death-inducing signaling complex (DISC) and pUL37 inhibits pro-apoptotic Bcl-2 members namely Bcl-2-associated X Protein (Bax) and Bcl-2 homologues antagonist/killer (Bak); thus, HCMV is hindering apoptosis through two distinct mechanisms (6, 61). Furthermore, HCMV has developed UL36 and UL37 proteins, which enhance the survival of infected cells; thus, stimulating viral dissemination within the host (37, 71). HCMV escapes complement attack by upregulating the host-encoded complement regulatory proteins (CRPs) (72) and by the ability of HCMV to integrate host cell-derived CRPs, CD55 and CD59 in its virions (6).

Lastly, HCMV produces the G-Protein-coupled receptors (GCRs) homologs US27, US28, UL33, and UL78 that might act as eliminators of chemotactic factors, thus hindering the inflammatory cells' accumulation at the viral infection site (61, 73). These viral approaches secure novel viral progeny production and facilitate the spread to other hosts (61). US28 is usually expressed in the early infection phase; it shows the highest homogeneity to the CC chemokine receptor CCR1. It also binds the CC chemokines RANTES, monocyte chemoattractant protein-1 (MCP-1), monocyte chemotactic protein-3 (MCP3), macrophage inflammatory protein-1 alpha (MIP-1α), and macrophage inflammatory protein-1 beta (MIP-1β), in addition to the membrane-associated CX3C chemokine, fractalkine (37, 61, 74). US28 expression results in the stimulation of phospholipase C and NF-κB signaling. The US28-fractalkine interaction has been involved in cell targeting and viral dissemination (61). The transcription of *US28* takes place during productive and latent HCMV infection, which might justify the dissemination of latent HCMV (75, 76). Moreover, HCMV encodes a homolog of the immunosuppressive cytokine IL-10 (UL111a) (62, 71); it likewise possesses potent immunosuppressive traits, including the inhibition of mitogen-stimulated peripheral blood mononuclear cells (PBMCs) proliferation in addition to the blockade of pro-inflammatory cytokine synthesis in PBMCs and monocyte (37, 61). CMV IL-10 binds to the cellular human receptor of IL-10 despite its minimal homology to the endogenous cellular IL-10 (61). In addition, HCMV establishes immune tolerance by inducing the transcription and release of TGF-β which inhibits anti-viral IFN-γ and TNF-α cytokine production and cytotoxic effector activities of HCMV specific Th1 cells (77). Further homologs encoded by HCMV are UL144 which is a viral TNF receptor and an effective IL-8-like chemokine (viral CXC-1) prompting the chemotaxis of peripheral blood neutrophils (UL146) (37). Lastly, HCMV strategies that modify the cellular infected environment to restrict immune identification are known to be widely expressed during lytic infection; however, recent evidence shows that viral genes' activity in preventing immune recognition is being remarkable even during latency phases (75). Recent data shows that the majority of the HCMV-encoded proteins and microRNAs (miRNAs) are expressed also during latent stages (75, 76) (**Table 1** and **Figure 1**).

HCMV COMPLICATIONS UNDER IMMUNOSUPPRESSION

The suppressive effects exerted by HCMV on the host immune system, HCMV persistence, dissemination, and reactivation result

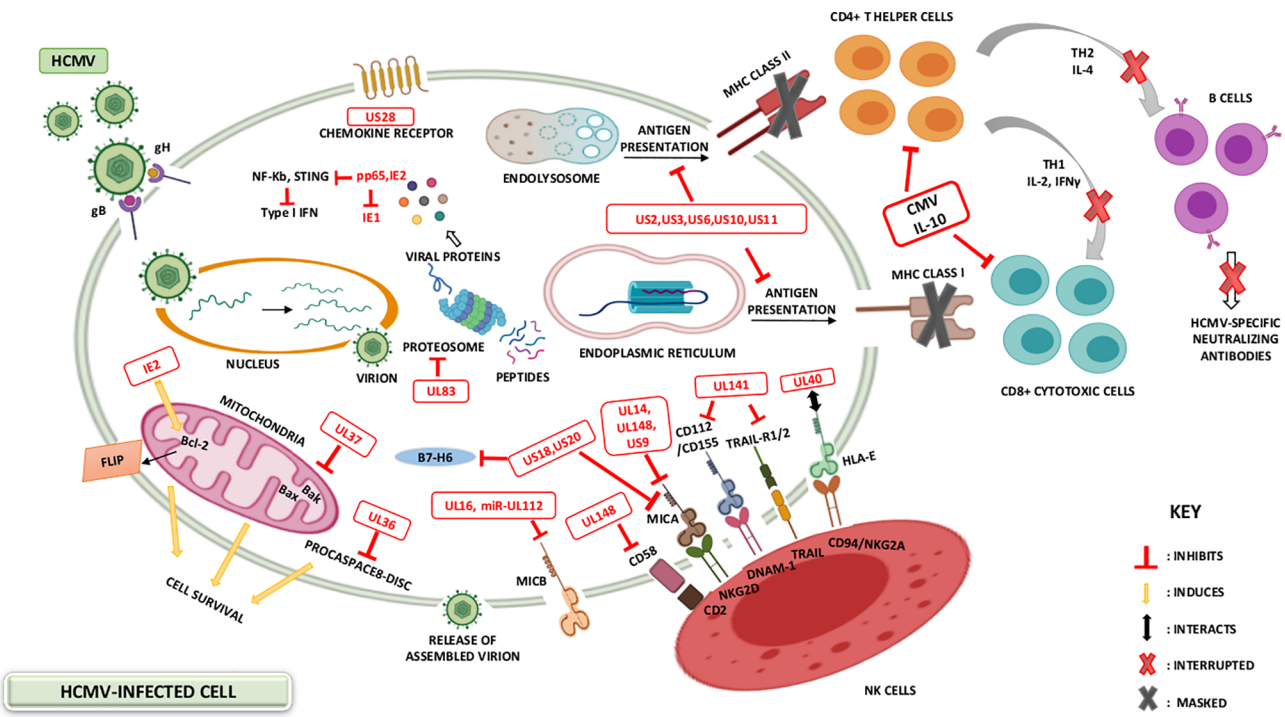

FIGURE 1 | HCMV-induced modulation of the host immune system. The battle between the host immunity and HCMV is permanent, with HCMV developing various mechanisms to evade the host immune system. Immunosuppression may be ascribed to the variety of immune modulators encoded by HCMV-specific gene products. HCMV viral genes prevent MHC Class I and II antigen presentation and thus interfere with interferon responses, NK cell recognition as well as $CD4^+$ and $CD8^+$ T-cell recognition. Additional HCMV genes for instance IL-10 homologue (UL111a), and viral proteins acting as receptors for host inflammatory cytokines (US28), further suppress the host immune responses.

in dire consequences. The severe and mortal complications resulting from HCMV reactivation mainly occur in immunosuppressed and seriously ill immunocompetent individuals in whom the HCMV infection is accompanied by prolonged hospitalization and/or mortality. Furthermore, the manipulation of the host immunity can result in superinfections with other herpesviruses or bacteria, and exacerbate SARS-CoV-2 infections that are benefiting from the weakened immune system (18, 19, 103, 104). Since HCMV infection is known as a prevalent congenital viral infection, it might generate viral hepatitis with jaundice in addition to long-lasting disabilities, including hearing and visual damage, neurological impairments, and mental retardation (103). Additionally, studies show that HCMV has been detected in tissue specimens from immunocompetent individuals with inflammatory diseases, including atherosclerosis, psoriasis (6, 105), rheumatoid arthritis (24), inflammatory bowel disease (IBD) (105), and systemic lupus erythematosus (SLE); it has been concerned in the development of these diseases (103). HCMV leads to the development of restenosis after coronary angioplasty, chronic rejection of organ transplantation, chronic graft-*versus*-host disease in recipients of bone marrow transplants (24). Such observations infer the presence of an association between HCMV and autoimmune diseases. Knowing that the HCMV chemokine receptor homolog, US28, is considered a major co-receptor for several HIV strains, it provokes cell fusion with several forms of viral envelope proteins in addition to stimulating HIV-1 entry into HCMV- infected cells (106). Several epidemiologic studies suggested that HCMV infection has been linked to an elevated risk of cardiovascular death, one of which revealed that CMV seropositivity has been significantly associated with cardiovascular mortality (*P-value*=0.007) (107). This association was confirmed by another study showing an "increased six-year cardiovascular mortality" (*P-value*=0.021) (108). Further findings showed that CMV seropositive elderly presented elevated cardiovascular mortality compared to CMV-negative ones; the subhazard ratio for cardiovascular mortality was 1.95 (95% CI: 1.29–2.96, *P-value*=0.002) (109). The association between HCMV and vascular diseases is verified by the transient presence of US28 in smooth muscle cells which induces chemokinesis and chemotaxis (61). Moreover, since HCMV infection has been involved in producing modifications among the total T-cell population and adversely affecting to the immune well-being of elderly, it stimulates the occurrence of numerous age-related syndromes, and decreases efficacy of vaccines (3, 12, 110). Additionally, in elderly, HCMV could contribute to inflammation-mediated vascular pathology which is evaluated by determining systemic inflammation markers (C-reactive protein, IL-6, and TNF) and it might also cause direct vascular damage (2).

HCMV ONCOMODULATION AND ITS SIGNIFICANCE IN TUMOR MICROENVIRONMENT

Alterations resulting from cancerous genetic and epigenetic instability provide recognizable antigens that are distinguished by the host immune system (111, 112). As cancer evolves, it can resist immune clearance by prompting tolerance in the presence of tumor-associated inflammatory cells (113). Consequently, a tumor microenvironment is generated and controlled by tumor-induced molecular and cellular interactions (114) in which immune cells not only fail to exert anti-tumor effector functions, but also promote tumor development (113). Since CMV possesses different cellular signaling pathways, encodes many genes that exhibit immunosuppressive effects, and may empower cancer hallmarks, it thus plays an essential role in generating cancerous cells and has a fundamental impact on the tumor microenvironment (1, 29, 115).

Some studies put extra emphasis on the indirect role of CMV in cancer (115, 116). For instance, Dey et al. suggested that the association between glioma and CMV is an "observational association" (117). However, the prevalence of HCMV is remarkably high in several cancer forms (26, 118). Several research groups showed that over 90% of breast, colon, and prostate cancer, rhabdomyosarcoma, hepatocellular cancer, salivary gland tumors, neuroblastoma and brain tumors were positive for HCMV nucleic acids and/or proteins (26). HCMV DNA was confirmed in 100% of breast cancer and 91% of sentinel lymph nodes samples from the metastatic group (119). Moreover, a study conducted by Taher et al., showed HCMV detection in 98% of breast cancer derived metastatic brain tumors, suggesting a potential link between HCMV and metastatic cancer (120). HCMV was considered as a potential therapeutic target in metastatic cancer due to the expression of HCMV-IE protein in 53% of breast cancer samples which therefore resulted in shorter overall survival, and the detection of HCMV DNA and transcripts in 92% and 80% of the used specimens respectively (120). Another study showed the inversely proportional relation between HCMV-IE1 presence and hormone receptor expression suggesting HCMV role in hormone receptor-negative breast cancer tumors (121). HCMV IE1 and pp65 were present in 82% and 78% of colorectal cancer samples, and in 80% and 92% of adenocarcinomas, respectively. In colon cancer cells, these HCMV-specific proteins contribute to the induction of Bcl-2 and COX-2 proteins thus promoting colon cancer progression (122). Cobbs et al. showed that HCMV-IE1 was expressed in all studied glioma biopsy specimens, in all grades (II-IV) (123). Over again, HCMV-IE and late proteins were expressed in 100% and 92% of primary neuroblastoma samples respectively; notably, HCMV proteins were detected in CD133 and CD44-positive neuroblastoma cells (118). HCMV DNA was detected in the peripheral blood of GBM patients (80%), suggesting either HCMV reactivation or viral DNA shedding from HCMV-tumor cells (124). In addition, HCMV was detected in all evaluated preneoplastic and neoplastic prostate lesions (125). In Hodgkin's disease cases, the HCMV infection frequency was 28.6% (126). Further, expression of HCMV was marked in the neoplastic epithelium of 97% of the carcinoma patients (127). 92% of the primary medulloblastoma cases expressed HCMV-IE protein while 73% expressed late viral proteins (128). Evident elevated survival rates were observed among HCMV positive glioblastoma patients who

TABLE 1 | HCMV gene products involved in immunomodulation and their oncogenic characteristics.

HCMV Gene Products	Mechanism of Action	Possible Oncogenic Characteristic
US2, US3, US6, US11	➢MHC class I expression impairment, reducing HCMV antigen presentation toward $CD8^+$ cells and evasion of $CD8^+$ T-cell immune responses, superinfection (60, 78) ➢US2 down regulates MHC class II and reduces HCMV antigen presentation to $CD4^+$ cells (79)	➢Preventing $CD8^+$ mediated cytotoxic tumor killing (80)
US18 and US20	➢Interfere with B7-H6 surface expression including endosomal degradation, evades NK cells' immune detection (81)	➢HCMV-immune evasion might indirectly affect tumor environment
***US28* (viral GPCR)**	➢Promotes chemotaxis (82, 83)	➢Cellular proliferation, tumor growth, enhanced angiogenesis and cell survival (84, 85)
UL16	➢Regulation of NK cell ligand NKG2D and impairing NK cells function (79)	➢Immune evasion, protects the cells from cytotoxic peptides-mediated lysis, and protects cancer cells from both NK and T-cells (80)
UL40	➢NK cell evasion (62) ➢HLA-E over expression (62), enhancing its interaction with the inhibitory receptor CD94/NKG2A (86)	➢HLA-E Over expression (1)
***UL83* (pp65)**	➢IE1 sequestration, repress proteasome processing, reduce NKp30 effect and delays antiviral gene expression (87)	➢Genomic mutation, immune evasion (84)
***UL122* (IE2)**	➢Overexpression of anti-apoptotic FLIP protein (60, 79)	➢Elevated immune suppression, cell proliferation, escaping growth suppressors and apoptosis (84)
***UL123* (IE1)**	➢Induction of TGF-β (82)	➢Cellular proliferation, genome instability and mutation, escaping growth suppressors, and ameliorated cell survival (84)
***UL82* (pp71)**	➢Inhibits antiviral response by binding to interferon stimulator gene (87, 88)	➢Cellular proliferation, escaping growth suppressors, and genomic mutation (84)
***UL111A* (cmvIL-10)**	➢Inhibits MHC class II expression and suppresses $CD4^+$ T-cell recognition (83, 89)	➢Immunosuppression, cellular proliferation, stimulates migration and metastasis, telomerase activation (84)
UL142	➢Inhibiting MICA (79, 90)	➢HCMV-immune evasion might indirectly affect tumor environment
UL36	➢Complexing with pro-caspase-8 thus suppressing its proteolytic stimulation and prompting its designation as viral inhibitor of caspase-8-induced apoptosis (vICA) (91, 92)	➢Enhanced cell survival
UL37	➢Inhibition of Bak and Bax protein, thus inhibiting apoptosis (79)	➢Enhanced cell survival
UL76	➢Activation of the DNA damage response thus inducing IL-8 expression (93)	➢Genome instability and mutation (84)
UL97	➢Forms a complex with pp65 and mediates immune evasion (5, 94)	➢Escaping growth suppressors (84)
UL141-UL144	➢Encodes for homolog of TNFR, hinders CD155 and CD112 expression (NK cell activating ligands) and the death receptor for the TNF family ligand TRAIL (5, 95)	➢HCMV-immune evasion might indirectly affect tumor environment
UL145	➢Depletion of helicase like transcription factor- (HLTF) through the recruitment of Cullin4/DDB ligase complex (96, 97)	➢Impeding innate immunity might indirectly affect tumor environment
UL146	➢Promotes neutrophil chemotaxis, immune escape (5, 98)	➢HCMV-immune evasion might indirectly affect tumor environment
UL148	➢CD58 Suppression; effective modulator of CTL function, amplify degranulation in cytotoxic T lymphocytes and NK cells against HCMV-infected cells (85)	➢HCMV-immune evasion might indirectly affect tumor environment
miR-UL112	➢Down regulation of MICB thus escaping NK cells, and decreased T-cell recognition (99, 100)	➢Exerts its oncogene function by directly targeting tumor suppressor candidate 3 (TUSC3) in GBM (101)
LncRNA	➢Function in both innate and adaptive immunity including the development, activation, and homeostasis of the immune system (102)	➢Cellular proliferation and transformation, facilitating signal transductions in cancer signaling pathways (84, 102), it's also involved in angiogenesis (85)

were on anti-viral therapy (valganciclovir) (120, 129). A 70% and 90% survival rate was proved with 6-month and continuous valganciclovir treatment, respectively (120). Despite the existing studies which describe the possible involvement of CMV in cancer, further large scale investigations are needed in addition to the necessity of novel epidemiologic studies knowing that the latter might be challenging to conduct especially among CMV-positive cancer patients.

HCMV infects multiple cell types including stem cells; referring to the fact that Thy-1 and platelet-derived growth factor receptor alpha (PDGFRα) stem cell markers enhance HCMV infection, stem cells are susceptible to HCMV infection (130, 131). Thus, stem cells serve as reservoirs for HCMV persistence and reactivation. The major stem cell regulator namely, Wnt tends to trigger HCMV transcriptional activation; hence, once HCMV disseminates to various body organs, viral expression occurs during patients' lifetime in stem cells (132). It is worth noting that the latter increases the chance of accumulation of genetic mutations; thus, stem cells lose control over their self-growth and renewal, act as a cancer source, and become susceptible to oncogenesis in the presence of inflammation and altered DNA repair pathways (133). In return, HCMV can support stem cells survival which would potentially elevate oncogenesis. Studies reveal the effect of

HCMV-IE1 protein in promoting the preservation of glioblastoma cancer stem cells through its induction of SRY-Box Transcription Factor 2 (SOX2), Nanog, Nestin, and octamer-binding transcription factor 4 (OCT3/4) where it's considered as a key regulator of glioblastoma stem-like phenotype (134). In GBM cells, the induction of transcription factors that are crucial for cancer stem cell persistence, cancer growth, and signaling pathways associated with the epithelial to mesenchymal (EMT) phenotype are stimulated by HCMV IE1 expression (134, 135). Many studies proved that cancer stem cells infected with HCMV possess a progression potential in contrast to HCMV-negative cancer stem cells. Some HCMV strains, for instance HCMV-DB and HCMV-BL, are capable of transforming human mammary epithelial cells and producing a "transcriptional profile" associated with DNA hypomethylation that resulted in enhanced proliferation, activation of cancer stem cell, and EMT process (136, 137). Likewise, HCMV was proven to induce an EMT phenotype in colorectal carcinoma cells accompanied with amplified tumor proliferation and cancer cell invasion (136). In addition, IE1 expression was detected in CMV transformed HMECs (CTH) cells which as well express embryonic stem cell markers (138). HCMV IE1 and IE2 gene expression in addition to UL76 genes prompt DNA mutagenesis, chromosome breakage, and genomic instability. Such expression of HCMV gene products could affect the pathways of p53 and Rb tumor suppressors, and other pathways that are responsible for DNA repair (27, 139, 140). Presuming the role of HCMV gene products in causing DNA damage directly and indirectly, and stimulating proliferation in stem cells, HCMV may have the potential to initiate and promote tumor formation. The oncomodulatory potential of HCMV catalyzes an oncogenic process by producing viral proteins, helping tumor cells to evade the immune system, and preventing and/or delaying cell death. The lack of HCMV specific cellular immune responses in these immune-privileged tumor sites would definitely enhance HCMV replication. On the other hand, cancer cells on their own can escape immune responses by diverse mechanisms. Thus, the combination of intrinsic cellular with viral immune escape machineries in cancer cells may offer an environment which enhances HCMV replication and boost cancer cells to evade from immune surveillance showing the bidirectional relationship between tumor cells and HCMV (25, 141). It's worth mentioning that HCMV can activate many of the tumor pathways' hallmarks including uncontrolled inflammation, myeloid cells' infiltration, immune modulation, angiogenesis, and metabolic reprogramming. Production of inflammatory cytokines including RANTES, MCP-1, MIP-1a, IFN-γ, TNF- α, IL-4, IL-18, and IL-8F is induced by HCMV (142, 143). The HCMV-US28 chemokine receptor strongly promotes the expression of the NF-kB, COX-2, IL-6, and p-STAT-3 which could initiate oncogenic pathways (144, 145). Upon HCMV infection of human cancer stem cells and in the presence of cmvIL-10, cancer stem cells can induce macrophage reprogramming "M2 phenotype" in the tumor microenvironment hence favoring the appearance of tumor-associated macrophages (TAMs) and enhancing other immunomodulatory, oncogenic, and angiogenic cytokines' expression such as STAT3 and vascular endothelial growth factor (VEGF) (146–148). Similar to US28, the cmvIL-10 chemokine which is known to be expressed in latency phase and tumor cells can enhance cancer cell invasion (149). In addition, HCMV can guarantee neutrophils and mononuclear cells survival, which can support a quick oncogenesis *via* the activation of an angiogenic switch (150, 151). Further, long non-coding RNAs (lncRNAs) were described as efficient players not only in facilitating signal transductions in tumor signaling pathways but also in promoting tumor evasion from immunosurveillance. It has been also shown that immune cells for instance, T-cells, B-cells, dendritic cells, macrophages, and myeloid cells control tumor immune responses *via* lncRNAs linked pathways (152, 153). In CTH cells, HCMV lncRNA4.9 was formerly detected in tumors isolated from xenograft NSG mice injected with CTH cells, as well as in human breast cancer biopsies (137). Moreover, several studies specified that modifications in gut permeability and intestinal microbiota translocation can stimulate chronic inflammation as well as causing auto-immune and neoplastic diseases (154, 155). Knowing that CMV presence was associated with upregulation of various cytokines, elevated epithelial gut damage, microbial translocation, and systemic inflammation (156, 157), it might play a part in eliciting carcinogenesis. All in all, these studies indicate that HCMV can be actively involved in enabling cancer progression and this is through inducing certain pathways that give rise to epigenetic modifications, and promoting the activation of cancer stem cell, angiogenesis, invasion, and an EMT phenotype (136, 158, 159). Certainly, one of the limitations for assessing the effect of HCMV on immunity and cancer progression is that the majority of the investigations were done on the high risk CMV-positive subpopulation which might involve diverse immunomodulation sociodemographic and environmental co-factors other than HCMV status as well as divergent lifestyles and medical history. Therefore, prospective studies are highly required to rule out other immunomodulatory factors and precisely evaluate the impact of CMV on host immunity.

Nonetheless, it is noteworthy that the oncogenic potency of HCMV clinical strains varies between low and high-risk strains. HCMV-DB and HCMV-BL have been classified as high-risk strains in which they possessed their oncogenic potentials in acutely infected human mammary epithelial cells (HMEC) *in vitro* showing sustained transformation. These high-risk strains were characterized by elevated *Myc* expression, PI3K/Akt pathway activation, and *p53* and *Rb* gene repression (138). With regard to immune responses, Myc suppresses immune surveillance by modulating the expression of the innate immune regulator (CD47, also known as integrin-associated protein) and the adaptive immune checkpoint namely, programmed death ligand 1 (PD-L1, also known as CD274 and B7-H1) (160–164). Further, Myc regulates thrombospondin-1 (161) and Type 1 IFN (165, 166). Hence, Myc initiates and maintains tumorigenesis through the modulation of immune regulatory molecules. PI3K/Akt activation induced inflammation and immunosuppression through nitric oxide synthase (NOS) overexpression; thus,

resulting in tumor initiation *via* the activated Notch pathway leading to tumor progression (167). On the other hand, suppression or mutation of p53 has been shown to decrease MHC-I presentation, increase STAT3 phosphorylation, upregulate PD-L1 *via* microRNA (miR34), elevate pro-inflammatory chemokine and cytokine production, and indirectly upregulate the expression of chemokine receptors (CXCR4 and CXCR5) (160, 168, 169). Loss of Rb leads to the increase in CCL2 and IL6 secretion and this is because of the elevated fatty acid oxidation (FAO) activity and enhanced mitochondrial superoxide (MS) production (170). Indeed, those molecular alterations have been linked to immune suppression in the tumor microenvironment indicating that only high-risk HCMV strains possessing oncomodulatory properties are potentially involved in the oncogenesis process as described previously (84, 138) (**Figure 2**). In line with the previously presented epidemiological studies, and since HCMV was confined within tumors correlating positively to poor prognosis, as well as the potential of HCMV gene products in regulating tumorigenic pathways and processes linked to cancer hallmarks, and finally the HCMV broad tissue tropism, we infer that HCMV possesses distinctive mechanisms in cancer progression (26, 29, 171).

THERAPEUTIC APPROACHES IN HIGH-RISK POPULATIONS

The fact that HCMV is highly prevalent in different cancer forms, opens up the possibility to manage such cancers with anti-HCMV medications. Currently, two major approaches are being chased; the first emphases on antiviral therapy while the other targets HCMV directed immunotherapy. The core approach to antiviral therapy involves the use of valganciclovir. The rationale behind using valganciclovir is suppressing HCMV replication in HCMV-positive glioblastoma (GBM) cells leading to the repression of virus-mediated tumor-promoting strategies. Despite its viral replication suppression, valganciclovir doesn't eradicate the virus. Thus, short-term valganciclovir treatment wouldn't be ideal for treating glioma patients, necessitating long-term treatment to maintain the tumor suppressive properties (116). Interestingly, valganciclovir treatment outcome was improved in combination with celecoxib (COX-2 specific inhibitor). Since glioblastoma, medulloblastoma, and neuroblastoma tumors show high expression of cyclooxygenase-2 (COX-2) and nonsteroidal anti-inflammatory drugs (NSAIDs) levels, COX-2 and PGE2 inhibitors possess a profound effect on tumor growth. The two inhibitors

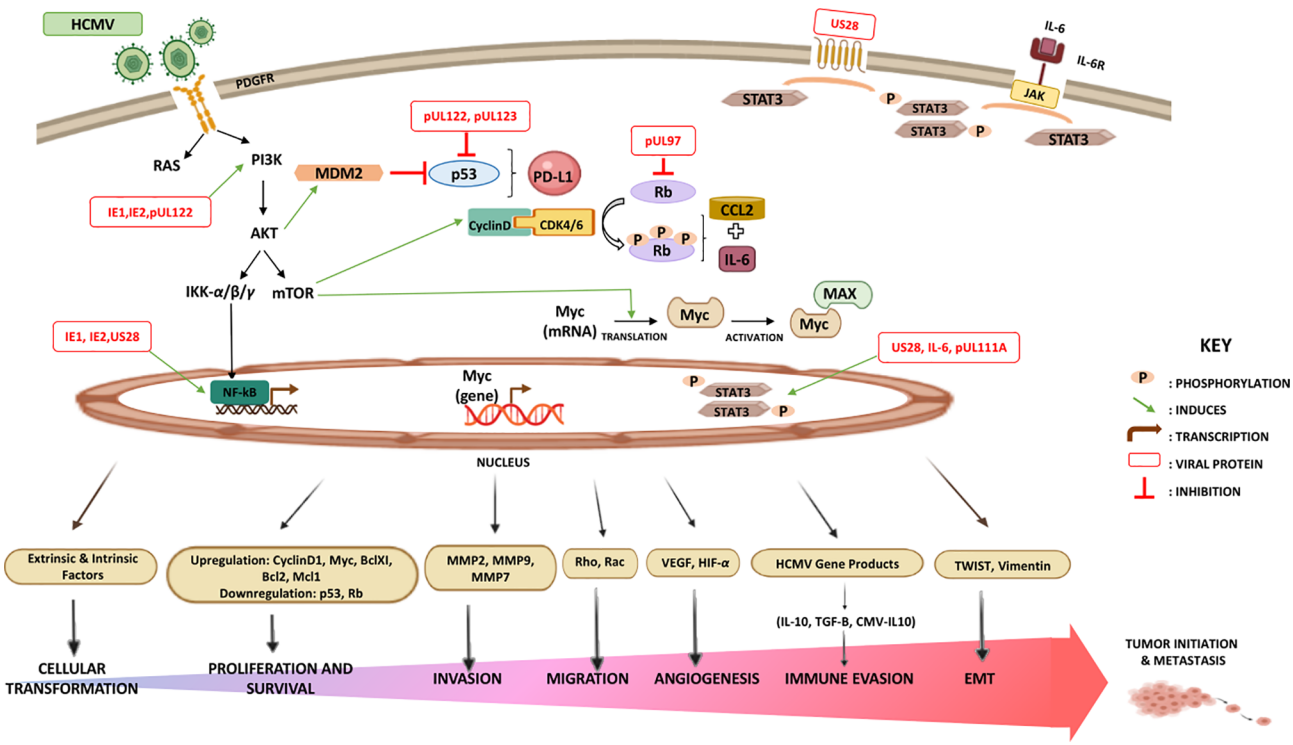

FIGURE 2 | Major signaling pathways stimulated by HCMV that modulate the immune landscape. HCMV-infected cells produce elevated levels of interleukin-6 (IL-6) that activates signal transduction *via* IL-6 receptor (IL6R)-STAT3 axis. US28, an active chemokine receptor, also plays a major role in activating STAT3 in cancer cells. The combination of STAT3 activation and the impact of HCMV on cancer cell apoptosis, invasion, migration, adhesion, angiogenesis, and immunogenicity significantly exacerbates malignancy. In contrast to p53 and Rb, the upregulation of Akt, Myc, PD-L1, and CCL2 strongly exerts immunosuppressive and oncomodulatory effects. HCMV-induced alterations in the TME may contribute to oncomodulation.

competently block HCMV replication and limiting the US28-expressing tumor cell growth. Therefore, the significant effects behind the use of aspirin and other NSAIDs in tumor prevention investigations could be somewhat due to the suppression of HCMV replication in pre-malignant lesions (80). The existing link between CMV and cancer creates a new avenue for immunotherapeutic strategies that target CMV such as, adoptive T-cell transfer and vaccine approaches (172). During adoptive lymphocyte transfer (ALT), autologous T-cells are expanded and activated *ex vivo* against the tumor. After that, they are transferred into patients where lymphodepletion stimulates a substantial proliferation of T-cells and intensifies tumor-specific immunity (173, 174). There exist various ongoing clinical studies assessing the effectiveness of adoptive T-cell therapy using HCMV-specific T-cells, or DCs with CMV-pp65 RNA in order to vaccinate GBM patients. CMV-specific T-cells, especially pp65-specific T-cells, favorably infiltrate glioblastoma tumors and were able to stimulate glioblastoma cells' killing (173). The fact that a high percentage of GMB samples were HCMV-positive has led to potential immunotherapy targets for GBM treatment. HCMV-specific proteins (IE1, pp65, and gB) are being investigated for the development of immunotherapy targets (116, 174). Interestingly, a study showed that CMV-stimulated NK cells and γδ-T-cells might have antineoplastic potential and CMV reactivation has been associated with minimal risk for relapsed leukemia in hematopoietic stem-cell transplantation (HSCT) patients (173). Since oncolytic virotherapy has been recognized as a promising approach for treating cancers in recent years, the use of "oncolytic CMV therapy" in combination with anti-tumor medications, immune checkpoint inhibitors (targeting CTLA4 and PD-L1), epigenetic therapeutics, or as "HCMV/HSV-1 oncolytic virus" could be regarded as one of the most intriguing antitumor approaches (175).

Major approaches used to target Myc are mostly targeting *Myc* gene transcription (JQ1, AZD5153, GSK525762, dBET1, THZ1, Roscovitine, Flavopiridol, QN-1, and APTO-253), inhibiting Myc mRNA translation (MLN0128, Silvestrol, eFT226, BTYNB, Rapamycin, MK2206, BEZ235), targeting Myc oncoprotein stability (Volasertib, TD19, P22077, MLN8237, BI 6727, and BI 2536), controlling Myc-Max interactions (Omomyc and MYCMI-6), and blocking Myc's accessibility to other genomic targets (Sulfopin) (176–178). Further, bromodomain and extraterminal protein (BET) inhibitor, JQ1, decreased expression of PD-L1 and CD47 resulting in the recruitment of T-cells. Hence, drugs targeting Myc-associated pathways may be used to modify the expression of immune checkpoints (179). Furthermore, the PI3K/AKT pathway is activated in cancer; thus, identifying AKT inhibitors that can block PI3K/AKT signaling could attenuate tumor growth and recover immune responses. AKT inhibitors are classified in Synthetic (MK-2206, AZD5363, GDC-0068, Perifosine) and natural AKT inhibitors (Resveratrol or grape powder, Ginger root extract, Sulforaphane) (180, 181). Few drugs in clinical use or preclinical assessment have been verified to directly or indirectly target PI3K signaling such as BEZ235, Ly294002, Rapamycin, CCI779, PX-866, SF1124, PX316, Miltefosine, and Perifosine (178, 181). Reactivating tumor suppressors is a substantial pharmacological challenge; restoring p-53 activity stimulated innate immunity particularly DC activation, and it also promoted adaptive immunity. Nutlin-3a, mouse double minute 2 homolog (MDM2) inhibitor, induces local p53 activation in the TME resulting in MDM2-mediated tumor cell apoptosis even in the presence of a sustained Notch activity (168, 182, 183). Re-expression of p53 was stimulated by Tamoxifen injections causing massive apoptosis (179). T-cell responses were driven by using p53 vaccines (ALVAC-p53 and MVAp53) or synthetic long peptides of p53 (169). Moreover, the highly selective cyclin-dependent kinases 4/6 (CDK4/6) inhibitors (Palbociclib, Ribociclib, and Abemaciclib) were proved to avert RB phosphorylation thus regulating MHC presentation, IFN-γ response, and IL-6 signaling (184–186). Carlumab, a human IgG1 monoclonal antibody, inhibited CCL2 and it consequently showed promising effects in both solid tumors and metastatic resistant prostate cancer. A distinct approach to CCL2/CCR2 interference, was hindering CCR2 using MLN1202 in bone metastasis (187). It has been shown that CCL2 knockout prompted marked suppression of TAMs-associated inflammatory cytokines (188); in addition, CCL2-CCR2 blockade exhibited tumor-suppressive function by blocking inflammatory monocyte recruitment within the tumor (170) (**Figure 3**).

Additionally, because of HCMV's ability to establish latency and reactivate, CMV vaccines are presently being developed for clinical use. To prevent HCMV infection in tumor-independent settings, the development of an effective HCMV vaccine has been investigated despite being a struggle for a couple of years. Few have already granted the approval to a phase III clinical trial thus possessing promising outcomes (189). The investigated anti-CMV vaccine types include the live-attenuated (phase 2), recombinant subunit (phase 2), virus vectored phase (1, 2), chimeric peptidic (phase 2), enveloped virus-like particles (phase 1), plasmid-based (phase 3), and mRNA (phase 2) vaccines (190). mRNA-1647, a CMV vaccine covering six mRNAs that encodes pentamer and gB protein, is designed for CMV prophylaxis; a phase 3 study will be initiated to assess the prevention of primary CMV infection in women of childbearing age (ClinicalTrials.gov Identifier: NCT04232280). Recent data showed that HCMV could perhaps induce transformation by enhancing the expression of viral genes (for example, *UL69* gene). The presence of *UL69* gene in CTH cell cultures and UL69 DNA in the majority of breast cancer biopsies indicates a potential significance of *UL69* as a target in developing HCMV-vaccine (191). The usage of HCMV vaccines for the treatment of cancer patients generally and GBM patients in specific might be of high therapeutic value especially that HCMV has been shown to express oncomodulatory functions. Letermovir, an FDA approved novel terminase inhibitor, is currently used for CMV prophylaxis as it selectively compromises CMV replication. It's characterized by its high potency compared to ganciclovir and limited toxicity profile (192). There exists an ongoing phase 2 clinical trial that aim to assess the anti-inflammatory potential of letermovir in adults with HIV and asymptomatic CMV being on

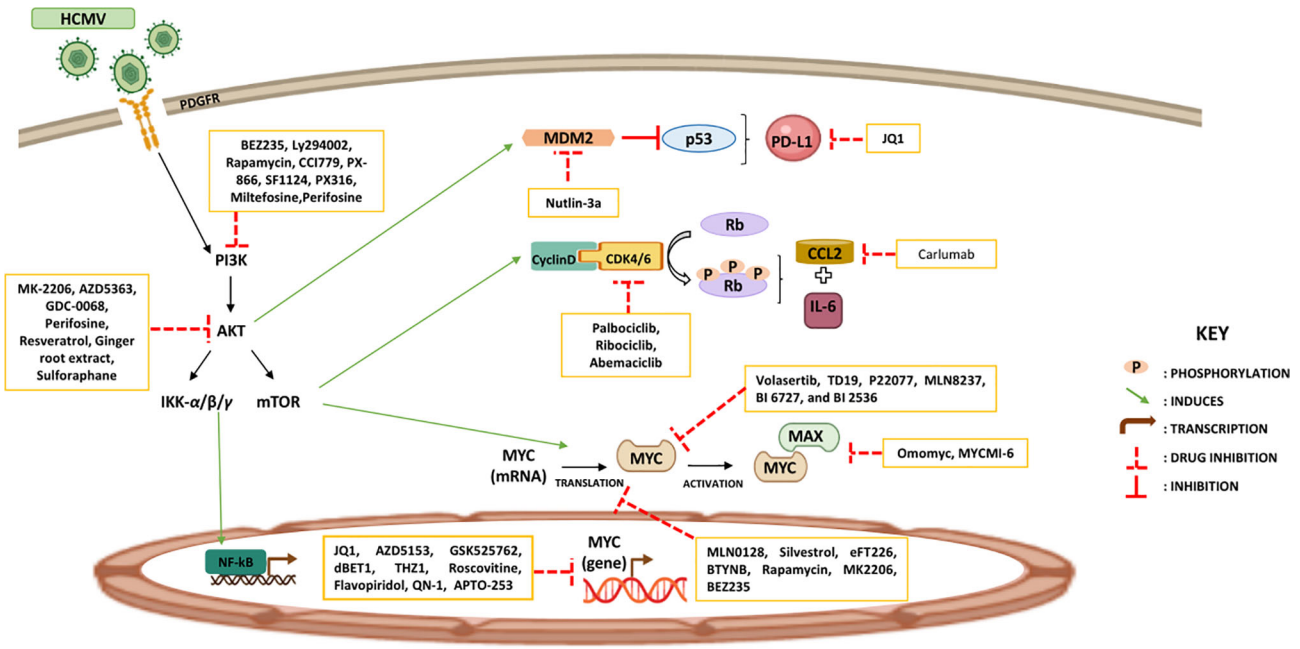

FIGURE 3 | Potential therapeutic approaches reinstating tumor immunity. Certain therapeutic strategies are used to increase tumor suppressor proteins and reduce oncoproteins' expression with the aim of restoring the immune response against tumors.

antiretroviral therapy-mediated suppression (ClinicalTrials.gov, Identifier: NCT04840199). Further investigations will be a welcome addition to evaluate the use of letermovir in averting CMV recurrence and treatment as well as to reverse letermovir resistance.

CONCLUSION

Overall, HCMV-induced amplification of immune evasion mechanisms mediates oncomodulation and enables tumors to further escape immune surveillance and develop immune tolerance favoring other malignant phenotypes. HCMV, infecting many cell types, induces a pro-inflammatory environment and conquers specific immune responses thus creating an immunosuppressive TME. Nevertheless, getting to know viral immune evasion mechanisms will aid in understanding aspects of cellular as well as immunological function, and contribute to the enhancement of immunotherapies' outcome and antiviral agents eliminating the virus from tumor tissues which could improve patient's immune responses and suppress tumor progression. Taking into consideration the profound effects of HCMV on the quality of life, there remain further experimental studies to be performed in order to design effective interventions including vaccines or other approaches that reinforce immune homeostasis and maintain the adapted immune response to aging.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Cox M, Kartikasari AER, Gorry PR, Flanagan KL, Plebanski M. Potential Impact of Human Cytomegalovirus Infection on Immunity to Ovarian Tumours and Cancer Progression. *Biomedicines* (2021) 9:351. doi: 10.3390/biomedicines9040351
2. Klenerman P, Oxenius A. T Cell Responses to Cytomegalovirus. *Nat Rev Immunol* (2016) 16:367–77. doi: 10.1038/nri.2016.38
3. Sansoni P, Vescovini R, Fagnoni FF, Akbar A, Arens R, Chiu Y-L, et al. New Advances in CMV and Immunosenescence. *Exp Gerontol* (2014) 55:54–62. doi: 10.1016/j.exger.2014.03.020
4. Foster H, Ulasov IV, Cobbs CS. Human Cytomegalovirus-Mediated Immunomodulation: Effects on Glioblastoma Progression. *Biochim Biophys Acta (BBA) - Rev Cancer* (2017) 1868:273–6. doi: 10.1016/j.bbcan.2017.05.006
5. Jackson SE, Mason GM, Wills MR. Human Cytomegalovirus Immunity and Immune Evasion. *Virus Res* (2011) 157:151–60. doi: 10.1016/j.virusres.2010.10.031

6. Lepiller Q, Aziz Khan K, Di Martino V, Herbein G. Cytomegalovirus and Tumors: Two Players for One Goal-Immune Escape. *TOVJ* (2011) 5:60–9. doi: 10.2174/1874357901105010060
7. Huygens A, Dauby N, Vermijlen D, Marchant A. Immunity to Cytomegalovirus in Early Life. *Front Immunol* (2014) 5:552. doi: 10.3389/fimmu.2014.00552
8. La Rosa C, Diamond DJ. The Immune Response to Human CMV. *Future Virol* (2012) 7:279–93. doi: 10.2217/fvl.12.8
9. Stagno S, Pass RF, Dworsky ME, Henderson RE, Moore EG, Walton PD, et al. Congenital Cytomegalovirus Infection: The Relative Importance of Primary and Recurrent Maternal Infection. *N Engl J Med* (1982) 306:945–9. doi: 10.1056/NEJM198204223061601
10. Dollard SC, Grosse SD, Ross DS. New Estimates of the Prevalence of Neurological and Sensory Sequelae and Mortality Associated With Congenital Cytomegalovirus Infection. *Rev Med Virol* (2007) 17:355–63. doi: 10.1002/rmv.544
11. Lanzieri TM, Dollard SC, Josephson CD, Schmid DS, Bialek SR. Breast Milk-Acquired Cytomegalovirus Infection and Disease in VLBW and Premature Infants. *PEDIATRICS* (2013) 131:e1937–45. doi: 10.1542/peds.2013-0076
12. Aiello A, Accardi G, Candore G, Caruso C, Colomba C, Di Bona D, et al. Role of Immunogenetics in the Outcome of HCMV Infection: Implications for Ageing. *IJMS* (2019) 20:685. doi: 10.3390/ijms20030685
13. Solana R, Tarazona R, Aiello AE, Akbar AN, Appay V, Beswick M, et al. CMV and Immunosenescence: From Basics to Clinics. *Immun Ageing* (2012) 9:23. doi: 10.1186/1742-4933-9-23
14. Pawelec G, Derhovanessian E. Role of CMV in Immune Senescence. *Virus Res* (2011) 157:175–9. doi: 10.1016/j.virusres.2010.09.010
15. Jergović M, Contreras NA, Nikolich-Žugich J. Impact of CMV Upon Immune Aging: Facts and Fiction. *Med Microbiol Immunol* (2019) 208:263–9. doi: 10.1007/s00430-019-00605-w
16. Moniz P, Brito S, Póvoa P. SARS-CoV-2 and Cytomegalovirus Co-Infections—A Case Series of Critically Ill Patients. *JCM* (2021) 10:2792. doi: 10.3390/jcm10132792
17. Le Balc'h P, Pinceaux K, Pronier C, Seguin P, Tadié J-M, Reizine F. Herpes Simplex Virus and Cytomegalovirus Reactivations Among Severe COVID-19 Patients. *Crit Care* (2020) 24:530. doi: 10.1186/s13054-020-03252-3
18. Söderberg-Nauclér C. Does Reactivation of Cytomegalovirus Contribute to Severe COVID-19 Disease? *Immun Ageing* (2021) 18:12. doi: 10.1186/s12979-021-00218-z
19. Xie L, Yi K, Li Y. SARS_CoV2 RBD Gene Transcription Cannot be Driven by CMV Promoter. *Virology* (2021) 558:22–7. doi: 10.1016/j.virol.2021.02.010
20. Wilski NA, Snyder CM. From Vaccine Vector to Oncomodulation: Understanding the Complex Interplay Between CMV and Cancer. *Vaccines* (2019) 7:62. doi: 10.3390/vaccines7030062
21. Aiello AE, Chiu Y-L, Frasca D. How Does Cytomegalovirus Factor Into Diseases of Aging and Vaccine Responses, and by What Mechanisms? *GeroScience* (2017) 39:261–71. doi: 10.1007/s11357-017-9983-9
22. Royston L, Isnard S, Lin J, Routy J-P. Cytomegalovirus as an Uninvited Guest in the Response to Vaccines in People Living With HIV. *Viruses* (2021) 13:1266. doi: 10.3390/v13071266
23. Poloni C, Szyf M, Cheishvili D, Tsoukas CM. Are the Healthy Vulnerable? Cytomegalovirus Seropositivity in Healthy Adults is Associated With Accelerated Epigenetic Age and Immune-Dysregulation. *J Infect Dis* (2021), jiab365. doi: 10.1093/infdis/jiab365
24. Soderberg-Naucler C. Does Cytomegalovirus Play a Causative Role in the Development of Various Inflammatory Diseases and Cancer? *J Intern Med* (2006) 259:219–46. doi: 10.1111/j.1365-2796.2006.01618.x
25. Cinatl J, Scholz M, Doerr HW. Role of Tumor Cell Immune Escape Mechanisms in Cytomegalovirus-Mediated Oncomodulation. *Med Res Rev* (2005) 25:167–85. doi: 10.1002/med.20018
26. Nauclér CS, Geisler J, Vetvik K. The Emerging Role of Human Cytomegalovirus Infection in Human Carcinogenesis: A Review of Current Evidence and Potential Therapeutic Implications. *Oncotarget* (2019) 10:4333–47. doi: 10.18632/oncotarget.27016
27. Shen Y, Zhu H, Shenk T. Human Cytomegalovirus IE1 and IE2 Proteins are Mutagenic and Mediate "Hit-and-Run" Oncogenic Transformation in Cooperation With the Adenovirus E1A Proteins. *Proc Natl Acad Sci* (1997) 94:3341–5. doi: 10.1073/pnas.94.7.3341
28. Soroceanu L, Cobbs CS. Is HCMV a Tumor Promoter? *Virus Res* (2011) 157:193–203. doi: 10.1016/j.virusres.2010.10.026
29. Geisler J, Touma J, Rahbar A, Söderberg-Nauclér C, Vetvik K. A Review of the Potential Role of Human Cytomegalovirus (HCMV) Infections in Breast Cancer Carcinogenesis and Abnormal Immunity. *Cancers* (2019) 11:1842. doi: 10.3390/cancers11121842
30. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, et al. Genetic Content of Wild-Type Human Cytomegalovirus. *J Gen Virol* (2004) 85:1301–12. doi: 10.1099/vir.0.79888-0
31. Cook CH. Cytomegalovirus Reactivation in "Immunocompetent" Patients: A Call for Scientific Prophylaxis. *J Infect Dis* (2007) 196:1273–5. doi: 10.1086/522433
32. Gerna G, Sarasini A, Patrone M, Percivalle E, Fiorina L, Campanini G, et al. Human Cytomegalovirus Serum Neutralizing Antibodies Block Virus Infection of Endothelial/Epithelial Cells, But Not Fibroblasts, Early During Primary Infection. *J Gen Virol* (2008) 89:853–65. doi: 10.1099/vir.0.83523-0
33. Revello MG, Zavattoni M, Furione M, Fabbri E, Gerna G. Preconceptional Primary Human Cytomegalovirus Infection and Risk of Congenital Infection. *J Infect Dis* (2006) 193:783–7. doi: 10.1086/500509
34. Scrivano L, Sinzger C, Nitschko H, Koszinowski UH, Adler B. HCMV Spread and Cell Tropism are Determined by Distinct Virus Populations. *PloS Pathog* (2011) 7:e1001256. doi: 10.1371/journal.ppat.1001256
35. Cheung AKL, Abendroth A, Cunningham AL, Slobedman B. Viral Gene Expression During the Establishment of Human Cytomegalovirus Latent Infection in Myeloid Progenitor Cells. *Blood* (2006) 108:3691–9. doi: 10.1182/blood-2005-12-026682
36. Sinzger C, Digel M, Jahn G. Cytomegalovirus Cell Tropism. In: TE Shenk and MF Stinski, editors. *Human Cytomegalovirus Current Topics in Microbiology and Immunology*. Berlin, Heidelberg: Springer Berlin Heidelberg. (2008). p. 63–83. doi: 10.1007/978-3-540-77349-8_4
37. Crough T, Khanna R. Immunobiology of Human Cytomegalovirus: From Bench to Bedside. *Clin Microbiol Rev* (2009) 22:76–98. doi: 10.1128/CMR.00034-08
38. Khairallah C, Déchanet-Merville J, Capone M. γδ T Cell-Mediated Immunity to Cytomegalovirus Infection. *Front Immunol* (2017) 8:105. doi: 10.3389/fimmu.2017.00105
39. Compton T, Kurt-Jones EA, Boehme KW, Belko J, Latz E, Golenbock DT, et al. Human Cytomegalovirus Activates Inflammatory Cytokine Responses *via* CD14 and Toll-Like Receptor 2. *J Virol* (2003) 77:4588–96. doi: 10.1128/JVI.77.8.4588-4596.2003
40. Boehme KW, Guerrero M, Compton T. Human Cytomegalovirus Envelope Glycoproteins B and H Are Necessary for TLR2 Activation in Permissive Cells. *J Immunol* (2006) 177:7094–102. doi: 10.4049/jimmunol.177.10.7094
41. Iversen A-C, Norris PS, Ware CF, Benedict CA. Human NK Cells Inhibit Cytomegalovirus Replication Through a Noncytolytic Mechanism Involving Lymphotoxin-Dependent Induction of IFN-β. *J Immunol* (2005) 175:7568–74. doi: 10.4049/jimmunol.175.11.7568
42. Wu Z, Sinzger C, Reichel JJ, Just M, Mertens T. Natural Killer Cells Can Inhibit the Transmission of Human Cytomegalovirus in Cell Culture by Using Mechanisms From Innate and Adaptive Immune Responses. *J Virol* (2015) 89:2906–17. doi: 10.1128/JVI.03489-14
43. Moretta A, Marcenaro E, Parolini S, Ferlazzo G, Moretta L. NK Cells at the Interface Between Innate and Adaptive Immunity. *Cell Death Differ* (2008) 15:226–33. doi: 10.1038/sj.cdd.4402170
44. Picarda G, Benedict CA. Cytomegalovirus: Shape-Shifting the Immune System. *JI* (2018) 200:3881–9. doi: 10.4049/jimmunol.1800171
45. Manandhar, Hò, Pump, Blasczyk, Bade-Doeding. Battle Between Host Immune Cellular Responses and HCMV Immune Evasion. *IJMS* (2019) 20:3626. doi: 10.3390/ijms20153626
46. Lim EY, Jackson SE, Wills MR. The CD4+ T Cell Response to Human Cytomegalovirus in Healthy and Immunocompromised People. *Front Cell Infect Microbiol* (2020) 10:202. doi: 10.3389/fcimb.2020.00202
47. Jackson SE, Sedikides GX, Mason GM, Okecha G, Wills MR. Human Cytomegalovirus (HCMV)-Specific CD4+ T Cells Are Polyfunctional and Can Respond to HCMV-Infected Dendritic Cells *In Vitro*. *J Virol* (2017) 91:e02128–16. doi: 10.1128/JVI.02128-16

48. Manley TJ, Luy L, Jones T, Boeckh M, Mutimer H, Riddell SR. Immune Evasion Proteins of Human Cytomegalovirus do Not Prevent a Diverse CD8+ Cytotoxic T-Cell Response in Natural Infection. *Blood* (2004) 104:1075–82. doi: 10.1182/blood-2003-06-1937
49. Hegde NR, Dunn C, Lewinsohn DM, Jarvis MA, Nelson JA, Johnson DC. Endogenous Human Cytomegalovirus gB is Presented Efficiently by MHC Class II Molecules to CD4+ CTL. *J Exp Med* (2005) 202:1109–19. doi: 10.1084/jem.20050162
50. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly Targeted Human Cytomegalovirus-Specific CD4+ and CD8+ T Cells Dominate the Memory Compartments of Exposed Subjects. *J Exp Med* (2005) 202:673–85. doi: 10.1084/jem.20050882
51. Northfield J, Lucas M, Jones H, Young NT, Klenerman P. Does Memory Improve With Age? CD85j (ILT-2/LIR-1) Expression on CD8+ T Cells Correlates With 'Memory Inflation' in Human Cytomegalovirus Infection. *Immunol Cell Biol* (2005) 83:182–8. doi: 10.1111/j.1440-1711.2005.01321.x
52. Pawelec G, Derhovanessian E, Larbi A. Immunosenescence and Cancer. *J Geriatric Oncol* (2010) 1:20–6. doi: 10.1016/j.jgo.2010.04.002
53. Poizot-Martin I, Allavena C, Duvivier C, Cano CE, Guillouet de Salvador F, Rey D, et al. CMV+ Serostatus Associates Negatively With CD4:CD8 Ratio Normalization in Controlled HIV-Infected Patients on cART. *PloS One* (2016) 11:e0165774. doi: 10.1371/journal.pone.0165774
54. Ballegaard V, Brændstrup P, Pedersen KK, Kirkby N, Stryhn A, Ryder LP, et al. Cytomegalovirus-Specific T-Cells are Associated With Immune Senescence, But Not With Systemic Inflammation, in People Living With HIV. *Sci Rep* (2018) 8:3778. doi: 10.1038/s41598-018-21347-4
55. Olsson J, Wikby A, Johansson B, Löfgren S, Nilsson B-O, Ferguson FG. Age-Related Change in Peripheral Blood T-Lymphocyte Subpopulations and Cytomegalovirus Infection in the Very Old: The Swedish Longitudinal OCTO Immune Study. *Mech Ageing Dev* (2001) 121:187–201. doi: 10.1016/S0047-6374(00)00210-4
56. Terrazzini N, Kern F. Cell-Mediated Immunity to Human CMV Infection: A Brief Overview. *F1000Prime Rep* (2014) 6. doi: 10.12703/P6-28
57. Bughio F, Elliott DA, Goodrum F. An Endothelial Cell-Specific Requirement for the UL133-UL138 Locus of Human Cytomegalovirus for Efficient Virus Maturation. *J Virol* (2013) 87:3062–75. doi: 10.1128/JVI.02510-12
58. Hewitt EW. The MHC Class I Antigen Presentation Pathway: Strategies for Viral Immune Evasion. *Immunology* (2003) 110:163–9. doi: 10.1046/j.1365-2567.2003.01738.x
59. Gilbert MJ, Riddell SR, Li CR, Greenberg PD. Selective Interference With Class I Major Histocompatibility Complex Presentation of the Major Immediate-Early Protein Following Infection With Human Cytomegalovirus. *J Virol* (1993) 67:3461–9. doi: 10.1128/jvi.67.6.3461-3469.1993
60. Gilbert MJ, Riddell SR, Plachter B, Greenberg PD. Cytomegalovirus Selectively Blocks Antigen Processing and Presentation of its Immediate–Early Gene Product. *Nature* (1996) 383:720–2. doi: 10.1038/383720a0
61. Vossen M, Westerhout E, Söderberg-Nauclér C, Wiertz E. Viral Immune Evasion: A Masterpiece of Evolution. *Immunogenetics* (2002) 54:527–42. doi: 10.1007/s00251-002-0493-1
62. Patel M, Vlahava V-M, Forbes SK, Fielding CA, Stanton RJ, Wang ECY. HCMV-Encoded NK Modulators: Lessons From *In Vitro* and *In Vivo* Genetic Variation. *Front Immunol* (2018) 9:2214. doi: 10.3389/fimmu.2018.02214
63. Sun J, Lanier L. The Natural Selection of Herpesviruses and Virus-Specific NK Cell Receptors. *Viruses* (2009) 1:362–82. doi: 10.3390/v1030362
64. Ulbrecht M, Martinozzi S, Grzeschik M, Hengel H, Ellwart JW, Pla M, et al. Cutting Edge: The Human Cytomegalovirus *UL40* Gene Product Contains a Ligand for HLA-E and Prevents NK Cell-Mediated Lysis. . *J Immunol* (2000) 164:5019–22. doi: 10.4049/jimmunol.164.10.5019
65. Tomasec P. Surface Expression of HLA-E, an Inhibitor of Natural Killer Cells, Enhanced by Human Cytomegalovirus Gpul40. *Science* (2000) 287:1031–3. doi: 10.1126/science.287.5455.1031
66. Spencer JV, Lockridge KM, Barry PA, Lin G, Tsang M, Penfold MET, et al. Potent Immunosuppressive Activities of Cytomegalovirus- Encoded Interleukin-10. *J Virol* (2002) 76:1285–92. doi: 10.1128/JVI.76.3.1285-1292.2002
67. Fielding CA, Aicheler R, Stanton RJ, Wang ECY, Han S, Seirafian S, et al. Two Novel Human Cytomegalovirus NK Cell Evasion Functions Target MICA for Lysosomal Degradation. *PloS Pathog* (2014) 10:e1004058. doi: 10.1371/journal.ppat.1004058
68. Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M, et al. Host Immune System Gene Targeting by a Viral miRNA. *Science* (2007) 317:376–81. doi: 10.1126/science.1140956
69. Weller M, Malipiero U, Aguzzi A, Reed JC, Fontana A. Protooncogene Bcl-2 Gene Transfer Abrogates Fas/APO-1 Antibody-Mediated Apoptosis of Human Malignant Glioma Cells and Confers Resistance to Chemotherapeutic Drugs and Therapeutic Irradiation. *J Clin Invest* (1995) 95:2633–43. doi: 10.1172/JCI117965
70. Chiou S-H, Yang Y-P, Lin J-C, Hsu C-H, Jhang H-C, Yang Y-T, et al. The Immediate Early 2 Protein of Human Cytomegalovirus (HCMV) Mediates the Apoptotic Control in HCMV Retinitis Through Up-Regulation of the Cellular FLICE-Inhibitory Protein Expression. *J Immunol* (2006) 177:6199–206. doi: 10.4049/jimmunol.177.9.6199
71. Powers C, DeFilippis V, Malouli D, Früh K. Cytomegalovirus Immune Evasion. In: TE Shenk and MF Stinski, editors. *Human Cytomegalovirus Current Topics in Microbiology and Immunology*. Berlin, Heidelberg: Springer Berlin Heidelberg (2008). p. 333–59. doi: 10.1007/978-3-540-77349-8_19
72. Spiller OB, Morgan BP, Tufaro F, Devine DV. Altered Expression of Host-Encoded Complement Regulators on Human Cytomegalovirus-Infected Cells. *Eur J Immunol* (1996) 26:1532–8. doi: 10.1002/eji.1830260719
73. Margulies BJ, Browne H, Gibson W. Identification of the Human Cytomegalovirus G Protein-Coupled Receptor Homologue Encoded by UL33 in Infected Cells and Enveloped Virus Particles. *Virology* (1996) 225:111–25. doi: 10.1006/viro.1996.0579
74. Slinger E, Maussang D, Schreiber A, Siderius M, Rahbar A, Fraile-Ramos A, et al. HCMV-Encoded Chemokine Receptor US28 Mediates Proliferative Signaling Through the IL-6-STAT3 Axis. *Sci Signaling* (2010) 3:ra58–8. doi: 10.1126/scisignal.2001180
75. Jackson SE, Redeker A, Arens R, van Baarle D, van den Berg SPH, Benedict CA, et al. CMV Immune Evasion and Manipulation of the Immune System With Aging. *GeroScience* (2017) 39:273–91. doi: 10.1007/s11357-017-9986-6
76. Beisser PS, Laurent L, Virelizier J-L, Michelson S. Human Cytomegalovirus Chemokine Receptor Gene US28 Is Transcribed in Latently Infected THP-1 Monocytes. *J Virol* (2001) 75:5949–57. doi: 10.1128/JVI.75.13.5949-5957.2001
77. Michelson S, Alcami J, Kim SJ, Danielpour D, Bachelerie F, Picard L, et al. Human Cytomegalovirus Infection Induces Transcription and Secretion of Transforming Growth Factor Beta 1. *J Virol* (1994) 68:5730–7. doi: 10.1128/jvi.68.9.5730-5737.1994
78. Hansen SG, Powers CJ, Richards R, Ventura AB, Ford JC, Siess D, et al. Evasion of CD8+ T Cells Is Critical for Superinfection by Cytomegalovirus. *Science* (2010) 328:102–6. doi: 10.1126/science.1185350
79. Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral Subversion of the Immune System. *Annu Rev Immunol* (2000) 18:861–926. doi: 10.1146/annurev.immunol.18.1.861
80. Johnsen JI, Baryawno N, Söderberg-Nauclér C. Is Human Cytomegalovirus a Target in Cancer Therapy? *Oncotarget* (2011) 2:1329–38. doi: 10.18632/oncotarget.383
81. Charpak-Amikam Y, Kubsch T, Seidel E, Oiknine-Djian E, Cavaletto N, Yamin R, et al. Human Cytomegalovirus Escapes Immune Recognition by NK Cells Through the Downregulation of B7-H6 by the Viral Genes US18 and US20. *Sci Rep* (2017) 7:8661. doi: 10.1038/s41598-017-08866-2
82. Patro ARK. Subversion of Immune Response by Human Cytomegalovirus. *Front Immunol* (2019) 10:1155. doi: 10.3389/fimmu.2019.01155
83. McSharry B, Avdic S, Slobedman B. Human Cytomegalovirus Encoded Homologs of Cytokines, Chemokines and Their Receptors: Roles in Immunomodulation. *Viruses* (2012) 4:2448–70. doi: 10.3390/v4112448
84. Herbein G. The Human Cytomegalovirus, From Oncomodulation to Oncogenesis. *Viruses* (2018) 10:408. doi: 10.3390/v10080408
85. Ye L, Qian Y, Yu W, Guo G, Wang H, Xue X. Functional Profile of Human Cytomegalovirus Genes and Their Associated Diseases: A Review. *Front Microbiol* (2020) 11:2104. doi: 10.3389/fmicb.2020.02104
86. Rossini G, Cerboni C, Santoni A, Landini MP, Landolfo S, Gatti D, et al. Interplay Between Human Cytomegalovirus and Intrinsic/Innate Host

Responses: A Complex Bidirectional Relationship. *Mediators Inflamm* (2012) 2012:1–16. doi: 10.1155/2012/607276
87. Fu Y-Z, Su S, Gao Y-Q, Wang P-P, Huang Z-F, Hu M-M, et al. Human Cytomegalovirus Tegument Protein UL82 Inhibits STING-Mediated Signaling to Evade Antiviral Immunity. *Cell Host Microbe* (2017) 21:231–43. doi: 10.1016/j.chom.2017.01.001
88. Kalejta RF, Shenk T. The Human Cytomegalovirus UL82 Gene Product (Pp71) Accelerates Progression Through the G 1 Phase of the Cell Cycle. *J Virol* (2003) 77:3451–9. doi: 10.1128/JVI.77.6.3451-3459.2003
89. Cheung AKL, Gottlieb DJ, Plachter B, Pepperl-Klindworth S, Avdic S, Cunningham AL, et al. The Role of the Human Cytomegalovirus UL111A Gene in Down-Regulating CD4+ T-Cell Recognition of Latently Infected Cells: Implications for Virus Elimination During Latency. *Blood* (2009) 114:4128–37. doi: 10.1182/blood-2008-12-197111
90. Ashiru O, Bennett NJ, Boyle LH, Thomas M, Trowsdale J, Wills MR. NKG2D Ligand MICA Is Retained in the Cis-Golgi Apparatus by Human Cytomegalovirus Protein Ul142. *J Virol* (2009) 83:12345–54. doi: 10.1128/JVI.01175-09
91. McCormick AL, Roback L, Livingston-Rosanoff D, St. Clair C. The Human Cytomegalovirus UL36 Gene Controls Caspase-Dependent and -Independent Cell Death Programs Activated by Infection of Monocytes Differentiating to Macrophages. *J Virol* (2010) 84:5108–23. doi: 10.1128/JVI.01345-09
92. Skaletskaya A, Bartle LM, Chittenden T, McCormick AL, Mocarski ES. Goldmacher VS. A Cytomegalovirus-Encoded Inhibitor of Apoptosis That Suppresses Caspase-8 Activation. *Proc Natl Acad Sci* (2001) 98:7829–34. doi: 10.1073/pnas.141108798
93. Costa H, Nascimento R, Sinclair J, Parkhouse RME. Human Cytomegalovirus Gene UL76 Induces IL-8 Expression Through Activation of the DNA Damage Response. *PloS Pathog* (2013) 9: e1003609. doi: 10.1371/journal.ppat.1003609
94. Prichard MN, Britt WJ, Daily SL, Hartline CB, Kern ER. Human Cytomegalovirus UL97 Kinase Is Required for the Normal Intranuclear Distribution of Pp65 and Virion Morphogenesis. *J Virol* (2005) 79:15494–502. doi: 10.1128/JVI.79.24.15494-15502.2005
95. Benedict CA. A CMV Vaccine: TREATing Despite the TRICKs. *Expert Rev Vaccines* (2013) 12:1235–7. doi: 10.1586/14760584.2013.844653
96. Nightingale K, Lin K-M, Ravenhill BJ, Davies C, Nobre L, Fielding CA, et al. High-Definition Analysis of Host Protein Stability During Human Cytomegalovirus Infection Reveals Antiviral Factors and Viral Evasion Mechanisms. *Cell Host Microbe* (2018) 24:447–60.e11. doi: 10.1016/j.chom.2018.07.011
97. Le-Trilling VTK, Becker T, Nachshon A, Stern-Ginossar N, Schöler L, Voigt S, et al. The Human Cytomegalovirus Pul145 Isoforms Act as Viral DDB1-Cullin-Associated Factors to Instruct Host Protein Degradation to Impede Innate Immunity. *Cell Rep* (2020) 30:2248–60.e5. doi: 10.1016/j.celrep.2020.01.070
98. Hu L, Wen Z, Chen J, Chen Y, Jin L, Shi H, et al. The Cytomegalovirus UL146 Gene Product Vcxcl1 Promotes the Resistance of Hepatic Cells to CD8+ T Cells Through Up-Regulation of PD-L1. *Biochem Biophys Res Commun* (2020) 532:393–9. doi: 10.1016/j.bbrc.2020.08.060
99. De Pelsmaeker S, Romero N, Vitale M, Favoreel HW. Herpesvirus Evasion of Natural Killer Cells. *J Virol* (2018) 92:e02105–17. doi: 10.1128/JVI.02105-17
100. Nachmani D, Lankry D, Wolf DG, Mandelboim O. The Human Cytomegalovirus microRNA miR-UL112 Acts Synergistically With a Cellular microRNA to Escape Immune Elimination. *Nat Immunol* (2010) 11:806–13. doi: 10.1038/ni.1916
101. Liang Q, Wang K, Wang B, Cai Q. HCMV-Encoded miR-UL112-3p Promotes Glioblastoma Progression *via* Tumour Suppressor Candidate 3. *Sci Rep* (2017) 7:44705. doi: 10.1038/srep44705
102. Wu M, Fu P, Qu L, Liu J, Lin A. Long Noncoding RNAs, New Critical Regulators in Cancer Immunity. *Front Oncol* (2020) 10:550987. doi: 10.3389/fonc.2020.550987
103. Gredmark-Russ S, Söderberg-Nauclér C. Dendritic Cell Biology in Human Cytomegalovirus Infection and the Clinical Consequences for Host Immunity and Pathology. *Virulence* (2012) 3:621–34. doi: 10.4161/viru.22239
104. Handous I, Achour B, Marzouk M, Rouis S, Hazgui O, Brini I, et al. Co-Infections of Human Herpesviruses (CMV, HHV-6, HHV-7 and EBV) in non-Transplant Acute Leukemia Patients Undergoing Chemotherapy. *Virol J* (2020) 17:37. doi: 10.1186/s12985-020-01302-4
105. Rahbar A, Boström L, Lagerstedt U, Magnusson I, Söderberg-Naucler C, Sundqvist V-A. Evidence of Active Cytomegalovirus Infection and Increased Production of IL-6 in Tissue Specimens Obtained From Patients With Inflammatory Bowel Diseases. *Inflamm Bowel Dis* (2003) 9:154–61. doi: 10.1097/00054725-200305000-00002
106. Pleskoff O. Identification of a Chemokine Receptor Encoded by Human Cytomegalovirus as a Cofactor for HIV-1 Entry. *Science* (1997) 276:1874–8. doi: 10.1126/science.276.5320.1874
107. Wang H, Peng G, Bai J, He B, Huang K, Hu X, et al. Cytomegalovirus Infection and Relative Risk of Cardiovascular Disease (Ischemic Heart Disease, Stroke, and Cardiovascular Death): A Meta-Analysis of Prospective Studies Up to 2016. *JAHA* (2017) 6:e005025. doi: 10.1161/JAHA.116.005025
108. Spyridopoulos I, Martin-Ruiz C, Hilkens C, Yadegarfar ME, Isaacs J, Jagger C, et al. CMV Seropositivity and T-Cell Senescence Predict Increased Cardiovascular Mortality in Octogenarians: Results From the Newcastle 85 + Study. *Aging Cell* (2016) 15:389–92. doi: 10.1111/acel.12430
109. Savva GM, Pachnio A, Kaul B, Morgan K, Huppert FA, Brayne C, et al. The Medical Research Council Cognitive Function and Ageing Study. Cytomegalovirus Infection is Associated With Increased Mortality in the Older Population. *Aging Cell* (2013) 12:381–7. doi: 10.1111/acel.12059
110. Fülöp T, Larbi A, Pawelec G. Human T Cell Aging and the Impact of Persistent Viral Infections. *Front Immunol* (2013) 4:271. doi: 10.3389/fimmu.2013.00271
111. Macaluso M, Paggi MG, Giordano A. Genetic and Epigenetic Alterations as Hallmarks of the Intricate Road to Cancer. *Oncogene* (2003) 22:6472–8. doi: 10.1038/sj.onc.1206955
112. You JS, Jones PA. Cancer Genetics and Epigenetics: Two Sides of the Same Coin? *Cancer Cell* (2012) 22:9–20. doi: 10.1016/j.ccr.2012.06.008
113. Gonzalez H, Hagerling C, Werb Z. Roles of the Immune System in Cancer: From Tumor Initiation to Metastatic Progression. *Genes Dev* (2018) 32:1267–84. doi: 10.1101/gad.314617.118
114. Whiteside TL. The Tumor Microenvironment and its Role in Promoting Tumor Growth. *Oncogene* (2008) 27:5904–12. doi: 10.1038/onc.2008.271
115. Schuessler A, Walker DG, Khanna R. Cytomegalovirus as a Novel Target for Immunotherapy of Glioblastoma Multiforme. *Front Oncol* (2014) 4:275. doi: 10.3389/fonc.2014.00275
116. Hochhalter CB, Carr C, O'Neill BE, Ware ML, Strong MJ. The Association Between Human Cytomegalovirus and Glioblastomas: A Review. *NN* (2017) 4:96. doi: 10.20517/2347-8659.2017.10
117. Dey M, Ahmed AU, Lesniak MS. Cytomegalovirus and Glioma: Putting the Cart Before the Horse. *J Neurol Neurosurg Psychiatry* (2015) 86:191–9. doi: 10.1136/jnnp-2014-307727
118. Wolmer-Solberg N, Baryawno N, Rahbar A, Fuchs D, Odeberg J, Taher C, et al. Frequent Detection of Human Cytomegalovirus in Neuroblastoma: A Novel Therapeutic Target?: Human Cytomegalovirus in Neuroblastoma. *Int J Cancer* (2013) 133:2351–61. doi: 10.1002/ijc.28265
119. Taher C, de Boniface J, Mohammad A-A, Religa P, Hartman J, Yaiw K-C, et al. High Prevalence of Human Cytomegalovirus Proteins and Nucleic Acids in Primary Breast Cancer and Metastatic Sentinel Lymph Nodes. *PloS One* (2013) 8:e56795. doi: 10.1371/journal.pone.0056795
120. Taher C, Frisk G, Fuentes S, Religa P, Costa H, Assinger A, et al. High Prevalence of Human Cytomegalovirus in Brain Metastases of Patients With Primary Breast and Colorectal Cancers. *Trans Oncol* (2014) 7:732–40. doi: 10.1016/j.tranon.2014.09.008
121. Rahbar A, Touma J, Costa H, Davoudi B, Bukholm IR, Sauer T, et al. Low Expression of Estrogen Receptor-α and Progesterone Receptor in Human Breast Cancer Tissues Is Associated With High-Grade Human Cytomegalovirus Protein Expression. *Clin Breast Cancer* (2017) 17:526–35.e1. doi: 10.1016/j.clbc.2017.04.013
122. Harkins L, Volk AL, Samanta M, Mikolaenko I, Britt WJ, Bland KI, et al. Specific Localisation of Human Cytomegalovirus Nucleic Acids and Proteins in Human Colorectal Cancer. *Lancet* (2002) 360:1557–63. doi: 10.1016/S0140-6736(02)11524-8

123. Cobbs C, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, et al. Human Cytomegalovirus Infection and Expression in Human Malignant Glioma. *Cancer Res* (2002) 62:3347–50.
124. Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, et al. Sensitive Detection of Human Cytomegalovirus in Tumors and Peripheral Blood of Patients Diagnosed With Glioblastoma. *Neuro-Oncology* (2008) 10:10–8. doi: 10.1215/15228517-2007-035
125. Samanta M, Harkins L, Klemm K, Britt WJ, Cobbs CS. High Prevalence of Human Cytomegalovirus in Prostatic Intraepithelial Neoplasia and Prostatic Carcinoma. *J Urol* (2003) 170:998–1002. doi: 10.1097/01.ju.0000080263.46164.97
126. Huang G, Yan Q, Wang Z, Chen X, Zhang X, Guo Y, et al. Human Cytomegalovirus in Neoplastic Cells of Epstein-Barr Virus Negative Hodgkin's Disease. *Int J Oncol* (2002) 21:31–6.
127. Harkins LE, Matlaf LA, Soroceanu L, Klemm K, Britt WJ, Wang W, et al. Detection of Human Cytomegalovirus in Normal and Neoplastic Breast Epithelium. *HerpesVi* (2010) 1:8. doi: 10.1186/2042-4280-1-8
128. Baryawno N, Rahbar A, Wolmer-Solberg N, Taher C, Odeberg J, Darabi A, et al. Detection of Human Cytomegalovirus in Medulloblastomas Reveals a Potential Therapeutic Target. *J Clin Invest* (2011) 121:4043–55. doi: 10.1172/JCI57147
129. Söderberg-Nauclér C, Rahbar A, Stragliotto G. Survival in Patients With Glioblastoma Receiving Valganciclovir. *N Engl J Med* (2013) 369:985–6. doi: 10.1056/NEJMc1302145
130. Li Q, Wilkie AR, Weller M, Liu X, Cohen JI. THY-1 Cell Surface Antigen (CD90) Has an Important Role in the Initial Stage of Human Cytomegalovirus Infection. *PloS Pathog* (2015) 11:e1004999. doi: 10.1371/journal.ppat.1004999
131. Soroceanu L, Akhavan A, Cobbs CS. Platelet-Derived Growth Factor-α Receptor Activation is Required for Human Cytomegalovirus Infection. *Nature* (2008) 455:391–5. doi: 10.1038/nature07209
132. Kapoor A, He R, Venkatadri R, Forman M, Arav-Boger R. Wnt Modulating Agents Inhibit Human Cytomegalovirus Replication. *Antimicrob Agents Chemother* (2013) 57:2761–7. doi: 10.1128/AAC.00029-13
133. Li J-W, Yang D, Yang D, Chen Z, Miao J, Liu W, et al. Tumors Arise From the Excessive Repair of Damaged Stem Cells. *Med Hypotheses* (2017) 102:112–22. doi: 10.1016/j.mehy.2017.03.005
134. Soroceanu L, Matlaf L, Khan S, Akhavan A, Singer E, Bezrookove V, et al. Cytomegalovirus Immediate-Early Proteins Promote Stemness Properties in Glioblastoma. *Cancer Res* (2015) 75:3065–76. doi: 10.1158/0008-5472.CAN-14-3307
135. Cobbs CS, Soroceanu L, Denham S, Zhang W, Kraus MH. Modulation of Oncogenic Phenotype in Human Glioma Cells by Cytomegalovirus IE1–Mediated Mitogenicity. *Cancer Res* (2008) 68:724–30. doi: 10.1158/0008-5472.CAN-07-2291
136. Teo WH, Chen H-P, Huang JC, Chan Y-J. Human Cytomegalovirus Infection Enhances Cell Proliferation, Migration and Upregulation of EMT Markers in Colorectal Cancer-Derived Stem Cell-Like Cells. *Int J Oncol* (2017) 51:1415–26. doi: 10.3892/ijo.2017.4135
137. Moussawi FA, Kumar A, Pasquereau S, Tripathy MK, Karam W, Diab-Assaf M, et al. The Transcriptome of Human Mammary Epithelial Cells Infected With the HCMV-DB Strain Displays Oncogenic Traits. *Sci Rep* (2018) 8:12574. doi: 10.1038/s41598-018-30109-1
138. Nehme Z, Pasquereau S, Haidar Ahmad S, Coaquette A, Molimard C, Monnien F, et al. Polyploid Giant Cancer Cells, Stemness and Epithelial-Mesenchymal Plasticity Elicited by Human Cytomegalovirus. *Oncogene* (2021) 40:3030–46. doi: 10.1038/s41388-021-01715-7
139. Fortunato EA, Spector DH. Viral Induction of Site-Specific Chromosome Damage. *Rev Med Virol* (2003) 13:21–37. doi: 10.1002/rmv.368
140. Siew V-K, Duh C-Y, Wang S-K. Human Cytomegalovirus UL76 Induces Chromosome Aberrations. *J BioMed Sci* (2009) 16:107. doi: 10.1186/1423-0127-16-107
141. Cinatl J, Scholz M, Kotchetkov R, Vogel J-U, Wilhelm Doerr H. Molecular Mechanisms of the Modulatory Effects of HCMV Infection in Tumor Cell Biology. *Trends Mol Med* (2004) 10:19–23. doi: 10.1016/j.molmed.2003.11.002
142. Clement M, Humphreys IR. Cytokine-Mediated Induction and Regulation of Tissue Damage During Cytomegalovirus Infection. *Front Immunol* (2019) 10:78. doi: 10.3389/fimmu.2019.00078
143. Streblow DN, Dumortier J, Moses AV, Orloff SL, Nelson JA. Mechanisms of Cytomegalovirus-Accelerated Vascular Disease: Induction of Paracrine Factors That Promote Angiogenesis and Wound Healing. In: TE Shenk and MF Stinski, editors. *Human Cytomegalovirus Current Topics in Microbiology and Immunology*. Berlin, Heidelberg: Springer Berlin Heidelberg (2008). p. 397–415. doi: 10.1007/978-3-540-77349-8_22
144. de Wit RH, Mujić-Delić A, van Senten JR, Fraile-Ramos A, Siderius M, Smit MJ. Human Cytomegalovirus Encoded Chemokine Receptor US28 Activates the HIF-1α/PKM2 Axis in Glioblastoma Cells. *Oncotarget* (2016) 7:67966–85. doi: 10.18632/oncotarget.11817
145. Maussang D, Langemeijer E, Fitzsimons CP, Stigter-van Walsum M, Dijkman R, Borg MK, et al. The Human Cytomegalovirus–Encoded Chemokine Receptor US28 Promotes Angiogenesis and Tumor Formation *via* Cyclooxygenase-2. *Cancer Res* (2009) 69:2861–9. doi: 10.1158/0008-5472.CAN-08-2487
146. Zheng X, Turkowski K, Mora J, Brüne B, Seeger W, Weigert A, et al. Redirecting Tumor-Associated Macrophages to Become Tumoricidal Effectors as a Novel Strategy for Cancer Therapy. *Oncotarget* (2017) 8:48436–52. doi: 10.18632/oncotarget.17061
147. Hollon TC, Price RL, Kwon C-H, Chiocca EA. Mutations in Glioblastoma Oncosuppressive Pathways Pave the Way for Oncomodulatory Activity of Cytomegalovirus. *OncoImmunology* (2013) 2:e25620. doi: 10.4161/onci.25620
148. Korbecki J, Gutowska I, Kojder I, Jeżewski D, Goschorska M, Łukomska A, et al. New Extracellular Factors in Glioblastoma Multiforme Development: Neurotensin, Growth Differentiation Factor-15, Sphingosine-1-Phosphate and Cytomegalovirus Infection. *Oncotarget* (2018) 9:7219–70. doi: 10.18632/oncotarget.24102
149. Valle Oseguera CA, Spencer JV. cmvIL-10 Stimulates the Invasive Potential of MDA-MB-231 Breast Cancer Cells. *PloS One* (2014) 9:e88708. doi: 10.1371/journal.pone.0088708
150. Nozawa H, Chiu C, Hanahan D. Infiltrating Neutrophils Mediate the Initial Angiogenic Switch in a Mouse Model of Multistage Carcinogenesis. *Proc Natl Acad Sci* (2006) 103:12493–8. doi: 10.1073/pnas.0601807103
151. Rahbar A, Cederarv M, Wolmer-Solberg N, Tammik C, Stragliotto G, Peredo I, et al. Enhanced Neutrophil Activity is Associated With Shorter Time to Tumor Progression in Glioblastoma Patients. *OncoImmunology* (2016) 5:e1075693. doi: 10.1080/2162402X.2015.1075693
152. Tai-Schmiedel J, Karniely S, Lau B, Ezra A, Eliyahu E, Nachshon A, et al. Human Cytomegalovirus Long Noncoding RNA4.9 Regulates Viral DNA Replication. *PloS Pathog* (2020) 16:e1008390. doi: 10.1371/journal.ppat.1008390
153. Denaro N, Merlano MC, Lo Nigro C. Long Noncoding RNAs as Regulators of Cancer Immunity. *Mol Oncol* (2019) 13:61–73. doi: 10.1002/1878-0261.12413
154. Tlaskalová-Hogenová H, Štěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, et al. The Role of Gut Microbiota (Commensal Bacteria) and the Mucosal Barrier in the Pathogenesis of Inflammatory and Autoimmune Diseases and Cancer: Contribution of Germ-Free and Gnotobiotic Animal Models of Human Diseases. *Cell Mol Immunol* (2011) 8:110–20. doi: 10.1038/cmi.2010.67
155. Francescone R, Hou V, Grivennikov SI. Microbiome, Inflammation, and Cancer. *Cancer J* (2014) 20:181–9. doi: 10.1097/PPO.0000000000000048
156. Ramendra R, Isnard S, Lin J, Fombuena B, Ouyang J, Mehraj V, et al. Cytomegalovirus Seropositivity Is Associated With Increased Microbial Translocation in People Living With Human Immunodeficiency Virus and Uninfected Controls. *Clin Infect Dis* (2020) 71:1438–46. doi: 10.1093/cid/ciz1001
157. Gianella S, Chaillon A, Mutlu EA, Engen PA, Voigt RM, Keshavarzian A, et al. Effect of Cytomegalovirus and Epstein–Barr Virus Replication on Intestinal Mucosal Gene Expression and Microbiome Composition of HIV-Infected and Uninfected Individuals. *AIDS* (2017) 31:2059–67. doi: 10.1097/QAD.0000000000001579
158. Al-Koussa H, Atat OE, Jaafar L, Tashjian H, El-Sibai M. The Role of Rho GTPases in Motility and Invasion of Glioblastoma Cells. *Analytical Cell Pathol* (2020) 2020:1–9. doi: 10.1155/2020/9274016
159. Shimamura M, Murphy-Ullrich JE, Britt WJ. Human Cytomegalovirus Induces TGF-β1 Activation in Renal Tubular Epithelial Cells After Epithelial-To-Mesenchymal Transition. *PloS Pathog* (2010) 6:e1001170. doi: 10.1371/journal.ppat.1001170

160. Casey SC, Baylot V, Felsher DW. MYC: Master Regulator of Immune Privilege. *Trends Immunol* (2017) 38:298–305. doi: 10.1016/j.it.2017.01.002
161. Rakhra K, Bachireddy P, Zabuawala T, Zeiser R, Xu L, Kopelman A, et al. CD4+ T Cells Contribute to the Remodeling of the Microenvironment Required for Sustained Tumor Regression Upon Oncogene Inactivation. *Cancer Cell* (2010) 18:485–98. doi: 10.1016/j.ccr.2010.10.002
162. Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. Mast Cells are Required for Angiogenesis and Macroscopic Expansion of Myc-Induced Pancreatic Islet Tumors. *Nat Med* (2007) 13:1211–8. doi: 10.1038/nm1649
163. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, et al. CD47 Is an Adverse Prognostic Factor and Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells. *Cell* (2009) 138:286–99. doi: 10.1016/j.cell.2009.05.045
164. Casey SC, Tong L, Li Y, Do R, Walz S, Fitzgerald KN, et al. MYC Regulates the Antitumor Immune Response Through CD47 and PD-L1. *Science* (2016) 352:227–31. doi: 10.1126/science.aac9935
165. Schlee M, Hölzel M, Bernard S, Mailhammer R, Schuhmacher M, Reschke J, et al. C-MYC Activation Impairs the NF-κb and the Interferon Response: Implications for the Pathogenesis of Burkitt's Lymphoma. *Int J Cancer* (2007) 120:1387–95. doi: 10.1002/ijc.22372
166. Stark GR, Kerr IM, Williams BRG, Silverman RH, Schreiber RD. How Cells Respond To Interferons. *Annu Rev Biochem* (1998) 67:227–64. doi: 10.1146/annurev.biochem.67.1.227
167. Villegas SN, Gombos R, García-López L, Gutiérrez-Pérez I, García-Castillo J, Vallejo DM, et al. PI3K/Akt Cooperates With Oncogenic Notch by Inducing Nitric Oxide-Dependent Inflammation. *Cell Rep* (2018) 22:2541–9. doi: 10.1016/j.celrep.2018.02.049
168. Cui Y, Guo G. Immunomodulatory Function of the Tumor Suppressor P53 in Host Immune Response and the Tumor Microenvironment. *IJMS* (2016) 17:1942. doi: 10.3390/ijms17111942
169. Blagih J, Buck MD, Vousden KH. P53, Cancer and the Immune Response. *J Cell Sci* (2020) 133:jcs237453. doi: 10.1242/jcs.237453
170. Li F, Kitajima S, Kohno S, Yoshida A, Tange S, Sasaki S, et al. Retinoblastoma Inactivation Induces a Protumoral Microenvironment *via* Enhanced CCL2 Secretion. *Cancer Res* (2019) 79:3903–15. doi: 10.1158/0008-5472.CAN-18-3604
171. Vishnu P, Aboulafia DM. The Oncogenicity of Human Cytomegalovirus. In: P Price, Makwana N and S Brunt, editors. *Manifestations of Cytomegalovirus Infection* (2013). doi: 10.5772/55051
172. Mitchell DA, Sampson JH. Toward Effective Immunotherapy for the Treatment of Malignant Brain Tumors. *Neurotherapeutics* (2009) 6:527–38. doi: 10.1016/j.nurt.2009.04.003
173. Quinn M, Erkes DA, Snyder CM. Cytomegalovirus and Immunotherapy: Opportunistic Pathogen, Novel Target for Cancer and a Promising Vaccine Vector. *Immunotherapy* (2016) 8:211–21. doi: 10.2217/imt.15.110
174. Rahman M, Dastmalchi F, Karachi A, Mitchell D. The Role of CMV in Glioblastoma and Implications for Immunotherapeutic Strategies. *OncoImmunology* (2019) 8:e1514921. doi: 10.1080/2162402X.2018.1514921
175. Herbein G, Nehme Z. Tumor Control by Cytomegalovirus: A Door Open for Oncolytic Virotherapy? *Mol Ther - Oncolytics* (2020) 17:1–8. doi: 10.1016/j.omto.2020.03.004
176. Wang C, Zhang J, Yin J, Gan Y, Xu S, Gu Y, et al. Alternative Approaches to Target Myc for Cancer Treatment. *Sig Transduct Target Ther* (2021) 6:117. doi: 10.1038/s41392-021-00500-y
177. Hashiguchi T, Bruss N, Best S, Lam V, Danilova O, Paiva CJ, et al. Cyclin-Dependent Kinase-9 Is a Therapeutic Target in MYC-Expressing Diffuse Large B-Cell Lymphoma. *Mol Cancer Ther* (2019) 18:1520–32. doi: 10.1158/1535-7163.MCT-18-1023
178. Chen H, Liu H, Qing G. Targeting Oncogenic Myc as a Strategy for Cancer Treatment. *Sig Transduct Target Ther* (2018) 3:5. doi: 10.1038/s41392-018-0008-7
179. Lozano G. Restoring P53 in Cancer: The Promises and the Challenges. *J Mol Cell Biol* (2019) 11:615–9. doi: 10.1093/jmcb/mjz063
180. Song M, Bode AM, Dong Z, Lee M-H. AKT as a Therapeutic Target for Cancer. *Cancer Res* (2019) 79:1019–31. doi: 10.1158/0008-5472.CAN-18-2738
181. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT Pathway for Cancer Drug Discovery. *Nat Rev Drug Discov* (2005) 4:988–1004. doi: 10.1038/nrd1902
182. Guo G, Yu M, Xiao W, Celis E, Cui Y. Local Activation of P53 in the Tumor Microenvironment Overcomes Immune Suppression and Enhances Antitumor Immunity. *Cancer Res* (2017) 77:2292–305. doi: 10.1158/0008-5472.CAN-16-2832
183. Beverly LJ, Felsher DW, Capobianco AJ. Suppression of P53 by Notch in Lymphomagenesis: Implications for Initiation and Regression. *Cancer Res* (2005) 65:7159–68. doi: 10.1158/0008-5472.CAN-05-1664
184. Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, et al. PD 0332991, a Selective Cyclin D Kinase 4/6 Inhibitor, Preferentially Inhibits Proliferation of Luminal Estrogen Receptor-Positive Human Breast Cancer Cell Lines *In Vitro*. *Breast Cancer Res* (2009) 11:R77. doi: 10.1186/bcr2419
185. Knudsen ES, Wang JYJ. Targeting the RB-Pathway in Cancer Therapy. *Clin Cancer Res* (2010) 16:1094–9. doi: 10.1158/1078-0432.CCR-09-0787
186. Hutcheson J, Witkiewicz AK, Knudsen ES. The RB Tumor Suppressor at the Intersection of Proliferation and Immunity: Relevance to Disease Immune Evasion and Immunotherapy. *Cell Cycle* (2015) 14:3812–9. doi: 10.1080/15384101.2015.1010922
187. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 Signaling Axis in Cancer Metastasis. *Oncotarget* (2016) 7:28697–710. doi: 10.18632/oncotarget.7376
188. Yang H, Zhang Q, Xu M, Wang L, Chen X, Feng Y, et al. CCL2-CCR2 Axis Recruits Tumor Associated Macrophages to Induce Immune Evasion Through PD-1 Signaling in Esophageal Carcinogenesis. *Mol Cancer* (2020) 19:41. doi: 10.1186/s12943-020-01165-x
189. Zhong J, Khanna R. Vaccine Strategies Against Human Cytomegalovirus Infection. *Expert Rev Anti-Infect Ther* (2007) 5:449–59. doi: 10.1586/14787210.5.3.449
190. Scarpini S, Morigi F, Betti L, Dondi A, Biagi C, Lanari M. Development of a Vaccine Against Human Cytomegalovirus: Advances, Barriers, and Implications for the Clinical Practice. *Vaccines* (2021) 9:551. doi: 10.3390/vaccines9060551
191. Haidar Ahmad S, Al Moussawi F, El Baba R, Nehme Z, Pasquereau S, Kumar A, et al. Identification of UL69 Gene and Protein in Cytomegalovirus-Transformed Human Mammary Epithelial Cells. *Front Oncol* (2021) 11:627866. doi: 10.3389/fonc.2021.627866
192. Shigle TL, Handy VW, Chemaly RF. Letermovir and its Role in the Prevention of Cytomegalovirus Infection in Seropositive Patients Receiving an Allogeneic Hematopoietic Cell Transplant. *Ther Adv Hematol* (2020) 11:2040620720937150. doi: 10.1177/2040620720937150

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GLOSSARY

ALT	Adoptive lymphocyte transfer
BAK	Bcl-2 homologues antagonist/killer
BAX	BCL2-Associated X Protein
Bcl-2	B-cell lymphoma 2
BET	Bromodomain and extraterminal protein
CDK	Cyclin-dependent kinases
COVID-19	Coronavirus disease 2019
COX-2	Cyclooxygenase-2
CRPs	Complement regulatory proteins
CTH	CMV transformed HMECs
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DISC	Death-inducing signaling complex
dsDNA	Double-stranded DNA
EBV	Epstein-Barr virus
EMT	Epithelial to mesenchymal transition
EPCR	Endothelial protein C receptor
FAO	Fatty acid oxidation
FLIP	Fas-associated death-domain-like IL-1β-converting enzyme-inhibitory proteins
gB	Glycoproteins B
GBM	Glioblastoma
GCRs	G-Protein-coupled receptors
gH	Glycoprotein H
HCC	Hepatocellular carcinoma
HCMV	Human Cytomegalovirus
HHV-5	Human herpesvirus 5
HLTF	Helicase like transcription factor
HMEC	Human mammary epithelial cells
HSCT	Hematopoietic stem-cell transplantation
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IFNs	Type I interferons
IL	Interleukin
IL6R	IL-6 receptor
JAK	Janus kinase
lncRNAs	Long non-coding RNAs
MCP-1	Monocyte chemoattractant protein-1
MCP3	Monocyte chemotactic protein-3
MDM2	Mouse double minute 2 homolog
MHC	Major histocompatibility complex
MICA	MHC Class I Polypeptide-Related Sequence A
MICB	MHC class I chain-related gene B
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
miRNAs	microRNAs
MS	Mitochondrial superoxide
NK	Natural killer
NKG2D	Natural Killer Group 2D
NOS	Nitric oxide synthase
NSAIDs	Nonsteroidal anti-inflammatory drugs
Oct-4	Octamer-binding transcription factor 4
PBMCs	Peripheral blood mononuclear cells
PDGFRα	Platelet-derived growth factor receptor alpha
PD-L1	Programmed death-ligand 1
PI3K/AKT	Phosphatidylinositol-3-kinase and protein kinase B
SLE	Systemic lupus erythematosus
SOX2	SRY-Box Transcription Factor 2
STAT	Signal transducer and activator of transcription
TAMS	Tumor-associated macrophages
Th	T-helper
TLR2	Toll-like receptors 2
TME	Tumor microenvironment
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor-alpha
TUSC3	Tumor suppressor candidate 3
UL	Unique long
ULBPs	UL16 binding proteins
US	Unique short
VEGF	Vascular endothelial growth factor
vICA	Viral inhibitor of caspase-8-induced apoptosis
γδ T-cells	Gamma-delta T-cells

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REVIEW
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Cytomegalovirus in Haematological Tumours

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The exquisite coupling between herpesvirus and human beings is the result of millions of years of relationship, coexistence, adaptation, and divergence. It is probably based on the ability to generate a latency that keeps viral activity at a very low level, thereby apparently minimising harm to its host. However, this evolutionary success disappears in immunosuppressed patients, especially in haematological patients. The relevance of infection and reactivation in haematological patients has been a matter of interest, although one fundamentally focused on reactivation in the post-allogeneic stem cell transplant (SCT) patient cohort. Newer transplant modalities have been progressively introduced in clinical settings, with successively more drugs being used to manipulate graft composition and functionality. In addition, new antiviral drugs are available to treat CMV infection. We review the immunological architecture that is key to a favourable outcome in this subset of patients. Less is known about the effects of herpesvirus in terms of mortality or disease progression in patients with other malignant haematological diseases who are treated with immuno-chemotherapy or new molecules, or in patients who receive autologous SCT. The absence of serious consequences in these groups has probably limited the motivation to deepen our knowledge of this aspect. However, the introduction of new therapeutic agents for haematological malignancies has led to a better understanding of how natural killer (NK) cells, CD4+ and CD8+ T lymphocytes, and B lymphocytes interact, and of the role of CMV infection in the context of recently introduced drugs such as Bruton tyrosine kinase (BTK) inhibitors, phosphoinosytol-3-kinase inhibitors, anti-BCL2 drugs, and even CAR-T cells. We analyse the immunological basis and recommendations regarding these scenarios.

Keywords: CMV, inflammation, lymphoma, transplantation, immunotherapy, CAR-T-cells

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INTRODUCTION

Human cytomegalovirus (CMV) is a DNA virus belonging to the herpesvirus family. Its transmission, through saliva, sexual contact, blood and breast milk, makes it highly prevalent, and the seroprevalence increases with age (1). The various studies carried out so far estimate a seroprevalence between 30% and more than 90%, depending on the population under study. This variation may be largely ascribed to age and socio-economic characteristics.

The implications of acute infection are of little relevance in terms of severity of infection and complications, as described below. However, the interest in healthy populations lies in its chronification and latency, and thereby in the development of an immune response that accompanies the host throughout its life, modulating its immune system through mechanisms that are not yet fully understood.

CMV infection plays a very important role in some population groups, such as immunosuppressed patients, and especially haematological patients, since acute infection causes significant morbidity and mortality in such patients.

The objective is to review the knowledge of CMV infection, and to understand its immunological effects in healthy individuals, in general, and in haematological patients.

CMV INFECTION

Primoinfection and Latent Phase

The infection is usually asymptomatic in adults, although sometimes it occurs in the setting of a mononucleosis-like syndrome. CMV is able to induce a persistent infection throughout the host lifetime. This is due to its ability to remain latent in some cells. It appears that CD34+ cells and CD14+ monocytes and macrophages as well as dendritic cells may constitute the fundamental reservoir (2–4). However, viral DNA has also been found in other cells of the immune system, as well as in epithelial and endothelial cells (3, 5–7) as they get also infected and which would also explain the mechanisms by which it is transmitted (**Figure 1**).

In the infectious phase (and endogenous reactivation), the initial participation of proteins encoded by immediate-early (IE) genes is key, since they strongly activate the expression of the genes that consolidate the infectious stage (7, 8). These IE genes are expressed in cells that allow it (permissive), which are, apparently, differentiated cells (9). This expression is subject to the activation of the major immediate-early promoter (MIEP) protein. There are a variety of cellular factors that repress the activity of the MIEP in non-permissive cells (2, 10, 11). The terminal differentiation of these cells, which initially does not allow the reactivation of CMV, could decrease the expression of these factors, leaving the promoter complex active, thereby permitting viral reactivation (12, 13).

CMV infection induces specific IgA, IgG, and IgM production. Neutralizing IgGs appear early after infection and are permanently detectable thereafter. IgA can be detected for several months and even years after primoinfection (14). Cellular response is crucial in CMV infection control, CD4+ and CD8+ T lymphocytes are directed to pp65 protein and IE1 protein. When this control is well set, infection usually follows an indolent course (15).

Chronic Infection and Possible Oncogenic Role

Long-term effects on immune system have been described despite the indolent course of CMV infection in the immunocompetent population. CMV has an impact on the T cell pool (and leaves a fingerprint) by large expansions of the CMV-specific memory pool and expansion of terminally differentiated T cells/*effector* T cells. This might impair the immunological response to neoantigens as well as the number of IL-2 and IL-4-producing CD8+ memory T lymphocytes in the elderly. CMV-specific CD8+ T lymphocytes producing IFN-γ, might contribute to a proinflammatory status, but this is

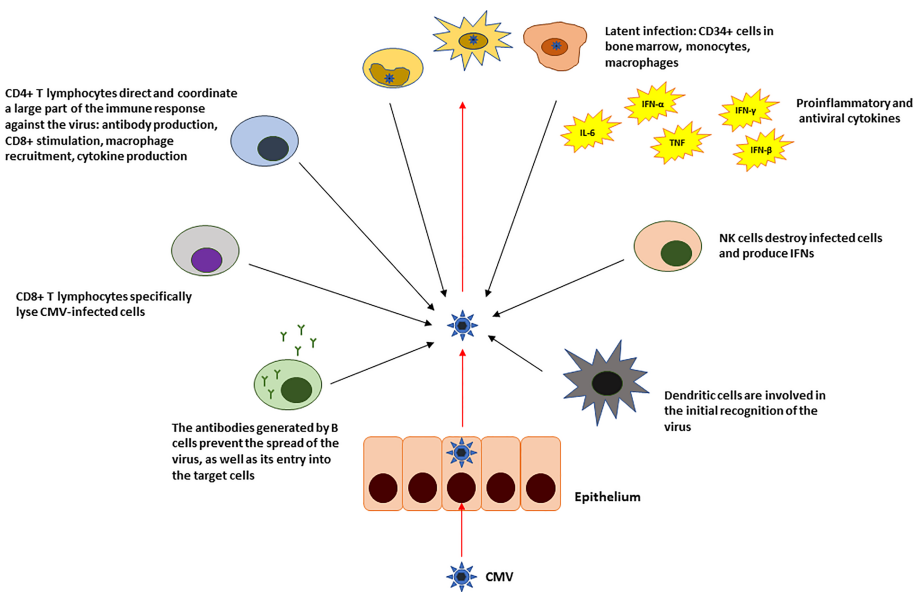

FIGURE 1 | Summary of the innate and adaptive immune response against CMV in immunocompetent individuals.

probably less clear. Therefore, some aging-associated processes might be accelerated due to latent CMV infection (15, 16). However, the role of CMV on aging is, in any case, a current matter of debate. And a recent extensive review of this matter by Jackson et al. has revealed that, according to existing data, there is only limited evidence supportive of the formation and maintenance of a large population of CMV specific CD8+ T cells, known as "memory inflation", as a mechanism of *immunosenescence* (17).

Potential oncogenicity has also been studied, yielding controversial results in solid and haematological tumours. This may be because the multiple causes, over and above many of the fundamental processes responsible for the development of solid and haematological tumours, make it difficult to assess the specific value of the individual primary causes.

The oncogenic role of other viruses and pathogens (hepatitis C and B, papilloma, and the bacterium *Helicobacter pylori*) is better established in the context of some solid neoplasms, such as the liver in the case of the first two, the cervix and larynx in the second, and stomach cancer in the case of *H. pylori*.

The association between CMV infection and numerous solid tumours in relation to its oncogenic and immunomodulatory roles has also been sought and found. In the case of colon cancer, the association between CMV and the development of cancer varies from study to study (18–21), whereby some find an association but others confirm the absence of one. A recent and very detailed review of US28 potential roles in aherosclerotic disease and cancer demonstrates the difficulty of attributing the causation of US28 in carcinogenesis and its role in atherosclerotic disease (22). In this field, the results of the investigations are now very numerous and not always concordant, so they must be integrated in order to establish consistent concepts regarding the functions of this protein. This could later relate it to CMV infection, and this molecule might then be used as a therapeutic target or disease marker and targeting US28 might prevent CMV disease and could benefit immunosuppressed individuals, including transplant patients (23).

CMV is expressed in most human glioma samples (24). However, correlation with peripheral blood CMV detection in glioblastoma patients is variable (25). The low incidence of glioblastoma cases compared with the high prevalence of CMV infection makes it difficult to explain the initiating role of CMV in the development of this neoplasm. In addition, anti-CMV treatment with antiviral drugs such as foscarnet or valganciclovir has not definitively been shown to improve survival in patients with glioblastoma multiforme (26–28). The efficacy of anti-CMV immunotherapy (29) may be due to targeting of CMV-expressing cells that drive tumour growth, activation of other immune cells that cause additional killing of CMV-negative cells, or cross-priming after killing of CMV-positive tumour cells. Therefore, a proposed role for CMV in gliomagenesis is most likely to be associated with an as yet undefined event (30), although it seems that it might be supported by CMV's oncoimmunomodulatory role.

Although CMV is the virus whose impact seems to be the most significant with respect to T lymphocyte deregulation, it has not been given so much importance from the haematological point of view, and very little is known about its oncogenic role. In fact, very few studies have analysed the influence of CMV on haematological pathology. In contrast, the association of the Epstein–Barr virus with the development of lymphomas is well established (31–34). The fact that the EBV tropism occurs in the B lymphocytes, where it remains latent (35–37), may make the relationship much more direct for the etiological study. In the case of CMV, no indirect association has been sought, perhaps because, although not directly, the effect on, or damage caused to the functionality of B lymphocytes and, especially T lymphocytes, by chronic infection and successive reactivations indirectly affects degree of predisposition to lymphomagenesis.

Although no prospective studies have been carried out, at least three retrospective studies have analysed the influence of the virus in populations that develop lymphomas. Two of the studies did not find a higher seroprevalence in patients with T cell lymphomas compared to the age-controlled population (38, 39). However, another study (40) found a very high seroprevalence in patients with mycosis fungoides and Sézary syndrome (**Table 1**).

The expression of proteins and transcription factors of the virus has also been observed in some series of patients. Specifically, in a series of Iranian patients, the expression of UL138 mRNAs (as latent infection markers) and IE1 proteins (as reactivation markers) was studied by RT-PCR in patients with Hodgkin and non-Hodgkin lymphomas. The expression of UL138 mRNAs was found to be expressed in 20% of the T cell lymphomas in the series (41). Of note, this observation remains to be explained since T cells are not infected by CMV; casualty seems to be difficult to demonstrate here due to a possible indirect effect; indeed we are yet to know if a potential immune dysregulation motivated by a chronic exposure to the antigen might be responsible of this association.

CMV IN THE HAEMATOLOGICAL SETTING

CMV in Allogeneic Stem Cell Transplantation

Definitions Regarding CMV Infection

Definitions of CMV infection and disease were initially developed and published as part of the proceedings of the 4th International CMV Conference in Paris in 1993 and have been progressively updated, most recently in 2020 (42–45).

Infection involves the detection of CMV in biological samples. When monitoring patients after transplant, it is usually determined in blood. In these cases, it is worthwhile differentiating whether the infection is detected by finding the antigen (antigenemia), growth in cell culture (viremia), or detecting DNA (DNAemia).

Primary infection takes place in seronegative patients, while reactivation refers to virus detection in previously seropositive patients. Recurrent infection refers to the detection of CMV in a patient with evidence of infection, but when there has been a 4-week infection-free gap between the two determinations.

TABLE 1 | CMV role in lymphoma development.

Study	Aim of study	Results
Gupta et al. (38)	Seroprevalence SS/MF vs Non-SS/MF	SS/MF 60.4 % (N=53) Non-SS/MF 61.5% (N=26)
Ballanger et al. (39)	Seroprevalence SS, MF& control group	Control group 37% (N=124) MF 66.67% (N=27) SS 42.86% (N=21) p=0.009
	PCR in affected tissue	CMV was not detected in diagnostic biopsies. CMV was detected in two SS skin biopsies realized at an advanced stage
Herne et al. (40)	Seroprevalence SS/MF vs bone marrow donors	Control group 57.3% (N=1322) MF/SS 97.4% (N=116) p<0.05
	Subanalysis with age-matched subgroups	CTCL 93% (N=32) Control group 53.6% (N=1103) p<0.05
Mehravaran et al. (41)	PCR in affected tissue IE1 (active replication) Nested-PCR in affected tissue UL138 (latency) Hodgkin and No Hodgkin	IE1 detected in 1/25 Non-HL UL138 in 5/25 Non-HL and 1/25 HL

SS, Sézary Syndrome; MF, Mycosis Fungoides; CTCL, Cutaneous T-cell lymphoma; HL, Hodgkin lymphoma.

Reinfection refers to a new infection by a different viral strain, while reactivation is established when the same viral strain, of endogenous origin is involved.

CMV disease involves the conjunction of signs and/or symptoms that indicate organ involvement (pulmonary, gastrointestinal, hepatic, retinal, renal, myocardial, encephalic, pancreatic, etc.) together with the detection of CMV (using one or more of the validated techniques) in the affected organ or tissue.

T Lymphocyte Reconstitution and CMV: Cause and Consequence

T lymphocyte reconstitution has an initial thymus-independent phase, during which we observe the antigen-driven expansion of T lymphocytes infused with the graft (**Figure 2**). The second phase is thymus-dependent. Naïve T lymphocytes derived from the donor with a diverse T lymphocyte receptor (TCR) repertoire expand, although very slowly, so it takes years to complete the reconstitutions of this subset (46). However, the process cannot always be completed in this way because thymic function is conditioned in many allogeneic SCT recipients, thymic involutes in old patients and graft-versus-host disease (GVHD) damages epithelial thymus cells (47). When this occurs, the thymic-independent pathway rapidly generates CD8+T lymphocytes (48), resulting in an inversion of the CD4:CD8 ratio that can persist for years (49). It also leads to peripheral expansion of memory T lymphocytes (CD45RO+CD27+/CD45RO+CD27-) since generation of naïve T lymphocytes (CD45RA+/CD28+) from prethymic progenitors depends on a functional thymus (50). The differences between precursor sources, conditioning regimens and donor features are a consequence of the absolute numbers of CD4+ and CD8+ T lymphocytes infused. In any case, the absolute number of CD4+ T lymphocytes (i.e., regulatory T lymphocytes (Tregs) and conventional CD4+ T lymphocytes), remain at unrecovered levels up to 2 years after hematopoietic stem-cell transplantation (HCT). CD8+ T lymphocytes can recover faster, but also depend on the conditioning regimen and immunosuppressors used. The TCR repertory is considered to drive and be the result of disease control and GVHD activity. With regard to disease control, a study including umbilical cord blood donors (UCDs) and matched related or unrelated donors using *in vivo* T cell depletion (TCD) showed that patients who remained in remission had greater TCR diversity. A broader TCR spectrum could have an antitumoral role. A narrower TCR spectrum is in turn observed in those with GVHD, which would presumably be related to preferential expansion of particular T lymphocyte clones (51). In contrast, another study reported that grade 2–3 acute GVHD is associated with greater TCR diversity (52). These differences might be due to the high variability among the conditioning regimens, immunosuppressive therapy, and donor source (53).

Activity of T lymphocytes (CD4+ and CD8+) is essential for the control of CMV infection (15, 53, 54). The correct reconstitution of CD8+ and CD4+ T lymphocytes is associated with the control of CMV infection (55). CMV-specific CD8+ T lymphocyte reconstitution is usually delayed by around 3 months after transplantation. There have been reports of prompt recovery, which is thought to be based on the receptor's ability to bring about T lymphocyte lymphopoiesis (56). Knowing the HLA-typing of the donor, the source of the graft and the type of conditioning are essential for this rapid recovery and therefore the early control of the infection: HLA-typing facilitates the reestablishment, which is faster in identical donors without mismatch, but slower the more T depletion there is. Receptor immunity also influences infection control until immune reconstitution is established, especially in patients on reduced intensity regimens (57).

The relation between T lymphocyte reconstitution and CMV infection is bidirectional, and while delayed T lymphocyte reestablishment compromises anti-CMV protection, prompt reactivation of this virus conditions the characteristics of the recovery (58). CMV expands effector memory T lymphocytes, causing a linked contraction of all naïve T lymphocytes, including putative CD31+ thymic emigrants (59).

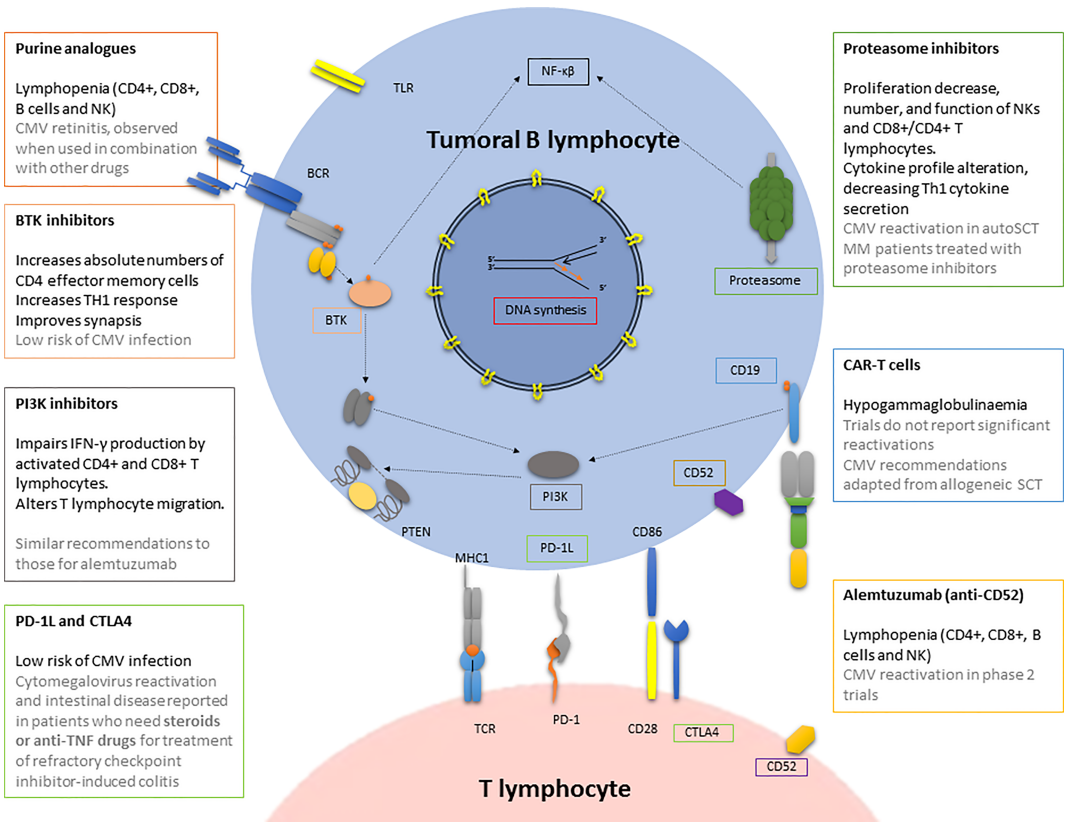

FIGURE 2 | CMV infection/reactivation in the context of antitumoral drugs used in haematological patients. BCR, B cell receptor; BTK, Bruton tyrosine kinase; CAR-T cells, chimeric antigen receptor T cell; CMV, cytomegalovirus; CTLA4, cytotoxic T-lymphocyte antigen 4; MHC, major histocompatibility complex; NK, natural killer; PI3K, phosphatidyl inositol 3 kinase; PD, programmed death; PD-1L, programmed death-ligand 1; PTEN, phosphatase and tensin homologue; SCT, stem cell transplantation; TLR, toll-like receptor.

Donor Source Role

With respect to the source of the graft, patients who receive an allogeneic umbilical cord blood (UCB) transplant are at the highest risk of CMV infection because of the type of cells present in UCB. In this type of transplant, T lymphocytes are immature, which lower the risk of developing GVHD, but could increase the risk of severe infections (60). UCB contains Tregs, which are more potent suppressors than adult Treg (61). On the other hand, this is consistent with the high dose of anti-thymocyte globulin employed in the conditioning regimen, which is needed to prevent mainly graft rejection but also the effects of GVHD. In summary, T lymphocyte reconstitution is delayed, and this is considered the main cause of the increased risk of infection, particularly by CMV, in this cohort (62).

Patients who receive CD34+ (positively selected) allogeneic SCT or T lymphocyte-depleted allogeneic progenitors belong to the high-risk group for CMV infection because they lack the mature and naïve T lymphocytes that should make cellular immunity reconstitution possible.

The faster engraftment of peripheral blood progenitors compared with bone marrow and cord blood progenitors, and the concordant faster immune reconstitution demonstrated *in vitro* (63) and *in vivo* (64), lead us to expect that better immune reconstitution against CMV would reduce the incidence of CMV disease in this group. Several studies have compared the sources and found mixed results for the risk of CMV reactivation in peripheral blood and bone marrow. For example, a non-randomized study of 158 patients showed that the incidence of CMV reactivation (monitoring antigenemia) and CMV interstitial pneumonia were lower in the peripheral blood than in the bone marrow group (65). Another randomized study (n = 172) showed the opposite relationship, with a higher incidence of CMV infection in unmodified peripheral blood SCT recipients (66). This latter theory of a higher risk of CMV infection in peripheral blood than in UCB and bone marrow has been described (63). However, another study found no such differences (64).

This risk of CMV infection drops to an intermediate level as time goes by, as long as anti-T drugs or high-dose steroids are not added.

The influence of the HLA discrepancy between donor and recipient is highly nuanced or dependent on the type of conditioning and immunosuppressive treatment used since these are adjusted based on these discrepancies. The greater

the discrepancy, the greater the risk of viral reactivation, but as explained above, this could be a response to the confluence of a more powerful immunosuppressor therapy and the anti-T-lymphocyte agents used.

Patients undergoing haploidentical allogeneic hematopoietic SCT (Haplo-HSCT) have been considered to be at higher risk of CMV reactivation than those receiving HLA-matched allografts due to impaired CMV-specific T lymphocyte reconstitution. Analysis of this and monitoring the CMV DNA load in parallel with CMV-specific IFN-γ-producing CD8+ and CD4+ T lymphocytes revealed that CMV was reactivated approximately as often in PTCy-haplo and HLA-matched recipients, and that CMV-specific T lymphocyte counts were similar in the two groups at most of the times examined. These findings suggest that the two groups reconstitute CMV-specific T lymphocyte immunity in a similar fashion (67).

Immunosuppressors and Anti-T Lymphocyte Agents

Steroids and anti-T lymphocyte agents are the cornerstone of the prevention and treatment of graft rejection and, fundamentally, of the development of graft-versus-recipient disease, the appearance of which considerably increases transplant-related mortality (not associated with relapse) (TRM). These data are well established, and by way of example, in one of the most recent series of patients treated with novel immunosuppressive treatment options, an increase in mortality of 5 to 16% has been described in patients with or without GVHD (68).

Patients who receive an allogeneic transplant from any source and who are treated with high-dose steroids or who receive anti-T lymphocyte agents, such as the aforementioned anti-thymocyte globulin, but also high doses of cyclophosphamide are also at high risk. This drug is necessarily employed in haploidentical transplant after progenitor infusion to avoid the GVHD that would accompany a half-discrepant haplotype. However, T lymphocyte *in vivo* depletion with cyclofosfamide is being used increasingly often in patients at high risk of GVHD when they undergo non-haploidentical transplants.

Cyclophosphamide

The effects of cyclophosphamide as a T lymphocyte depletion regimen were first studied in the haploidentical transplant setting (69). Its benefits were then extended to other mismatched, or even matched, donors (70). Cyclophosphamide is used as an agent that depletes donor T lymphocytes *in vivo*. For this purpose, it is administered during the first days of infusing the progenitor cells – the post-transplant-cyclophosphamide (PTCy) strategy. The mechanism by which cyclophosphamide modifies the T lymphocyte response and thereby reduces GVHD has been studied and extrapolated based on murine models of skin graft rejections. However, there has been some disagreement about whether the mechanisms involved are similar.

Nunes et al. developed a murine major histocompatibility complex (MHC)-haploidentical HCT model (B6C3F1 → B6D2F1) that is equivalent to the clinical HCT setting. They described how PTCy, a non-T lymphocyte-cycle-dependent alkylator, affects both highly and lowly proliferative host-alloreactive donor T lymphocytes. After infusion of progenitors, host-alloreactive donor T lymphocytes become activated, proliferative, and give rise to an inflammatory environment. Between post-transplant days +3 and +7, there is continued high-level proliferation of host-alloreactive donor CD8+ effector T lymphocytes and reduced, but continued proliferation of the surviving host-alloreactive donor CD4+ T lymphocytes, both effector and Tregs (70). This time schedule and pattern of proliferation is important for determining when to apply PTCy, because the decrease in host-alloreactive donor CD4+ effector T lymphocyte proliferation is needed to prevent GVHD (71). Around day +5, the functionality of surviving host-alloreactive donor effector T lymphocytes becomes impaired. The severity of this increases over time, and the apparently rapid effect of PTCy is enhanced by preferential reconstitution of donor CD4+ Tregs between days +7 and +21, which suppresses the host-alloreactive donor effector T lymphocytes. Meanwhile, host-non-alloreactive donor T lymphocytes maintain the slow proliferation, so the relative proportion of alloreactive donor T lymphocytes ends up increasing. The dynamics after these first stages might change over time and due to antigenic stimulation.

It has been observed that the regulatory lymphocytes of patients who receive PTCy recover quickly during the post-transplant period; as little as 1 month after the transplant, they are already at levels similar to the baseline of the donor, even when the transplant patient still exhibits lymphopenia (72). In patients receiving PTCy as the sole prophylaxis of GVHD in identical transplants, it has been observed that recipients' TCR level after infusion of the progenitors is lower than that of the donor in the first moments. However, beyond the first 3 months, it begins to resemble the donor's repertoire more closely, and in CMV-positive cases, the number and repertoire increasingly resemble those of the donor (73).

PTCy continues to prove to be one of the most beneficial agents for the control of GVHD and even of relapse. In a prospective multi-centre, randomized phase II clinical trial, regimens of (i) tacrolimus, mycophenolate mofetil, and cyclophosphamide, (ii) tacrolimus, methotrexate, and bortezomib, and (iii) tacrolimus, methotrexate, and maraviroc were compared against standard tacrolimus and methotrexate (74). Only the PTCy-containing regimen resulted in superior GVHD-free (severe acute and chronic), relapse-free survival. However, this benefit might alter when CMV infection appears. As previously stated, an increased CMV infection is associated with Haplo-HSCT receiving PTCy (HaploCy). However, the specific roles of the allograft source and the use of PTCy in CMV infection and disease are unresolved. A recent analysis of patients reported to the Center for International Blood and Marrow Transplant Research (CIBMTR) has addressed this aspect by comparing the cumulative incidence of CMV infection at day 180 in three cohorts: one that had received HaploCy (42%), a second group of sibling SCTs with PTCy (37%) and a third cohort of sibling SCTs with calcineurin inhibitor-based (23%) prophylaxis for AML/ALL/MDS. PTCy,

regardless of donor, was associated with a higher incidence of CMV infection. The study also concluded that CMV infection could negate the cGVHD protective benefit of PTCy (75).

Methotrexate

Methotrexate is an antitumor and immunosuppressive drug. It is a structural analogue of folic acid; it blocks purine synthesis by inhibiting numerous regulatory enzymes. It does not have protumoral activity, unlike alkylating drugs, so it is of particular interest in the context of patients undergoing multiple therapies with potential induction of secondary tumours. It targets the S phase of the cell cycle, which determines that its action is largely confined to highly proliferative cells. In the early post-transplant period, it predominantly acts on highly proliferative alloreactive lymphocytes. Its use in the context of marrow transplantation dates back to 1970, when Donald Thomas described its role in controlling GVHD in dogs (76). Since then, its use has been maintained with dose modifications and optimizations in its combinations. In combination with a calcineurin inhibitor, it has been the standard of care for immunosuppression in myeloablative matched hematopoietic cell transplants.

Calcineurin Inhibitors

Calcineurin inhibitors stop downstream signalling of the T cell receptor (TCR) of naïve and memory T lymphocytes. This makes them highly effective at suppressing alloimmunity after SCT (77). They have undesirable collateral effects on anti-infectious and tumour-protective immunity, and reactivation of latent herpes viruses including CMV is frequent (78).

Anti-Thymocyte Globulin (ATG)

Low-dose ATG in transplants from high-risk alternative donors reduces GVHD and transplant-related death. All four randomized ATG trials undertaken demonstrated protection against GVHD, and three of them found no detrimental effect on survival (79–81). Two ATG formulations, derived from horse and rabbit, have different mechanisms of action, effects on Tregs, and depths of induced lymphopenia.

A direct association has consistently been found in both formulations between the use of ATG and the occurrence of viral infections, particularly CMV. This association has recently been validated (72).

Steroids

Patients being treated with high-dose steroids in the setting of GVHD, had significantly fewer activated CMV-specific T lymphocytes, both CD8+/IFN-γ+ and CD4+/IFN-γ+ at all developmental stages after allo-SCT. Reconstitution of CMV-specific CD4+ and CD8+ activated lymphocytes was observed at +180 days post-transplant, which was 80 days later than what was observed in the non-steroid counterpart (83). This work explains how and why steroid treatment increases the risk of CMV infection in patients who, because of their serological status and graft source, would otherwise not be at high risk of reactivation.

Some other groups have analysed how GVHD treatment affects T lymphocyte functionality. Young patients with active GVHD (based mainly on steroids) do not have adequate levels of activated CMV-specific CD4+and CD8+T lymphocytes, and do not produce IFN-γ and IL-2 (51). The lack of control of CMV reactivation after allogeneic SCT has been shown to respond to an impaired function of antigen-specific CD8+ T lymphocytes, whereas the amount of CMV-specific T lymphocytes does not have such a marked impact (84).

Monitoring CMV Infection

Serological determination of CMV-specific antibodies is important for determining a patient's risk of CMV infection after transplantation (85). However, it is worth noting that in polytransfused patients (as exemplified by many haematological patients awaiting an allogeneic transplant), the serological status may be an artefact of a passive immunization mechanism. Discrepancies in CMV have been observed in up to 29%-33% of patients when this has been analysed (86, 87). Routinely determined serostatus is still currently used as a criterion for estimating CMV reactivation risk before transplant.

Without prophylaxis, the disease of 80% of patients who are serologically positive for CMV would reactivate after allogeneic transplantation. Strategies to prevent the development of CMV disease have emerged in recent decades, based on antiviral prophylaxis and CMV viremia monitoring (before developing the disease), and treatment before the disease causes organ damage (pre-emptive therapy). Serological status is not appropriate for the purpose of detecting CMV infection. In turn, detection of CMV in blood is the recommended strategy for preventing CMV disease (88). It can be detected with pp65 antigen in leukocytes, or by the polymerase chain reaction (PCR) technique, which is more sensitive (89) and therefore the most frequently used. The presence of antigen in peripheral blood or high loads of DNA are both predictive of CMV disease in these patients (90, 91). Likewise, patients who are to receive SCT must receive leukodepleted, filtered and irradiated products.

Treatment Options for CMV Infection: Improving Anti-CMV Reconstitution

To decide the best therapy for each patient, we must consider whether the patient has received antiviral prophylaxis, the risk profile for CMV disease, T lymphocyte reconstitution (both general and CMV-specific), viral load and potential drug resistance (92).

Effective agents to control CMV infection have been notably toxic. The three main drugs used in recent years are ganciclovir/valganciclovir, foscarnet and cidofovir. Ganciclovir is an analogue of nucleoside 2'-deoxyguanosine, which functions as a competitive inhibitor with deoxyguanosine triphosphate (dGTP) used by DNA polymerase of viruses for its replication. Foscarnet reversibly blocks the pyrophosphate-binding site of viral polymerase in a non-competitive manner and inhibits the separation of pyrophosphate from deoxynucleotide triphosphates, 100 times more strongly than its action against cellular DNA polymerase α. Cidofovir, in its active form of

cidofovir diphosphate, prevents viral replication by selectively inhibiting viral DNA polymerases. It also inhibits human DNA polymerases, but up to 600 times weaklier. Ganciclovir induces haematological toxicity, in which neutropenia contributes to the development of opportunistic bacterial and fungal infections (93, 94). Foscarnet and cidofovir cause kidney damage and require strict analytical control that usually requires the patient's admission to hospital to receive the treatment (95, 96). They are often used in the context of pre-emptive therapy, and, in fact, when oral agents such as valganciclovir are used as primary prophylaxis, there is no significant benefit (97). The strategy used to date has therefore been pre-emptive therapy, except in patients with a high risk of CMV disease, for whom alternative strategies are warranted. This scenario might soon change with the introduction of letermovir, which is known to reduce CMV reactivation and decrease all-cause mortality, but without being significantly toxic. In fact, it has performed similarly to the placebo in the phase 3 trials (98).

Letermovir inhibits the CMV DNA terminase complex, which is required for cleavage and encapsidation of the resulting viral DNA, thereby interfering with virion formation and without significant toxicities compared to placebo (98). This is extremely important since both ganciclovir, cidofovir and foscarnet have well known hematologic and renal toxicities that frequently limitate their use. The rationale for its potential benefit in comparison with other drugs is a different therapeutic target, that could overcome the resistance observed in the clinic (92, 99). There is an urgent need of drugs that effectively treat CMV reactivation, both in patients who are refractory to ganciclovir and in those who do not admit additional toxicities induced by the antivirals used. For this reason its use has been approved by some regulatory agencies and in the coming years we will verify the real impact on the clinic of transplant patients, fundamentally.

To improve T lymphocyte recovery and CMV control, strategies that aim to improve thymus function could be key. These include protection of thymic epithelium, thymopoiesis enhancement and increasing the number of T lymphoid precursors (47). These *in vivo* strategies have been highly diverse, including the use of specific lymphokines, growth factors and hormones, among others. On the other hand, cellular therapies have also been developed. Among these, infusion of *ex vivo*-expanded virus-specific cytotoxic T lymphocytes (CMV-VSTs) has been of particular note (100). These specific cytotoxic lymphocytes have been used either with the original donor source or with partially matched donors. In general, this strategy has proved efficacious in post-transplant CMV reactivation and disease (101, 102). There appear to be correlations (based on retrospective studies) between the baseline CD4+ level (the recipient's previous immunity) and the rate and duration of engraftment and treatment success, probably because the CD4+ component is essential for mediating the engraftment and activity of the effector cells (103).

Attempts have been made to vaccinate against CMV, the most recent using techniques being based on DNA vaccines as well as peptide-based CMV conjugated with TLR agonists (104). The most advanced vaccines are those combined from CMV glycoprotein B (gB) with the adjuvant MF59. Its use has been tested in transplant patients to prevent post-transplant CMV disease, and in seronegative pregnant women or adolescents. In the first cohort (phase 2 study) of vaccinated *vs* placebo (105), a decrease in viremia was demonstrated in vaccinated patients proportional to the antibody load generated, although we know that the humoral response constitutes only one line of defence against the CMV, the results were not negligible, and confirmation of efficacy is awaited in phase 3 studies. In the cohort of young women, the vaccine was safe and immunogenic, although with an efficacy of 45% after 2 doses, therefore which was considered insufficient to continue the line of research (105). It is highly likely that the advances in DNA and RNA vaccine technology during 2020 and 2021 will change the spectrum of vaccines, and that the landscape will change in the years to come.

CMV in Other Haematological Settings: CMV Reactivation and Drugs in Haematological Malignancies

CMV and Autologous Stem Cell Transplantation

The role of CMV reactivation or infection has been much less extensively studied in patients receiving an autologous transplant than in those receiving an allogeneic transplant. From the perspective of the treatment of haematological disease, which usually have high short-term mortality rates, CMV reactivations have been studied to determine whether or not the disease will develop. The implications of these reactivations for the immune system in the medium and long term have been considered less important. In fact, the available series confirm that reactivation is a relatively frequent event in patients receiving autologous parental transplants. CMV reactivates in up to 41% of patients during the post-transplant period. Anyway, the rate of CMV disease remains low (106). Those patients receiving high doses of steroids, irradiation, purine analogues or alemtuzumab would require more attention (107).

CMV and Lymphoproliferative Disorders

The immunosuppression observed in many haematological tumours is conditioned by the underlying disease itself, but also fundamentally by the type of treatment used. Lymphoid pathology (acute and chronic) reveals a fundamental involvement of the B and T lymphoid compartments. This creates a tendency to develop viral and fungal infections, as well as infections borne by some intracellular parasites, such as *Pneumocystis jirovecii*. The cellular immunosuppression observed in this group of patients determines many of the prophylaxis strategies, which are sometimes conditioned to the treatment, but in others are more typical of the immune defect that generates the disease.

Thus, trimethoprim/sulfamethoxazole prophylaxis and the use of acyclovir in lymphoproliferative syndromes are quite widespread.

Acute and chronic myeloid pathologies, such as acute leukaemias or myelodysplastic syndromes, involve the myeloid compartment. Neutropenia mainly conditions bacterial and fungal infections. Their long evolution, the use of purine analogues in treatment regimens, or the frequent consolidation

with allogeneic transplantation in this group causes CMV infection to be a concern in more advanced stages of the disease. CMV serostatus and non-relapse mortality rate after transplant are quite well established in acute myeloid leukaemias (108). In turn, recent explorations of series featuring other pathologies such as diffuse large B-cell lymphoma (DLBCL) have not been able to demonstrate such an association (109).

B-cell chronic lymphocytic leukaemias (B-CLL) is the most frequent chronic leukaemia in western countries. It has been suggested that this disease features a CD8+ T lymphocyte expansion that increases as the disease advances. Analysis of specific immune responses with tetrameric CMV-peptide complexes showed that patients exhibiting such an expansion, actually have an increase of CMV-specific CD8+CD45RA +CD27- T lymphocytes. This change is actually specific to CMV-seropositive patients and might reflect the greater effort required to control CMV reactivations (110). Similarly, another analysis of CMV in CLL patients has revealed that the expanded CMV-specific response observed in CLL patients apparently arises with the onset of chemotherapy and stabilizes thereafter (111). Some researchers have called attention to the possible consequence of redirecting autologous CMV-specific cytotoxic T lymphocytes (CTLs) towards B-CLL cells for cancer immunotherapy (112). CMV infection in patients with CLL usually occurs in the context of treatment with purine inhibitors, alemtuzumab, or even alkylating agents such as chlorambucil, and the disease itself. Of all the mechanisms involved in CLL immunosuppression, which falls beyond the scope of this review, hypogammaglobulinemia seems to be of great importance. It has been thoroughly described in CLL and has recently been associated with a shorter time until the next treatment (113).

CMV and Purine Analogues

All purine analogues mimic metabolic purines. Of them, fludarabine is the most extensively used in hematologic cancer. Fludarabine inhibits multiple DNA polymerases, DNA primase, and DNA ligase I, and is S phase-specific (since these enzymes are highly active during DNA replication). Fludarabine acts particularly on T cells, and is therefore very toxic to this compartment.

Normal T lymphocyte function is essential to the control of CMV reinfection. The use of agents such as purine analogues, that have a very potent T lymphocyte suppressor profile while being highly effective for treating chronic lymphoproliferative disorders (114), are highly relevant to the development of CMV reactivations.

Cases of CMV retinitis have been documented in patients with low-grade lymphomas treated with rituximab, fludarabine and steroids (115). Previous serological status was not available for most patients, probably because there is not a specific approach with CMV seropositivity outside transplant scenarios. Despite being a very rare event even in the seropositive population, the sequelae were adverse (blindness) in 86% of patients. CMV monitoring, and clinical observation of possible infection and reactivation, must be considered when these regimens are used.

CMV and Alemtuzumab

Alemtuzumab is a monoclonal antibody which acts on the CD52 protein found on the surface of peripheral blood and lymph node lymphocytes.

CMV reactivations in patients receiving alemtuzumab treatment for CLL are common, with a rate of around 20% in the phase II study with the drug that was evaluated in 78 patients (116). However, viral load monitoring and pre-emptive treatment manages to prevent the disease in most cases (117).

Some studies have even proposed the use of primary prophylaxis with valganciclovir in patients receiving alemtuzumab in whom the drug has a high efficacy (118). However, the main concern with using prophylactic valganciclovir is the additional myelosuppression beyond the already significant amount produced by the alemtuzumab-based regimens themselves. An attempt to reduce cost and toxicity by using lower weekly doses (119) produced lower toxicity and acceptable efficacy. However, due to the aforementioned concerns, primary prophylaxis is not common practice in this group of patients.

CMV and New Agents in the Treatment of Lymphoproliferative Syndromes

Many drugs have been introduced into the therapeutic arsenal to treat lymphoproliferative syndromes, in particular, CLL. However, CMV seropositivity in the era of new therapies has not led to reduced survival or relapse in CLL patients when prospectively compared with seronegative patients (120).

Bruton Tyrosine Kinase Inhibitors

CMV infection is not common in the case of a BTK inhibitor such as ibrutinib, although some cases have been reported (121). Although these are infrequent, we should always be aware of this possibility among immunocompromised patients, particularly those who have been treated with new agents, because this is a curable condition and early therapy seems to be critical if a good outcome is to be achieved.

PI3K Inhibitors

Recommendations for the management and prophylaxis of CMV reactivation have been established in patients treated with idelalisib. These patients can develop a serious infectious disease, with a high risk of CMV reactivation and the involvement of other opportunistic germs (122). It has been shown how idelalisib impairs IFN-γ production by activating T lymphocytes in CLL-treated patients, highlighting the importance of PI3Kδ in this process. Idelalisib inhibits T lymphocytes in relation to generic TCR stimulation and in response to virus-specific stimulation. The CD4+ and CD8+ T lymphocyte subsets both seem to be affected. This might explain the higher rate of CMV reactivations in those CLL patients who are treated with it. Finally, idelalisib has also been shown to alter T lymphocyte migration *in vitro* (123).

The high risk of CMV reactivation in this therapy group has meant that the guidelines have been adapted to those for the use of alemtuzumab (124). CMV-seronegative patients should receive CMV-negative or filtered blood products (this is blood,

platelet, and plasma transfusions. Progenitor cell transplantation products are not irradiated due to the need to maintain the viability of the infused cells). Seropositive patients should be periodically tested for CMV. Idelalisib should be discontinued and ganciclovir or valganciclovir pre-emptively initiated in patients with positive CMV PCR/antigen and symptoms consistent with CMV infection, as well as in patients with fever and no clear source, and for whom quantitative CMV testing is unavailable, and in asymptomatic patients with a rising viral load (125).

Proteasome Inhibitors

Bortezomib and the next-generation proteasome inhibitors have been cornerstones of multiple myeloma (MM) treatment for several years. Although sufficiently relevant to be considered one of the causes of the increase in survival in this group of patients, they have also entailed infectious risks that should be highlighted. Bortezomib was reported to increase the risk of varicella-zoster virus (VZV) reactivation by up to four times (126), and a high incidence of CMV reactivation in fit patients receiving autologous transplantation due MM after treatment with bortezomib-based regimens was recently reported. Of 80 patients who underwent autologous SCT after bortezomib-based therapies, seven (4.1%) received an antiviral treatment for a symptomatic CMV reactivation and one died (127). Although no specific cause has yet been determined, *in vitro* studies have demonstrated that bortezomib can reduce proliferation, number, and function of natural killer cells and CD8+/CD4+ T and alter the cytokine profile, in particular decreasing Th1 cytokine secretion (128).

In the setting of allogeneic transplantation, bortezomib induces preferential apoptosis among alloreactive T lymphocytes (decreasing Th1 response) by inhibiting nuclear factor-κB (NF-κB) activation, whilst unstimulated T lymphocytes are barely affected. This might explain its potential use as a drug for GVHD treatment.

All these mechanisms could help explain the increased incidence of Herpesviridae family viruses (of which VZV is the best known) in MM patients and might contribute to the increased susceptibility to CMV reactivation in MM patients treated with bortezomib-based regimens followed by ASCT (129).

CMV and CAR-T-Cells

One of the most significant innovations in recent years has been the treatment with the CD19-directed chimerical antigen receptor T lymphocyte. Currently, data regarding the rate and type of viral infections in patients receiving this treatment are scarce. However, there are already some data on the incidence of CMV infections and reactivation in real clinical practice. In a retrospective series of 60 patients with aggressive lymphoma treated with CAR-T, who received antiviral prophylaxis for herpes simplex virus (HSV) according to the recommendations of each hospital, 10 viral infections were documented in the first 30 days, of which two corresponded to CMV reactivations, without organ affectation. During days 31-180, another reactivation by CMV occurred, again without organ disease. None of them, therefore, compromised the life of the patient (130).

Considering now the patients with ALL, data were collected from 83 patients up to 21 years of age who had received CAR-T therapy for this reason (131). Sixteen of them developed viral infections within the first 28 days; these were caused mainly by respiratory viruses, without specifying infection by CMV in these reported cases. Between days 29 and 90, seven patients got viral infections, all of which were due to respiratory viruses.

There are documented cases of CMV reactivation in the first month and during the first three months. Previous therapies, disease stage and patient basal characteristics seem to be crucial.

Regarding prophylaxis against viral infections, there are no unique international recommendations, and these recommendations are heterogeneous (132). The European recommendations are based on data from allogeneic transplant recipients (133). In general, antiviral prophylaxis is established with acyclovir or valacyclovir at least up to one year after CAR-T infusion, or until a CD4+ T lymphocyte level greater than 0.2 x 10^9/L is documented (134). In paediatrics, nonspecific immunoglobulins are also frequently used to maintain total IgG levels above 0.4 g/L (135). With respect to monitoring, the EBMT guidelines recommend that PCR be performed when clinically indicated (133).

CONCLUSIONS

The relationships between CMV infection and haematological pathologies are well known. Fundamentally, as a result of the important repercussions from the management of the infection and reactivation of the virus in the post-allogeneic transplant. However, there are many other situations that give rise to severe immunosuppression, either due to the haematological pathology itself or to the treatments used, which should increase vigilance concerning the complications derived from infection by this virus. Thus, it is necessary to study the effect of new drugs on the immune system and so adapt CMV prophylaxis and infection monitoring to different treatment schemes and situations, now that new anti-CMV drugs with fewer secondary effects are available for this purpose.

In contrast, knowledge about the role of this virus in the development of haematological diseases, in other words, its oncogenic or oncomodulatory potential, is much more limited. The difficulty in finding it may be because CMV infection is associated with age and the fact that its main effect is to bring about the dysfunction of T lymphocytes. T cell lymphomas are quite rare. The search for causality in the more commonly occurring counterpart B-cell lymphomas involves looking for indirect causes arising from the basic relationship between T and B lymphocytes.

Living with this herpesvirus is a situation that has arisen from years of evolution, which has apparently produced a balanced and tolerable relationship. This would imply that its effect, does not directly or indirectly limit survival or favour oncogenesis. In the forthcoming era of medicine we are approaching, there will

be infinite possibilities for producing antiviral agents with very low toxicity and for immunizing against the most prevalent microorganisms. These promising possibilities should inspire an exhaustive study of the real effects of CMV and other microorganisms on the oncogenesis and mortality of individuals and populations. It seems clear that the ultimate answer about the oncogenic role of the virus will come from a prospective design approach that will allow us to determine whether human beings who are not infected have a lower risk of developing haematological neoplasms or other conditions than those who are infected.

AUTHOR CONTRIBUTIONS

The authors' contributions were as follows: SA-Á and EC designed the study, SA-Á and EC wrote the manuscript, and MM-G and RA-A reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Staras SAS, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of Cytomegalovirus Infection in the United States, 1988-1994. *Clin Infect Dis* (2006) 43(9):1143–51. doi: 10.1086/508173
2. Sinclair J, Sissons P. Latency and Reactivation of Human Cytomegalovirus. *J Gen Virol* (2006) 87(7):1763–79. doi: 10.1099/vir.0.81891-0
3. Mendelson M, Monard S, Sissons P, Sinclair J. Detection of Endogenous Human Cytomegalovirus in CD34+ Bone Marrow Progenitors. *J Gen Virol* (1996) 77(12):3099–102. doi: 10.1099/0022-1317-77-12-3099
4. Hahn G, Revello MG, Patrone M, Percivalle E, Campanini G, Sarasini A, et al. Human Cytomegalovirus UL131-128 Genes Are Indispensable for Virus Growth in Endothelial Cells and Virus Transfer to Leukocytes. *J Virol* (2004) 78(18):10023–33. doi: 10.1128/jvi.78.18.10023-10033.2004
5. Maidji E, Somsouk M, Rivera JM, Hunt PW, Stoddart CA. Replication of CMV in the Gut of HIV-Infected Individuals and Epithelial Barrier Dysfunction. *PloS Pathog* (2017) 13(2). doi: 10.1371/journal.ppat.1006202
6. Jarvis MA, Nelson JA. Human Cytomegalovirus Persistence and Latency in Endothelial Cells and Macrophages. *Curr Opin Microbiol* (2002) 5(4):403–7. doi: 10.1016/S1369-5274(02)00334-X
7. Fortunato EA, Sanchez V, Yen JY, Spector DH. Infection of Cells with Human Cytomegalovirus during S Phase Results in a Blockade to Immediate-Early Gene Expression That Can Be Overcome by Inhibition of the Proteasome. *J Virol* (2002) 76(11):5369–79. doi: 10.1128/jvi.76.11.5369-5379.2002
8. Stenberg RM, Fortney J, Barlow SW, Magrane BP, Nelson JA, Ghazal P. Promoter-Specific Trans Activation and Repression by Human Cytomegalovirus Immediate-Early Proteins Involves Common and Unique Protein Domains. *J Virol* (1990) 64(4):1556–65. doi: 10.1128/jvi.64.4.1556-1565.1990
9. Sissons JGP, Bain M, Wills MR. Latency and Reactivation of Human Cytomegalovirus. *J Infect* (2002) 44(2):73–7. doi: 10.1053/jinf.2001.0948
10. Sinclair J, Sissons P. Latent and Persistent Infections of Monocytes and Macrophages. *Intervirology* (1996) 39(5-6):293–301. doi: 10.1159/000150501
11. Sinclair J, Baillie J, Bryant L, Caswell R. Human Cytomegalovirus Mediates Cell Cycle Progression Through G1 Into Early S Phase in Terminally Differentiated Cells. *J Gen Virol* (2000) 81(Pt 6):1553–65. doi: 10.1099/0022-1317-81-6-1553
12. Baskar JF, Smith PP, Nilaver G, Jupp RA, Hoffmann S, Peffer NJ, et al. The Enhancer Domain of the Human Cytomegalovirus Major Immediate-Early Promoter Determines Cell Type-Specific Expression in Transgenic Mice. *J Virol* (1996) 70(5):3207–14. doi: 10.1128/jvi.70.5.3207-3214.1996
13. Meier JL, Keller MJ, McCoy JJ. Requirement of Multiple cis-Acting Elements in the Human Cytomegalovirus Major Immediate-Early Distal Enhancer for Viral Gene Expression and Replication. *J Virol* (2002) 76(1):313–26. doi: 10.1128/jvi.76.1.313-326.2002
14. Nielsen SL, Sorensen I, Andersen HK. Kinetics of Specific Immunoglobulins M, E, A, and G in Congenital, Primary, and Secondary Cytomegalovirus Infection Studied by Antibody-Capture Enzyme-Linked Immunosorbent Assay. *J Clin Microbiol* (1988) 26(4):654–61. doi: 10.1128/jcm.26.4.654-661.1988
15. Terrazzini N, Kern F. Cell-Mediated Immunity to Human CMV Infection: A Brief Overview. *F1000Prime Rep* (2014) 6:28. doi: 10.12703/P6-28
16. Almanzar G, Schwaiger S, Jenewein B, Keller M, Herndler-Brandstetter D, Würzner R, et al. Long-Term Cytomegalovirus Infection Leads to Significant Changes in the Composition of the CD8+ T-Cell Repertoire, Which May Be the Basis for an Imbalance in the Cytokine Production Profile in Elderly Persons. *J Virol* (2005) 79(6):3675–83. doi: 10.1128/jvi.79.6.3675-3683.2005
17. Moro-García MA, López-Iglesias F, Marcos-Fernández R, Bueno-García E, Díaz-Molina B, Lambert JL, et al. More Intensive CMV-Infection in Chronic Heart Failure Patients Contributes to Higher T-Lymphocyte Differentiation Degree. *Clin Immunol* (2018) 192:20–9. doi: 10.1016/j.clim.2018.03.015
18. Hart H, Neill WA, Norval M. Lack of Association of Cytomegalovirus With Adenocarcinoma of the Colon. *Gut* (1982) 23(1):21–30. doi: 10.1136/gut.23.1.21
19. Boguszaková L, Hirsch I, Brichácek B, Faltýn J, Fric P, Dvoráková H, et al. Absence of Cytomegalovirus, Epstein-Barr Virus, and Papillomavirus DNA From Adenoma and Adenocarcinoma of the Colon. *Acta Virol* (1988) 32 (4):303–8.
20. Bricháček B, Hirsch I, Závadová H, Procházka M, Faltýn J, Vonka V. Absence of Cytomegalovirus DNA from Adenocarcinoma of the Colon. *Intervirology* (1980) 14:223–7. doi: 10.1159/000149187
21. Huang ES, Roche JK. Cytomegalovirus DNA and Adenocarcinoma of the Colon: Evidence for Latent Viral Infection. *Lancet* (1978) 311:957–60. doi: 10.1016/S0140-6736(78)90248-9
22. Krishna BA, Miller WE, O'Connor CM. US28: HCMV's Swiss Army Knife. *Viruses* (2018) 10(8):445. doi: 10.3390/v10080445
23. Maussang D, Verzijl D, Van Walsum M, Leurs R, Holl J, Pleskoff O, et al. Human Cytomegalovirus-Encoded Chemokine Receptor US28 Promotes Tumorigenesis. *Proc Natl Acad Sci USA* (2006) 103:13068–73. doi: 10.1073/pnas.0604433103
24. Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, et al. Sensitive Detection of Human Cytomegalovirus in Tumors and Peripheral Blood of Patients Diagnosed With Glioblastoma. *Neuro Oncol* (2008) 10:10–8. doi: 10.1215/15228517-2007-035
25. Dziurzynski K, Chang SM, Heimberger AB, Kalejta RF, Dallas SRMG, Smit M, et al. Consensus on the Role of Human Cytomegalovirus in Glioblastoma. *Neuro Oncol* (2012) 14:246–55. doi: 10.1093/neuonc/nor227
26. Stragliotto G, Rahbar A, Solberg NW, Lilja A, Taher C, Orrego A, et al. Effects of Valganciclovir as an add-on Therapy in Patients with Cytomegalovirus-Positive Glioblastoma: A Randomized, Double-Blind, Hypothesis-Generating Study. *Int J Cancer* (2013) 133:1204–13. doi: 10.1002/ijc.28111

27. Söderberg-Naucler C, Peredo I, Rahbar A, Hansson F, Nordlund A, Stragliotto G. Use of Cox Regression With Treatment Status as a Time-Dependent Covariate to Re-Analyze Survival Benefit Excludes Immortal Time Bias Effect in Patients With Glioblastoma Who Received Prolonged Adjuvant Treatment With Valganciclovir. *Int J Cancer* (2014) 135:248–9. doi: 10.1002/ijc.28663
28. Hadaczek P, Ozawa T, Soroceanu L, Yoshida Y, Matlaf L, Singer E, et al. Cidofovir: A Novel Antitumor Agent for Glioblastoma. *Clin Cancer Res* (2013) 19:6473–83. doi: 10.1158/1078-0432.CCR-13-1121
29. Prins RM, Cloughesy TF, Liau LM. Cytomegalovirus Immunity after Vaccination with Autologous Glioblastoma Lysate. *N Engl J Med* (2008) 359:539–41. doi: 10.1056/nejmc0804818
30. Rahman M, Dastmalchi F, Karachi A, Mitchell D. The role of CMV in Glioblastoma And Implications for Immunotherapeutic Strategies. *Oncoimmunology* (2019) 8(1):e1514921. doi: 10.1080/2162402X.2018.1514921
31. Deacon EM, Pallesen G, Niedobitek G, Crocker J, Brooks L, Rickinson AB, et al. Epstein-Barr Virus and Hodgkin's Disease: Transcriptional Analysis of Virus Latency in the Malignant Cells. *J Exp Med* (1993) 177:339–49. doi: 10.1084/jem.177.2.339
32. Vockerodt M, Yap LF, Shannon-Lowe C, Curley H, Wei W, Vrzalikova K, et al. The Epstein-Barr virus and the Pathogenesis of Lymphoma. *J Pathol* (2015) 235:312–22. doi: 10.1002/path.4459
33. Shannon-Lowe C, Rickinson AB, Bell AI. Epstein-Barr Virus-Associated Lymphomas. *Philos Trans R Soc B Biol Sci* (2017) 372(1732):20160271. doi: 10.1098/rstb.2016.0271
34. Murray PG, Young LS. An Etiological Role for the Epstein-Barr Virus in the Pathogenesis of Classical Hodgkin Lymphoma. *Blood* (2019) 134:591–6. doi: 10.1182/blood.2019000568
35. Qu L, Rowe DT. Epstein-Barr Virus Latent Gene Expression in Uncultured Peripheral Blood Lymphocytes. *J Virol* (1992) 66:3715–24. doi: 10.1128/jvi.66.6.3715-3724.1992
36. Tierney RJ, Steven N, Young LS, Rickinson AB. Epstein-Barr Virus Latency in Blood Mononuclear Cells: Analysis of Viral Gene Transcription During Primary Infection and in the Carrier State. *J Virol* (1994) 68:7374–85. doi: 10.1128/jvi.68.11.7374-7385.1994
37. Chen H, Smith P, Ambinder RF, Hayward SD. Expression of Epstein-Barr virus BamHI-A Rightward Transcripts in Latently Infected B cells From Peripheral Blood. *Blood* (1999) 93(9):3026–32.
38. Gupta RK, Ramble J, Tong CY, Whittaker S, MacMahon E. Cytomegalovirus Seroprevalence is Not Higher in Patients With Mycosis Fungoides/Sezary Syndrome. *Blood* (2006) 107(3):1241–2.
39. Ballanger F, Bressollette C, Volteau C, Planche L, Dreno B. Cytomegalovirus: Its Potential Role in the Development of Cutaneous T-cell Lymphoma. *Exp Dermatol* (2009) 18:574–6. doi: 10.1111/j.1600-0625.2008.00817.x
40. Herne KL, Talpur R, Breuer-McHam J, Champlin R, Duvic M. Cytomegalovirus Seropositivity is Significantly Associated With Mycosis Fungoides and Sézary Syndrome. *Blood* (2003) 101:2132–6. doi: 10.1182/blood-2002-07-2247
41. Mehravaran H, Makvandi M, Zade AS, Neisi N, Kiani H, Radmehr H, et al. Association of Human Cytomegalovirus With Hodgkin's Disease and Non-Hodgkin's Lymphomas. *Asian Pacific J Cancer Prev* (2017) 18:593–7. doi: 10.22034/APJCP.2017.18.3.593
42. Ljungman P, De Bock R, Cordonnier C, Einsele H, Engelhard D, Grundy J, et al. Practices for Cytomegalovirus Diagnosis, Prophylaxis and Treatment In Allogeneic Bone Marrow Transplant Recipients: A Report From the Working Party for Infectious Diseases of the EBMT. *Bone Marrow Transplant* (1993) 12(4):399–403.
43. Reusser P, Cordonnier C, Einsele H, Engelhard D, Link D, Locasciulli A, et al. European Survey of Herpesvirus Resistance to Antiviral Drugs in Bone Marrow Transplant Recipients. Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation (EBMT). *Bone Marrow Transplant* (1996) 17(5):813–7.
44. Styczyński J, Tridello G, Koster L, Iacobelli S, van Biezen A, van der Werf S, et al. Death After Hematopoietic Stem Cell Transplantation: Changes Over Calendar Year Time, Infections and Associated Factors. *Bone Marrow Transplant* (2020) 55(1):126–36. doi: 10.1038/s41409-019-0624-z
45. Nava T, Ansari M, Dalle JH, de Heredia CD, Güngör T, Trigoso E, et al. Supportive Care During Pediatric Hematopoietic Stem Cell Transplantation: Beyond Infectious Diseases. A Report From Workshops on Supportive Care of the Pediatric Diseases Working Party (PDWP) of the European Society for Blood and Marrow Transplantation (EBMT). *Bone Marrow Transplant* (2020) 55(6):1126–36. doi: 10.1038/s41409-020-0818-4
46. Ringhoffer S, Rojewski M, Döhner H, Bunjes D, Ringhoffer M. T-Cell Reconstitution After Allogeneic Stem Cell Transplantation: Assessment by Measurement of the sjTREC/βTREC Ratio and Thymic Naïve T Cells. *Haematologica* (2013) 98:1600–8. doi: 10.3324/haematol.2012.072264
47. Clave E, Busson M, Douay C, De Latour RP, Berrou J, Rabian C, et al. Acute Graft-Versus-Host Disease Transiently Impairs Thymic Output in Young Patients After Allogeneic Hematopoietic Stem Cell Transplantation. *Blood* (2009) 113:6477–84. doi: 10.1182/blood-2008-09-176594
48. Mackall CL, Fleisher TA, Brown MR, Andrich MP, Chen CC, Feuerstein IM, et al. Distinctions Between CD8+ and CD4+ T-Cell Regenerative Pathways Result In Prolonged T-Cell Subset Imbalance After Intensive Chemotherapy. *Blood* (1997) 89(10):3700–7.
49. Forman SJ, Nöcker P, Gallagher M, Zaia J, Wright C, Bolen J, et al. Pattern of T Cell Reconstitution Following Allogeneic Bone Marrow Transplantation For Acute Hematological Malignancy. *Transplantation* (1982) 34:96–8. doi: 10.1097/00007890-198208000-00007
50. Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T-Cell Regeneration After Bone Marrow Transplantation: Differential CD45 Isoform Expression on Thymic-Derived Versus Thymic-Independent Progeny. *Blood* (1993) 82(8):2585–94.
51. Yew PY, Alachkar H, Yamaguchi R, Kiyotani K, Fang H, Yap KL, et al. Quantitative Characterization of T-Cell Repertoire in Allogeneic Hematopoietic Stem Cell Transplant Recipients. *Bone Marrow Transplant* (2015) 50:1227–34. doi: 10.1038/bmt.2015.133
52. Van Heijst JWJ, Ceberio I, Lipuma LB, Samilo DW, Wasilewski GD, Gonzales AMR, et al. Quantitative Assessment of T Cell Repertoire Recovery After Hematopoietic Stem Cell Transplantation. *Nat Med* (2013) 19:372–7. doi: 10.1038/nm.3100
53. Mehta RS, Rezvani K. Immune reconstitution post allogeneic transplant and the impact of immune recovery on the risk of infection. *Virulence* (2016) 7:901–16. doi: 10.1080/21505594.2016.1208866
54. Pera A, Vasudev A, Tan C, Kared H, Solana R, Larbi A. CMV Induces Expansion of Highly Polyfunctional CD4+ T cell Subset Coexpressing CD57 and CD154. *J Leukoc Biol* (2017) 101(2):555–66. doi: 10.1189/jlb.4A0316-112R
55. Jeitziner SM, Walton SM, Torti N, Oxenius A. Adoptive Transfer of Cytomegalovirus-Specific Effector CD4+ T Cells Provides Antiviral Protection From Murine CMV Infection. *Eur J Immunol* (2013) 43 (11):2886–95. doi: 10.1002/eji.201343690
56. Lilleri D, Gerna G, Zelini P, Chiesa A, Rognoni V, Mastronuzzi A, et al. Monitoring of Human Cytomegalovirus and Virus-Specific T-Cell Response In Young Patients Receiving Allogeneic Hematopoietic Stem Cell Transplantation. *PloS One* (2012) 7(7):e41648. doi: 10.1371/journal.pone.0041648
57. Abate D, Cesaro S, Cofano S, Fiscon M, Saldan A, Varotto S, et al. Diagnostic Utility Of Human Cytomegalovirus-Specific T-Cell Response Monitoring in Predicting Viremia in Pediatric Allogeneic Stem-Cell Transplant Patients. *Transplantation* (2012) 93:536–42. doi: 10.1097/TP.0b013e31824215db
58. Sellar RS, Vargas FA, Henry JY, Verfuerth S, Charrot S, Beaton B, et al. CMV Promotes Recipient T-Cell Immunity Following Reduced-Intensity T-Cell-Depleted HSCT, Significantly Modulating Chimerism Status. *Blood* (2015) 125(4):731–9. doi: 10.1182/blood-2014-07-589150
59. Suessmuth Y, Mukherjee R, Watkins B, Koura DT, Finstermeier K, Desmarais C, et al. CMV Reactivation Drives Posttransplant T-Cell Reconstitution and Results in Defects in the Underlying TCRβ Repertoire. *Blood* (2015) 125(25):3835–50. doi: 10.1182/blood-2015-03-631853
60. Crisinel PA, Duval M, Crisinel DT, Mallette B, Bellier N, Vachon MF, et al. Risk of Cytomegalovirus Infection and Disease After Umbilical Cord Blood Transplantation in Children. *Can J Infect Dis Med Microbiol* (2013) 24(1): e11–5. doi: 10.1155/2013/159691
61. Godfrey WR, Spoden DJ, Ge YG, Baker SR, Liu B, Levine BL, et al. Cord blood CD4+CD25+-Derived T Regulatory Cell Lines Express FoxP3 Protein and Manifest Potent Suppressor Function. *Blood* (2005) 105:750–8. doi: 10.1182/blood-2004-06-2467
62. Cornetta K, Laughlin M, Carter S, Wall D, Weinthal J, Delaney C, et al. Umbilical Cord Blood Transplantation in Adults: Results of the Prospective

Cord Blood Transplantation (COBLT). *Biol Blood Marrow Transplant* (2005) 11:149–60. doi: 10.1016/j.bbmt.2004.11.020
63. Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H. Improved Immune Reconstitution After Allotransplantation of Peripheral Blood Stem Cells Instead of Bone Marrow. *Blood* (1996) 88(7):2775–9.
64. Roberts MM, To LB, Gillis D, Mundy J, Rawling C, Ng K, et al. Immune Reconstitution Following Peripheral Blood Stem Cell Transplantation, Autologous Bone Marrow Transplantation and Allogeneic Bone Marrow Transplantation. *Bone Marrow Transplant* (1993) 12(5):469–75.
65. Trenschel R, Ross S, Hüsing J, Ottinger H, Elmaagacli A, Roggendorf M, et al. Reduced Risk of Persisting Cytomegalovirus pp65 Antigenemia and Cytomegalovirus Interstitial Pneumonia Following Allogeneic PBSCT. *Bone Marrow Transplant* (2000) 25:665–72. doi: 10.1038/sj.bmt.1702216
66. Tong J, Sun Z, Liu H, Geng L, Zheng C, Tang B, et al. Risk Factors of CMV Infection in Patients After Umbilical Cord Blood Transplantation: A Multicenter Study in China. *Chin J Cancer Res* (2013) 25:695–703. doi: 10.3978/j.issn.1000-9604.2013.11.08
67. Huntley D, Giménez E, Pascual MJ, Remigia MJ, Amat P, Vázquez L, et al. Reconstitution of Cytomegalovirus-Specific T-Cell Immunity Following Unmanipulated Haploidentical Allogeneic Hematopoietic Stem Cell Transplantation With Posttransplant Cyclophosphamide. *Bone Marrow Transplant* (2020) 55(7):1347–56. doi: 10.1038/s41409-020-0865-x
68. Yu J, Parasuraman S, Shah A, Weisdorf D. Mortality, Length of Stay and Costs Associated With Acute Graft-Versus-Host Disease During Hospitalization For Allogeneic Hematopoietic Stem Cell Transplantation. *Curr Med Res Opin* (2019) 35(6):983–8. doi: 10.1080/03007995.2018.1551193
69. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-Haploidentical Bone Marrow Transplantation for Hematologic Malignancies Using Nonmyeloablative Conditioning and High-Dose, Posttransplantation Cyclophosphamide. *Biol Blood Marrow Transplant* (2008) 14:641–50. doi: 10.1016/j.bbmt.2008.03.005
70. Nunes NS, Kanakry CG. Mechanisms of Graft-Versus-Host Disease Prevention by Post-Transplantation Cyclophosphamide: An Evolving Understanding. *Front Immunol* (2019) 10:2668. doi: 10.3389/fimmu.2019.02668
71. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Kanakry CG. Optimized Timing of Post-Transplantation Cyclophosphamide in MHC-Haploidentical Murine Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant* (2020) 26:230–41. doi: 10.1016/j.bbmt.2019.09.030
72. Kanakry CG, Ganguly S, Zahurak M, Bolaños-Meade J, Thoburn C, Perkins B, et al. Aldehyde Dehydrogenase Expression Drives Human Regulatory T Cell Resistance to Posttransplantation Cyclophosphamide. *Sci Transl Med* (2013) 5(211):211ra157. doi: 10.1126/scitranslmed.3006960
73. Kanakry CG, Coffey DG, Towlerton AM, Vulic A, Storer BE, Chou J, et al. Origin and Evolution of the T Cell Repertoire After Posttransplantation Cyclophosphamide. *JCI Insight* (2016) 1(5):e86252. doi: 10.1172/jci.insight.86252
74. Bolaños-Meade J, Reshef R, Fraser R, Fei M, Abhyankar S, Al-Kadhimi Z, et al. Three Prophylaxis Regimens (Tacrolimus, Mycophenolate Mofetil, and Cyclophosphamide; Tacrolimus, Methotrexate, and Bortezomib; or Tacrolimus, Methotrexate, and Maraviroc) Versus Tacrolimus and Methotrexate For Prevention of Graft-Versus-Host Disease With Haemopoietic Cell Transplantation With Reduced-Intensity Conditioning: A Randomised Phase 2 Trial With a Non-Randomised Contemporaneous Control Group (BMT CTN 1203). *Lancet Haematol* (2019) 6:e132–43. doi: 10.1016/S2352-3026(18)30221-7
75. Goldsmith SR, Abid MB, Auletta JJ, Bashey A, Beitinjaneh A, Castillo P, et al. Posttransplant Cyclophosphamide is Associated With Increased Cytomegalovirus Infection: A CIBMTR Analysis. *Blood* (2021) 137(23):3291–305. doi: 10.1182/blood.2020009362
76. Storb R, Epstein RB, Graham TC, Thomas ED. Methotrexate Regimens For Control of Graft-Versus-Host Disease in Dogs With Allogeneic Marrow Grafts. *Transplantation* (1970) 9(3):240–6. doi: 10.1097/00007890-197003000-00007
77. Ekberg H, Bernasconi C, Tedesco-Silva H, Vítko S, Hugo C, Demirbas A, et al. Calcineurin Inhibitor Minimization in The Symphony Study: Observational Results 3 Years After Transplantation. *Am J Transplant* (2009) 9:1876–85. doi: 10.1111/j.1600-6143.2009.02726.x
78. Fisher RA. Cytomegalovirus Infection and Disease in the New Era of Immunosuppression Following Solid Organ Transplantation. *Transpl Infect Dis* (2009) 11:195–202. doi: 10.1111/j.1399-3062.2009.00372.x
79. Bacigalupo A, Lamparelli T, Barisione G, Bruzzi P, Guidi S, Alessandrino PE, et al. Thymoglobulin Prevents Chronic Graft-versus-Host Disease, Chronic Lung Dysfunction, and Late Transplant-Related Mortality: Long-Term Follow-Up of a Randomized Trial in Patients Undergoing Unrelated Donor Transplantation. *Biol Blood Marrow Transplant* (2006) 12:560–5. doi: 10.1016/j.bbmt.2005.12.034
80. Socié G, Schmoor C, Bethge WA, Ottinger HD, Stelljes M, Zander AR, et al. Chronic Graft-Versus-Host Disease: Long-Term Results From A Randomized Trial On Graft-Versus-Host Disease Prophylaxis With or Without Anti-T-cell Globulin ATG-Fresenius. *Blood* (2011) 117:6375–82. doi: 10.1182/blood-2011-01-329821
81. Kröger N, Solano C, Wolschke C, Bandini G, Patriarca F, Pini M, et al. Antilymphocyte Globulin for Prevention of Chronic Graft-Versus-Host Disease. *N Engl J Med* (2016) 374:43–53. doi: 10.1056/nejmoa1506002
82. Figgins B, Hammerstrom A, Ariza-Heredia E, Oran B, Milton DR, Yeh J. Characterization of Viral Infections after Antithymocyte Globulin–Based Conditioning in Adults Undergoing Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2019) 25:1837–43. doi: 10.1016/j.bbmt.2019.05.020
83. Suárez-Lledó M, Martínez-Cibrián N, Gutiérrez-García G, Dimova-Svetoslavova V, Marcos MA, Martín-Antonio B, et al. Deleterious Effect of Steroids on Cytomegalovirus Infection Rate after Allogeneic Stem Cell Transplantation Depends on Pretransplant Cytomegalovirus Serostatus of Donors and Recipients. *Biol Blood Marrow Transplant* (2018) 24:2088–93. doi: 10.1016/j.bbmt.2018.05.001
84. Özdemir E, St John LS, Gillespie G, Rowland-Jones S, Champlin RE, Molldrem JJ, et al. Cytomegalovirus Reactivation Following Allogeneic Stem Cell Transplantation is Associated With the Presence of Dysfunctional Antigen-Specific CD8+ T Cells. *Blood* (2002) 100:3690–7. doi: 10.1182/blood-2002-05-1387
85. Ljungman P, Hakki M, Boeckh M. Cytomegalovirus in Hematopoietic Stem Cell Transplant Recipients. *Hematol Oncol Clin North Am* (2011) 25(1):151–69. doi: 10.1016/j.hoc.2010.11.011
86. Perry DA, Hakki M. Assessment of a Cytomegalovirus Serology Dual-Testing Strategy in Hematopoietic Stem Cell Transplant Recipients. *Transpl Infect Dis* (2016) 18:809–14. doi: 10.1111/tid.12591
87. Giulieri S, Manuel O. QuantiFERON®-CMV Assay for the Assessment of Cytomegalovirus Cell-Mediated Immunity. *Expert Rev Mol Diagn* (2011) 11:17–25. doi: 10.1586/erm.10.109
88. Meyers JD, Ljungman P, Fisher LD. Cytomegalovirus Excretion as a Predictor of Cytomegalovirus Disease After Marrow Transplantation: Importance of Cytomegalovirus Viremia. *J Infect Dis* (1990) 162(2):373–80. doi: 10.1093/infdis/162.2.373
89. Boeckh M, Huang M, Ferrenberg J, Stevens-Ayers T, Stensland L, Nichols WG, et al. Optimization of Quantitative Detection of Cytomegalovirus DNA in Plasma by Real-Time PCR. *J Clin Microbiol* (2004) 42(3):1142–8. doi: 10.1128/JCM.42.3.1142-1148.2004
90. Boeckh M, Bowden RA, Goodrich JM, Pettinger M, Meyers JD. Cytomegalovirus Antigen Detection in Peripheral Blood Leukocytes After Allogeneic Marrow Transplantation. *Blood* (1992) 80(5):1358–64.
91. Einsele H, Hebart H, Kauffmann-Schneider C, Sinzger C, Jahn G, Bader P, et al. Risk Factors for Treatment Failures in Patients Receiving PCR-Based Preemptive Therapy for CMV Infection. *Bone Marrow Transplant* (2000) 25(7):757–63. doi: 10.1038/sj.bmt.1702226
92. Einsele H, Ljungman P, Boeckh M. How I Treat CMV Reactivation After Allogeneic Hematopoietic Stem Cell Transplantation. *Blood* (2020) 135(19):1619–29. doi: 10.1182/blood.2019000956
93. Reusser P, Einsele H, Lee J, Volin L, Rovira M, Engelhard D, et al. Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Randomized Multicenter Trial of Foscarnet Versus Ganciclovir For Preemptive Therapy of Cytomegalovirus Infection After Allogeneic Stem Cell Transplantation. *Blood* (2002) 99(4):1159–64. doi: 10.1182/blood.v99.4.1159
94. Robin M, Porcher R, De Castro Araujo R, de Latour RP, Devergie A, Rocha V, et al. Risk Factors For Late Infections After Allogeneic Hematopoietic

Stem Cell Transplantation From a Matched Related Donor. *Biol Blood Marrow Transplant* (2007) 13(11):1304–12. doi: 10.1016/j.bbmt.2007.07.007
95. Mattes FM, Hainsworth EG, Geretti AM, Nebbia G, Prentice G, Potter M, et al. A Randomized, Controlled Trial Comparing Ganciclovir to Ganciclovir Plus Foscarnet (Each at Half Dose) for Preemptive Therapy of Cytomegalovirus Infection in Transplant Recipients. *J Infect Dis* (2004) 189(8):1355–61. doi: 10.1086/383040
96. Reusser P. Challenges and Options in the Management of Viral Infections After Stem Cell Transplantation. *Support Care Cancer* (2002) 10(3):197–203. doi: 10.1007/s005200100299
97. Boeckh M, Nichols WG, Chemaly RF, Papanicolaou GA, Wingard JR, Xie H, et al. Valganciclovir for the Prevention of Complications of Late Cytomegalovirus Infection After Allogeneic Hematopoietic Cell Transplantation: A Randomized Trial. *Ann Intern Med* (2015) 162(1):1–10. doi: 10.7326/M13-2729
98. Marty FM, Ljungman P, Chemaly RF, Maertens J, Dadwal SS, Duarte RF, et al. Letermovir Prophylaxis for Cytomegalovirus in Hematopoietic-Cell Transplantation. *N Engl J Med* (2017) 377(25):2433–44. doi: 10.1056/NEJMoa1706640
99. Kilgore JT, Becken B, Varga MG, Parikh S, Prasad V, Lugo D, et al. Use of Letermovir for Salvage Therapy for Resistant Cytomegalovirus in a Pediatric Hematopoietic Stem Cell Transplant Recipient. *J Pediatr Infect Dis Soc* (2020) 9(4):486–9. doi: 10.1093/jpids/piz050
100. Barrett AJ, Bollard CM. The Coming of Age of Adoptive T-Cell Therapy For Viral Infection After Stem Cell Transplantation. *Ann Transl Med* (2015) 3 (5):62. doi: 10.3978/j.issn.2305-5839.2015.01.18
101. O'Reilly RJ, Prockop S, Hasan AN, Koehne G, Doubrovina E. Virus-Specific T-Cell Banks for 'off the Shelf' Adoptive Therapy of Refractory Infections. *Bone Marrow Transplant* (2016) 51(9):1163–72. doi: 10.1038/bmt.2016.17
102. Prockop SE, Hasan A, Koehne G, Doubrovina E, Sauter CS, Barker JN, et al. Third Party Donor Derived CMV Specific T Cells for the Treatment of Refractory CMV Viremia and Disease after Hematopoietic Stem Cell Transplant. *Blood* (2014) 124:184–4. doi: 10.1182/blood.v124.21.184.184
103. Fabrizio VA, Irene Rodriguez-Sanchez M, Mauguen A, Dahi PB, Doubrovina E, O'Reilly RJ, et al. Adoptive Therapy with CMV-Specific Cytotoxic T Lymphocytes Depends on Baseline CD41 Immunity to Mediate Durable Responses. *Blood Adv* (2021) 5:496–503. doi: 10.1182/bloodadvances.2020002735
104. Kharfan-Dabaja MA, Boeckh M, Wilck MB, Langston AA, Chu AH, Wloch MK, et al. A Novel Therapeutic Cytomegalovirus DNA Vaccine in Allogeneic Haemopoietic Stem-Cell Transplantation: A Randomised, Double-Blind, Placebo-Controlled, Phase 2 Trial. *Lancet Infect Dis* (2012) 12:290 299. doi: 10.1016/S1473-3099(11)70344-9
105. Nakamura R, La Rosa C, Longmate J, Drake J, Slape C, Zhou Q, et al. Viraemia, Immunogenicity, and Survival Outcomes of Cytomegalovirus Chimeric Epitope Vaccine Supplemented With PF03512676 (CMVPepVax) in Allogeneic Haemopoietic Stem-Cell Transplantation: Randomised Phase 1b Trial. *Lancet Haematol* (2016) 3:e87–98. doi: 10.1016/S2352-3026(15)00246-X
106. Marchesi F, Pimpinelli F, Ensoli F, Mengarelli A. Cytomegalovirus Infection in Hematologic Malignancy Settings Other Than The Allogeneic Transplant. *Hematol Oncol* (2018) 36(2):381–91. doi: 10.1002/hon.2453
107. Hebart H, Schröder A, Löffler J, Klingebiel T, Martin H, Wassmann B, et al. Cytomegalovirus Monitoring By Polymerase Chain Reaction of Whole Blood Samples From Patients Undergoing Autologous Bone Marrow or Peripheral Blood Progenitor Cell Transplantation. *J Infect Dis* (1997) 175:1490–3. doi: 10.1086/516484
108. Zhang YL, Zhu Y, Xiao Q, Wang L, Liu L, Luo XH. Cytomegalovirus Infection is Associated With AML Relapse After Allo-HSCT: A Meta-Analysis of Observational Studies. *Ann Hematol* (2019) 98(4):1009–20. doi: 10.1007/s00277-018-3585-1
109. Mariotti J, Maura F, Spina F, Roncari L, Dodero A, Farina L, et al. Impact of Cytomegalovirus Replication and Cytomegalovirus Serostatus on the Outcome of Patients With B Cell Lymphoma After Allogeneic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2014) 20(6):885–90. doi: 10.1016/j.bbmt.2014.02.015
110. Mackus WJM, Frakking FNJ, Grummels A, Gamadia LE, De Bree GJ, Hamann D, et al. Expansion of CMV-Specific CD8+CD45RA+CD27- T Cells in B-Cell Chronic Lymphocytic Leukemia. *Blood* (2003) 102:1057–63. doi: 10.1182/blood-2003-01-0182
111. Pourgheysari B, Bruton R, Parry H, Billingham L, Fegan C, Murray J, et al. The Number of Cytomegalovirus-Specific CD4+ T Cells is Markedly Expanded in Patients With B-Cell Chronic Lymphocytic Leukemia and Determines the Total CD4+ T-Cell Repertoire. *Blood* (2010) 116:2968–74. doi: 10.1182/blood-2009-12-257147
112. Mous R, Savage P, Remmerswaal EBM, van Lier RAW, Eldering E, van Oers MHJ. Redirection of CMV-Specific CTL Towards B-CLL via CD20-Targeted HLA/CMV Complexes. *Leukemia* (2006) 20:1096–102. doi: 10.1038/sj.leu.2404185
113. Parikh SA, Leis JF, Chaffee KG, Call TG, Hanson CA, Ding W, et al. Hypogammaglobulinemia in Newly Diagnosed Chronic Lymphocytic Leukemia: Natural History, Clinical Correlates, and Outcomes. *Cancer* (2015) 121:2883–91. doi: 10.1002/cncr.29438
114. Morra E, Nosari A, Montillo M. Infectious Complications in Chronic Lymphocytic Leukaemia. *Hematol Cell Ther* (1999) 41(4):145–51. doi: 10.1007/s00282-999-0145-0
115. Chan TSY, Cheung CYM, Yeung IYL, Hwang YY, Gill H, Wong IY, et al. Cytomegalovirus Retinitis Complicating Combination Therapy With Rituximab and Fludarabine. *Ann Hematol* (2015) 94:1043–7. doi: 10.1007/s00277-014-2296-5
116. Ferrajoli A, O'Brien SM, Cortes JE, Giles FJ, Thomas DA, Faderl S, et al. Phase II Study of Alemtuzumab in Chronic Lymphoproliferative Disorders. *Cancer* (2003) 98:773–8. doi: 10.1002/cncr.11551
117. Laurenti L, Piccioni P, Cattani P, Cingolani A, Efremov D, Chiusolo P, et al. Cytomegalovirus Reactivation During Alemtuzumab Therapy for Chronic Lymphocytic Leukemia: Incidence and Treatment With Oral Ganciclovir. *Haematologica* (2004) 89(10):1248–52.
118. O'Brien S, Ravandi F, Riehl T, Wierda W, Huang X, Tarrand J, et al. Valganciclovir Prevents Cytomegalovirus Reactivation in Patients Receiving Alemtuzumab-Based Therapy. *Blood* (2008) 111:1816–9. doi: 10.1182/blood-2007-03-080010
119. Hwang YY, Cheung WWW, Leung AYH, Tse E, Au WY, Kwong YL. Valganciclovir thrice Weekly for Prophylaxis Against Cytomegalovirus Reactivation During Alemtuzumab Therapy. *Leukemia* (2009) 23:800–1. doi: 10.1038/leu.2008.282
120. Parry HM, Damery S, Hudson C, Maurer MJ, Cerhan JR, Pachnio A, et al. Cytomegalovirus Infection Does Not Impact on Survival or Time to First Treatment in Patients With Chronic Lymphocytic Leukemia. *Am J Hematol* (2016) 91:776–81. doi: 10.1002/ajh.24403
121. Reddy Y, Baig M, Kalva N, Puli S, Dhillon S. Cytomegalovirus Proctitis in a Patient with Chronic Lymphocytic Leukemia on Ibrutinib Therapy: A Case Report. *Cureus* (2020) 12(4):e7837. doi: 10.7759/cureus.7837
122. Greenwell IB, Ip A, Cohen JB. PI3K Inhibitors: Understanding Toxicity Mechanisms and Management. *Oncol (Williston Park)* (2017) 31(11):821–8.
123. Martinelli S, Maffei R, Fiorcari S, Quadrelli C, Zucchini P, Benatti S, et al. Idelalisib impairs T-Cell-Mediated Immunity in Chronic Lymphocytic Leukemia. *Haematologica* (2018) 103:e598–601. doi: 10.3324/haematol.2017.187070
124. Cuneo A, Barosi G, Danesi R, Fagiuoli S, Ghia P, Marzano A, et al. Management of Adverse Events Associated With Idelalisib Treatment in Chronic Lymphocytic Leukemia And Follicular Lymphoma: A Multidisciplinary Position Paper. *Hematol Oncol* (2019) 37:3–14. doi: 10.1002/hon.2540
125. Cheah CY, Fowler NH. Idelalisib in the Management of Lymphoma. *Blood* (2016) 128:331–6. doi: 10.1182/blood-2016-02-702761
126. Chanan-Khan A, Sonneveld P, Schuster MW, Stadtmauer EA, Facon T, Harousseau JL, et al. Analysis of Herpes Zoster Events Among Bortezomib-Treated Patients in the Phase III APEX Study. *J Clin Oncol* (2008) 26:4784–90. doi: 10.1200/JCO.2007.14.9641
127. Marchesi F, Mengarelli A, Giannotti F, Tendas A, Anaclerico B, Porrini R, et al. High Incidence of Post-Transplant Cytomegalovirus Reactivations in Myeloma Patients Undergoing Autologous Stem Cell Transplantation After Treatment With Bortezomib-Based Regimens: A Survey From the Rome Transplant Network. *Transpl Infect Dis* (2014) 16:158–64. doi: 10.1111/tid.12162

128. Blanco B, Pérez-Simón JA, Sánchez-Abarca LI, Carvajal-Vergara X, Mateos J, Vidriales B, et al. Bortezomib Induces Selective Depletion of Alloreactive T Lymphocytes and Decreases the Production of Th1 Cytokines. *Blood* (2006) 107:3575–83. doi: 10.1182/blood-2005-05-2118
129. Basler M, Lauer C, Beck U, Groettrup M. The Proteasome Inhibitor Bortezomib Enhances the Susceptibility to Viral Infection. *J Immunol* (2009) 183:6145–50. doi: 10.4049/jimmunol.0901596
130. Wudhikarn K, Palomba ML, Pennisi M, Garcia-Recio M, Flynn JR, Devlin SM, et al. Infection During the First Year in Patients Treated With CD19 CAR T Cells for Diffuse Large B Cell Lymphoma. *Blood Cancer J* (2020) 10 (8):79. doi: 10.1038/s41408-020-00346-7
131. Vora SB, Waghmare A, Englund JA, Qu P, Gardner RA, Hill JA. Infectious Complications Following CD19 Chimeric Antigen Receptor T-cell Therapy for Children, Adolescents, and Young Adults. *Open Forum Infect Dis* (2020) 7(5):ofaa121. doi: 10.1093/ofid/ofaa121
132. Mahmoudjafari Z, Alexander M, Roddy J, Shaw R, Shigle TL, Timlin C, et al. American Society for Transplantation and Cellular Therapy Pharmacy Special Interest Group Position Statement on Pharmacy Practice Management and Clinical Management for COVID-19 in Hematopoietic Cell Transplantation and Cellular Therapy Patients in the United States. *Biol Blood Marrow Transplant* (2020) 26:1043–9. doi: 10.1016/j.bbmt.2020.04.005
133. Yakoub-Agha I, Chabannon C, Bader P, Basak GW, Bonig H, Ciceri F, et al. Management of Adults and Children Undergoing Chimeric Antigen Receptor T-Cell Therapy: Best Practice Recommendations of the European Society for Blood and Marrow Transplantation (EBMT) and the Joint Accreditation Committee of ISCT and EBMT (JACIE). *Haematologica* (2020) 105:297–316. doi: 10.3324/haematol.2019.229781
134. Styczyński J. Infections Following Car-T Cells Therapy: Current State-Of-The-Art Review and Recommendations. *Acta Haematol Pol* (2020) 51:11–6. doi: 10.2478/ahp-2020-0004
135. Vora SB, Brothers AW, Waghmare A, Englund JA. Antiviral Combination Therapy for Cytomegalovirus Infection in High-Risk Infants. *Antivir Ther* (2018) 23:505–11. doi: 10.3851/IMP3238

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REVIEW
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CMV Infection and CMV-Specific Immune Reconstitution Following Haploidentical Stem Cell Transplantation: An Update

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Haploidentical stem cell transplantation (haploSCT) has advanced to a common procedure for treating patients with hematological malignancies and immunodeficiency diseases. However, cure is seriously hampered by cytomegalovirus (CMV) infections and delayed immune reconstitution for the majority of haploidentical transplant recipients compared to HLA-matched stem cell transplantation. Three major approaches, including *in vivo* T-cell depletion (TCD) using antithymocyte globulin for haploSCT (*in vivo* TCD-haploSCT), *ex vivo* TCD using CD34 + positive selection for haploSCT (*ex vivo* TCD-haploSCT), and T-cell replete haploSCT using posttransplant cyclophosphamide (PTCy-haploSCT), are currently used worldwide. We provide an update on CMV infection and CMV-specific immune recovery in this fast-evolving field. The progress made in cellular immunotherapy of CMV infection after haploSCT is also addressed. Groundwork has been prepared for the creation of personalized avenues to enhance immune reconstitution and decrease the incidence of CMV infection after haploSCT.

Keywords: cytomegalovirus, infection, immune reconstitution, haploidentical, stem cell transplantation

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INTRODUCTION

HLA-haploidentical stem cell transplantation (haploSCT) has spread rapidly worldwide in recent years. HLA-haploidentical donors sharing a single HLA haplotype with transplant recipients are almost always available, so haploSCT can be performed for patients who are lacking HLA-matched donors and/or are urgently needing transplantation. The major approaches for T-cell depletion are *in vivo* T-cell depletion using antithymocyte globulin (ATG) (*in vivo* TCD-haploSCT), *ex vivo* T-cell depletion (TCD) using CD34 + positive selection (*ex vivo* TCD-haploSCT), and T-cell replete haploSCT using posttransplant cyclophosphamide (PTCy-haploSCT). Compared with HLA-identical sibling transplantation, patients undergoing haploSCT usually receive more intensive immunosuppressors to guarantee engraftment and later prevent graft-versus-host disease (GVHD). Therefore, these patients always have impaired immune reconstitution after transplantation and a high incidence of CMV infection and CMV disease (**Figure 1**). As the use of haploidentical transplantation has increased substantially, we summarize current data on CMV infection and its immune reconstitution after haploSCT during the last decade.

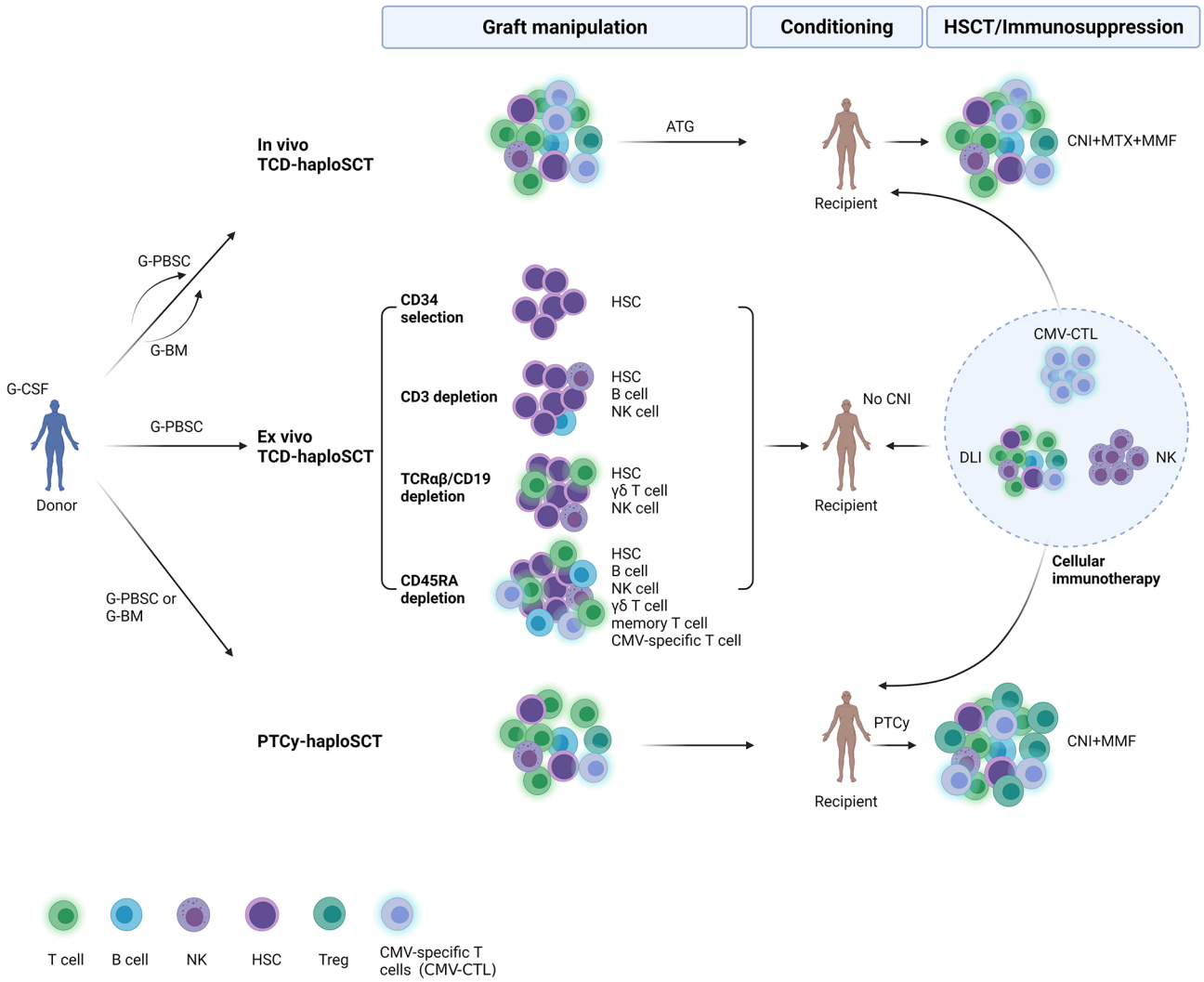

FIGURE 1 | Overview of immune reconstitution to cytomegalovirus and cellular immunotherapy after three major approaches of haploidentical stem cell transplantation (haploSCT). In vivo TCD-haploSCT, in vivo T-cell depletion (TCD) using antithymocyte globulin for haploSCT; Ex vivo TCD-haploSCT, ex vivo TCD using CD34 + positive selection for haploSCT; PTCy-haploSCT, T-cell replete haploSCT using posttransplant cyclophosphamide. G-CSF, granulocyte-colony stimulating factor; G-PBSC, G-CSF primed peripheral blood stem cells; G-BM, G-CSF primed bone marrow; HSC, hematopoietic stem cell; CMV, cytomegalovirus; CNI, calcineurin inhibitors; MTX, methotrexate; MMF, mycophenolate mofetil; DLI, donor lymphocyte infusion; NK cell, natural killer cell; Treg, regulatory T cell; HSCT, hematopoietic stem cell transplantation. Created with BioRender (https://biorender.com/).

INCIDENCE OF CYTOMEGALOVIRUS INFECTION AFTER haploSCT

In Vivo TCD-haploSCT (Anti-Thymocytic Globulin/ATG-Based)

Using the Beijing protocol at Peking University (1–7), there was a high incidence of CMV reactivation early after haploSCT (59.5-66%), whereas the rate of CMV disease was actually low (2.92-17%). CMV DNAemia was initially detected after a median of 35 days with a mean duration of positivity of 15 days (5, 6). Most (91.2%) cases of CMV gastroenteritis developed within 100 days, whereas most (90.3%) cases of CMV retinitis were late onset with the cumulative incidence of CMV retinitis at 2.3% one year (a median onset of 167 days) after haploSCT (6, 7). Einat Shmueli et al. from Israel designed a conditioning protocol for haploSCT including fludarabine, thiotepa, anti-thymocytic globulin, and total body irradiation (8). After receiving preemptive therapy, the incidence of CMV infection was 66.7% in haploSCT, and 11.6% of haploSCT transplant recipients with CMV reactivation developed CMV disease. Importantly, drug-resistance mutations and clinically suspected resistance were discovered only in haploSCT recipients (8), favoring prophylactic over preemptive treatment in high-risk patients and highlighting the need for better anti-CMV drugs.

It remains unclear whether primary disease affects CMV infection after haploSCT. Lan-Ping Xu et al. from Peking University conducted studies to confirm the feasibility of haploidentical transplantation in patients with severe aplastic anemia (SAA) as salvage therapy (9–12). CMV viremia occurred in 51.7~84.00% of SAA patients. However, no difference in the rates of early CMV disease between haploidentical patients and matched related patients was found (9, 10). Consistently, several centers in China obtained similar results for SAA patients (13–15). The haploSCT cohorts with AML, MDS, or Ph+ ALL, including haplo-cord-HSCT, had higher CMV viremia than the HLA-matched HSCT cohorts (16–19), but the incidence of CMV disease was not significantly different between the two groups. Even in pediatric patients with MDS or patients with relapsed/refractory acute lymphoblastic leukemia after CAR-T therapy who underwent haploSCT, the incidence of CMV reactivation/infection was less than 60%, and very few patients developed CMV disease (20, 21).

Using a similar protocol, several transplant centers have reported promising results for unmanipulated haploidentical peripheral blood stem cell transplantation (PBSCT) (22, 23) or cotransplantation of unrelated cord blood (UCB) (24–26) or mesenchymal stem cells (MSCs) (27, 28). The 1-year cumulative incidence of CMV DNAemia in patients with hematologic malignancies was 23.5-41.7% in the matched sibling donor (MSD)-SCT group versus 62.1-81.0% in the haploSCT group with peripheral blood stem cells (PBSCs) (29, 30). The median time to the onset of CMV DNAemia in the haploSCT group was 33 days (range, 10–159 days) with the 1-year cumulative incidence of CMV disease at 7.9% (95% CI, 3.6–14.3%) (29). In addition, a total of 19.4%-92% of these patients experienced CMV reactivation after combination of haploSCT with UCB or MSCs (24–28). There was no statistical significance in the incidence of CMV viremia in terms of haplo-cord SCT *vs* HLA-matched donor SCT (MD-SCT) or haplo-cord SCT *vs* haploSCT (24–26).

As the use of ATG as a regimen for *in vivo* TCD and immunosuppressants is limited by impaired immune restoration and a high risk of severe infections, researchers are working on their impact after haploSCT. Peking University performed a study comparing 6 mg/kg ATG versus 10 mg/kg ATG in patients who underwent haploSCT (31). The 1-year cumulative incidence of CMV reactivation was similar between the ATG-6 and ATG-10 groups[(75.0% (66.8–83.2%) *vs* 78.6% (75.2–82.0%)]. Another multicenter study investigated the impact of 7.5 mg/kg and 10.0 mg/kg rabbit ATG on GVHD and virus reactivation after haploSCT (32). The 1-year incidence of CMV DNAemia was higher in the 10.0 mg/kg group [83.4% (77.5-87.9)] than in the 7.5 mg/kg group [73.4% (67.2-79.4)], whereas the 2-year incidence of CMV-associated diseases was also higher in the 10.0 mg/kg group [5.9% (3.2–9.7%)] than in the 7.5 mg/kg group [1.5% (0.4–4.0%)]. Yu Wang et al. recently extended follow-up from this original trial (33). They found that patients undergoing haploSCT benefit from 7.5 mg/kg ATG compared to 10.0 mg/kg ATG based on a balance between GVHD and infection control. The data supports ATG (7.5 mg/kg) is potentially the standard regimen in this platform. Researchers from Japan and the Republic of Korea later performed haploSCT using low-dose thymoglobulin at 5 mg/kg (34, 35). CMV reactivated in 41.67% and 72.7% of patients, but CMV disease developed in 0 and 19.4% of patients, respectively. A recent report from the Republic of Korea indicated that the cumulative incidence of CMV DNAemia at 3 years was 45.7% (30.7-59.4) for ATG (5-10 mg/kg)-based haploSCT (36). Moreover, a short-term tacrolimus regimen for the prophylaxis of GVHD in haploSCT did not increase the incidence of CMV infection compared with the Cyclosporine A regimen (39.5% *vs*. 37.5%, p = 0.783) (37).

Ex Vivo TCD-haploSCT

CD34+ selection was initially used as a method for TCD, but it resulted in delayed immune reconstitution and a high incidence of opportunistic infections and nonrelapse mortality. The *ex vivo* TCD techniques have developed from CD34+ selection, CD3+ cell depletion, and αβ+/CD19+ cell depletion to recent CD45RA+ depletion. Compared with CD34+ cell selection, after CD3+ cell depletion, the graft has more natural killer (NK) cells, monocytes, and other immunomodulating cells with better outcomes. Sameh Gaballa et al. retrospectively compared data on patients undergoing a two-step (a fixed T cell infusion followed 2 days later by cyclophosphamide, and then a CD34-selected stem cell product infused) haploidentical or matched related PBSCT for high-risk hematological malignancies and aplastic anemia (38). Compared with the matched related PBSCT group (matched related, 19%), the 100-day cumulative incidence of CMV viremia was higher in the haploidentical group (haploidentical, 67%). The median time to develop CMV reactivation was 26 days in the haploSCT group and 36 days in the matched related PBSCT group.

The cumulative incidence of CMV DNAemia in patients with acute leukemia was 73.5-81% after *ex vivo* αβ T cell-depleted haploSCT (39, 40). No patient developed CMV disease or died (39). A more recent study explored the role of interim-foscarnet prophylaxis in preventing CMV infection after *ex vivo* αβ T cell-depleted haploSCT in children between May 2012 and May 2018 (41). Forty (50.8%) of 81 patients developed CMV reactivation at a median of 41.3 days (range, 13–132) after haploSCT. The median duration of CMV reactivation was 28.5 days (range, 1–179), and the peak PCR level was 3.82 log copies/mL (range, 2.85–6.03) (41). In nonmalignant disease, ganciclovir/foscarnet significantly decreased CMV reactivation incidence (43.7% *vs*. 78.3%), whereas the prophylaxis strategy had no significant impact in patients with hematological malignancies. No significant difference was found in the rate of CMV disease according to prophylaxis method. It suggests that this intensified antiviral strategy may be necessary for αβ T cell-depleted haploSCT patients with nonmalignant disease who require higher doses of ATG.

Through TCR α+β+/CD19+ cell-depleted haploSCT, it is feasible to transfer to the transplant recipient both donor hematopoietic stem cells (HSCs) and hematopoietic progenitors as well as NK and γδ T cells, which could protect

against leukemia and life-threatening infections, including posttransplant lymphoproliferative disease (PTLD). A total of 7.27-75% of patients undergoing TCR α+β+/CD19+ cell depleted HSCT experienced CMV reactivation (42–46). Most patients experienced CMV viremia during the first month after haploSCT (days +1 to +24) (45). In a report including three sickle cell disease and 11 thalassemia patients, Gaziev J et al. stated that viral reactivation occurred in the vast majority of patients after TCR α+β+/CD19+ cell–depleted haploSCT, with CMV reactivation in 64%, although no cases of CMV were noted (47).

After removal of potentially alloreactive CD45RA+ cell depletion, memory T cells, including virus-specific T cells left in grafts, could shorten viremia and reduce GVHD (48). B M Triplett et al. reported data from 17 patients with poor-prognosis hematologic malignancy who underwent haploSCT with CD45RA-depleted grafts after a reduced intensity conditioning regimen without TBI or serotherapy (49). Three patients of 17 received anti-CMV treatment after CMV reactivation. None of the patients experienced CMV disease, and all of them cleared CMV viremia without donor lymphocyte infusion (DLI). Early T-cell reconstitution was directly linked to the CD45RA-depleted graft content. This group then compared 41 patients receiving CD3-depleted (CD3dep recipients) grafts with 26 receiving CD45RA-depleted grafts (CD45dep recipients) after haploSCT (50). CD3dep recipients were more likely to develop CMV reactivation—23 (56%) *vs* 5 (19%). All CD3dep recipients with CMV received treatment, and eight (36%) were also infused with donor lymphocytes for CMV, whereas CMV treatment was needed for only three of the five CD45RAdep recipients. Although three CD3dep recipients died with active CMV viremia, CMV was not detected in CD45RAdep recipients at the time of death. It seems that CD45RA-depleted haploSCT confers enhanced T-cell recovery and reduced infection without increase in severe GVHD among these *ex vivo* TCD methods.

PTCy-haploSCT

PTCy is a method of *in vivo* T cell depletion that mainly acts on alloreactive T cells after haploSCT. CMV reactivation was noticed in 42%-69.2% of patients who underwent PTCy-haploSCT (51–58). A total of 2.8%-4.5% of patients experienced CMV-associated disease (51, 52). CMV reactivation occurred at a median time of 35-39 days (51, 52, 57). The median time to first episode of CMV DNAemia was 33 days (range, –7 to 123 days) after haploSCT (58). Moreover, the CMV DNA peak load was remarkably higher in haploSCT recipients, but the mortality by days 180 and 365 did not differ among comparison groups (55). García-Cadenas Irene et al. studied the impact of HLA donor matching on infection in patients receiving PTCy-based alloSCT (59). They found that haploSCT recipients had a higher incidence of CMV infection/reactivation at 18 months than other transplant modalities [(61% (95% CI: 41–74%) *vs*. 44% (95% CI: 31–54%)], whereas lethal infections were uncommon across all these groups. In their study, severe infections were common in transplant patients using PTCy. A more recent CIBMTR analysis reported (51) that PTCy increased the risk of CMV infection among CMV-seropositive recipients in both haploSCT and matched sibling donor HSCT compared with calcineurin inhibitor–based sibling donor transplantation, suggesting intensive CMV prevention strategies in all receiving PTCy. This is supported by the fact that an intensified method to prevent CMV reactivation correlated with a lower incidence of CMV reactivation (67% intensified group versus 81% traditional group) and less CMV disease (0% hybrid/intermediate dose versus 8% traditional dose) without increased toxicity after PTCy-haploSCT compared with a traditional antiviral prophylaxis regimen (60).

Primary disease and conditioning regimen could also impact CMV infection after PTCy-haploSCT. CMV reactivation post engraftment was noted in 43.7% and 62% of transplant recipients with primary immune deficiency disorders (PIDs) (61) and relapsed/refractory SAA (62) undergoing PTCy-haploSCT, respectively. R V Raj et al. then investigated the effect of conditioning intensity on the incidence of viral infection after PTCy-haploSCT (63). Their study found that challenging viral infections after haploSCT cause significant morbidity in this patient population. It appears that the incidence of viral complications is higher following myeloablative doses of busulfan-containing conditioning regimens (63). Emmanuel Katsanis et al. recently performed a single center phase I study substituting day +4 PTCy with bendamustine (PT-BEN) following myeloablative conditioning and T-cell replete haploidentical bone marrow transplantation (64). CMV reactivation was notably less common in trial patients receiving PTCy/BEN, with one out of eight at-risk (seropositive recipient and/or seropositive donor) of experiencing CMV reactivation, whereas 71.4% of the at-risk PTCy patients reactivated CMV.

Compared with bone marrow (BM) as a graft source, PBSCs could yield higher CD34+ cell counts but were possibly accompanied by increased GVHD; however, no difference in GVHD was observed in haploSCT (65). A total of 46-68% of patients with PTCy-haploSCT and PBSC grafts had posttransplant CMV viremia (65–69). The median time to viremia was 24 days (range: 3–68). CMV disease occurred in 17-28.8% of patients with CMV viremia (65, 68). Sirolimus with micophenolate mofetil (MMF) has recently been regarded as an alternative to calcineurin inhibitor-containing approaches, as this combination has a decreased risk of acute renal failure, decreased incidence of CMV reactivation, and better regulatory T cell reconstitution. Some groups have introduced PTCy plus sirolimus and MMF (PT-CY-Sir-MMF) as GVHD prophylaxis in allo-HSCT, regardless of donor type (70, 71). CMV DNAemia occurred in 52-63% of patients after haploSCT. The cumulative incidence of CMV DNAemia in patients who received pre-emptive antiviral therapy at one year was 39% (95% CI, 31–47%), and the 1-year cumulative incidence of CMV disease was 2.6% (95% CI, 0.09–5%) (70).

ATG+ PTCy-haploSCT

As ATG is usually used to reduce the risk of graft rejection and GVHD, it is assumed that ATG combined with PTCy in T-cell replete-haploSCT would minimize GVHD risk but not impact engraftment and risk of relapse. Princes Margaret Cancer Centre from Canada established unmanipulated haploidentical PBSC

transplantation following RIC with ATG (total 4.5 mg/kg), PTCy (cyclophosphamide 50 mg/kg/day i.v. on days +3 and +4), and cyclosporine as a GVHD prevention strategy (72–74). CMV reactivation occurred in 74% of cases with CMV disease in 11.5% of cases (72). Cheng-Hsien Lin et al. retrospectively compared the cumulative incidence of CMV DNAemia, two-year OS, and leukemia-free survival rates in acute leukemia patients with MSD, matched unrelated donor (MUD), and haploidentical donor allografts (ATG: 2 mg·kg-1 day-1, from day -3 to day -2; PTCy) (75). The cumulative incidences of CMV DNAemia at day 180 in the haploidentical groups were 85.7%, which were higher than those in the MSD and MUD allo-HSCT groups. For the haploidentical groups, CMV DNAemia was detected at a median time of 29 days.

Yu Wang et al. from Peking University initiated a prospective study in patients with a standard-dose ATG/granulocyte colony-stimulating factor (G-CSF)-based regimen (ATG-PTCy) followed by low-dose PTCy (14.5 mg/kg on days 3 and 4) for haploSCT (76, 77). The 100-day cumulative incidence of CMV reactivation in the ATG-PTCy cohort was markedly higher than that in the ATG cohort (74% *vs* 30%), with a comparable incidence of CMV disease between the two cohorts (8% *vs* 8%) (77), indicating that dual T cell depletion with PTCy and ATG may bring about a higher incidence of CMV reactivation.

Comparison Among These Approaches

Published data have been inconsistent on the incidence of CMV reactivation and CMV disease after haploSCT (**Table 1**). It indicates that haploSCT carries a substantially higher risk for CMV infection compared with HLA-matched related or unrelated allo-HSCTs, but this seemed not to impact overall and non-relapse mortality. Hence, some data suggest the use of prophylactic anti-CMV antivirals when PTCy is used because a higher incidence of CMV reactivation was associated with the use of PTCy (51, 60). Surprisingly, a systematic review and meta-analysis of studies on haploSCT in idiopathic AA suggested that the addition of PTCy correlated with a lower risk of CMV viremia (10.4%) to a larger extent than MTX-containing (55.7%) and other (38.6%) regimens (79). The opposite results can be partly explained by the absence of an approved threshold of viral load to initiate anti-CMV treatment, considering the different transplant centers. The heterogeneous CMV serological status in the donor/recipient on account of geographical and ethnological characteristics is another possible explanation because the CMV seroprevalence is usually much higher (>=90%) in adult populations of China than in Europe and the USA (80–84). This issue could be better investigated in a future clinical trial.

Johanna Tischer et al. retrospectively compared the incidence of virus infections and outcome in two different haploSCT settings (78). The first approach was the combination of T cell repletion and T cell depletion (CD6 deletion) using ATG prior to haploSCT (cTCR/TCD group). The second was T cell repletion (TCR) using high-dose posttransplantation cyclophosphamide (TCR/PTCy group). CMV reactivation occurred more frequently in the cTCR/TCD group (57%) than in the TCR/PTCy group (31%). Furthermore, pre-emptive treatment of CMV reactivation was successful in the TCR/PTCy group, whereas CMV DNA became undetectable in only 50% of the cTCR/TCD group.

CMV-SPECIFIC IMMUNE RECONSTITUTION AND ITS ASSOCIATION WITH CMV REACTIVATION AFTER haploSCT

CMV-Specific T Cell (CTL)

We previously investigated CMV-specific T cell (CMV-CTL) reconstitution post *in vivo* TCD-haploSCT (85–87). The CD8+ T cell number in transplant recipients was comparable to that of controls at day 60 after transplantation. The median number of CMV-CTLs and their subsets was equal to those of the controls from day 30 to day 360. In addition, haploSCT recipients had a high frequency of CMV-CTLs with strong proliferation capacities and interferon-γ production at one year after transplantation (86). CMV-specific T cells with the central memory CD45RO+CD62L+ cell phenotype were significantly expanded when CMV was reactivated early after transplantation (87). Ruri Kato et al. demonstrated that there were considerably lower maximum numbers of CMV-CTLs in the CMV antigenemia resolved group than in the persistent group (median, 22.15 *vs*. 50 cells/μl) (88). Nevertheless, CMV-CTLs reached a peak more quickly in the resolved group than in the persistent group (median, 21 *vs*. 78 days) (88).

M Noviello et al. retrospectively explored either CD34 selection or posttransplant sirolimus as GVHD prophylaxis for haploSCT recipients (89). At 30 days, 21.7% of patients had CMV-specific T cells higher than 1 sfc/μL measured by enzyme-linked immunosorbent spot (ELISPOT), whereas CMV viremia occurred in only one patient who received anti-CMV treatment. At 90 days, 29.0% of patients reached this threshold, and no patients experienced clinically relevant viremia. At 180 days, 52.9% of patients finally reached the threshold, and none of them experienced CMV viremia. They found the protective value of 1 CMV sfc/μL against CMV reactivation posttransplant (89).

Dixie Huntley et al. performed a multicenter observational study to monitor CMV-specific T cell kinetics in PTCy-haploSCT patients and compared it with HLA-matched transplantation (58). In their analysis, CMV DNAemia developed at a similar frequency with equal numbers of CMV-specific T-cell at most time points examined between PTCy-haploSCT and MRD/MUD recipients. CMV DNAemia did not affect CMV-specific CD8+ and CD4+ T-cell reconstitution by the end of the follow-up period (day +180) in either allo-HSCT modality. They claimed that PTCy-haploSCT recipients may restore CMV-specific T-cell immunity to the same extent as HLA-matched allo-HSCT patients (58). The same group also reported that CMV infection was related to high levels of CD27 −CD28− T cells, which behave like Tregs (90). They found a suboptimal correlation between CMV-specific CD4+ or CD8+ T cells and Tregs in peripheral blood (PB), which was weaker in patients with CMV reactivation prior to immunological monitoring. This suggests that recovery of PB Tregs and that

TABLE 1 | Selected reports on CMV infection after haploidentical stem cell transplantation.

Group	Year	Country	haploSCT Sample size	Primary Disease (n)	Stem cell source (n)	Graft manipulation	Dose of ATG	Conditioning (n)	GVHD prophylaxis	Assays measuring CMV DNAemia	Cutoff values for CMV reactivation or reactivation needing PET	CMV reactivation	CMV disease	Clinical outcome/Comments	Reference
Y Wang et al.	2013	China	756	AML (321); ALL (299); CML (136)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY	CsA+MMF+short-term MTX	Real-time PCR or with a CMV pp65 antigenemia test	NR	100-day 64%	4%	2-year relapse (18%); 3-year OS (67%), LFS (63%), NRM (18%). More CMV-seropositive patients became antigenemia-positive than CMV-seronegative patients.	(4)
Y Chen et al.	2016	China	248	AL (201); CML (32); Others (15)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 or 1.5mg/kg×4d	Modified BUCY (241); TBI+CY +Me-CCNU (7)	CsA+MMF+short-term MTX	Real-time PCR (RT-PCR)	A viral load of >500 copies/ml for two consecutive readings 5 days apart	59.50%	6.85%	CMV DNAemia was found to be a poor prognostic factor in terms of NRM and OS. HBsAg seropositivity was associated with an increased risk of cytomegalovirus DNAemia.	(5)
CH Yan et al.	2020	China	1466	AML (801); ALL (490); MDS (175)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY (1416); TBI+CY +Me-CCNU (50)	CsA+MMF+short-term MTX	Automated, real-time, quantitative PCR assay	A detection threshold of >1000 copies/ml was defined as positive	64.80%	1-year CMVR 2.3%	CMVR was a rare complication after haploidentical HSCT but that the risk was greater in patients with multiple risk factors.	(6)
XY Meng el al.	2020	China	3862	AML (36); ALL (51); MDS (14); CML (4); SAA (2); Others (6)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY or TBI+CY+Me-CCNU BUCY (SAA)	CsA+MMF+short-term MTX	Real-time PCR	A limit of detection of 509 IU/mL	NR	2.92%	1 year NRM 34.9% in patients with CMV diseases	(7)
LP Xu et al.	2016	China	101	SAA	BM +PBSC (100); BM (1)	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	BUCY	CsA+MMF+short-term MTX	NR	NR	68.30%	1%	3-year OS (89.0%); FFS (86.8%)	(9)
LP Xu et al.	2017	China	89	SAA (69); VSAA (20)	BM +PBSC (78); BM (9); PBSC (2)	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	BUCY	CsA+MMF+short-term MTX	NR	NR	51.70%	1.12%	3-year OS (86.1 ± 3.7%); FFS (85.0 ± 3.9%)	(10)
LP Xu et al.	2018	China	51	SAA	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	BUCY	CsA+MMF+short-term MTX	NR	NR	84.00 ± 0.29%	1.96%	1- and 3-year OS 83.5 ± 5.4% (the probabilities of FFS were equal to the OS)	(11)
LP Xu et al.	2017	China	52 pediatric patients	SAA (32); VSAA (20)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	BUCY	CsA+MMF+short-term MTX	NR	NR	69.20%	NR	3-year OS (84.5 ± 5.0%); FFS (82.7 ± 5.2%)	(12)
Y Lu et al.	2018	China	41	SAA	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 7.5 mg/kg (total dose) ATG-F 20mg/kg (total dose)	BUCY	Tacro+MMF+short-term MTX	PCR	Higher than 500 copies/mL	65.90%	4.88%	3-year OS (80.3 ± 5.1%); FFS (76.4 ± 5.1%); GFFS (79.0 ± 8.6%)	(13)
L Liu et al.	2020	China	146	SAA (75); VSAA (71); SAA with PNH clone (15)	BM (15); PBSC (4); BM + PBSC (127)	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	BUCY	CsA+MMF+short-term MTX	Real-time PCR	NR	42.47%	2.05%	4-year OS (81.4 ± 3.3%); GFFS (69.2 ± 3.9%)	(24)
Z Liu et al.	2017	China	44	SAA (31); VSAA (13)	BM +PBSC +MSCs	*in vivo* TCD-haploSCT	r-ATG 3.125 mg/kg×4d	BUCY	CsA+MMF+short-term MTX	NR	NR	65.90%	0	2-year OS 77.3%	(27)
Z Wang et al.	2014	China	17 children and adolescents	SAA (11); VSAA (5); 2nd HSCT (1)	BM +PBSC +MSC	*in vivo* TCD-haploSCT	r-ATG 5mg/kg×4d (-4 to -1); ALG 20mg/kg/day d-4 to -1	Flu+BUCY	CsA+MMF+short-term MTX +basiliximab	Real-time PCR	NR	82.30%	0	1-year OS 71.60 ± 17.00%	(14)
L Gao et al.	2014	China	26	SAA (16); VSAA (10)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Flu+CY	CsA+MMF+short-term MTX	PCR	NR	23.08%	3.85%	TRM 3.8% (100-day), 11.5% (1-year), 15.4% (2-year); OS 84.6% (follow-up of 1313.2 days)	(15)
Y Lu et al.	2021	China	377	AML	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 7.5-10mg/kg; ATG-F 20mg/kg	Modified BUCY, n=118; Intensified BU-based MAC, n=259	CsA+MMF+short-term MTX	Real-time quantitative PCR	NR	67.4 ± 5.1%	1.06%	3-year OS 74.9 ± 2.4%; LFS 73.8 ± 4.8%; relapse rates 14.3 ± 4.0%; NRM 12.3 ± 3.5%	(16)
Jiafu Huang et al.	2020	China	75 patients aged over 50 years	AML (60); MDS (15)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 7.5-10mg/kg	BUCY or BF or TBI+CY	CsA+MMF+short-term MTX	PCR	NR	64.00%	4.00%	2-year relapse 27.0% ± 5.6%; PFS 59.3% ± 5.8%; OS 63.0% ± 5.8%; GRFS 42.6% ± 5.9%	(17)

(Continued)

TABLE 1 | Continued

Group	Year	Country	haploSCT Sample size	Primary Disease (n)	Stem cell source (n)	Graft manipulation	Dose of ATG	Conditioning (n)	GVHD prophylaxis	Assays measuring CMV DNAemia	Cutoff values for CMV reactivation or reactivation needing PET	CMV reactivation	CMV disease	Clinical outcome/Comments	Reference
P Suo et al.	2020	China	27	MDS	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY	CsA+MMF+short-term MTX	Quantitative PCR	PET was given when a single CMV DNA > 1000 copies/mL or 600 copies/mL were observed twice.	59.30%	0	3-year DFS and 3-year OS 81.9%	(20)
P Ke et al.	2018	China	48	MDS	BM (9); PBSC (1); BM +PBSC (38); coinfusion of the cord blood	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY	CsA+MMF+short-term MTX	NR	NR	42%	0	2-year OS 64%; RFS 56%; relapse 12%; NRM 33%	(19)
L Gao et al.	2015	China	47	Ph+ ALL	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	TBI+Ara-C+CY	CsA+MMF+short-term MTX	NR	NR	38.30%	8.51%	2-year OS 63.8%; LFS 59.5%	(18)
H Zhao et al.	2020	China	55	ALL	BM +PBSC or PBSC	*in vivo* TCD-haploSCT	NR	BUCY+TBI or nonmyeloablative regimens	NR	NR	NR	56.10%	NR	2-year LFS 65.6%; OS 77.0%	(21)
L Gao et al.	2017	China	174	AML (73); ALL (61); CML (22); MDS (18)	BM +PBSC	*in vivo* TCD-haploSCT	ATG-F 5mg/kg×4d	CCNU+BU+CY +Ara-C (AML, CML and MDS) CY+TBI+Ara-C (ALL)	CsA/Tacro+MMF +short-term MTX	PCR	NR	39.5% (Short-term Tacro); 37.5% (CsA)	NR	2-year OS 59.3% (Short-term Tacro), 55.7% (CsA); 2-year DFS 65.1% (Short-term Tacro), 61.4% (CsA)	(37)
Y Wang et al.	2014	China	224	AML (106); ALL (91); CML (14); MDS (13)	BM +PBSC	*in vivo* TCD-haploSCT	r-ATG 1.5 mg/kg×4d, n=112; r-ATG 2.5 mg/kg×4d, n=112	Modified BUCY, n=218; TBI based regimen, n=6	CsA+MMF+short-term MTX	Real-time Taqman CMV DNA PCR	>600 copies/mL	1-year 75.0% (ATG-6) and 78.6% (ATG-10)	0.89% (ATG-6) and 5.36% (ATG-10)	1-year relapse 7.6% (ATG-6), 4.6% (ATG-10); NRM 8.1% (ATG-6), 10.3% (ATG-10); OS 88.4% (ATG-6), 87.0% (ATG-10); DFS 84.3% (ATG-6); 86.0% (ATG-10)	(31)
S Kako et al.	2017	Japan	12	AML (5); ALL (1); CMML (1); Ph+ ALL (2); NHL (1); LCS (1); PMF (1)	PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×2d (-4 to -3)	BU+Mel, n=2; CY +TBI, n=6; Flu +Mel+TBI, n=3; Flu+BU+TBI, n=1	CsA+short-term MTX	NR	NR	41.67%	0	1-year OS 33.3%, PFS 24.3%, RR 59.0%, and NRM 16.7%	(34)
GJ Min et al.	2020	Korea	186	AML	BM or PBSC	*in vivo* TCD-haploSCT	r-ATG 1.25 mg/kg×4d	Flu+BU+TBI	CsA+short-term MTX	Real-time quantitative-PCR	NR	72.70%	19.40%	OS 52.3% (mismatched) and 55.3% (matched); GRFS 40.6% (mismatched) and 42.2% (matched); Relapse 22.5% (mismatched) and 8.6% (matched); NRM 28.9% (mismatched) and 27.1% (matched)	(35)
L Zhu et al.	2015	China	25	AML (7); ALL (17); Bi-lineage AL (1)	BM +PBSC +MSC (21) or BM +MSC (4)	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d (-4 to -1)	BU+Ara-C+CY	CsA+MMF+short-term MTX	NR	NR	92%	NR	14-month OS 53.28%	(28)
J Xu et al.	2020	China	72	T-ALL	BM or PBSC or BM +PBSC combined with CB	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d (-4 to -1)	Modified BUCY	CsA+MMF+short-term MTX	PCR	NR	19.40%	NR	3-year OS (66.6 ± 6.2)%; RFS (62.0 ± 6.5)%; relapse (24.2 ± 6.4)%; NRM (16.9 ± 5.1)%	(25)
J Wang et al.	2019	China	139	AML (100); ALL (39)	BM +PBSC or BM +PBSC +UCB	*in vivo* TCD-haploSCT	ATG-F 5 mg/kg×4d	BUCY+Me-CCNU+FLAG/CLAG, n=96; TBI +CY+Me-CCNU +FLAG/CLAG, n=43	CsA+MMF+short-term MTX	Real-time PCR	NR	100-day 59.8% (Cord-HaploSCT) and 47.6% (HaploSCT)	2.88%	2-year relapse 25.9% (Cord-HaploSCT) and 53.2% (HaploSCT); NRM 38.8% (Cord-HaploSCT) and 24.6% (HaploSCT); OS 35.5% (Cord-HaploSCT) and 22.7% (HaploSCT); PFS 35.5% (Cord-HaploSCT) and 17.9% (HaploSCT)	(26)

(Continued)

TABLE 1 | Continued

Group	Year	Country	haploSCT Sample size	Primary Disease (n)	Stem cell source (n)	Graft manipulation	Dose of ATG	Conditioning (n)	GVHD prophylaxis	Assays measuring CMV DNAemia	Cutoff values for CMV reactivation or reactivation needing PET	CMV reactivation	CMV disease	Clinical outcome/Comments	Reference
XN Gao et al.	2020	China	110	AML (58); MDS (6); CML (4); MDS/MPN (1); ALL (38), NHL (3), PCL (1)	PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY, n=95; TBI+CY, n=3; Flu+BU, n=4; BU+FLAG, n=8	CsA+MMF+short-term MTX	Real-time quantitative PCR	CMV DNA loads exceeded 1000 copies/mL	1-year 55.0%	1-year 7.9%	3-year NRM 30.5% (CMV DNAemia+) and 13.7% (CMV DNAemia-); 3-year OS 55.0% (CMV DNAemia+) and 60.4% (CMV DNAemia-)	(29)
HH Li et al.	2017	China	94	AML (46); Therapy-related AML (6); MDS transformed AML (5); MDS-refractory anemia with excess blast (1); ALL (26); CML (5); Lymphoma (5)	PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/kg×4d	Modified BUCY, n=60; TBI+CY, n=28; BF, n=6	CsA+MMF+short-term MTX	NR	NR	1-year 62.1%	1-year 8.1%	3-year NRM 24.0% (HaploSCT) and 10.2% (MSD); relapse 39.0% (HaploSCT) and 22.6% (MSD); DFS 45.7% (HaploSCT) and 78.9% (MSD)	(30)
E Shmueli et al.	2014	Israel	102	Congenital disease; SAA; hematological malignancy; solid tumor	NR	*in vivo* TCD-haploSCT	ATG*	Flu+TT+TBI	NR	Real-time PCR	Higher than 50 copies/mL	66.70%	11.6%	The high rate of drug resistance as interlinked with severe disease in haplo-HSCT recipients.	(8)
SS Park et al.	2021	Korea	46	SAA	PBSC	*in vivo* TCD-haploSCT	r-ATG 5-10 mg/kg	TBI+Flu	Tacro+short-term course MTX	NR	NR	45.70%	NR	3-year OS 84.4%; 3-year TRM 11.2%	(36)
A Bertaina et al.	2014	Italy	23	SCID (8); SAA (4); FA (4); IPEX (1); CAMT (1); SDS (1); UNC13D-mutated HLH (1); DOCK-8-mutated HIEs (1); Osteopetrosis (1); Thalassemia (1)	PBSC	*ex vivo* TCD-haploSCT (αβ+ T and CD19+ B cells depletion)	r-ATG 4 mg/Kg×3d (-5 to -3)	BU+TT+Flu, n=3; Treo+TT+Flu, n=4; Treo+Flu, n=8; Flu+CY ± TBI, n=8	No posttransplantation pharmacologic GVHD prophylaxis	NR	NR	38% (CMV and adenovirus)	4.35%	The 2-year probability of both OS and DFS was 91.1%	(42)
AE Hammerstrom et al.	2018	USA	86	Leukemia (75); Lymphoma (8); MM (1); AA (2)	BM (83); PBSC (3)	PTCy-haploSCT	No	Mel+TT+Flu	MMF+Tacro	pp65 CMV antigenemia assay or PCR.	CMV antigenemia with ≥1 cell/million or detectable CMV DNA	Traditional 81%; Hybrid 53%; Intermediatedose 71%	8% (Traditional), 0% (Hybrid), and 0% (Intermediate dose)	100-day NRM 0 (Traditional), 13% (Hybrid), and 13% (Intermediate dose); 100-day OS 100% (Traditional), 80% (Hybrid), and 87% (Intermediate dose)	(60)
R Mitchell et al.	2019	Australia	19	Primary immunodeficiency disease; HLH; FA; AML; ALL	PBSC; BM	*ex vivo* TCD-haploSCT (αβ+ T and CD19+ B cells depletion)	ATG*	Treo+Flu+TT; Bu+Flu+TT; Treo+Flu; Bu+CY+Flu; Flu+CY; Flu+Mel+TT; TBI+Flu+Mel+TT	MMF (n=11) or CsA (=3) or combination CsA/MMF (n=5), or no prophylaxis (n=1)	CMV PCR screening	NR	50.00%	5.26%	100-day TRM 0% and 1-year TRM 15%; 5-years OS 80%	(43)
SH Kang et al.	2021	Korea	81	Malignant disease (45); Nonmalignant disease (36)	PBSC	*ex vivo* TCD-haploSCT (αβ T lymphocyte depletion)	Malignant disease r-ATG (2 mg/kg at -8d and 1 mg/kg at -7d); Nonmalignant disease r-ATG (2.5 mg/kg/day, -8d to -6d)	Flu+CY+TBI	NR	Quantitative real-time PCR	>2.49 log copies/mL	50.8% (GCV/FCV 44.4% *vs* GCV 62.6%)	15.4%; no significant difference in the incidence of CMV disease according to prophylaxis method	Interim-FCV prophylaxis effectively prevented CMV reactivation in those undergoing αβ T cell-depleted haploSCT.	(41)
I Airoldi et al.	2015	USA	27	ALL (9); AML (6); SCID (4); FA (3); Hyper-IgE syndrome (1); Refractory cytopenia of childhood (2); Kostmann syndrome (1); Osteopetrosis (1); SDS (1)	PBSC	*ex vivo* TCD-haploSCT (TCR-αβ+/CD19+ lymphocytes depletion)	No	TBI+TT+Mel; TBI+TT+CY; TBI+TT+Flu; Treo+TT+Mel; BU+TT+Flu; BU+CY+Mel; Treo+TT+Flu; Treo+Flu; TBI+CY+Flu; BU+Flu	No posttransplantation pharmacologic GVHD prophylaxis	NR	NR	55.50%	NR	81.5% survived at last follow-up	(44)
L Kaynar et al.	2017	Turkey	34	AML (24); ALL (10)	PBSC	*ex vivo* TCD-haploSCT (TcRαβ-depletion)	ATG-F 30 mg/kg (-12 to -9)	Flu+TT+Mel	MMF	PCR	NR	73.5% (AML 66.7%; ALL 90.0%)	0	1-year DFS 42%; OS 54%	(39)
HF Nazir et al.	2020	Oman	12	FHLH	PBSC	*ex vivo* TCD-haploSCT (CD3/CD19 depletion)	ATG-F 10 mg/kg (-6 to -3)	Treo+TT+Flu +Rituximab	CsA or Tacro or No pharmcologic prophylaxis	PCR	CMV viral load exceeded 500 copies/mL	75.00%	16.67%	3-year DFS 58.3%	(45)

(Continued)

TABLE 1 | Continued

Group	Year	Country	haploSCT Sample size	Primary Disease (n)	Stem cell source (n)	Graft manipulation	Dose of ATG	Conditioning (n)	GVHD prophylaxis	Assays measuring CMV DNAemia	Cutoff values for CMV reactivation or reactivation needing PET	CMV reactivation	CMV disease	Clinical outcome/Comments	Reference
F Erbey et al.	2018	Turkey	21	ALL (14); AML (7)	PBSC	*ex vivo* TCD-haploSCT (TcRαβ-depletion)	r-ATG 20mg/kg (-13 to -9)	Flu+TT+Mel	MMF with or without CsA	PCR screening	NR	81.00%	NR	5-year OS 71.1%; RFS 86.9%; TRM 16.3%	(40)
S Gaballa et al.	2016	USA	50	AML (27); MDS or MPD (3); ALL (14); NHL (5); AA (1)	DLI + CD34-selected stem cell	PTCy-haploSCT	No	TBI (12 Gy over 4 day)	Tacro+MMF	PCR	NR	100-day 67%	0	3-year OS 70%; PFS 68%; NRM 10%	(38)
R Crocchiolo et al.	2015	Italy	70	HL (35); NHL (20); MM (2); AL (11); CLL (2)	BM (66); PBSC (4)	PTCy-haploSCT	No	NMA, n=48; RIC, n=16; MAC, n=6	Tacro/CsA+MMF	PCR	Threshold of CMV viremia for PET was 3300 copies/mL	54.00%	4.29%	2-year OS 48%, TRM 26%	(53)
J Gaziev et al.	2018	USA	54	Thalassemia (45); Sickle cell anemia (7); HbS-b thalassemia (2)	PBSC and/or BM	*ex vivo* TCD-haploSCT (CD34 selection of PBSCs and BM, n=32; CD34 selection of PBSCs and CD3/CD19 depletion of BM, n = 8; TCRαβ/CD19 depletion of PBSCs, n = 14)	r-ATG 12.5 mg/kg over 4 days, n=6; ATG-F 50 to 25 mg/kg over 5 days, n=48	BUTT10CY200 preceded by HuAzFlu or BUTT10CY200 preceded by Flu with/without Rituximab prophylaxis	CsA +methylprednisolone or CsA+MMF	reverse-transcription PCR	NR	64.00%	0	OS 78% (TCR group) and 84% (CD34 group); DFS 69% (TCR group) and 39% (CD34 group)	(47)
L Prezioso et al.	2019	Italy	59	AML (32); ALL (6); NHL (6); HL (8); MF (4); MDS (2); MM (1); PCL (1)	PBSC (24); CD34+ (35)	*ex vivo* TCD-haploSCT (αβTCR/CD19+ depletion or selection of the CD34+ cells)	r-ATG 1.5 mg/kg ×4d (-9 to -6)	Flu+TT	No posttransplantation pharmacologic GVHD prophylaxis	PCR	NR	7.27%	1.69%	2-year OS 50.8%	(46)
D Huntley et al.	2020	Spain	118	AL (43); CL (9); Lymphoma (26) MDS/MM/ Myelofibrosis (25); Other (15)	PBSC (110); BM (8)	PTCy-haploSCT	Only one patient received ATG	MAC,n=35; RIC, n=83	CsA or Tacro	RealTime CMV PCR	31 IU/ml or 137 IU/ml at different centers	63.90%	4.50%	1-year OS 70.3%	(55)
LJ Arcuri et al.	2020	USA	87	SAA	BM (81); PBSC (3); BM +PBSC (3)	PTCy-haploSCT	12 patients received r-ATG	Flu+CY+TBI	CsA+MMF or Tacro +MMF	Positive antigenemia or PCR	NR	100-day 61%, 1-year 62%, 2-year 62%	NR	2-year OS 79%; 2-year EFS 70%	(62)
M Slade et al.	2017	USA	104	AML (70); ALL (11); MDS (11); Other (12)	PBSC	PTCy-haploSCT	NR	MAC, n=43; NMA, n=61	CsA+MMF or Tacro +MMF	PCR	>40 000 IU/mL	55.00%	15%	51% survived at last follow-up	(69)
E Katsanis et al.	2020	USA	17	AL,CML, NHL	BM	PTCy/BEN-haploSCT (9); PTCy-haploSCT (8)	No	TBI+Flu or BU +Flu+Mel	MMF+Tacro	PCR	NR	12.5% in PTCy-BEN with 71.4% in PTCy	NR	2-year OS 83.3% in PTCy-BEN with 58.3% in PTCy	(64)
GC Irene et al.	2021	Spain	40	AL/MDS (28); MPN (1); Lymphoid malignancies (9); Others (2)	PBSC or BM	PTCy-haploSCT	No	RIC,n=1;MAC, n=39;	Tacro	Quantitative PCR	PET: a level of DNAemia of >1000 IU/ml in one blood sample or two consecutive samples with a level of >500 IU/mL	18-month 61%	2.50%	18-month OS 71.3%; PFS 67.4% with no differences by donor type	(59)
RV Raj et al.	2016	USA	43	AML/MDS (27); ALL (5); Myeloma (4); NHL/HL (4); Others (3)	BM (22); PBSC (21)	PTCy-haploSCT	No	Flu+CY+TBI, n=23; Flu+Bu +CY, n=15; Flu +Mel+TBI, n=5	Tacro+MMF	Quantitative nucleic acid amplified tests (NAAT)	NR	RIC with 40% in MAC	0 (RIC) and 7% (MAC)	NR	(63)
SR Goldsmith et al.	2016	USA	138	AML (93); MDS (15); Other (30)	PBSC	PTCy-haploSCT	No	MAC, n=58; RIC, n=80	Tacro+MMF or other	Real-time qPCR	NR	58.00%	16.67%	Post-transplant CMV viremia was not associated with a statistical difference in overall survival	(65)
J Montoro et al.	2020	Spain	42	AL (15); MM (5); Lymphoproliferative disorders (13); MDS (5); MPD (4)	BM (5); PBSC (37)	PTCy-haploSCT	No	TBF-MAC, n=9; TBF-RIC, n=2; BU+Flu+CY, n=11	MMF+Sirolimus	Quantitative real-time PCR assays	NR	52.00%	2.38%	1-year NRM 14%; EFS 75%; OS 82%; GRFS 47%. A higher cumulative incidence of CMV DNAemia requiring pre-emptive antiviral therapy in the haploidentical cohort.	(70)
N Cieri et al.	2015	Italy	40	AML (22); ALL (5); MDS (1); CML (1); HL (6); NHL (5)	PBSC	PTCy-haploSCT	No	Flu+Treo+Mel	MMF+Sirolimus	Quantitative PCR	PET was started when CMV DNA copy number was more than 1000	63.00%	15%	1-year OS 56%; DFS 48%	(71)

(Continued)

TABLE 1 | Continued

Group	Year	Country	haploSCT Sample size	Primary Disease (n)	Stem cell source (n)	Graft manipulation	Dose of ATG	Conditioning (n)	GVHD prophylaxis	Assays measuring CMV DNAemia	Cutoff values for CMV reactivation or reactivation needing PET	CMV reactivation	CMV disease	Clinical outcome/Comments	Reference
											copies/mL or increased more than.5 log in peripheral blood plasma.				
N Stocker et al.	2020	France	19	AML (10); MPN (1); MDS (1); ALL (4); NHL (3)	PBSC	PTCy-haploSCT	2.5 mg/kg, n=3; 5 mg/kg, n=16	RTC, n=13; TT +etoposide+CY +RIC, n=6	CsA+MMF	Quantitative PCR	PET was initiated when CMV was above 1000 IU/mL	46.00%	NR	2-year Relapse 19% (Control group) and 19% (PTCy group); PFS 73% (Control group) and 70% (PTCy group); OS 78% (Control group) and 79% (PTCy group)	(67)
Crocchiolo R et al.	2016	Italy and France	207	AL (44); HL (54); NHL (61); MM (13); MDS/ MPS (25); Drepanocytosis (1)	PBSC (111); BM (96)	PTCy-haploSCT	NR	NMA/RIC, n=181; MAC, n=26	NR	NR	NR	42.00%	1.45%	Two-year OS 62% (Cohort 1); 65% (Cohort 2); 50% (Cohort 3); 42% (Cohort 4)	(56)
SR Goldsmith et al.	2021	USA	757	AML/ALL/MDS	BM or PBSC	PTCy-haploSCT	No	MAC or RIC/ NMA	Tacro or CsA	PCR	NR	180-day 42%	100-day 2.8%	2-year mortaligy 49.5%	(51)
Y Lu et al.	2018	China	41	SAA (28)/VSAA (13)	BM +PBSC	*in vivo* TCD-haploSCT	ATG-r 7.5 mg/ kg, n=42; ATG-F 20 mg/kg, n=47	BU+Flu+CY	Tacro+MMF+short-term MTX	PCR	Higher than 500 copies/mL in plasma	65.90%	4.88%	3-year OS 80.3% ± 5.1%; 3-year FFS 76.4% ± 5.1%	(13)
W-R Huang et al.	2016	China	130	AML; ALL; CML; Lymphoma	PBSC	*in vivo* TCD-haploSCT	r-ATG 2.5 mg/ kg/day -5d to -2d	Modified BUCY, n=90; Modified BF, n=32; TBI +CY, n=8	CsA+MMF+short-term MTX	PCR	NR	1-year 61.0 ± 5.3%	1-year 8.0% ± 2.9%	3-year OS 45.6% ± 5.6%; LFS 44.2% ± 5.9%	(23)
BM Triplett et al.	2015	USA	17	ALL (6); AML (9); MLL (1); MDS (1)	PBSC	*ex vivo* T-cell depletion (CD45RA-depletion)	No	TLI+Flu+CY+TT +Mel	Sirolimus or MMF	PCR	NR	17.65%	0	76.5% survived at a median of 223 days	(49)
BM Triplett et al.	2018	USA	67	ALL (28); AML (22); MLL (4); MDS (8); Lymphoma (3); CML (2)	PBSC	*ex vivo* T-cell depletion (CD3-depletion,n=41; CD45RA-depletion, n=26)	No	CD3-depleted: Flu+TT+Mel +OKT3 (n = 21) or alemtuzumab (n=20)+Rituximab CD45RA-depleted: Flu+TT +Mel+lymphoid irradiation+CY	a short (<60 days) course of MMF	Quantitative PCR	NR	CD3-depleted 56%, CD45RA-depleted 19%	NR	180-day mortality CD3dep recipients 22% *vs* CD45RAdep recipients 15.4%	(50)
A Fayard et al.	2019	France	381	AL/MDS (208); HL/ NHL (115); MPN (31); MM/solitary plasmacytoma (15); chronic leukemia (10); bone marrow failure syndrome (2)	BM (103); PBSC (278)	PTCy-haploSCT	No	RIC, n=307; MAC, n=73	an anticalcineurin +MMF	A single pp65 antigen-positive leukocyte or a positive viremia in peripheral blood	NR	48.80%	4.50%	Median of PFS 19.9 months; Median of OS 33.5 months	(52)
A Esquirol et al.	2021	Spain	236	AML (76); MDS (39); ALL (22); NHL (39); HL (31); CLL (8); CML/MPN (12); MM (5); biphenotypic acute leukemia (2); aplasia (1); prolymphocytic leukemia (1)	BM (45); PBSC (191)	PTCy-haploSCT	NR	Flu+BU; Flu+Bu +CY; TBF; Other	CsA+MMF or Tacro alone	PCR	>1000 IU/mL	69.00%	2.12%	12-month OS 64%; 12-month PFS 57%	(54)
Monzr M. Al Malki et al.	2017	USA	119	Acute leukemia (80); bone marrow failure (15); lymphoma (11); chronic leukemia (6); hemoglobinopathies (5); MM (2)	PBSC (81); BM (38)	PTCy-haploSCT	NR	MAC, n=46; RIC/ NMA, n=73	Tacro/MMF	PCR	NR	100-day 69.2%	0	CMV reactivation was not associated with OS, RFS, relapse incidence, or NRM.	(57)
D Huntley et al.	2020	Spain	71	Acute leukemia (24); Chronic leukemia (6); Lymphoma (15);	PBSC (65); BM (6)	PTCy-haploSCT	No	MAC, n=17; RIC, n=54	Tacro-based, n=41; MMF-based, n=15	Real-time PCR	Higher than 600 IU/ml or higher than IU/ml at different centers	59.70%	4.23%	PTCy-haploSCT recipients may reconstitute CMV-specific T-cell immunity to the same extent as	(58)

(Continued)

TABLE 1 | Continued

Group	Year	Country	haploSCT Sample size	Primary Disease (n)	Stem cell source (n)	Graft manipulation	Dose of ATG	Conditioning (n)	GVHD prophylaxis	Assays measuring CMV DNAemia	Cutoff values for CMV reactivation or reactivation needing PET	CMV reactivation	CMV disease	Clinical outcome/Comments	Reference
				Myelofibrosis/MDS (18); Other (5)										patients undergoing HLA-matched allo-HSCT	
R Uppuluri et al.	2019	India	16	Primary immune deficiency disorder	BM (6); PBSC (10)	PTCy-haploSCT	NR	Flu+Mel, n=5; Flu +Treo, n=3; Treo +Flu+TBI, n=3; Treo+Flu, n=1; Flu+Treo+TBI, n=4	NR	NR	NR	43.70%	6.25%	Overall mortality 37.5%; OS 62.5%; Cytokine release syndrome (CRS) 75%	(61)
SR Solomon et al.	2015	USA	30	AML (16); ALL (6); CML (5); MDS (1); NHL (2)	PBSC	PTCy-haploSCT	No	Flu+TBI	Tacro+MMF	Quantitative CMV PCR	PET was initiated if viral reactivation was detected (higher than 400 copies/mL)	58.00%	0	2-year OS 78%; 2-year DFS 73%	(66)
C Oltolini et al.	2020	Italy	145	Myeloid disorders (106); Lymphoid disorders (39)	PBSC	PTCy-haploSCT	No	MAC, n=110; RIC, n=35	sirolimus+MMF, n=141; CsA+MMF, n=3	PCR	PET was started when plasmatic CMVDNA higher than 1000 copies/mL or increased >0.5 log.	61% (68%, haploSCT)	13.79%	Relapse 44%	(68)
AD Law et al.	2018	Canada	50	AML (28); MDS (8); MPN (6); ALL (2); Lymphoma (5); BPDCN (1)	PBSC	PTCy-haploSCT	r-ATG 4.5 mg/kg	Flu+BU+TBI	CsA	NR	NR	74%	8%	1-year OS 48.1%; NRM 38.2%	(72)
MQ Salas et al.	2020	Canada	52	AML (29); MDS (8); MPN (5); ALL (3); Lymphoproliferative disease (6); BPDCN (1)	PBSC	PTCy-haploSCT	r-ATG 4.5 mg/kg	Flu+BU+TBI	CsA	Quantitative PCR	>200 copies/ml	58%	4%	1-year OS 58.8 (44–70.9)%; 1-year RFS 53.3 (38.8–65.8)%	(73)
J Tischer et al.	2015	Germany	55	AML (33); CML (2); ALL (7); SAA (1); NHL (14); CLL (2)	BM +PBSC	*ex vivo* T-cell depletion (cTCR/TCD: CD6-depleted G-CSF-mobilized peripheral blood stem cells); PTCy-haploSCT	cTCR/TCD: r-ATG 20 mg/kg for 5 days; TCR/PTCY: No ATG	RIC or MAC	CsA+MTX or Tacro +MMF or MMF	Quantitative real-time PCR	NR	cTCR/TCD 42.9%; TCR/PTCy 14.8%	7.14% (cTCR/TCD) and 0 (TCR/PTCy)	cTCR/TCD: 1-year OS 39%, RFS 38%; TCR/PTCY: 1-year OS 59%; RFS 55%	(78)

HaploSCT, haploidentical stem cell transplantation; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; AL, acute leukemia; MDS, myelodysplastic syndromes; AA, aplastic anemia; SAA, severe aplastic anemia; VSAA, very severe aplastic anemia; Ph+, Philadelphia chromosome-positive; PNH, paroxysmal nocturnal hemoglobinuria; CMML, chronic myelomonocytic leukemia; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction; PET, preemptive therapy; LCS, Langerhans cell sarcoma; PMF, primary myelofibrosis; BPDCN, blastic plasmacytoid dendritic cell neoplasm; PCL, plasma cell leukemia; SCID, severe combined immunodeficiency; FA, Fanconi anemia; IPEX, immunodeficiency with polyendocrinopathy and enteropathy X-linked; CAMT, congenital amegakaryocytic thrombocytopenia; SDS, Shwachmann-Diamond syndrome; HLH, hemophagocytic lymphohistiocytosis; UNC13D-mutated HLH, UNC13D-mutated hemophagocytic lymphohistiocytosis; DOCK-8-mutated HIEs, DOCK-8–mutated hyper-IgE syndrome; FHLH, familial hemophagocytic lymphohistiocytosis; MPD, myeloproliferative disease; HL, Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; CL, chronic leukemia; MPN, myeloproliferative neoplasm; MPS, myeloproliferative syndrome; MLL, mixed lineage leukemia; BM, bone marrow; PBSC, peripheral blood stem cells; HSCT, hematopoietic stem cell transplant; MSC, mesenchymal stem cell; CB, cord blood; UCB, umbilical cord blood; DLI, donor lymphocyte infusion; TCD, T-cell depletion; PTCy, posttransplant cyclophosphamide; ATG, anti-thymocyte globulin; ATG-F, ATG-Fresenius; r-ATG, ATG-Genzyme; BU, busulfan; CY, cyclophosphamide; BUCY, busulfan cyclophosphamide regimen; CCNU, lomustine; Me-CCNU, simustine; Ara-c, cytosine arabinoside; BF, busulfan fludarabine regimen; FLAG, fludarabine+ cytarabine + granulocyte colony-stimulating factor; CLAG, cladribine + cytarabine + granulocyte colony-stimulating factor; Flu, fludarabine; TT, thiotepa; Treo, treosulfan; Mel, melphalan; Az, azathioprine; Hu, hydroxyurea; TBI, total body irradiation; TBF, thiotepa busulfan fludarabine; MAC, myeloablative conditioning, NMA, non-myeloablative; RIC, reduced-intensity conditioning; RTC, reduced toxicity conditioning; TLI, total lymphoid irradiation; CMV, cytomegalovirus; CsA, cyclosporine A; Tacro, tacrolimus; MMF, mycophenolate mofetil; MTX, methotrexate; GCV, ganciclovir; FCV, foscarnet; TCR, T-cell-replete; HLA, human leukocyte antigen; CMVR, cytomegalovirus retinitis; RRM, relapse-related mortality; OS, overall survival; LFS, leukemia-free survival; NRM, non-relapse mortality; TRM, transplant-related mortality; GVHD, graft-versus-host disease; aGVHD, acute graft-versus-host disease; FFS, failure-free survival; GFFS, GVHD-free and relapse-free survival; GRFS, GVHD-free relapse-free survival; PFS, progression-free survival; EFS, event-free survival; DFS, disease-free survival; RFS, relapse-free survival; RR, relapse rate; MSD, matched sibling donor; NR, not reported.

**The dose of ATG is not mentioned in the paper.*

of CMV-specific T-cell subsets show distinct kinetics, particularly after CMV reactivation.

More recently, Jasper J. P. van Beek et al. conducted longitudinal analysis of high-dimensional T-cell immunophenotypes in 21 recipients of PTCy-haploSCT (91). CMV-specific T-cells were primed early after PTCy-haploSCT and initially showed a proliferating/activated phenotype, that was quickly replaced by a terminal effector phenotype, while uncontrolled viral replication associated with lower abundance of distinct CMV-specific CD4+ T-cell immunophenotypes, hinting at a possible role of these cells in CMV control. CMV-specific T-cell features were similar to those of the CMV-seropositive donor one year posttransplantation, implying reestablishment of physiological homeostasis.

NK

NK cells similarly play an essential role in defense against infections and leukemia relapse after hapoSCT. Fengyan Jin et al. explored NK cell dynamics in 29 patients after haploSCT between August 2011 and November 2014 (92). IFNγ-producing NK cells expanded in 19 patients after CMV reactivation, and the percentages of IFNγ-producing NK cells in these patients greatly increased from day 60 to 180 after transplantation compared to those of their donors. The percentage of KIR-expressing NK cells and IFNγ-producing NKG2C+ NK cells was significantly higher in haploSCT recipients with CMV reactivation than in those without CMV reactivation. Moreover, CMV reactivation was associated with expansion of the $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cell subset between days 30 and 90 after haploSCT (93). Patients with increased $CD56^{bright}CD16^{dim/-}DNAM1^{+}$ NK cells also had a remarkably higher CMV viral load (93).

Letizia Muccio et al. reported that CMV reactivation boosted the arrival of mature NK cells in pediatric patients with hematological malignancies receiving HLA-haploSCT after removal of both αβT cells and CD19 B cells (94). A memory-like NK cell subset expressing NKG2C and CD57 progressively expanded in most children. NKG2C+CD57+ NK cells were detected by month 3 after allo-SCT and expanded until at least month 12. These cells characteristically expressed high levels of killer Ig-like receptors (KIRs) and leukocyte inhibitory receptor 1 (LIR-1) and low levels of Siglec-7, NKG2A and interleukin-18Rα. Additionally, they poorly secreted interferon-γ in response to interleukin-12 and interleukin-18. The compromised response to these cytokines as well as their highly differentiated profile may reflect their skewing toward immune control of human cytomegalovirus.

Xiang-Yu Zhao et al. from Peking University previously found that donor-recipient KIR ligand matching decreased CMV reactivation and refractory CMV infection by day 100 post-transplantation (95). This indicates that donor-recipient KIR ligand matching might improve the NK cell licensing process and promote NK cell-mediated control of CMV reactivation. The same group then prospectively assessed NK cell reconstitution in patients undergoing matched sibling transplantation and haploSCT (96). CD107a was increasingly expressed in NK cells after versus before CMV reactivation at days 60, 100, and 180 after transplantation, but CMV reactivation did not impact the maturation process of NK cells after transplantation. In addition, KIR expression and NKp30 expression were lower on NK cells in patients with CMV reactivation than in those without CMV reactivation at day 30. The NK-to-T-cell (NK/T) ratio was persistently higher in patients with CMV reactivation than in those without CMV reactivation from 30 days to one year after haploSCT.

An emerging report from Elisa Zaghi et al. demonstrated impaired adaptive NK cells expanded after CMV reactivation in PTCy-haploSCT (97). By a longitudinal single-cell computational profiling of multiparametric flow cytometry, they found that CMV accelerates NK cell immune recovery with the expansion of $CD158b1b2j^{pos}/NKG2A^{neg}/NKG2C^{pos}/NKp30^{lo}$ NK cells. The number of this subset is associated with CMV reactivation, further increases in recipients with multiple viral reactivations and persists for months after the infection. The transcriptional characteristics of FACS-sorted $CD158b1b2j^{pos}$ NK cells confirmed the capacity of CMV to deregulate NKG2C, NKG2A, and NKp30 gene expression, thus mediating the expansion of NK cells with adaptive traits. These results imply that the dysfunction/exhaustion of "adaptive" KIR^{pos} NK cells in patients with CMV reactivated is induced, at least partially, by the CMV-induced expression of checkpoint inhibitors.

γδ T

Fifty pediatric patients undergoing αβ T cell-depleted haploSCT between August 2012 and December 2015 were analyzed (98). CMV reactivation developed in 19 transplantations at a median of 30 days (range, 13-318 days) after haploSCT. Higher γδ T cells were observed in patients without CMV reactivation than in patients with CMV reactivation at day 30 (197.8 ± 153.9 *vs* 53.9 ± 58.7). There was a significantly higher incidence of CMV reactivation in patients with a low percentage of γδ T cells at day 30 than in patients with a high percentage of γδ T cells (78.0 ± 15.3% *vs* 22.2 ± 13.9%). No difference in day 30 γδ T cells was found between patients with and without CMV disease.

Irma Airoldi et al. prospectively monitored the functional and phenotypic characteristics of γδ T cells up to 7 months after αβ+ T cells and CD19+ B cells depleted haploSCT in 27 children (44). They reported that γδ T cells are the foremost T-cell population in patients during the first weeks and are mainly derived from the graft content and expanded *in vivo* after transplantation. Central memory cells predominated very early after haploSCT for both the Vδ1 and the Vδ2 subsets. Vδ1 cells are specifically expanded in patients with CMV reactivation and are more cytotoxic than those of children without reactivation.

CMV-specific T-cell, NK cell, and γδ T-cell are vital to immune control of CMV infection post haploSCT, but it seems that γδ T-cell is more likely responsible for viral reactivation in the context of *ex vivo* TCD-haploSCT. Although NK cells and γδ T cells are the first lymphocytes that recover after transplantation, CMV-specific T cells are dominant in number in case of viral infection. The majority of studies state that impaired T-cell and NK-cell reconstitution and increased risk of CMV infection after haploSCT, so seeking factors influencing CMV-specific immune reconstitution and interventions to improve immune reconstitution is urgent at the moment. Although data from Dixie Huntley et al. supported similar

incidence of CMV infection and restored CMV-specific T cells after PTCy-haploSCT compared to MRD/MUD transplantation (58), the scarce number of MUD and MRD recipients and more sirolimus used in PTCy-haploSCT group preclude any definitive conclusion and further studies are warranted to validate their findings.

CELLULAR IMMUNOTHERAPY OF CMV INFECTION

Delayed CMV-specific immune reconstitution has been consistently associated with the development of CMV infection and CMV disease after allo-SCT. Accordingly, adoptive transfer of CMV-specific T cells has been employed to treat CMV infection. Several clinical trials and case reports have confirmed the safety and efficacy of this strategy for the prophylaxis and treatment of CMV infection after haploSCT. **Table 2** lists cellular approaches currently in clinical trials and serves as evidence that CMV-targeting immune-based interventions could provide a safe, novel treatment option while offering clinical benefit to CMV reactivated recipients after haploSCT.

Therapeutical CMV-Specific T-Cell Approaches

Feuchtinger T et al. treated 18 patients after allo-SCT from HLA–mismatched/haploidentical or HLA–matched unrelated donors with polyclonal CMV-specific T cells (99). These T cells were generated by isolation of interferon-γ–producing cells after stimulation with pp65 antigen. Patients with refractory CMV disease or viremia received a mean of 21×10^3/kg pp65-specific T cells. CMV infection was cleared, or viral burden was significantly decreased in 83% of these patients, even in patients with CMV encephalitis. Viral control was related to improved antiviral T-cell reconstitution and *in vivo* expansion of CMV-specific T cells in 12 of 16 evaluable cases without inducing GVHD or acute side effects.

In another CMV infection refractory cohort (100), 27 of 32 treated patients after haploSCT cleared CMV within four weeks after adoptive T-cell therapy without recurrence. After cellular transfer, CMV-specific T cells expanded *in vivo* with improved cytokine production and proliferation ability. In addition, the expression of programmed death-1 (PD-1) on CMV-specific T cells was reduced. In the early effective group, patients who cleared viremia within four weeks after T-cell infusion, CMV-specific CD8+ IFN-γ+ and CD4+ IFN-γ+ T cells were rapidly and massively expanded *in vivo*, whereas in the late effective group, there was no significant expansion of CMV-specific T cells. Xiang-Yu Zhao et al. further evaluated the safety and efficacy of donor-derived CMV-specific cytotoxic T cells (CTLs) as a first-line therapy for CMV infection after haploSCT (101). They observed that first-line therapy with CTLs significantly reduced the incidence of CMV infection with lower 1-year treatment-related mortality and better 1-year overall survival. Moreover, first-line therapy with CTLs promoted the recovery of CTLs in patients, which correlated with CMV clearance.

A case report described two patients with drug-resistant CMV encephalitis after haploSCT successfully received donor CMV-specific CTLs (102). In the first case, a 27-year-old male developed CMV encephalitis during ganciclovir maintenance treatment after haploSCT. After administering foscarnet and donor CMV-specific CTLs, CMV-DNA of his cerebrospinal fluid (CSF) was negative by RT-PCR, and the lesions on brain magnetic resonance imaging (MRI) were reduced. Another case, a 57-year-old female, also experienced CMV encephalitis during maintenance treatment with ganciclovir after haploSCT. After intrathecal treatment with donor CMV-specific CTLs, the CMV load of the CSF was reduced.

Prophylactic DLI

Prophylactic and therapeutic DLI are administered to improve posttransplant immune restoration to reduce both infectious complications and disease relapse. Michael Maschan et al. investigated low-dose memory (CD45RA-depleted) donor lymphocyte infusion (mDLI) after αβ T-cell depleted HSCT (103–105). The incidence of CMV reactivation was 45-50% in the experimental mDLI arm and 54-55% in the control arm. The median duration of CMV viremia was 3 weeks (range, 1-9) in the prospective cohort and 4 weeks (range, 1-26) in the historical cohort (105). Memory DLI was associated with improved CMV-specific T-cell reconstitution in a subcohort of CMV IgG seropositive recipients. Analysis of a subcohort of CMV seropositive recipients indicated remarkably better CMV-specific T-cell reconstitution on day 30 in the experimental arm (104). Compared to that of the historical cohort, restoration of CMV-specific immunity at day 30 was significantly enhanced in the prospective cohort (40% versus 25%) (105). Luca Castagna et al. prospectively evaluated a CD45RA+ depleted DLI in terms of reducing viral infection early after PTCy-haploSCT (106). CMV reactivation occurred in 28% of patients. Although the majority of the patients received the planned three infusions, only one patient developed grade 2 acute GVHD, and two patients had moderate chronic GVHD.

Therapeutic DLI

Park HJ et al. reported the successful treatment of refractory CMV colitis after PTCy-haploSCT using CD45RA+ depleted DLI (107). After failure of ganciclovir and foscarnet, granulocyte colony-stimulating factor-primed, CD45RA+ depleted DLI was administered to treat refractory CMV colitis. CMV pp65-specific CTLs were found in recipients four weeks after DLI. Meanwhile, diffuse wall thickening involving the entire colon was also normalized in the abdominal CT scan.

As manipulated DLI approaches are still not widely used due to high cost and intensive labor, unmanipulated donor lymphocytes (U-DLIs), if feasible by harvesting CTLs directly from the peripheral blood of seropositive donors, are used for refractory or relapsed patients with CMV infection. Researchers from Turkey enrolled five pediatric patients receiving U-DLI for CMV infection after transplantation (108). Among them, three patients underwent haploSCT. One patient who was

TABLE 2 | Ongoing clinical trials using cytomegalovirus-specific cellular immunotherapy for allo-SCT patients including haploidentical SCT (accessed on 5 Oct 2021, ClinicalTrials.gov).

Intervention	Patients	Enrollment	Phase	Duration	NCT number	Status
Donor-derived viral specific T-cells (VSTs)	Stem cell transplant recipients who have evidence of viral infection or reactivation	450	Phase 1/ Phase 2	2014-2024	NCT02048332	Recruiting
HLA-matched VSTs	EBV, CMV, adenovirus, and BK infections post allogeneic SCT	47	Phase 1	2021-2024	NCT04013802	Recruiting
Multivirus (CMV, EBV, AdV)-specific T cells	Chemo-refractory viral infections after allo-HSCT	149	Phase 3	2019-2022	NCT04832607	Recruiting
Third party donor derived CMVpp65 specific T-cells	CMV Infection or persistent CMV viremia after allogeneic hematopoietic stem cell transplantation	41	Phase 2	2014-2022	NCT02136797	Recruiting
Adaptive NK cells infusion post transplantation	CMV infection in patients post haploidentical transplantation	30	Not Applicable	2020-2021	NCT04320303	Recruiting
CMV-specific T cells	Relapsing or therapy refractory CMV infection after allogeneic stem cell transplantation	20	Phase 2	2016-2022	NCT03067155	Recruiting
CMV cytotoxic T cells (CTLs) manufactured with the Miltenyi CliniMACS Prodigy Cytokine Capture System	Refractory cytomegalovirus (CMV) infection post allogeneic hematopoietic stem cell transplantation (AlloHSCT), with primary immunodeficiencies (PID) or post solid organ transplant	20	Phase 2	2018-2023	NCT03266640	Recruiting
Direct infusions of donor-derived virus-specific T-cells using the Cytokine Capture System	Recipients of hematopoietic stem cell transplantation with post-transplant viral infections	12	Phase 2	2014-2022	NCT02007356	Recruiting
Emergency access to CMV pp65/IE-1 specific cytotoxic T lymphocytes	Recipients of allogeneic stem cell transplants with persistent or therapy refractory Infections	20	Phase 1	2008-2014	NCT00769613	Active, not recruiting
Viral specific T-Lymphocytes by Cytokine Capture System (CCS)	Infection with adenovirus, cytomegalovirus or Epstein-Barr Virus after hematopoietic cell transplantation or solid organ transplantation and in patients with compromised immunity	25	Phase 1/ Phase 2	2021-2028	NCT04364178	Recruiting
CMV specific adoptive t-cells	Opportunistic cytomegalovirus infection occurring after stem cell transplant	20	Early Phase 1	2016-2022	NCT02982902	Recruiting
Virus specific T-cell (VST) infusion	Enhancing T-cell reconstitution before or after hematopoietic stem cell transplantation	60	Phase 1/ Phase 2	2018-2023	NCT03475212	Active, not recruiting
CMV-specific T-cells	CMV in pediatric and adult immunocompromised patients or recipients of allogeneic stem cell transplantation	20	Phase 1	2020-2026	NCT03798301	Recruiting
Allogeneic cytomegalovirus-specific cytotoxic T lymphocytes	CMV reactivation or infection in participants who have undergone stem cell transplant or solid organ transplant	10	Early Phase 1	2020-2021	NCT03665675	Recruiting
Adoptive cell immunotherapy	Prophylaxis of cytomegalovirus infection in haploidentical transplantation of hematopoietic progenitors	15	Phase 2	2021-2022	NCT04056533	Not yet recruiting
Adoptive transfer of selected cytomegalovirus-specific cytotoxic T lymphocytes (CMV-CTL)	Patients at risk of CMV Disease after allogeneic stem cell transplantation (SCT)	78	Phase 2	2009-2013	NCT00986557	Recruiting
Donor derived cytomegalovirus specific T lymphocytes	Treatment of cytomegalovirus infection after allogeneic hematopoietic stem cell transplantation	30	Phase 4	2016-2021	NCT03004261	Recruiting

transplanted from an unrelated donor received U-DLI from his haploidentical mother. CMV titers were dramatically reduced after U-DLI in these patients.

SUMMARY AND OUTLOOK

Despite the use of prophylactic or preemptive treatments, CMV infection remains an obstacle for successful haploSCT and the improvement of immunologic reconstitution is the primary strategy for infection prevention. A higher rate of CMV reactivation occurred early after haploSCT compared to HLA-matched HSCT, but CMV disease rates were low after haploSCT, particularly in *in vivo* TCD-haploSCT and PTCy-haploSCT. It results from expansion of CMV-specific central memory T-cells in the setting of CMV antigenemia or acceptable CMV-specific T-cell reconstitution. Traditional *ex vivo* TCD-haploSCT successfully prevents lethal GVHD without any posttransplantation immunosuppression, but the small number of T cells in the graft results in impaired immune recovery, which could be overcome by novel *ex vivo* TCD-haploSCT and adoptive cellular therapy. *In vivo* TCD-haploSCT and PTCy-haploSCT indicated low treatment-related mortality (TRM) and an acceptable safety profile, which appears to compare favorably with *ex vivo* TCD-haploSCT in terms of infections. However, synergistic immunosuppression by PTCy and ATG has led to a higher incidence of CMV infection. We now have a better understanding of CMV reactivation and immune reconstitution post haploSCT. Our data demonstrate that novel *ex vivo* TCD techniques followed by prophylactic and therapeutic DLI, a low dose of ATG, an intensified antiviral prophylaxis regimen, sirolimus-containing immunosuppressors and CMV-specific cellular immunotherapy can boost immune recovery and decrease the incidence of CMV reactivation. Furthermore, the

majority of patients receiving the RIC regimen might be less susceptible to infections (63). In this context, it would be essential to perform a prospective study comparing the risk of infectious complications after *in vivo* TCD-haploSCT *vs. ex vivo* TCD-haploSCT or PTCy-haploSCT in patients who received a similar conditioning regimen.

CMV reactivation is associated with delayed immune reconstitution, although this reactivation could also leave a profound imprint on the recovering T cell compartment long-term following allo-SCT (91, 109, 110). Several studies have reported that CMV serostatus and CMV reactivation may be more predictive of T-cell restoration after allo-SCT than GVHD, highlighting the deep impact of this virus on reconstituting T-cells, considering the high incidence of CMV reactivation after haploSCT. More importantly, CMV infection is increasingly recognized as an immunomodulator in cancer patients (111), even in the context of allo-SCT, which is associated with a decreased risk of leukemia relapse, although it is still conflicting (112–115). There is evidence of a bidirectional relationship between CMV reactivation and acute GVHD (116, 117). We should take these into account and balance the merit and disadvantage of taking steps to enhance CMV-specific immune reconstitution and decrease CMV infection.

AUTHOR CONTRIBUTIONS

X-HL wrote the first draft of the manuscript, conducted the literature search, reviewed the abstracts, performed analysis and contributed to the final draft. YZ contributed to revising the manuscript and provided scientific input. Y-TC and L-PS conducted the literature search. LL revised and wrote the final draft, and contributed to the analysis. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Lu DP, Dong L, Wu T, Huang XJ, Zhang MJ, Han W, et al. Conditioning Including Antithymocyte Globulin Followed by Unmanipulated HLA-Mismatched/Haploidentical Blood and Marrow Transplantation can Achieve Comparable Outcomes With HLA-Identical Sibling Transplantation. *Blood* (2006) 107:3065–73. doi: 10.1182/blood-2005-05-2146
2. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W, et al. Haploidentical Hematopoietic Stem Cell Transplantation Without *In Vitro* T-Cell Depletion for the Treatment of Hematological Malignancies. *Bone Marrow Transplant* (2006) 38:291–7. doi: 10.1038/sj.bmt.1705445
3. Xiao-Jun H, Lan-Ping X, Kai-Yan L, Dai-Hong L, Yu W, Huan C, et al. Partially Matched Related Donor Transplantation can Achieve Outcomes Comparable With Unrelated Donor Transplantation for Patients With Hematologic Malignancies. *Clin Cancer Res* (2009) 15:4777–83. doi: 10.1158/1078-0432.CCR-09-0691
4. Wang Y, Liu D-H, Liu K-Y, Xu L-P, Zhang X-H, Han W, et al. Long-Term Follow-Up of Haploidentical Hematopoietic Stem Cell Transplantation Without *In Vitro* T Cell Depletion for the Treatment of Leukemia. *Cancer* (2013) 119:978–85. doi: 10.1002/cncr.27761
5. Chen Y, Xu LP, Liu KY, Chen H, Chen YH, Zhang XH, et al. Risk Factors for Cytomegalovirus DNAemia Following Haploidentical Stem Cell Transplantation and Its Association With Host Hepatitis B Virus Serostatus. *J Clin Virol* (2016) 75:10–5. doi: 10.1016/j.jcv.2015.12.003
6. Yan CH, Wang Y, Mo XD, Sun YQ, Wang FR, Fu HX, et al. Incidence, Risk Factors, and Outcomes of Cytomegalovirus Retinitis After Haploidentical Hematopoietic Stem Cell Transplantation. *Bone Marrow Transplant* (2020) 55:1147–60. doi: 10.1038/s41409-020-0790-z
7. Meng X-Y, Fu H-X, Zhu X-L, Wang J-Z, Liu X, Yan C-H, et al. Comparison of Different Cytomegalovirus Diseases Following Haploidentical Hematopoietic Stem Cell Transplantation. *Ann Hematol* (2020) 99:2659–70. doi: 10.1007/s00277-020-04201-4
8. Shmueli E, Or R, Shapira MY, Resnick IB, Caplan O, Bdolah-Abram T, et al. High Rate of Cytomegalovirus Drug Resistance Among Patients Receiving Preemptive Antiviral Treatment After Haploidentical Stem Cell Transplantation. *J Infect Dis* (2014) 209:557–61. doi: 10.1093/infdis/jit475
9. Xu L-P, Wang S-Q, Wu D-P, Wang J-M, Gao S-J, Jiang M, et al. Haplo-Identical Transplantation for Acquired Severe Aplastic Anaemia in a Multicentre Prospective Study. *Br J Haematol* (2016) 175:265–74. doi: 10.1111/bjh.14225
10. Xu L-P, Jin S, Wang S-Q, Xia L-H, Bai H, Gao S-J, et al. Upfront Haploidentical Transplant for Acquired Severe Aplastic Anemia: Registry-Based Comparison With Matched Related Transplant. *J Hematol Oncol* (2017) 10:25. doi: 10.1186/s13045-017-0398-y
11. Xu LP, Xu ZL, Wang FR, Mo XD, Han TT, Han W, et al. Unmanipulated Haploidentical Transplantation Conditioning With Busulfan, Cyclophosphamide and Anti-Thymoglobulin for Adult Severe Aplastic Anaemia. *Bone Marrow Transplant* (2018) 53:188–92. doi: 10.1038/bmt.2017.237
12. Xu LP, Zhang XH, Wang FR, Mo XD, Han TT, Han W, et al. Haploidentical Transplantation for Pediatric Patients With Acquired Severe Aplastic Anemia. *Bone Marrow Transplant* (2017) 52:381–7. doi: 10.1038/bmt.2016.281
13. Lu Y, Sun R-J, Zhao Y-L, Xiong M, Cao X-Y, Zhang J-P, et al. Unmanipulated Haploidentical Hematopoietic Stem Cell Transplantation Achieved Outcomes Comparable With Matched Unrelated Donor Transplantation in Young Acquired Severe Aplastic Anemia. *Biol Blood Marrow Transplant* (2018) 24:1881–7. doi: 10.1016/j.bbmt.2018.05.015
14. Wang Z, Zheng X, Yan H, li D, Wang H. Good Outcome of Haploidentical Hematopoietic SCT as a Salvage Therapy in Children and Adolescents With Acquired Severe Aplastic Anemia. *Bone Marrow Transplant* (2014) 49:1481–5. doi: 10.1038/bmt.2014.187
15. Gao L, Li Y, Zhang Y, Chen X, Gao L, Zhang C, et al. Long-Term Outcome of HLA-Haploidentical Hematopoietic SCT Without *In Vitro* T-Cell Depletion for Adult Severe Aplastic Anemia After Modified Conditioning and Supportive Therapy. *Bone Marrow Transplant* (2014) 49:519–24. doi: 10.1038/bmt.2013.224
16. Lu Y, Zhao YL, Zhang JP, Xiong M, Cao XY, Liu DY, et al. Comparable Outcomes Among Unmanipulated Haploidentical, Matched Unrelated, and Matched Sibling Donors in BU-Based Myeloablative Hematopoietic Stem Cell Transplantation for Intermediate and Adverse Risk Acute Myeloid Leukemia in Complete Remission: A Single-Center Study. *Ann Hematol* (2021) 100(6):1579–91. doi: 10.1007/s00277-020-04355-1
17. Huang J, Huang F, Fan Z, Xu N, Xuan L, Liu H, et al. Haploidentical Related Donor vs Matched Sibling Donor Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic

Syndrome Aged Over 50 Years: A Single-Center Retrospective Study. *Cancer Med* (2020) 9:6244–55. doi: 10.1002/cam4.3290
18. Gao L, Zhang C, Gao L, Liu Y, Su Y, Wang S, et al. Favorable Outcome of Haploidentical Hematopoietic Stem Cell Transplantation in Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia: A Multicenter Study in Southwest China. *J Hematol Oncol* (2015) 8:90. doi: 10.1186/s13045-015-0186-5
19. Ke P, Bao XB, Hu XH, Zhuang J, Wu XJ, Liu YJ, et al. Myeloablative Conditioning Regimens With Combined of Haploidentical and Cord Blood Transplantation for Myelodysplastic Syndrome Patients. *Bone Marrow Transplant* (2018) 53:162–8. doi: 10.1038/bmt.2017.229
20. Suo P, Wang S, Xue Y, Cheng Y, Kong J, Yan C, et al. Unmanipulated Haploidentical Hematopoietic Stem Cell Transplantation for Children With Myelodysplastic Syndrome. *Pediatr Transplant* (2020) 24:e13864. doi: 10.1111/petr.13864
21. Zhao H, Wei J, Wei G, Luo Y, Shi J, Cui Q, et al. Pre-Transplant MRD Negativity Predicts Favorable Outcomes of CAR-T Therapy Followed by Haploidentical HSCT for Relapsed/Refractory Acute Lymphoblastic Leukemia: A Multi-Center Retrospective Study. *J Hematol Oncol* (2020) 13:42. doi: 10.1186/s13045-020-00873-7
22. Luo Y, Xiao H, Lai X, Shi J, Tan Y, He J, et al. T-Cell-Replete Haploidentical HSCT With Low-Dose Anti-T-Lymphocyte Globulin Compared With Matched Sibling HSCT and Unrelated HSCT. *Blood* (2014) 124:2735–43. doi: 10.1182/blood-2014-04-571570
23. Huang WR, Li HH, Gao CJ, Bo J, Li F, Dou LP, et al. Haploidentical, Unmanipulated G-CSF-Primed Peripheral Blood Stem Cell Transplantation for High-Risk Hematologic Malignancies: An Update. *Bone Marrow Transplant* (2016) 51:1464–9. doi: 10.1038/bmt.2016.166
24. Liu L, Zhang Y, Jiao W, Zhou H, Wang Q, Qiu H, et al. Combination of Haploidentical Haematopoietic Stem Cell Transplantation With an Unrelated Cord-Blood Unit in Patients With Severe Aplastic Anemia: A Report of 146 Cases. *Bone Marrow Transplant* (2020) 55:2017–25. doi: 10.1038/s41409-020-0874-9
25. Xu J, Zhao R, Yang L, Gong H, Ma S, Chen J, et al. Haploidentical Stem Cells Combined With a Small Dose of Umbilical Cord Blood Transplantation Exert Similar Survival Outcome of HLA-Matched Stem Cells Transplantation in T-Cell Acute Lymphoblastic Leukemia. *Bone Marrow Transplant* (2020) 55:1197–9. doi: 10.1038/s41409-019-0666-2
26. Wang J, Wang Z, Wei W, Zhang W, Zhang T, Cheng H, et al. Cord Haploidentical Non-In Vitro T Cell Depletion Allogeneic Hematopoietic Stem Cell Transplantation Reduces Relapse of Refractory Acute Leukemia. *Biol Blood Marrow Transplant* (2019) 25:121–8. doi: 10.1016/j.bbmt.2018.09.002
27. Liu Z, Zhang Y, Xiao H, Yao Z, Zhang H, Liu Q, et al. Cotransplantation of Bone Marrow-Derived Mesenchymal Stem Cells in Haploidentical Hematopoietic Stem Cell Transplantation in Patients With Severe Aplastic Anemia: An Interim Summary for a Multicenter Phase II Trial Results. *Bone Marrow Transplant* (2017) 52:704–10. doi: 10.1038/bmt.2016.347
28. Zhu L, Wang Z, Zheng X, Ding L, Han D, Yan H, et al. Haploidentical Hematopoietic Stem Cell Transplant With Umbilical Cord-Derived Multipotent Mesenchymal Cell Infusion for the Treatment of High-Risk Acute Leukemia in Children. *Leuk Lymphoma* (2015) 56:1346–52. doi: 10.3109/10428194.2014.939970
29. Gao XN, Lin J, Wang LJ, Li F, Li HH, Wang SH, et al. Risk Factors and Associations With Clinical Outcomes of Cytomegalovirus Reactivation After Haploidentical Versus Matched-Sibling Unmanipulated PBSCT in Patients With Hematologic Malignancies. *Ann Hematol* (2020) 99:1883–93. doi: 10.1007/s00277-020-04156-6
30. Li H-H, Li F, Gao C-J, Huang W-R, Bo J, Dou L-P, et al. Similar Incidence of Severe Acute GVHD and Less Severe Chronic GVHD in PBSCT From Unmanipulated, Haploidentical Donors Compared With That From Matched Sibling Donors for Patients With Haematological Malignancies. *Br J Haematol* (2017) 176:92–100. doi: 10.1111/bjh.14331
31. Wang Y, Fu HX, Liu DH, Xu LP, Zhang XH, Chang YJ, et al. Influence of Two Different Doses of Antithymocyte Globulin in Patients With Standard-Risk Disease Following Haploidentical Transplantation: A Randomized Trial. *Bone Marrow Transplant* (2014) 49:426–33. doi: 10.1038/bmt.2013.191
32. Lin R, Wang Y, Huang F, Fan Z, Zhang S, Yang T, et al. Two Dose Levels of Rabbit Antithymocyte Globulin as Graft-Versus-Host Disease Prophylaxis in Haploidentical Stem Cell Transplantation: A Multicenter Randomized Study. *BMC Med* (2019) 17:156. doi: 10.1186/s12916-019-1393-7
33. Wang Y, Liu Q-F, Lin R, Yang T, Xu Y-J, Mo X-D, et al. Optimizing Antithymocyte Globulin Dosing in Haploidentical Hematopoietic Cell Transplantation: Long-Term Follow-Up of a Multicenter, Randomized Controlled Trial. *Sci Bull* (2021). doi: 10.1016/j.scib.2021.06.002
34. Kako S, Akahoshi Y, Harada N, Nakano H, Kameda K, Ugai T, et al. HLA-Mismatched Haploidentical Transplantation Using Low-Dose Anti-Thymocyte Globulin (ATG: Thymoglobulin). *Hematology* (2017) 22:129–35. doi: 10.1080/10245332.2016.1231968
35. Min GJ, Kim HJ, Kim TG, Hyun YS, Hyun SJ, Baek IC, et al. Specific Donor HLA Allotypes as Predictors of Cytomegalovirus Disease Risk in Acute Myeloid Leukemia. *Hla* (2020) 96:445–55. doi: 10.1111/tan.13966
36. Park SS, Min GJ, Park S, Lee SE, Yoon JH, Shin SH, et al. Comparable Outcomes Between Unrelated and Haploidentical Stem Cell Transplantation in Adult Patients With Severe Aplastic Anemia. *Transplantation* (2021) 105 (5):1097–105. doi: 10.1097/tp.0000000000003342
37. Gao L, Liu J, Zhang Y, Chen X, Gao L, Zhang C, et al. Low Incidence of Acute Graft-Versus-Host Disease With Short-Term Tacrolimus in Haploidentical Hematopoietic Stem Cell Transplantation. *Leuk Res* (2017) 57:27–36. doi: 10.1016/j.leukres.2017.02.006
38. Gaballa S, Palmisiano N, Alpdogan O, Carabasi M, Filicko-O'Hara J, Kasner M, et al. A Two-Step Haploidentical Versus a Two-Step Matched Related Allogeneic Myeloablative Peripheral Blood Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2016) 22:141–8. doi: 10.1016/j.bbmt.2015.09.017
39. Kaynar L, Demir K, Turak EE, Öztürk ÇP, Zararsız G, Gönen ZB, et al. Tcrαβ-Depleted Haploidentical Transplantation Results in Adult Acute Leukemia Patients. *Hematology* (2017) 22:136–44. doi: 10.1080/10245332.2016.1238182
40. Erbey F, Akçay A, Atay D, Ovalı E, Öztürk G. Comparison of Outcomes After HLA-Matched Unrelated and αβ T-Cell-Depleted Haploidentical Hematopoietic Stem Cell Transplantation for Children With High-Risk Acute Leukemia. *Pediatr Transplant* (2018) 22:e13192. doi: 10.1111/petr.13192
41. Kang SH, Yoo JW, Seo JK, Kim H, Koh KN, Choi ES, et al. The Role of Interim-Foscarnet Prophylaxis in Preventing Cytomegalovirus Infection After *Ex Vivo* αβ T Cell-Depleted Haploidentical Hematopoietic Cell Transplant in Children. *Bone Marrow Transplant* (2021) 56(2):505–7. doi: 10.1038/s41409-020-01020-z
42. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, et al. HLA-Haploidentical Stem Cell Transplantation After Removal of αβ+ T and B Cells in Children With Nonmalignant Disorders. *Blood* (2014) 124:822–6. doi: 10.1182/blood-2014-03-563817
43. Mitchell R, Cole T, Shaw PJ, Mechinaud F, O'Brien T, Fraser C. TCR α+β+/CD19+ Cell–Depleted Hematopoietic Stem Cell Transplantation for Pediatric Patients. *Pediatr Transplant* (2019) 23:e13517. doi: 10.1111/petr.13517
44. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, et al. γδ T-Cell Reconstitution After HLA-Haploidentical Hematopoietic Transplantation Depleted of TCR-αβ+/CD19+ Lymphocytes. *Blood* (2015) 125:2349–58. doi: 10.1182/blood-2014-09-599423
45. Nazir HF, Ba Alawi FS, Al Hosni S, Al Rawas A, Dennison D. T Cell Depleted Haploidentical Hematopoietic Stem Cell Transplantation for Patients With Familial Hemophagocytic Lymphohistiocytosis Who Do Not Have Matched Family Donors: Experience in Oman. *Biol Blood Marrow Transplant* (2020) 26:1119–23. doi: 10.1016/j.bbmt.2020.02.014
46. Prezioso L, Manfra I, Bonomini S, Schifano C, Segreto R, Monti A, et al. Haploidentical Hematopoietic Stem Cell Transplantation in Adults Using the αβtcr/CD19-Based Depletion of G-CSF-Mobilized Peripheral Blood Progenitor Cells. *Bone Marrow Transplant* (2019) 54:698–702. doi: 10.1038/s41409-019-0608-z
47. Gaziev J, Isgrò A, Sodani P, Paciaroni K, De Angelis G, Marziali M, et al. Haploidentical HSCT for Hemoglobinopathies: Improved Outcomes With Tcrαβ(+)/CD19(+)-Depleted Grafts. *Blood Adv* (2018) 2:263–70. doi: 10.1182/bloodadvances.2017012005

48. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of Acute Leukemia Patients Transplanted With Naive T Cell–Depleted Stem Cell Grafts. *J Clin Invest* (2015) 125:2677–89. doi: 10.1172/JCI81229
49. Triplett BM, Shook DR, Eldridge P, Li Y, Kang G, Dallas M, et al. Rapid Memory T-Cell Reconstitution Recapitulating CD45RA-Depleted Haploidentical Transplant Graft Content in Patients With Hematologic Malignancies. *Bone Marrow Transplant* (2015) 50:968–77. doi: 10.1038/bmt.2014.324
50. Triplett BM, Muller B, Kang G, Li Y, Cross SJ, Moen J, et al. Selective T-Cell Depletion Targeting CD45RA Reduces Viremia and Enhances Early T-Cell Recovery Compared With CD3-Targeted T-Cell Depletion. *Transplant Infect Dis* (2018) 20:e12823. doi: 10.1111/tid.12823
51. Goldsmith SR, Abid MB, Auletta JJ, Bashey A, Beitinjaneh A, Castillo P, et al. Posttransplant Cyclophosphamide Is Associated With Increased Cytomegalovirus Infection: A CIBMTR Analysis. *Blood* (2021) 137:3291–305. doi: 10.1182/blood.2020009362
52. Fayard A, Daguenet E, Blaise D, Chevallier P, Labussière H, Berceanu A, et al. Evaluation of Infectious Complications After Haploidentical Hematopoietic Stem Cell Transplantation With Post-Transplant Cyclophosphamide Following Reduced-Intensity and Myeloablative Conditioning: A Study on Behalf of the Francophone Society of Stem Cell Transplantation and Cellular Therapy (SFGM-Tc). *Bone Marrow Transplant* (2019) 54:1586–94. doi: 10.1038/s41409-019-0475-7
53. Crocchiolo R, Bramanti S, Vai A, Sarina B, Mineri R, Casari E, et al. Infections After T-Replete Haploidentical Transplantation and High-Dose Cyclophosphamide as Graft-Versus-Host Disease Prophylaxis. *Transplant Infect Dis* (2015) 17:242–9. doi: 10.1111/tid.12365
54. Esquirol A, Pascual MJ, Kwon M, Pérez A, Parody R, Ferra C, et al. Severe Infections and Infection-Related Mortality in a Large Series of Haploidentical Hematopoietic Stem Cell Transplantation With Post-Transplant Cyclophosphamide. *Bone Marrow Transplant* (2021) 56:2432–44. doi: 10.1038/s41409-021-01328-4
55. Huntley D, Giménez E, Pascual MJ, Hernández-Boluda JC, Gago B, Vázquez L, et al. Incidence, Features, and Outcomes of Cytomegalovirus DNAemia in Unmanipulated Haploidentical Allogeneic Hematopoietic Stem Cell Transplantation With Post-Transplantation Cyclophosphamide. *Transpl Infect Dis* (2020) 22:e13206. doi: 10.1111/tid.13206
56. Crocchiolo R, Castagna L, Furst S, Devillier R, Sarina B, Bramanti S, et al. The Patient's CMV Serological Status Affects Clinical Outcome After T-Cell Replete Haplo-HSCT and Post-Transplant Cyclophosphamide. *Bone Marrow Transplant* (2016) 51:1134–6. doi: 10.1038/bmt.2016.69
57. Al Malki MM, Dadwal S, Yang D, Mokhtari S, Cao T, Gendzekhadze K, et al. High Incidence of CMV Reactivation After Haploidentical Donor Hematopoietic Cell Transplantation Using High-Dose Post-Transplant Cyclophosphamide, and Its Impact on Transplant Outcomes. *Blood* (2017) 130:4494–4. doi: 10.1182/blood.V130.Suppl_1.4494.4494
58. Huntley D, Giménez E, Pascual MJ, Remigia MJ, Amat P, Vázquez L, et al. Reconstitution of Cytomegalovirus-Specific T-Cell Immunity Following Unmanipulated Haploidentical Allogeneic Hematopoietic Stem Cell Transplantation With Posttransplant Cyclophosphamide. *Bone Marrow Transplant* (2020) 55:1347–56. doi: 10.1038/s41409-020-0865-x
59. Irene G-C, Albert E, Anna B-V, Rahinatu A, Silvana N, Silvana S, et al. Patterns of Infection and Infectious-Related Mortality in Patients Receiving Post-Transplant High Dose Cyclophosphamide as Graft-Versus-Host-Disease Prophylaxis: Impact of HLA Donor Matching. *Bone Marrow Transplant* (2021) 56(4):818–27. doi: 10.1038/s41409-020-01092-x
60. Hammerstrom AE, Lombardi LR, Pingali SR, Rondon G, Chen J, Milton DR, et al. Prevention of Cytomegalovirus Reactivation in Haploidentical Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2018) 24:353–8. doi: 10.1016/j.bbmt.2017.09.018
61. Uppuluri R, Sivasankaran M, Patel S, Swaminathan VV, Ramanan KM, Ravichandran N, et al. Haploidentical Stem Cell Transplantation With Post-Transplant Cyclophosphamide for Primary Immune Deficiency Disorders in Children: Challenges and Outcome From a Tertiary Care Center in South India. *J Clin Immunol* (2019) 39:182–7. doi: 10.1007/s10875-019-00600-z
62. Arcuri LJ, Nabhan SK, Cunha R, Nichele S, Ribeiro AAF, Fernandes JF, et al. Impact of CD34 Cell Dose and Conditioning Regimen on Outcomes After Haploidentical Donor Hematopoietic Stem Cell Transplantation With Post-Transplantation Cyclophosphamide for Relapsed/Refractory Severe Aplastic Anemia. *Biol Blood Marrow Transplant* (2020) 26:2311–7. doi: 10.1016/j.bbmt.2020.09.007
63. Raj RV, Hari P, Pasquini M, Epperla N, D'Souza A, Fenske T, et al. Impact of Haploidentical Hematopoietic Cell Transplantation Conditioning Intensity on the Incidence and Severity of Post-Transplantation Viral Infections. *Bone Marrow Transplant* (2016) 51:1602–4. doi: 10.1038/bmt.2016.216
64. Katsanis E, Maher K, Roe DJ, Simpson RJ. Progressive Substitution of Posttransplant Cyclophosphamide With Bendamustine: A Phase I Study in Haploidentical Bone Marrow Transplantation. *eJHaem* (2020) 1:286–92. doi: 10.1002/jha2.20
65. Goldsmith SR, Slade M, DiPersio JF, Westervelt P, Lawrence SJ, Uy GL, et al. Cytomegalovirus Viremia, Disease, and Impact on Relapse in T-Cell Replete Peripheral Blood Haploidentical Hematopoietic Cell Transplantation With Post-Transplant Cyclophosphamide. *Haematologica* (2016) 101:e465–8. doi: 10.3324/haematol.2016.149880
66. Solomon SR, Sizemore CA, Sanacore M, Zhang X, Brown S, Holland HK, et al. Total Body Irradiation–Based Myeloablative Haploidentical Stem Cell Transplantation Is a Safe and Effective Alternative to Unrelated Donor Transplantation in Patients Without Matched Sibling Donors. *Biol Blood Marrow Transplant* (2015) 21:1299–307. doi: 10.1016/j.bbmt.2015.03.003
67. Stocker N, Gaugler B, Labopin M, Farge A, Ye Y, Ricard L, et al. High-Dose Post-Transplant Cyclophosphamide Impairs γδ T-Cell Reconstitution After Haploidentical Haematopoietic Stem Cell Transplantation Using Low-Dose Antithymocyte Globulin and Peripheral Blood Stem Cell Graft. *Clin Trans Immunol* (2020) 9:e1171. doi: 10.1002/cti2.1171
68. Oltolini C, Greco R, Galli L, Clerici D, Lorentino F, Xue E, et al. Infections After Allogenic Transplant With Post-Transplant Cyclophosphamide: Impact of Donor HLA Matching. *Biol Blood Marrow Transplant* (2020) 26:1179–88. doi: 10.1016/j.bbmt.2020.01.013
69. Slade M, Goldsmith S, Romee R, DiPersio JF, Dubberke ER, Westervelt P, et al. Epidemiology of Infections Following Haploidentical Peripheral Blood Hematopoietic Cell Transplantation. *Transpl Infect Dis* (2017) 19:e12629. doi: 10.1111/tid.12629
70. Montoro J, Piñana JL, Hernández-Boluda JC, Hernani R, Lorenzo I, Pérez A, et al. Uniform Graft-Versus-Host Disease Prophylaxis With Posttransplant Cyclophosphamide, Sirolimus, and Mycophenolate Mofetil Following Hematopoietic Stem Cell Transplantation From Haploidentical, Matched Sibling and Unrelated Donors. *Bone Marrow Transplant* (2020) 55:2147–59. doi: 10.1038/s41409-020-0921-6
71. Cieri N, Greco R, Crucitti L, Morelli M, Giglio F, Levati G, et al. Post-Transplantation Cyclophosphamide and Sirolimus After Haploidentical Hematopoietic Stem Cell Transplantation Using a Treosulfan-Based Myeloablative Conditioning and Peripheral Blood Stem Cells. *Biol Blood Marrow Transplant* (2015) 21:1506–14. doi: 10.1016/j.bbmt.2015.04.025
72. Law AD, Salas MQ, Lam W, Michelis FV, Thyagu S, Kim DDH, et al. Reduced-Intensity Conditioning and Dual T Lymphocyte Suppression With Antithymocyte Globulin and Post-Transplant Cyclophosphamide as Graft-Versus-Host Disease Prophylaxis in Haploidentical Hematopoietic Stem Cell Transplants for Hematological Malignancies. *Biol Blood Marrow Transplant* (2018) 24:2259–64. doi: 10.1016/j.bbmt.2018.07.008
73. Salas MQ, Prem S, Atenafu EG, Datt Law A, Lam W, Al-Shaibani Z, et al. Dual T-Cell Depletion With ATG and PTCy for Peripheral Blood Reduced Intensity Conditioning Allo-HSCT Results in Very Low Rates of GVHD. *Bone Marrow Transplant* (2020) 55:1773–83. doi: 10.1038/s41409-020-0813-9
74. Salas MQ, Prem S, Atenafu EG, Law AD, Lam W, Al-Shaibani Z, et al. Reduced Intensity Allogeneic Stem Cell Transplant With Anti-Thymocyte Globulin and Post-Transplant Cyclophosphamide in Acute Myeloid Leukemia. *Eur J Haematol* (2019) 103:510–8. doi: 10.1111/ejh.13321
75. Lint CH, Su YJ, Hsu CY, Wang PN, Teng CL. Haploidentical Allogeneic Hematopoietic Stem Cell Transplantation Increases the Risk of Cytomegalovirus Infection in adult Patients With Acute Leukemia. *Transpl Infect Dis* (2019) 21(4):e13096.
76. Wang Y, Chang Y-J, Chen L, Xu L-P, Bian Z-L, Zhang X-H, et al. Low-Dose Post-Transplant Cyclophosphamide can Mitigate GVHD and Enhance the G-CSF/ATG Induced GVHD Protective Activity and Improve

Haploidentical Transplant Outcomes. *OncoImmunology* (2017) 6:e1356152. doi: 10.1080/2162402X.2017.1356152
77. Wang Y, Wu D-P, Liu Q-F, Xu L-P, Liu K-Y, Zhang X-H, et al. Low-Dose Post-Transplant Cyclophosphamide and Anti-Thymocyte Globulin as an Effective Strategy for GVHD Prevention in Haploidentical Patients. *J Hematol Oncol* (2019) 12:88. doi: 10.1186/s13045-019-0781-y
78. Tischer J, Engel N, Fritsch S, Prevalsek D, Hubmann M, Schulz C, et al. Virus Infection in HLA-Haploidentical Hematopoietic Stem Cell Transplantation: Incidence in the Context of Immune Recovery in Two Different Transplantation Settings. *Ann Hematol* (2015) 94:1677–88. doi: 10.1007/s00277-015-2423-y
79. ElGohary G, El Fakih R, de Latour R, Risitano A, Marsh J, Schrezenmeier H, et al. Haploidentical Hematopoietic Stem Cell Transplantation in Aplastic Anemia: A Systematic Review and Meta-Analysis of Clinical Outcome on Behalf of the Severe Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (SAAWP of EBMT). *Bone Marrow Transplant* (2020) 55:1906–17. doi: 10.1038/s41409-020-0897-2
80. Li J-J, Huang X, Wang H, Guo X-Y, Ge S-X, Zhang J. Baseline Antibody Level may Help Predict the Risk of Active Human Cytomegalovirus Infection in a HCMV Seropositive Population. *Eur J Clin Microbiol Infect Dis* (2017) 36:863–8. doi: 10.1007/s10096-016-2873-8
81. Lu S-C, Chin L-T, Wu F-M, Hsieh G-J, Haung S-P, Chen J-C, et al. Seroprevalence of CMV Antibodies in a Blood Donor Population and Premature Neonates in the South-Central Taiwan. *Kaohsiung J Med Sci* (1999) 15:603–10.
82. Zuhair M, Smit GSA, Wallis G, Jabbar F, Smith C, Devleesschauwer B, et al. Estimation of the Worldwide Seroprevalence of Cytomegalovirus: A Systematic Review and Meta-Analysis. *Rev Med Virol* (2019) 29:e2034. doi: 10.1002/rmv.2034
83. Lachmann R, Loenenbach A, Waterboer T, Brenner N, Pawlita M, Michel A, et al. Cytomegalovirus (CMV) Seroprevalence in the Adult Population of Germany. *PloS One* (2018) 13:e0200267. doi: 10.1371/journal.pone.0200267
84. Antona D, Lepoutre A, Fonteneau L, Baudon C, Halftermeyer-Zhou F, Le Strat Y, et al. Seroprevalence of Cytomegalovirus Infection in France in 2010. *Epidemiol Infect* (2017) 145:1471–8. doi: 10.1017/S0950268817000103
85. Luo XH, Chang YJ, Huang XJ. Improving Cytomegalovirus-Specific T Cell Reconstitution After Haploidentical Stem Cell Transplantation. *J Immunol Res* (2014) 2014. doi: 10.1155/2014/631951
86. Luo XH, Huang XJ, Li D, Liu KY, Xu LP, Liu DH. Immune Reconstitution to Cytomegalovirus Following Partially Matched-Related Donor Transplantation: Impact of *In Vivo* T-Cell Depletion and Granulocyte Colony-Stimulating Factor-Primed Peripheral Blood/Bone Marrow Mixed Grafts. *Transplant Infect Dis* (2013) 15:22–33. doi: 10.1111/j.1399-3062.2012.00722.x
87. Luo X-H, Huang X-J, Liu K-Y, Xu L-P, Liu D-H. Protective Immunity Transferred by Infusion of Cytomegalovirus-Specific CD8+ T Cells Within Donor Grafts: Its Associations With Cytomegalovirus Reactivation Following Unmanipulated Allogeneic Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2010) 16:994–1004. doi: 10.1016/j.bbmt.2010.02.007
88. Kato R, Tamaki H, Ikegame K, Yoshihara S, Kaida K, Taniguchi K, et al. Early Detection of Cytomegalovirus-Specific Cytotoxic T Lymphocytes Against Cytomegalovirus Antigenemia in Human Leukocyte Antigen Haploidentical Hematopoietic Stem Cell Transplantation. *Ann Hematol* (2015) 94:1707–15. doi: 10.1007/s00277-015-2446-4
89. Noviello M, Forcina A, Veronica V, Crocchiolo R, Stanghellini MT, Carrabba M, et al. Early Recovery of CMV Immunity After HLA-Haploidentical Hematopoietic Stem Cell Transplantation as a Surrogate Biomarker for a Reduced Risk of Severe Infections Overall. *Bone Marrow Transplant* (2015) 50:1262–4. doi: 10.1038/bmt.2015.132
90. Huntley D, Giménez E, Pascual MJ, Vázquez L, Amat P, Remigia MJ, et al. Peripheral Blood Regulatory T Cells and Occurrence of Cytomegalovirus DNAemia After Unmanipulated Haploidentical Allogeneic Hematopoietic Stem Cell Transplantation With Posttransplant Cyclophosphamide. *Bone Marrow Transplant* (2020) 55:1493–6. doi: 10.1038/s41409-020-0950-1
91. van Beek JJP, Puccio S, Roberto A, De Paoli F, Graziano G, Salviato E, et al. Single-Cell Profiling Reveals the Dynamics of Cytomegalovirusspecific T-Cells in Haploidentical Hematopoietic Stem Cell Transplantation. *Haematologica* (2021) 106(10):2768–73. doi: 10.3324/haematol.2020.276352
92. Jin F, Lin H, Gao S, Wang H, Yan H, Guo J, et al. Characterization of Ifnγ-Producing Natural Killer Cells Induced by Cytomegalovirus Reactivation After Haploidentical Hematopoietic Stem Cell Transplantation. *Oncotarget* (2017) 8:51–63. doi: 10.18632/oncotarget.13916
93. Jang JE, Hwang DY, Chung H, Kim SJ, Eom JI, Jeung HK, et al. Early Cytomegalovirus Reactivation and Expansion of CD56(bright)CD16(dim/-) DNAM1(+) Natural Killer Cells Are Associated With Antileukemia Effect After Haploidentical Stem Cell Transplantation in Acute Leukemia. *Biol Blood Marrow Transplant* (2019) 25:2070–8. doi: 10.1016/j.bbmt.2019.06.008
94. Muccio L, Bertaina A, Falco M, Pende D, Meazza R, Lopez-Botet M, et al. Analysis of Memory-Like Natural Killer Cells in Human Cytomegalovirus-Infected Children Undergoing αβ+T and B Cell-Depleted Hematopoietic Stem Cell Transplantation for Hematological Malignancies. *Haematologica* (2016) 101:371–81. doi: 10.3324/haematol.2015.134155
95. Zhao XY, Luo XY, Yu XX, Zhao XS, Han TT, Chang YJ, et al. Recipient-Donor KIR Ligand Matching Prevents CMV Reactivation Post-Haploidentical T Cell-Replete Transplantation. *Br J Haematol* (2017) 177:766–81. doi: 10.1111/bjh.14622
96. Hu L-J, Cao X-H, Yu X-X, Liu X-F, Zhao X-S, Chang Y-J, et al. NK Cell Reconstitution Following Unmanipulated HLA-Mismatched/Haploidentical Transplantation Compared With Matched Sibling Transplantation. *Sci China Life Sci* (2020) 63:781–4. doi: 10.1007/s11427-018-9565-5
97. Zaghi E, Calvi M, Puccio S, Spata G, Terzoli S, Peano C, et al. Single-Cell Profiling Identifies Impaired Adaptive NK Cells Expanded After HCMV Reactivation in Haploidentical-HSCT. *JCI Insight* (2021) 6(12):e146973. doi: 10.1172/jci.insight.146973
98. Park M, Im HJ, Lee Y-J, Park N, Jang S, Kwon SW, et al. Reconstitution of T and NK Cells After Haploidentical Hematopoietic Cell Transplantation Using αβ T Cell-Depleted Grafts and the Clinical Implication of γδ T Cells. *Clin Transplant* (2018) 32:e13147. doi: 10.1111/ctr.13147
99. Feuchtinger T, Opherk K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, et al. Adoptive Transfer of Pp65-Specific T Cells for the Treatment of Chemorefractory Cytomegalovirus Disease or Reactivation After Haploidentical and Matched Unrelated Stem Cell Transplantation. *Blood* (2010) 116:4360–7. doi: 10.1182/blood-2010-01-262089
100. Pei XY, Zhao XY, Chang YJ, Liu J, Xu LP, Wang Y, et al. Cytomegalovirus-Specific T-Cell Transfer for Refractory Cytomegalovirus Infection After Haploidentical Stem Cell Transplantation: The Quantitative and Qualitative Immune Recovery for Cytomegalovirus. *J Infect Dis* (2017) 216:945–56. doi: 10.1093/infdis/jix357
101. Zhao X-Y, Pei X-Y, Chang Y-J, Yu X-X, Xu L-P, Wang Y, et al. First-Line Therapy With Donor-Derived Human Cytomegalovirus (HCMV)–specific T Cells Reduces Persistent HCMV Infection by Promoting Antiviral Immunity After Allogenic Stem Cell Transplantation. *Clin Infect Dis* (2019) 70:1429–37. doi: 10.1093/cid/ciz368
102. Ke P, Bao X, Zhou J, Li X, Zhuang J, He X, et al. Donor CMV-Specific Cytotoxic T Lymphocytes Successfully Treated Drug-Resistant Cytomegalovirus Encephalitis After Allogeneic Hematopoietic Stem Cell Transplantation. *Hematology* (2020) 25:43–7. doi: 10.1080/16078454.2019.1710945
103. Maschan M, Blagov S, Shelikhova L, Shekhovtsova Z, Balashov D, Starichkova J, et al. Low-Dose Donor Memory T-Cell Infusion After TCR Alpha/Beta Depleted Unrelated and Haploidentical Transplantation: Results of a Pilot Trial. *Bone Marrow Transplant* (2018) 53:264–73. doi: 10.1038/s41409-017-0035-y
104. Dunaikina M, Zhekhovtsova Z, Shelikhova L, Glushkova S, Nikolaev R, Blagov S, et al. Safety and Efficacy of the Low-Dose Memory (CD45RA-Depleted) Donor Lymphocyte Infusion in Recipients of αβ T Cell-Depleted Haploidentical Grafts: Results of a Prospective Randomized Trial in High-Risk Childhood Leukemia. *Bone Marrow Transplant* (2021) 56:1614–24. doi: 10.1038/s41409-021-01232-x
105. Shelikhova L, Glushkova S, Nikolaev R, Dunaikina M, Zhekhovtsova Z, Blagov S, et al. Serotherapy-Free Regimen Improves Non-Relapse Mortality and Immune Recovery Among the Recipients of αβ TCell–Depleted

Haploidentical Grafts: Retrospective Study in Childhood Leukemia. *Transplant Cell Ther* (2021) 27:330.e1–.e9. doi: 10.1016/j.jtct.2021.01.010
106. Castagna L, Valli V, Timofeeva I, Capizzuto R, Bramanti S, Mariotti J, et al. Feasibility and Efficacy of CD45RA+ Depleted Donor Lymphocytes Infusion After Haploidentical Transplantation With Post-Transplantation Cyclophosphamide in Patients With Hematological Malignancies. *Transplant Cell Ther* (2021) 27:478.e1–.e5. doi: 10.1016/j.jtct.2021.03.010
107. Park HJ, Hong KT, Yun SO, Ahn HY, Choi JY, Shin HY, et al. Successful Treatment of Refractory CMV Colitis After Haploidentical HSCT With Post-Transplant Cyclophosphamide Using CD45RA+ Depleted Donor Lymphocyte Infusion. *Bone Marrow Transplant* (2020) 55:1674–6. doi: 10.1038/s41409-019-0685-z
108. Uygun V, Karasu G, Daloğlu H, Öztürkmen S, Yalçın K, Çelen SS, et al. Use of Low Cell Dose for Unmanipulated Donor Lymphocyte for Management of Cytomegalovirus Infection: A Single-Center Experience. *Pediatr Transplant* (2020) 24:e13882. doi: 10.1111/petr.13882
109. Lugthart G, van Ostaijen-ten Dam MM, Jol - van der Zijde CM, van Holten TC, Kester MGD, Heemskerk MHM, et al. Early Cytomegalovirus Reactivation Leaves a Specific and Dynamic Imprint on the Reconstituting T Cell Compartment Long-Term After Hematopoietic Stem Cell Transplantation. *Biol Blood Marrow Transplant* (2014) 20:655–61. doi: 10.1016/j.bbmt.2014.01.018
110. Raphael I, Marie R, Helene M-T, Marc D, Marc B, Aliénor X, et al. Cytomegalovirus Shapes Long-Term Immune Reconstitution After Allogeneic Stem Cell Transplantation. *Haematologica* (2015) 100:114–23. doi: 10.3324/haematol.2014.113415
111. Luo XH, Meng Q, Rao M, Liu Z, Paraschoudi G, Dodoo E, et al. The Impact of Inflationary Cytomegalovirus-Specific Memory T Cells on Anti-Tumour Immune Responses in Patients With Cancer. *Immunology* (2018) 155:294–308. doi: 10.1111/imm.12991
112. Zhang Y-L, Zhu Y, Xiao Q, Wang L, Liu L, Luo X-H. Cytomegalovirus Infection is Associated With AML Relapse After Allo-HSCT: A Meta-Analysis of Observational Studies. *Ann Hematol* (2019) 98:1009–20. doi: 10.1007/s00277-018-3585-1
113. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M, et al. Early Human Cytomegalovirus Replication After Transplantation is Associated With a Decreased Relapse Risk: Evidence for a Putative Virus-Versus-Leukemia Effect in Acute Myeloid Leukemia Patients. *Blood* (2011) 118:1402–12. doi: 10.1182/blood-2010-08-304121
114. Green ML, Leisenring WM, Xie H, Walter RB, Mielcarek M, Sandmaier BM, et al. CMV Reactivation After Allogeneic HCT and Relapse Risk: Evidence for Early Protection in Acute Myeloid Leukemia. *Blood J Am Soc Hematol* (2013) 122:1316–24. doi: 10.1182/blood-2013-02-487074
115. Teira P, Battiwalla M, Ramanathan M, Barrett AJ, Ahn KW, Chen M, et al. Early Cytomegalovirus Reactivation Remains Associated With Increased Transplant-Related Mortality in the Current Era: A CIBMTR Analysis. *Blood* (2016) 127:2427–38. doi: 10.1182/blood-2015-11-679639
116. Cantoni N, Hirsch HH, Khanna N, Gerull S, Buser A, Bucher C, et al. Evidence for a Bidirectional Relationship Between Cytomegalovirus Replication and Acute Graft-Versus-Host Disease. *Biol Blood Marrow Transplant* (2010) 16:1309–14. doi: 10.1016/j.bbmt.2010.03.020
117. Lönnqvist B, Ringdén O, Wahren B, Gahrton G, Lundgren G. Cytomegalovirus Infection Associated With and Preceding Chronic Graft-Versus-Host Disease. *Transplantation* (1984) 38:465–8. doi: 10.1097/00007890-198411000-00004

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Dominant Antiviral CD8$^+$ T Cell Responses Empower Prophylactic Antibody-Eliciting Vaccines Against Cytomegalovirus

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Human cytomegalovirus (HCMV) is an ubiquitous herpesvirus that can cause serious morbidity and mortality in immunocompromised or immune-immature individuals. A vaccine that induces immunity to CMV in these target populations is therefore highly needed. Previous attempts to generate efficacious CMV vaccines primarily focused on the induction of humoral immunity by eliciting neutralizing antibodies. Current insights encourage that a protective immune response to HCMV might benefit from the induction of virus-specific T cells. Whether addition of antiviral T cell responses enhances the protection by antibody-eliciting vaccines is however unclear. Here, we assessed this query in mouse CMV (MCMV) infection models by developing synthetic vaccines with humoral immunity potential, and deliberately adding antiviral CD8$^+$ T cells. To induce antibodies against MCMV, we developed a DNA vaccine encoding either full-length, membrane bound glycoprotein B (gB) or a secreted variant lacking the transmembrane and intracellular domain (secreted (s)gB). Intradermal immunization with an increasing dose schedule of sgB and booster immunization provided robust viral-specific IgG responses and viral control. Combined vaccination of the sgB DNA vaccine with synthetic long peptides (SLP)-vaccines encoding MHC class I-restricted CMV epitopes, which elicit exclusively CD8$^+$ T cell responses, significantly enhanced antiviral immunity. Thus, the combination of antibody and CD8$^+$ T cell-eliciting vaccines provides a collaborative improvement of humoral and cellular immunity enabling enhanced protection against CMV.

Keywords: cytomegalovirus, prophylactic vaccination, DNA vaccination, antibody response, synthetic long peptides, T cells

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the β-herpesvirus family, is estimated to infect 60-80% of the world population. In healthy individuals, CMV establishes low-level viral persistence with little or no clinical symptoms with the exception of sporadically causing a mononucleosis-like illness (1). However, in immunocompromised individuals, including both solid organ and bone

marrow-transplantation patients and HIV-infected persons, HCMV infection often causes serious complications. Moreover, congenital HCMV infection in the immunological immature unborn and newborn babies can cause severe morbidity, lifelong invalidity and even mortality (2). Although treatment options such as antiviral drugs and cellular therapy are available against HCMV-associated disease, preventive strategies such as vaccines are highly desired. Antiviral drugs require prolonged treatment, are accompanied by significant toxicity, and viral resistance to the drug is not uncommon (3). Despite ongoing efforts, no licensed effective prophylactic or therapeutic HCMV vaccines are available yet.

Infection with CMV results in activation of basically all arms of the immune system. In-depth studies documented that innate, humoral and cellular immune responses play important roles in the control of CMV infection and disease (2, 4). The impact on the immune system, however, is highly dependent on the infectious dose (5–7). The contribution of antibodies for protection against and control of CMV is mainly associated with restricting viral dissemination, limiting recurrent infection and the severity of the disease (8–10). Mothers that have HCMV antibodies before conception, transmit infection to the fetus at a lower frequency than women with primary infections (11), and passive immunization with HCMV antibodies can protect against congenital HCMV infection in newborns (12). Moreover, if antibodies specific to HCMV upon primary maternal infection are of low avidity and poor neutralizing activity, a higher transmission of viral infection from mother to fetus occurs (13). The administration of HCMV-specific antibodies to transplant recipients also results in reduction of HCMV-associated disease (14). The majority of the antibodies with virus-neutralizing capacity bind to the CMV glycoproteins, used for host cell entry. Especially virus-neutralizing antibodies against glycoprotein B (gB), a major envelope glycoprotein involved in cell attachment and penetration, accounts for the neutralizing antibody response to HCMV (15, 16). Moreover, also non-neutralizing anti-gB antibodies have been shown to exhibit protective capacity (17–19), which may be caused by induction of antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (20).

In addition to the humoral response, the T cell-mediated immune response is another major mechanism for controlling and restricting CMV replication in hosts (21). Functional CMV-specific $CD8^+$ and $CD4^+$ T cells are activated and expanded during primary infection. The ensuing T cell response is characterized by the maintenance of large oligoclonal T cell populations that remain high or even increase over time (22, 23). This phenomenon, named memory inflation (24), is not unique to CMV infection, but this virus seems to be most effective in triggering memory inflation (25). The CMV-specific memory $CD8^+$ T cells have an advanced differentiated state and are able to lyse virus-infected cells and suppress intracellular virus replication by the secretion of IFN-γ and TNF (26). $CD4^+$ T cells also have direct effects on viral replication by secretion of IFN-γ, in addition to supporting antibody and memory $CD8^+$ T cell responses (27). Clinical data from transplant patients and HIV-infected individuals exposed a crucial role for $CD8^+$ and $CD4^+$ T cells in the control of HCMV (28–32). Moreover, the administration of CMV-specific $CD8^+$ T cells by adoptive transfer limits CMV disease in experimental CMV settings and in the clinic (33–36). Especially, the strong inflationary $CD8^+$ T cell responses against the immunogenic pp65 and IE1 CMV proteins are central in CMV control (37–39). In line with this, also the inflationary $CD8^+$ T cells in murine models have strong antiviral capacity (40, 41).

Based on the success of numerous prophylactic vaccines designed to elicit antibodies, several vaccines aiming to induce protective humoral responses against HCMV were developed (42). The recombinant monomeric gB vaccine adjuvanted with MF59 was clinically tested and demonstrated a 50% efficacy in prevention of HCMV infection in CMV-seronegative women (43, 44). Since the protective capacity of the gB vaccines is considered to primarily depend on the induction of antibodies (45), a possible cause for the lack of higher efficacy could be the absence of strong $CD8^+$ T cell responses by this vaccine. Accordingly, CMV vaccines were developed that induce both antibody and $CD8^+$ T cell responses (reviewed in (42, 46)). However, it remained unclear whether addition of $CD8^+$ T cell responses could actually aid antibody-mediated protection.

Previously, our group developed a synthetic long peptide (SLP) vaccine platform inducing robust and functional CMV-specific $CD8^+$ T cell responses, resulting in reduced viral replication upon challenge (40). These SLP vaccines did not elicit MCMV-specific antibody or $CD4^+$ T cell responses, indicating that vaccine-induced $CD8^+$ T cells can operate solely to control viral infection. In this study, we aimed to demonstrate whether these vaccine-induced $CD8^+$ T cell responses have an added value to antibody-eliciting vaccines. For this purpose, we developed DNA vaccines encoding gB, and subsequently analyzed the potency of a combinatorial DNA and SLP synthetic vaccine approach. We show that combined administration of a DNA vaccine eliciting a humoral response and an SLP vaccine eliciting antiviral $CD8^+$ T cell responses results in more efficient control of lytic MCMV infection, which unequivocally demonstrates the need for directing both CMV-specific B and T cell immunity to combat CMV-associated disease.

RESULTS

Booster Vaccination With DNA Vaccines Encoding Soluble gB Elicits Robust IgG Responses Against CMV

To develop effective vaccines eliciting antibody-based protection against CMV, we constructed several DNA vaccines encoding glycoprotein B (gB). This glycoprotein is expressed on the surface of mouse and human CMV and is directly involved in viral entry into host cells (47, 48). To compare antibody responses against the full-length, membrane bound form of gB and a soluble, secreted form of gB (sgB, lacking the transmembrane and intracellular domain), two different DNA

vaccines were tested (**Figures 1A, B**). The gB and sgB-encoding DNA vaccines were compared following administration in C57BL/6 mice *via* the intradermal (ID) and intramuscular (IM) route in a booster regimen. DNA vaccines encoding sgB administered either ID or IM resulted in an increase of the MCMV-specific IgG response upon booster vaccination, whereas the gB DNA vaccine elicited a lower IgG response that inferiorly responded to booster vaccination (**Figures 1C, D**). Moreover, vaccination *via* the ID route induced consistently a higher MCMV-specific IgG antibody response compared to IM vaccination, and this was observed after immunization with both the secreted and membrane bound forms of gB (**Figures 1C, D**). Evaluation of the IgG isotypes revealed that the IgG1 response was overall subordinate in all vaccination groups (**Figure 1E**), while IgG2b and most profoundly IgG2c (the C57BL/6 mice equivalent of IgG2a) levels where strongly induced by the sgB DNA vaccine compared to the gB DNA vaccine. Thus, booster vaccination *via* the ID route with sgB-encoding DNA vaccines results in superior MCMV-specific IgG antibody responses.

Dose-Escalating DNA-Based Vaccination Improves Antibody Responses and Viral Protection

Next, we determined whether the antibody-eliciting sgB DNA vaccine could provide viral protection against MCMV challenge in a dose-dependent manner. C57BL/6 mice were ID vaccinated in a prime-boost-boost regimen with either 10 or 60 μg of the sgB DNA vaccine to measure a low and high dose of the sgB DNA vaccine (49), and 20 days after the last booster vaccination, mice were infected with MCMV (**Figure 2A**). The high-dose sgB vaccine induced a slightly higher MCMV-specific IgG response (**Figure 2B**) and IgG end-point titer (**Figure 2C**). Consistently, whereas the low-dose sgB DNA vaccine resulted in a 4-fold reduction of the viral load in the liver, the high-dose sgB vaccine lowered the viral load 12-fold compared to unvaccinated (naïve) mice (**Figure 2D**). These results show that low and especially high dosages of the sgB vaccine induces antibody responses able to provide protection against viral challenge.

Short-interval vaccination schedules with an increasing dose, mimicking the increment of foreign antigens as occurs upon natural infection, leads to improved vaccine-specific $CD8^+$ T cell responses (50). To assess if such vaccine regimens could also improve the IgG response and associated viral protection of the sgB DNA vaccine, mice were immunized with increasing dosages (10, 20, 30 μg) at day 0, 3 and 6, respectively, or received a single dose vaccination. In both groups, the booster vaccines were provided as a single dose (**Figure 2E**). Dose escalation during prime did not significantly increase the MCMV-specific IgG response (**Figure 2F**) and the IgG end-point titer (**Figure 2G**). The increasing dosage priming effect nevertheless resulted in an increased reduction in the viral load in the liver upon viral challenge as compared to single dose priming (**Figure 2H**). Overall, these results show that using an increasing dose schedule with DNA vaccines during prime immunization improves protection against viral challenge.

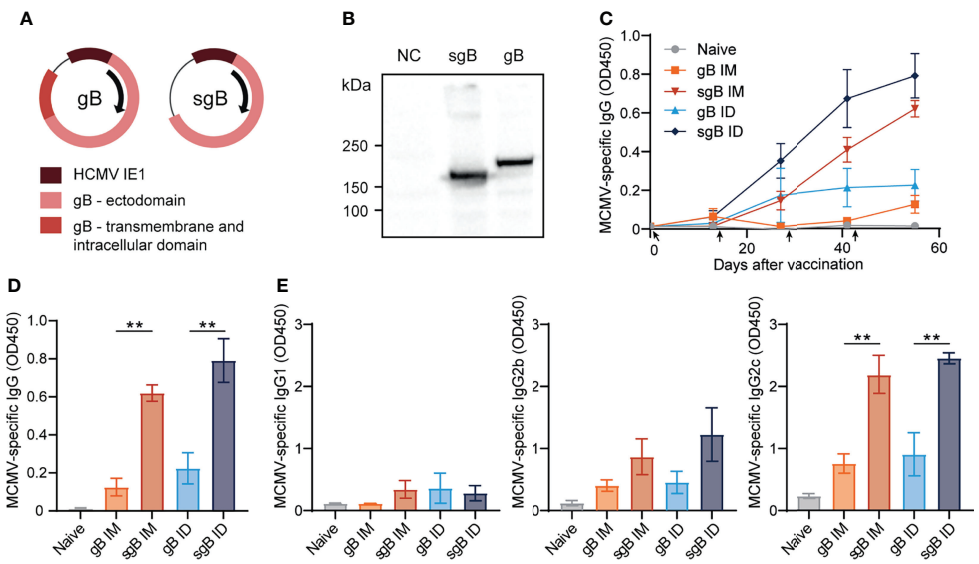

FIGURE 1 | Booster vaccination with DNA vaccines encoding soluble gB elicits robust IgG responses against CMV. **(A)** Schematic representation of the DNA vaccines encoding gB or a soluble version of gB (sgB). **(B)** Western blot showing expression of sgB and gB proteins following transfection of the DNA vaccines into B16F10 cells. Negative control (NC) only received the transfection agents. **(C)** Kinetic analysis of the MCMV-specific IgG response in serum. Mice were vaccinated intradermally (ID) or intramuscularly (IM) four times at a two-week interval with 10 μg of sgB or gB DNA vaccine. At different time points blood was taken and serum was extracted to analyze the MCMV-specific IgG antibody response. Data shown are mean values ± SEM (n=4 per group). Arrows indicate vaccine injection time-points (day 0, 14, 28, 42). **(D)** Presence of MCMV-specific IgG antibodies for the different vaccinations two weeks after the fourth vaccination. Experiments were performed twice with similar outcome. **(E)** Presence of MCMV-specific IgG1, IgG2b and IgG2c subclasses for the different vaccinations two weeks after the fourth vaccination. Experiments were performed twice with similar outcome. One-way ANOVA was used for statistical analysis. **P<0.01.

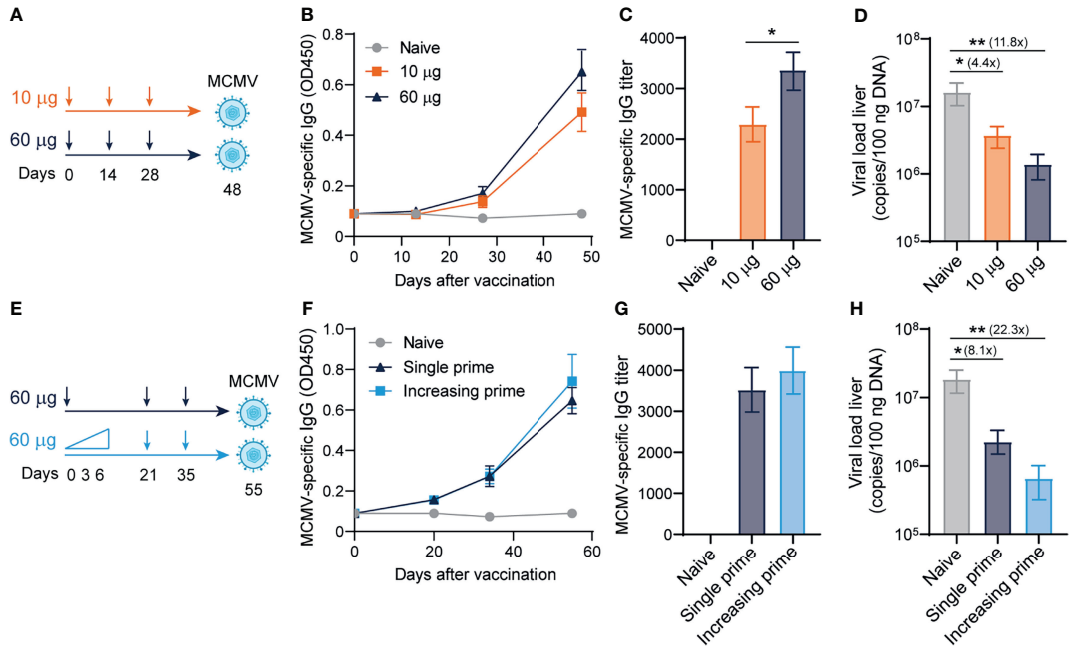

FIGURE 2 | Dose-escalating DNA-based vaccination improves antibody responses and viral protection. **(A)** Vaccination schedule for mice were vaccinated intradermally three times with different doses (10 ug and 60 ug) of the sgB DNA vaccine. Three weeks after the final vaccination, mice were challenged intraperitoneally with 5×10^4 PFU salivary gland-derived MCMV-Smith. **(B)** Kinetic analysis of the MCMV-specific IgG response in serum. Data shown are mean values ± SEM (n=8) **(C)** MCMV-specific endpoint binding IgG titers after three vaccinations. Data shown are mean values ± SEM (n=8). One-way ANOVA was used for statistical analysis. **(D)** At day 4 post-infection, livers were isolated and the viral genome copies were determined by PCR. The viral load is depicted as mean values ± SEM (n=8). **(E)** Vaccination schedule for mice vaccinated intradermally with either an increasing dose prime schedule (10 µg on day 0, 20 µg on day 3 and 30 µg on day 6) or a single prime dose of 60 µg on day 0, followed by two booster immunizations with a high dose (60 µg) of the sgB DNA vaccine. Three weeks after the final vaccination, mice were challenged intraperitoneally with 5×10^4 PFU salivary gland-derived MCMV-Smith. **(F)** Kinetic analysis of the MCMV-specific IgG response in serum. Data shown are mean values ± SEM (n=8) **(G)** MCMV-specific endpoint binding IgG titers after two booster vaccinations are shown and represent mean values ± SEM (n=8). One-way ANOVA was used for statistical analysis. **(H)** At day 4 post-infection, livers were isolated and the viral genome copies were determined by PCR. The viral load is depicted as mean values ± SEM (n=8). Experiments were performed twice with similar outcome. A Kruskal-Wallis test was used for statistical analysis. *P<0.05, **P<0.01.

Combining the sgB DNA Vaccine With Synthetic Long Peptide Vaccines Enhances M38-Specific CD8$^+$ T Cell Responses

Previously, we showed that synthetic long peptide (SLP) vaccines eliciting exclusively MCMV-specific CD8$^+$ T cell responses without the induction of antiviral CD4$^+$ T cells or antibodies enhanced protection against viral challenge (40). To determine whether the induction of robust antiviral CD8$^+$ T cell responses can improve antibody-mediated immunity, the antibody-eliciting sgB DNA vaccine was combined with the SLP vaccine eliciting CD8$^+$ T cells against the MHC class I restricted epitopes in M38 and m139. Mice were vaccinated in a prime-boost regimen with either the sgB DNA vaccine, the SLP vaccine or the combination of both vaccines (**Figure 3A**).

As expected, the SLP vaccine did not elicit MCMV-specific antibodies but exclusively provoked CD8$^+$ T cell responses while CD8$^+$ T cell responses were not detected after sgB vaccination (**Figures 3B–D, F**). Moreover, antibodies against the SLPs were also not induced (**Supplementary Figure 1B**). Mice that received both the DNA and SLP-based vaccines inducing the antibody and CD8$^+$ T cell responses, respectively, showed a similar gB-specific and MCMV-specific IgG response compared to mice that only received the DNA vaccine (**Figures 3B, C**). However, after prime SLP vaccination and after the booster, the combinatorial DNA/SLP vaccine improved the M38-specific CD8$^+$ T cell response compared to the SLP vaccine alone (**Figure 3D**), whereas the m139-specific CD8$^+$ T cell response was not affected, (**Figure 3F**). The combinatorial DNA/SLP vaccine induced a similar KLRG1$^+$CD62L$^-$ M38- and m139-specific CD8$^+$ T cell population compared to the SLP vaccine alone after boost vaccination (**Figures 3E, G**). M38-specific and m139-specific CD8$^+$ T cells induced upon either SLP or DNA/SLP vaccination did not upregulate the T cell exhaustion marker PD-1 (**Figures 3E, G**).

Next, we determined the role of CD4$^+$ T cells for the development of the vaccine-specific B and T cell responses. First, we established whether the gB DNA and/or SLP vaccines vaccine elicited a CD4$^+$ T cell response. Following sgB DNA vaccination, IFN-γ production of CD4$^+$ T cells was clearly detected

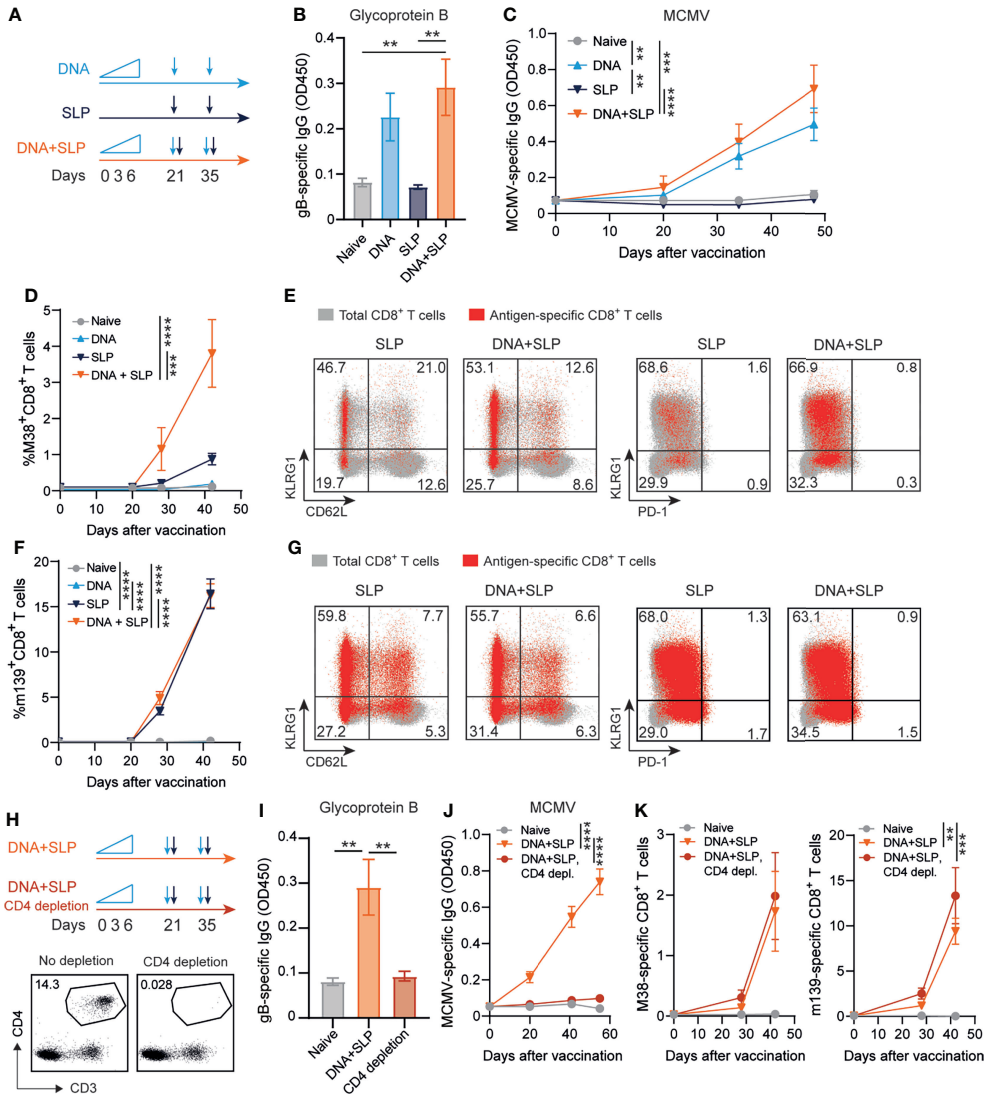

FIGURE 3 | Combining the sgB DNA vaccine with synthetic long peptide vaccines enhances antibody and CD8$^+$ T cell responses. **(A)** Vaccination schedule for mice vaccinated with the sgB DNA vaccine and/or the SLP vaccine. The sgB DNA vaccine was intradermally with an increasing dose prime schedule (10 µg on day 0, 20 µg on day 3 and 30 µg on day 6) followed by two booster immunizations with 60 µg. The SLP vaccine (synthetic long peptides containing the M38 and m139 class I epitopes, adjuvanted with CpG) was provided subcutaneously on day 21 and day 35. **(B)** Glycoprotein B-specific IgG response on day 72 after vaccination in blood. Data shown are mean values ± SEM (n=8). One-way ANOVA was used for statistical analysis. **(C)** Kinetics of the MCMV-specific IgG response in serum over time. Data shown are mean values ± SEM (n=8). One-way ANOVA on day 48 was used for statistical analysis. **(D, F)** Kinetic analysis of the M38- **(D)** and m139-specific **(F)** CD8$^+$ T cell response. Data shown are mean values ± SEM (n=8). One-way ANOVA on day 48 was used for statistical analysis. **(E, F)** Representative flow cytometry plots showing the KLRG1 vs CD62L or KLRG1 vs PD-1 cell-surface expression on the total CD8$^+$ T cells (gray) and M38- **(E)** and m139-specific **(G)** CD8$^+$ T cells (red) in blood at day 7 post-boost vaccination. Indicated percentages are representative values for each group. Experiments were performed twice with similar outcome. **(H)** Vaccination schedule for mice vaccinated with the sgB DNA vaccine and the SLP vaccine with and without CD4$^+$ T cell depletion during the vaccination period. The vaccines were administered as described in **(A)**. The CD4$^+$ T cell depleting antibody was administered s.c. every six days. Representative flow cytometry plot showing confirmation of CD4$^+$ T cell depletion in blood. **(I)** Glycoprotein B-specific IgG response on day 72 after vaccination in blood. Data shown are mean values ± SEM (n=9). One-way ANOVA was used for statistical analysis. **P<0.01. **(J)** Kinetics of the MCMV-specific IgG response in serum over time. Data shown are mean values ± SEM (n=9). One-way ANOVA on day 55 was used for statistical analysis. **(K)** Kinetic analysis of the M38- and m139-specific CD8$^+$ T cell response. Data shown are mean values ± SEM (n=8). One-way ANOVA on day 42 was used for statistical analysis. **P<0.01, ***P<0.001, ****P<0.0001.

after stimulation with peptides spanning the MCMV gB protein, whereas stimulation with the SLPs did not elicit reactivity (**Supplementary Figure 1C**). Moreover, SLP vaccination did not elicit IFN-γ production by the CD4$^+$ T cells after stimulation with gB protein or SLPs. To determine whether CD4$^+$ T cell help is critical for the vaccine-specific B and T cell responses, we depleted the CD4$^+$ T cells during the vaccination period (**Figure 3H**). CD4$^+$ T cell depletion resulted in the absence of gB-specific and MCMV-

specific IgG antibodies (**Figures 3I, J**). Depletion of the $CD4^+$ T cells did however not affect the height of the M38- and m139-specific $CD8^+$ T cell response (**Figure 3K**). Together, these results indicate that combining the sgB DNA vaccine with SLP vaccines results in similar levels of vaccines-specific antibody levels in a $CD4^+$ T cell dependent manner, and enhancement of the M38-specific $CD8^+$ T cell response.

The Combination of sgB DNA Vaccines With $CD8^+$ T Cell-Eliciting Synthetic Long Peptide Vaccines Is Superior in Protection Against MCMV

To determine the protective capacity of the combinatorial DNA/SLP vaccine, mice were challenged with MCMV *via* the intraperitoneal route, to mimic systemic infection that can occur upon organ transplantation of a CMV-positive donor into a CMV-negative recipient, or *via* the intranasal route, representing the natural route of CMV infection (51) (**Figure 4A**). The viral load in the liver after intraperitoneal challenge was 5-fold lower in the mice that received combinatorial vaccination as compared to single SLP vaccination, and 16-fold lower compared to single DNA vaccination (**Figure 4B**). Upon intranasal challenge, the combinatorial vaccine reduced the viral load in the liver 8-fold compared to SLP vaccination, and also 8-fold compared to DNA vaccination (**Figure 4C**). The protective capacity of the combinatorial SLP/DNA vaccine was also observed in the lungs of intranasally challenged mice. Here, the viral load of the combinatorial vaccine was 4 and 5-fold lower compared to single SLP and single DNA vaccination, respectively (**Figure 4C**).

Taken together, we show that the combinatorial DNA/SLP vaccine improves protection in different organs, compared to the single-arm vaccination strategies, indicating a synergistic effect of combining these vaccine platforms to enhance humoral as well as cellular responses against CMV.

DISCUSSION

Here we show in experimental CMV models that the effectivity of antibody-eliciting DNA vaccines against CMV infection *via* the intranasal and intraperitoneal route can be improved by the addition of $CD8^+$ T cell responses induced by SLP vaccines. This experimental study allowed the direct comparison of vaccine platforms inducing either a CMV-specific antibody or a $CD8^+$ T cell response or the combination thereof, and emphasized the importance of inducing both the humoral and cellular immune response to counteract CMV-associated disease. In this respect, vaccines such as V160, a conditionally replication-defective vaccine derived from the AD169 strain, are of interest because of the induction of both neutralizing antibody titers and cellular responses (52, 53).

The sgB antibody-eliciting DNA vaccine outperformed the gB DNA vaccine, which is in line with Lauterbach et al., who showed >10 fold higher IgG titers after DNA vaccines encoding soluble antigen compared to membrane bound antigen (54). This may be explained by trapping of sgB by follicular dendritic cells and subsequent presentation to B cells (55). Further optimization of the sgB antibody-eliciting DNA vaccine and $CD8^+$ T cell-eliciting SLP vaccine could be achieved by several possibilities. First, although gB vaccines elicit $CD4^+$ T cell responses (56, 57) (**Supplementary Figure 1C**) stronger induction of CMV-specific $CD4^+$ T cell responses with SLP vaccines for example has direct antiviral effects (58), and may facilitate both the $CD8^+$ T cell and antibody response (59). With respect to the latter, the sgB vaccine may already induce sufficient (selective) $CD4^+$ T cell help to facilitate the IgG response. The $CD4^+$ T cell help mediated by the gB vaccine is in our setting, however, not instrumental in the increment of the M38-specific $CD8^+$ T cell response after DNA/SLP booster vaccination. This elevation may be caused by an adjuvant effect of the DNA vaccine, which is coupled to immunostimulatory DNA sequences like

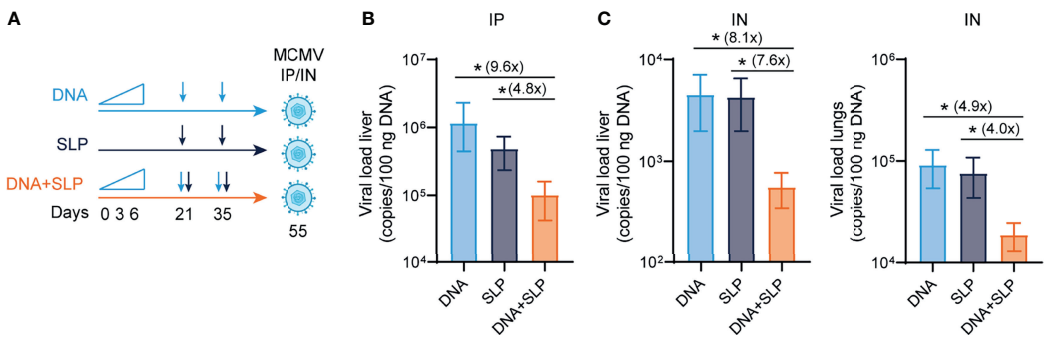

FIGURE 4 | The combination of sgB DNA vaccines with the $CD8^+$ T cell eliciting synthetic long peptide vaccines is superior in protection against MCMV. **(A)** Vaccination schedule for mice vaccinated with the sgB DNA vaccine and/or the SLP vaccine. The sgB DNA vaccine was intradermally with an increasing dose prime schedule (10 µg on day 0, 20 µg on day 3 and 30 µg on day 6) followed by two booster immunizations with 60 µg. The SLP vaccine (synthetic long peptides containing the M38 and m139 class I epitopes, adjuvanted with CpG) was provided subcutaneously on day 21 and day 35. Three weeks after the final vaccination, mice were challenged either intraperitoneally or intranasally with 5×10^4 PFU salivary gland-derived MCMV-Smith. **(B)** At day 5 post intraperitoneal infection, livers were isolated. The viral genome copies were determined by PCR. The viral load is depicted as mean values ± SEM (n=8). **(C)** At day 5 post intranasal infection, livers and lungs were isolated. The viral genome copies were determined by PCR. The viral load is depicted as mean values ± SEM are shown (n=8). Experiments was performed twice with similar outcome. A Kruskal-Wallis test was used for statistical analysis. *P<0.05.

unmethylated CpG motifs and cytoplasmic DNA sensors in the STING/IRF7 pathway (60, 61). In contrast to $CD4^+$ T cell responses, $CD8^+$ T cell responses, are not elicited by gB vaccines (57), which is in line with studies demonstrating the absence of immunodominant class I-restricted epitopes in the gB protein (62, 63). Moreover, the addition of an agonistic antibody to OX40, a costimulatory receptor on activated T cells, could be added to enhance $CD4^+$ and $CD8^+$ T cell-based protection (58). Furthermore, vaccines using the trimeric form of gB as an antigen instead of monomeric resulted in 50-fold times higher neutralizing titers (64), and neutralizing antibodies against the trimeric (gH/gL/gO) and pentameric complex (gH/gL/UL128/UL130/UL131A) have shown neutralizing activity and prevention of infection of epithelial, endothelial cells and fibroblasts (65–68). Thus, further enhancing antibody-mediated protection by optimizing gB-based vaccines and adding other immunogenic proteins are both advisable. Designing one vaccine platform inducing both strong antibody and T cell responses against CMV may, however, be challenging but combined formulations as used here may be an option for further development of synthetic vaccines.

Taken together, the findings here establish that deliberate induction of humoral and cellular immunity enables enhanced protection against herpesvirus infection. Especially, antibody-mediated protection can become more effective by addition of strong vaccine-induced $CD8^+$ T cell responses, thereby highlighting the importance of designing CMV vaccines that elicit both strong T and B cell responses.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France). Mice were maintained under specific-pathogen-free conditions at the Central Animal Facility of Leiden University Medical Center (LUMC). Mice were aged 8-10 weeks at the start of each experiment. All animal experiments were approved by the Animal Experiments Committee of the LUMC and performed according to the Dutch Experiments on Animals Act that serves as the implementation of the guidelines on the protection of experimental animals by the Council of Europe.

DNA Construct, Peptides and Vaccination

Full-length glycoprotein B (gB, NCBI Gene symbol MuHV1_gp059) was amplified by PCR from a BAC clone containing the Murid betaherpesvirus 1 genome (MCMV K181, pSM3fr-MCK-2fl). As forward primer, 5'-CCAAGCT GTCTAGAGCCGCCACC ATG GCA AGA AGA AAC GAA AGA GGA TGT C-3' containing a single Ser-to-Ala substitution at position 2 was used to introduce a consensus Kozak sequence. A reverse primer 5'- GT TTA CTT CTC GAA CTG AGG GTG AGA CCA AGC GCT GTA CTC GAA ATC GGA GTC CTC C-3' was used containing a Strep-tag (underlined, amino acid sequence SA-WSHPQFEK) and stop codon. To amplify a gene fragment encoding secreted glycoprotein (sgB), the same forward primer was used in combination with the reverse primer 5'- GT TTA CTT CTC GAA CTG AGG GTG AGA CCA AGC GCT AAA CGG GTT CGT CAG GAA GC-3' to generate a construct replacing the transmembrane and cytoplasmic domains (aa 787 to 937) with a Strep-tag. Both PCR fragments were assembled into a pVAX-based expression vector containing a HCMV IE1-promoter, a rabbit beta-globin poly-A signal and kanamycin resistance marker using the NEBuilder HiFi DNA Assembly kit (New England Biolabs, Ipswich, MA, USA). Plasmids were propagated in E. coli cultures and purified using Nucleobond Xtra maxi EF columns (Macherey-Nagel) according to manufacturer's instructions. DNA constructs were verified by double-stranded Sanger sequencing (Baseclear). For vaccination, plasmids were column-purified twice, each time using a fresh column, and dissolved at 3 mg/ml in Tris : EDTA buffer (1:0.1 mM). Mice were intradermally or intramuscularly vaccinated with 30 µL DNA-lipid nanoparticles containing cationic lipid SAINT-18 (provided by Synvolux Therapeutics) in a 1: 0.75 ratio (µg DNA: nmole SAINT-18) in 0.9% NaCl at the tail base.

Synthetic long peptides (SLP) containing MHC class I-restricted T cell epitopes from MCMV proteins M38 and m139 ($M38_{316-323}$ and $m139_{419-426}$) (40) were synthesized at the peptide facility of the LUMC. Mice were vaccinated subcutaneously at the tail base with a mixture of 50 µg of each SLP and 20 µg CpG (ODN 1826, *Invivogen*) in 50 µL PBS.

Western Blot Analysis

Expression of DNA vaccines was verified *in vitro* by transfection and Western blotting using a gB-specific antibody. Briefly, mouse B16F10 cells were seeded in a 6-wells plate and transfected the next day with 1 µg DNA and Saint-DNA transfection reagent (Synvolux Therapeutics) according to the manufacturer's instructions. After two days, cells were washed in PBS, and lysed in Laemmli buffer containing beta-mercaptoethanol. Equal amounts of total cell lysates were separated on a 10% polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Amersham Protran). Membranes where then incubated with a primary antibody specific for MCMV glycoprotein B (cat. MCBG11, Alpha Diagnostic Intl.) and HRP-conjugated swine anti-rabbit secondary antibody (Dako Agilent). Antibody binding was visualized by chemiluminescence using Clarity Western ECL substrate (Bio-Rad).

MCMV Preparation, Infection and Determination of Viral Load

MCMV-Smith was obtained from the American Type Culture Collection (ATCC VR-194; Manassas, VA, USA) and virus stocks were prepared from salivary glands of infected BALB/c mice. The viral titers of the produced virus stocks were determined by viral plaque assays with 3T3 mouse embryonic fibroblasts (MEFs) (ATCC). Age- and sex-matched mice were immunized intraperitoneally with 1×10^4 PFU MCMV. 20 days upon the last booster vaccination, mice were challenged with 5×10^4 PFU MCMV. At day 5 post MCMV challenge, viral loads in liver and lungs were determined by real-time PCR as described previously (5).

Serum Antibody Detection by ELISA

Blood of mice was collected *via* the tail vein. Serum was collected upon centrifugation and stored at -20°C until further use. MCMV-specific IgG levels were measured by ELISA as described before (5), and endpoint binding antibody titers were determined by calculating the dilution at which the OD450 was twice as high as the background of the assay. In brief, 96-well plates (Nunc MaxiSorp) were coated overnight at 4°C with tissue culture derived MCMV-Smith in bicarbonate buffer (pH 9.6) and washed with PBS. After blocking the plates for 1 h at 37°C with blocking buffer (PBS/5% milk powder) and washing with PBS containing 0.05% Tween, diluted sera (in PBS/1% milk powder) were added and incubated for 1h at 37°C. For the gB-specific IgG levels, 96-well plates (Nunc MaxiSorp) were coated overnight at 4°C with recombinant glycoprotein B (1 μg/mL, Alpha Diagnostic International) in bicarbonate buffer (pH 9.6), washed with PBS/0.05% Tween and blocked with PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween (blocking buffer) for 1 h at room temperature. Plates were washed with PBS/0.05% Tween and incubated with serial dilutions of mouse sera in blocking buffer and incubated for 1 h at room temperature. The M38- and m139-specific IgG levels were determined by coating streptavidin coated plates (Kaivogen) with 5 μg/mL biotinylated M38 and m139 SLP in bicarbonate buffer (pH 9.6) and washed with PBS/0.05% Tween and blocked with PBS/5% milk powder for 1 h at room temperature. Plates were washed with PBS/0.05% Tween and incubated with serial dilutions of mouse sera in PBS/1%BSA and incubated for 1 h at room temperature. For all ELISAs, plates were then washed with PBS/0.05% Tween, after which horseradish peroxidase (HRP)-conjugated IgG (diluted in PBS/1% milk powder was added) was incubated for 1 h at 37°C. To develop the plates, 50 μL of TMB 3,3=,5,5=tetramethylbenzidine) (Sigma-Aldrich) was added to each well and incubated for 10 minutes at room temperature. Plates were measured with a microplate reader (model 680; Bio-Rad) at 450 nm within 5 minutes after the reaction was stopped by the addition of 50 μL 1M H_2SO_4.

Flow Cytometry

Blood was collected *via* the tail vein and antigen-specific $CD8^+$ T cell responses in blood were evaluated with cell surface staining, performed as previously described (69). In brief, upon lysis of erythrocytes single-cell suspensions were incubated with fluorescently-labeled antibodies and MHC class I tetramers for 30 minutes at 4˚C. MHC class I tetramers specific for $M38_{316–323}$ and $m139_{419–426}$ MCMV epitopes were used to stain M38-and m139-specific $CD8^+$ T cells. Fluorochrome-conjugated antibodies specific for mouse CD3, CD4, CD8, CD62L, KLRG1, IFN-γ, and PD-1 were purchased from Biolegend or eBioscience. Dead cells were excluded with the use of 7-aminoactinomycinD (7-AAD) (Invitrogen). For examination of intracellular IFN-γ production, white blood cells were stimulated with long peptides for 8 h of which the last 6 h in presence of brefeldin A (Golgiplug; BD Pharmingen). Flow cytometric acquisition was performed on a BD Fortessa flow cytometer (BD Biosciences) or Aurora Cytek spectral analyzer, and samples were analyzed using FlowJo software (TreeStar).

Flow cytometric acquisition was performed on a LSR Fortessa cytometer (BD Biosciences) and samples were analyzed using FlowJo software (TreeStar).

$CD4^+$ T Cell Depletion

$CD4^+$ T cell depleting monoclonal antibodies (clone GK1.5, BioXcell) were administered intraperitoneally twice per week, starting 4 days before vaccination. For the first injection, mice received 150 μg per mouse, and $CD4^+$ T cell depletion was maintained with 50 μg per mouse.

Statistical Analysis

Significance between groups was evaluated by performing an unpaired Student's T test or ANOVA. To evaluate statistical significant difference of the viral load, a Kruskal-Wallis test was used. All statistical analyses were performed in Prism (Graphpad software). The level of statistical significance was set at $P<0.05$.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiments Committee of the LUMC.

AUTHOR CONTRIBUTIONS

IP designed and performed most of the experiments and data analysis, and wrote the manuscript. SD, DV, DB and AR assisted in experiments and reviewed the manuscript. WH and GZ designed and assisted in experiments and reviewed the manuscript. JB and FO provided conceptual input and reviewed the manuscript. RA designed and supervised the study, wrote the manuscript and provided funding for the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.680559/full#supplementary-material

Supplementary Figure 1 | (A) Representative flow cytometry gating of tetramer-positive $CD8^+$ T cells. **(B)** M38- and m139-specific IgG antibody response on day 72 upon vaccination. **(C)** Intracellular IFN-γ production of $CD4^+$ T cells in blood at day 79 after DNA, SLP or combined DNA/SLP vaccination. Blood single cell suspension was stimulated with the M38 SLP, m139 SLP, synthetic long peptides spanning the whole MCMV gB protein no peptide.

REFERENCES

1. Cannon MJ, Schmid DS, Hyde TB. Review of Cytomegalovirus Seroprevalence and Demographic Characteristics Associated With Infection. *Rev Med Virol* (2010) 20(4):202–13. doi: 10.1002/rmv.655
2. Crough T, Khanna R. Immunobiology of Human Cytomegalovirus: From Bench to Bedside. *Clin Microbiol Rev* (2009) 22(1):76–98. doi: 10.1128/CMR.00034-08
3. Kotton CN. Updates on Antiviral Drugs for Cytomegalovirus Prevention and Treatment. *Curr Opin Organ Transplant* (2019) 24(4):469–75. doi: 10.1097/MOT.0000000000000666
4. Picarda G, Benedict CA. Cytomegalovirus: Shape-Shifting the Immune System. *J Immunol* (2018) 200(12):3881–9. doi: 10.4049/jimmunol.1800171
5. Redeker A, Welten SP, Arens R. Viral Inoculum Dose Impacts Memory T-Cell Inflation. *Eur J Immunol* (2014) 44(4):1046–57. doi: 10.1002/eji.201343946
6. Trgovcich J, Kincaid M, Thomas A, Griessl M, Zimmerman P, Dwivedi V, et al. Cytomegalovirus Reinfections Stimulate CD8 T-Memory Inflation. *PloS One* (2016) 11(11):e0167097. doi: 10.1371/journal.pone.0167097
7. Redeker A, Remmerswaal EBM, van der Gracht ETI, Welten SPM, Hollt T, Koning F, et al. The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose. *Front Immunol* (2017) 8:1953. doi: 10.3389/fimmu.2017.01953
8. Jonjic S, Pavic I, Polic B, Crnkovic I, Lucin P, Koszinowski UH. Antibodies Are Not Essential for the Resolution of Primary Cytomegalovirus Infection But Limit Dissemination of Recurrent Virus. *J Exp Med* (1994) 179(5):1713–7. doi: 10.1084/jem.179.5.1713
9. Klenovsek K, Weisel F, Schneider A, Appelt U, Jonjic S, Messerle M, et al. Protection From CMV Infection in Immunodeficient Hosts by Adoptive Transfer of Memory B Cells. *Blood* (2007) 110(9):3472–9. doi: 10.1182/blood-2007-06-095414
10. Welten SP, Redeker A, Toes RE, Arens R. Viral Persistence Induces Antibody Inflation Without Altering Antibody Avidity. *J Virol* (2016) 90(9):4402–11. doi: 10.1128/JVI.03177-15
11. Fowler KB, Stagno S, Pass RF, Britt WJ, Boll TJ, Alford CA. The Outcome of Congenital Cytomegalovirus Infection in Relation to Maternal Antibody Status. *N Engl J Med* (1992) 326(10):663–7. doi: 10.1056/NEJM199203053261003
12. Nigro G, Adler SP, La TR, Best AM. Passive Immunization During Pregnancy for Congenital Cytomegalovirus Infection. *N Engl J Med* (2005) 353(13):1350–62. doi: 10.1056/NEJMoa043337
13. Boppana SB, Britt WJ. Antiviral Antibody Responses and Intrauterine Transmission After Primary Maternal Cytomegalovirus Infection. *J Infect Dis* (1995) 171(5):1115–21. doi: 10.1093/infdis/171.5.1115
14. Snydman DR, Werner BG, Heinze-Lacey B, Berardi VP, Tilney NL, Kirkman RL, et al. Use of Cytomegalovirus Immune Globulin to Prevent Cytomegalovirus Disease in Renal-Transplant Recipients. *N Engl J Med* (1987) 317(17):1049–54. doi: 10.1056/NEJM198710223171703
15. Britt WJ, Vugler L, Butfiloski EJ, Stephens EB. Cell Surface Expression of Human Cytomegalovirus (HCMV) Gp55-116 (Gb): Use of HCMV-Recombinant Vaccinia Virus-Infected Cells in Analysis of the Human Neutralizing Antibody Response. *J Virol* (1990) 64(3):1079–85. doi: 10.1128/jvi.64.3.1079-1085.1990
16. Marshall GS, Rabalais GP, Stout GG, Waldeyer SL. Antibodies to Recombinant-Derived Glycoprotein B After Natural Human Cytomegalovirus Infection Correlate With Neutralizing Activity. *J Infect Dis* (1992) 165(2):381–4. doi: 10.1093/infdis/165.2.381
17. Baraniak I, Kropff B, Ambrose L, McIntosh M, McLean GR, Pichon S, et al. Protection From Cytomegalovirus Viremia Following Glycoprotein B Vaccination Is Not Dependent on Neutralizing Antibodies. *Proc Natl Acad Sci USA* (2018) 115(24):6273–8. doi: 10.1073/pnas.1800224115
18. Nelson CS, Huffman T, Jenks JA, Cisneros de la Rosa E, Xie G, Vandergrift N, et al. HCMV Glycoprotein B Subunit Vaccine Efficacy Mediated by Nonneutralizing Antibody Effector Functions. *Proc Natl Acad Sci USA* (2018) 115(24):6267–72. doi: 10.1073/pnas.1800177115
19. Bootz A, Karbach A, Spindler J, Kropff B, Reuter N, Sticht H, et al. Protective Capacity of Neutralizing and Non-Neutralizing Antibodies Against Glycoprotein B of Cytomegalovirus. *PloS Pathog* (2017) 13(8):e1006601. doi: 10.1371/journal.ppat.1006601
20. Sedova ES, Scherbinin DN, Lysenko AA, Alekseeva SV, Artemova EA, Shmarov MM. Non-Neutralizing Antibodies Directed at Conservative Influenza Antigens. *Acta Naturae* (2019) 11(4):22–32. doi: 10.32607/20758251-2019-11-4-22-32
21. Polic B, Hengel H, Krmpotic A, Trgovcich J, Pavic I, Luccaronin P, et al. Hierarchical and Redundant Lymphocyte Subset Control Precludes Cytomegalovirus Replication During Latent Infection. *J Exp Med* (1998) 188(6):1047–54. doi: 10.1084/jem.188.6.1047
22. Vescovini R, Biasini C, Fagnoni FF, Telera AR, Zanlari L, Pedrazzoni M, et al. Massive Load of Functional Effector CD4+ and CD8+ T Cells Against Cytomegalovirus in Very Old Subjects. *J Immunol* (2007) 179(6):4283–91. doi: 10.4049/jimmunol.179.6.4283
23. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly Targeted Human Cytomegalovirus-Specific CD4+ and CD8+ T Cells Dominate the Memory Compartments of Exposed Subjects. *J Exp Med* (2005) 202(5):673–85. doi: 10.1084/jem.20050882
24. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, et al. Memory Inflation: Continuous Accumulation of Antiviral CD8+ T Cells Over Time. *J Immunol* (2003) 170(4):2022–9. doi: 10.4049/jimmunol.170.4.2022
25. O'Hara GA, Welten SP, Klenerman P, Arens R. Memory T Cell Inflation: Understanding Cause and Effect. *Trends Immunol* (2012) 33(2):84–90. doi: 10.1016/j.it.2011.11.005
26. van den Berg SPH, Pardieck IN, Lanfermeijer J, Sauce D, Klenerman P, van Baarle D, et al. The Hallmarks of CMV-Specific CD8 T-Cell Differentiation. *Med Microbiol Immunol* (2019) 208(3-4):365–73. doi: 10.1007/s00430-019-00608-7
27. Lim EY, Jackson SE, Wills MR. The CD4+ T Cell Response to Human Cytomegalovirus in Healthy and Immunocompromised People. *Front Cell Infect Microbiol* (2020) 10:202. doi: 10.3389/fcimb.2020.00202
28. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-Lymphocyte Response to Cytomegalovirus After Human Allogeneic Bone Marrow Transplantation: Pattern of Recovery and Correlation With Cytomegalovirus Infection and Disease. *Blood* (1991) 78(5):1373–80. doi: 10.1182/blood.V78.5.1373.1373
29. Reusser P, Cathomas G, Attenhofer R, Tamm M, Thiel G. Cytomegalovirus (CMV)-Specific T Cell Immunity After Renal Transplantation Mediates Protection From CMV Disease by Limiting the Systemic Virus Load. *J Infect Dis* (1999) 180(2):247–53. doi: 10.1086/314879
30. Gamadia LE, Remmerswaal EB, Weel JF, Bemelman F, van Lier RA, ten Berge IJ. Primary Immune Responses to Human CMV: A Critical Role for IFN-Gamma-Producing CD4+ T Cells in Protection Against CMV Disease. *Blood* (2003) 101(7):2686–92. doi: 10.1182/blood-2002-08-2502
31. Shlobin OA, West EE, Lechtzin N, Miller SM, Borja M, Orens JB, et al. Persistent Cytomegalovirus-Specific Memory Responses in the Lung Allograft and Blood Following Primary Infection in Lung Transplant Recipients. *J Immunol* (2006) 176(4):2625–34. doi: 10.4049/jimmunol.176.4.2625
32. Sinclair E, Tan QX, Sharp M, Girling V, Poon C, Natta MV, et al. Protective Immunity to Cytomegalovirus (CMV) Retinitis in AIDS Is Associated With CMV-Specific T Cells That Express Interferon- Gamma and Interleukin-2

and Have a CD8+ Cell Early Maturational Phenotype. *J Infect Dis* (2006) 194 (11):1537–46. doi: 10.1086/508997
33. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of Viral Immunity in Immunodeficient Humans by the Adoptive Transfer of T Cell Clones. *Science* (1992) 257(5067):238–41. doi: 10.1126/science.1352912
34. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, et al. Reconstitution of Cellular Immunity Against Cytomegalovirus in Recipients of Allogeneic Bone Marrow by Transfer of T-Cell Clones From the Donor. *N Engl J Med* (1995) 333(16):1038–44. doi: 10.1056/NEJM199510193331603
35. Reddehase MJ, Weiland F, Munch K, Jonjic S, Luske A, Koszinowski UH. Interstitial Murine Cytomegalovirus Pneumonia After Irradiation: Characterization of Cells That Limit Viral Replication During Established Infection of the Lungs. *J Virol* (1985) 55(2):264–73. doi: 10.1128/jvi.55.2.264-273.1985
36. Holtappels R, Bohm V, Podlech J, Reddehase MJ. CD8 T-Cell-Based Immunotherapy of Cytomegalovirus Infection: "Proof of Concept" Provided by the Murine Model. *Med Microbiol Immunol* (2008) 197 (2):125–34. doi: 10.1007/s00430-008-0093-2
37. Pipeling MR, West EE, Osborne CM, Whitlock AB, Dropulic LK, Willett MH, et al. Differential CMV-Specific CD8+ Effector T Cell Responses in the Lung Allograft Predominate Over the Blood During Human Primary Infection. *J Immunol* (2008) 181(1):546–56. doi: 10.4049/jimmunol.181.1.546
38. Bestard O, Lucia M, Crespo E, Van Liempt B, Palacio D, Melilli E, et al. Pretransplant Immediately Early-1-Specific T Cell Responses Provide Protection for CMV Infection After Kidney Transplantation. *Am J Transplant* (2013) 13(7):1793–805. doi: 10.1111/ajt.12256
39. Bunde T, Kirchner A, Hoffmeister B, Habedank D, Hetzer R, Cherepnev G, et al. Protection From Cytomegalovirus After Transplantation Is Correlated With Immediate Early 1-Specific CD8 T Cells. *J Exp Med* (2005) 201(7):1031–6. doi: 10.1084/jem.20042384
40. Panagioti E, Redeker A, van Duikeren S, Franken KL, Drijfhout JW, van der Burg SH, et al. The Breadth of Synthetic Long Peptide Vaccine-Induced CD8+ T Cell Responses Determines the Efficacy Against Mouse Cytomegalovirus Infection. *PloS Pathog* (2016) 12(9):e1005895. doi: 10.1371/journal.ppat.1005895
41. Pahl-Seibert MF, Juelch M, Podlech J, Thomas D, Deegen P, Reddehase MJ, et al. Highly Protective *In Vivo* Function of Cytomegalovirus IE1 Epitope-Specific Memory CD8 T Cells Purified by T-Cell Receptor-Based Cell Sorting. *J Virol* (2005) 79(9):5400–13. doi: 10.1128/JVI.79.9.5400-5413.2005
42. Schleiss MR, Permar SR, Plotkin SA. Progress Toward Development of a Vaccine Against Congenital Cytomegalovirus Infection. *Clin Vaccine Immunol* (2017) 24(12):e00268–17. doi: 10.1128/CVI.00268-17
43. Pass RF, Zhang C, Evans A, Simpson T, Andrews W, Huang ML, et al. Vaccine Prevention of Maternal Cytomegalovirus Infection. *N Engl J Med* (2009) 360 (12):1191–9. doi: 10.1056/NEJMoa0804749
44. Bernstein DI, Munoz FM, Callahan ST, Rupp R, Wootton SH, Edwards KM, et al. Safety and Efficacy of a Cytomegalovirus Glycoprotein B (Gb) Vaccine in Adolescent Girls: A Randomized Clinical Trial. *Vaccine* (2016) 34(3):313–9. doi: 10.1016/j.vaccine.2015.11.056
45. Jenks JA, Nelson CS, Roark HK, Goodwin ML, Pass RF, Bernstein DI, et al. Antibody Binding to Native Cytomegalovirus Glycoprotein B Predicts Efficacy of the Gb/MF59 Vaccine in Humans. *Sci Transl Med* (2020) 12(568): eabb3611. doi: 10.1126/scitranslmed.abb3611
46. Anderholm KM, Bierle CJ, Schleiss MR. Cytomegalovirus Vaccines: Current Status and Future Prospects. *Drugs* (2016) 76(17):1625–45. doi: 10.1007/s40265-016-0653-5
47. Heldwein EE, Krummenacher C. Entry of Herpesviruses Into Mammalian Cells. *Cell Mol Life Sci* (2008) 65(11):1653–68. doi: 10.1007/s00018-008-7570-z
48. Compton T, Nepomuceno RR, Nowlin DM. Human Cytomegalovirus Penetrates Host Cells by pH-Independent Fusion at the Cell Surface. *Virology* (1992) 191(1):387–95. doi: 10.1016/0042-6822(92)90200-9
49. Tondini E, Arakelian T, Oosterhuis K, Camps M, van Duikeren S, Han W, et al. A Poly-Neoantigen DNA Vaccine Synergizes With PD-1 Blockade to Induce T Cell-Mediated Tumor Control. *Oncoimmunology* (2019) 8 (11):1652539. doi: 10.1080/2162402X.2019.1652539
50. Johansen P, Storni T, Rettig L, Qiu Z, Der-Sarkissian A, Smith KA, et al. Antigen Kinetics Determines Immune Reactivity. *Proc Natl Acad Sci USA* (2008) 105(13):5189–94. doi: 10.1073/pnas.0706296105
51. Oduro JD, Redeker A, Lemmermann NA, Ebermann L, Marandu TF, Dekhtiarenko I, et al. Murine Cytomegalovirus Infection via the Intranasal Route Offers a Robust Model of Immunity Upon Mucosal CMV Infection. *J Gen Virol* (2015) 97(1):185–95. doi: 10.1099/jgv.0.000339
52. Wang D, Freed DC, He X, Li F, Tang A, Cox KS, et al. A Replication-Defective Human Cytomegalovirus Vaccine for Prevention of Congenital Infection. *Sci Transl Med* (2016) 8(362):362ra145. doi: 10.1126/scitranslmed.aaf9387
53. Adler SP, Lewis N, Conlon A, Christiansen MP, Al-Ibrahim M, Rupp R, et al. Phase 1 Clinical Trial of a Conditionally Replication-Defective Human Cytomegalovirus (CMV) Vaccine in CMV-Seronegative Subjects. *J Infect Dis* (2019) 220(3):411–9. doi: 10.1093/infdis/jiz141
54. Lauterbach H, Gruber A, Ried C, Cheminay C, Brocker T. Insufficient APC Capacities of Dendritic Cells in Gene Gun-Mediated DNA Vaccination. *J Immunol* (2006) 176(8):4600–7. doi: 10.4049/jimmunol.176.8.4600
55. Allen CD, Cyster JG. Follicular Dendritic Cell Networks of Primary Follicles and Germinal Centers: Phenotype and Function. *Semin Immunol* (2008) 20 (1):14–25. doi: 10.1016/j.smim.2007.12.001
56. Sabbaj S, Pass RF, Goepfert PA, Pichon S. Glycoprotein B Vaccine Is Capable of Boosting Both Antibody and CD4 T-Cell Responses to Cytomegalovirus in Chronically Infected Women. *J Infect Dis* (2011) 203(11):1534–41. doi: 10.1093/infdis/jir138
57. Dasari V, Smith C, Zhong J, Scott G, Rawlinson W, Khanna R. Recombinant Glycoprotein B Vaccine Formulation With Toll-Like Receptor 9 Agonist and Immune-Stimulating Complex Induces Specific Immunity Against Multiple Strains of Cytomegalovirus. *J Gen Virol* (2011) 92(Pt 5):1021–31. doi: 10.1099/vir.0.029413-0
58. Panagioti E, Boon L, Arens R, van der Burg SH. Enforced OX40 Stimulation Empowers Booster Vaccines to Induce Effective CD4+ and CD8+ T Cell Responses Against Mouse Cytomegalovirus Infection. *Front Immunol* (2017) 8:144. doi: 10.3389/fimmu.2017.00144
59. Zhong J, Khanna R. Delineating the Role of CD4+ T Cells in the Activation of CMV-Specific Immune Responses Following Immunization With Ad-Gbcmvpoly Vaccine: Implications for Vaccination of Immunocompromised Individuals. *J Gen Virol* (2010) 91(Pt 12):2994–3001. doi: 10.1099/vir.0.025742-0
60. Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, et al. Immunostimulatory DNA Sequences Necessary for Effective Intradermal Gene Immunization. *Science* (1996) 273(5273):352–4. doi: 10.1126/science.273.5273.352
61. Suschak JJ, Wang S, Fitzgerald KA, Lu S. A cGAS-Independent STING/IRF7 Pathway Mediates the Immunogenicity of DNA Vaccines. *J Immunol* (2016) 196(1):310–6. doi: 10.4049/jimmunol.1501836
62. Munks MW, Gold MC, Zajac AL, Doom CM, Morello CS, Spector DH, et al. Genome-Wide Analysis Reveals a Highly Diverse CD8 T Cell Response to Murine Cytomegalovirus. *J Immunol* (2006) 176(6):3760–6. doi: 10.4049/jimmunol.176.6.3760
63. Wills MR, Carmichael AJ, Mynard K, Jin X, Weekes MP, Plachter B, et al. The Human Cytotoxic T-Lymphocyte (CTL) Response to Cytomegalovirus Is Dominated by Structural Protein Pp65: Frequency, Specificity, and T-Cell Receptor Usage of Pp65-Specific CTL. *J Virol* (1996) 70(11):7569–79. doi: 10.1128/jvi.70.11.7569-7579.1996
64. Cui X, Cao Z, Wang S, Lee RB, Wang X, Murata H, et al. Novel Trimeric Human Cytomegalovirus Glycoprotein B Elicits a High-Titer Neutralizing Antibody Response. *Vaccine* (2018) 36(37):5580–90. doi: 10.1016/j.vaccine.2018.07.056
65. Freed DC, Tang Q, Tang A, Li F, He X, Huang Z, et al. Pentameric Complex of Viral Glycoprotein H Is the Primary Target for Potent Neutralization by a Human Cytomegalovirus Vaccine. *Proc Natl Acad Sci USA* (2013) 110(51): E4997–5005. doi: 10.1073/pnas.1316517110
66. Wen Y, Monroe J, Linton C, Archer J, Beard CW, Barnett SW, et al. Human Cytomegalovirus Gh/Gl/UL128/UL130/UL131A Complex Elicits Potently Neutralizing Antibodies in Mice. *Vaccine* (2014) 32(30):3796–804. doi: 10.1016/j.vaccine.2014.05.004

67. Macagno A, Bernasconi NL, Vanzetta F, Dander E, Sarasini A, Revello MG, et al. Isolation of Human Monoclonal Antibodies That Potently Neutralize Human Cytomegalovirus Infection by Targeting Different Epitopes on the Gh/Gl/UL128-131A Complex. *J Virol* (2010) 84(2):1005–13. doi: 10.1128/JVI.01809-09
68. Gerna G, Percivalle E, Perez L, Lanzavecchia A, Lilleri D. Monoclonal Antibodies to Different Components of the Human Cytomegalovirus (HCMV) Pentamer Gh/Gl/Pul128l and Trimer Gh/Gl/gO as Well as Antibodies Elicited During Primary HCMV Infection Prevent Epithelial Cell Syncytium Formation. *J Virol* (2016) 90(14):6216–23. doi: 10.1128/JVI.00121-16
69. Arens R, Loewendorf A, Redeker A, Sierro S, Boon L, Klenerman P, et al. Differential B7-CD28 Costimulatory Requirements for Stable and Inflationary Mouse Cytomegalovirus-Specific Memory CD8 T Cell Populations. *J Immunol* (2011) 186(7):3874–81. doi: 10.4049/jimmunol.1003231

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