VIRAL EVASION MECHANISMS OF THE HOST RESPONSE

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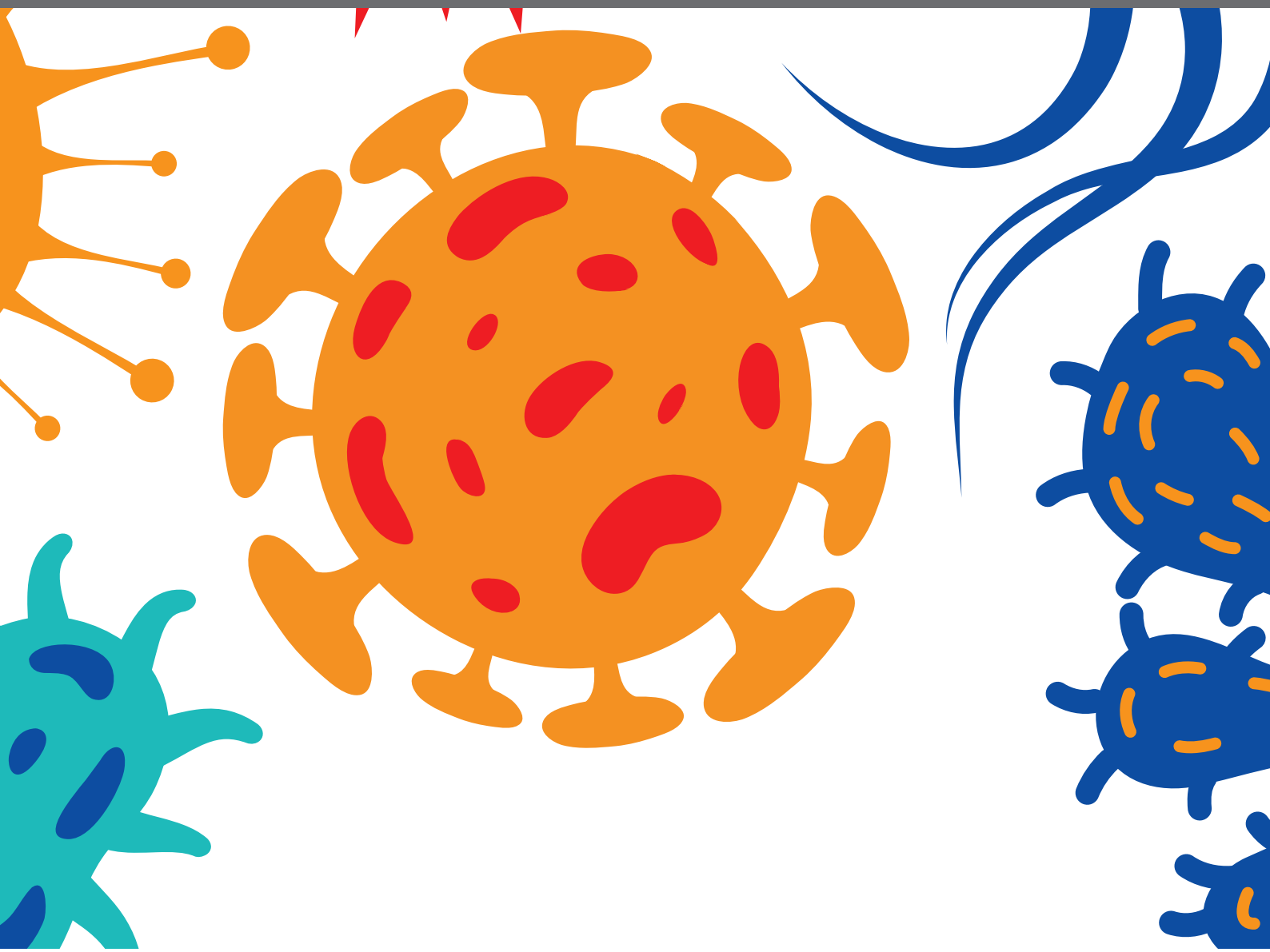

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VIRAL EVASION MECHANISMS OF THE HOST RESPONSE

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Editorial: Viral Evasion Mechanisms of the Host Response

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Keywords: DNA virus, RNA virus, MicroRNA, programmed cell death protein, microbiota, RNAstasis, cell metabolism

Editorial on the Research Topic

Viral Evasion Mechanisms of the Host Response

An essential function of the host response is to protect the organism against invading pathogens. At present, a multiplicity of mechanisms has been described on how the host sense and response to virus infections. Viruses are intracellular pathogens. Both RNA and DNA viruses have evolved mechanisms to evade host detection and to blunt both the host innate and adaptive immune responses. Considering viruses as pathogens with a relatively fast evolutionary rate, particularly RNA viruses, the result of host-virus coevolution depends on the rapid recognition and response by the host as well as on the evasion mechanism by the virus as a continuous struggle for escape/spread and immunity/clearance of virus from the host.

In this Special Research Topics issue on the recent advances in Viral Evasion Mechanisms of the Host Response, we compiled a total of twelve research and review articles. The special issue includes five Original Research Articles, five Review Articles, and two Mini Review Articles. Meanwhile five articles were dedicated to viral general mechanisms, seven were specifically focused on picornavirus, respiratory syncytial virus (RSV), Dengue virus (DENV), herpes simplex virus (HSV), and Influenza virus (IAV). The family *Picornaviridae* includes some of the most important RNA viruses for human and veterinary diseases as poliovirus, rhinovirus, and foot-and-mouth-disease virus, which comprised pioneer studies on the structural aspects of viral components. In their minireview, Cifuente and Moratorio summarize genetic variation mechanisms used by picornaviruses on structural changes involved in binding receptor and capsid antibody evasion of enteroviruses to ensure adaptation, spread and survival. The *Paramyxoviridae* family includes several important human RNA virus as Measles, Mumps, and RSV. In their article, Bakre et al. hypothesize that viral quasi-species enable RNA viruses to modulate host gene expression by regulating miRNA function via sequence complementarity or identity with the miRNA seed sites and consequently they test the hypothesis by analyzing Paramyxovirus transcripts that mimic or bind to host miRNAs by seed sequence and found that complex molecular interactions likely occurred at this host-virus interface. The human RSV (hRSV) belongs to the *Pneumoviridae* subfamily and is the leading cause of severe acute lower respiratory tract infections in humans at all ages and is the main cause of hospitalization due to pneumonia, asthma, and bronchiolitis in infants. Vaccine development against this pathogen has been delayed after the detrimental effects observed in children by vaccination with a formalin-inactivated hRSV preparation, which caused enhanced disease upon natural viral infection. In this issue, Acevedo et al., discuss the eventual role

of Fcγ receptor-mediated immunity underlying such disease exacerbation and immune response mechanisms involved in reinfections. Respiratory viral infections are associated to IgA nephropathy (IgAN). Here, Hu et al., using a RSV-induced IgAN exacerbation mouse model demonstrates that RSV activates C5a-C5aR1 axis and modulates the Th1, Th17, and Treg balance. C5aR1 inhibition alters both kidney damage and Th1, Th17, and Treg cell dysfunction supporting that blocking the C5a-C5aR1 axis might be a potential therapy for RSV-induced IgAN. The Dengue virus (DENV), a member of the *Flaviviridae* family, causes 400 million infections each year. Whereas, primary dengue infection by any of the four serotypes is asymptomatic or mild, secondary infection with a heterotypic serotype is associated with hemorrhagic fever suggesting that pre-existing immunity to DENV play a role for enhanced secondary infections. Ripoll et al. carried out molecular simulations guided by previous *in vitro* experiments and structural studies to explore the role of antibody fine-specificity, viral conformation, and maturation state on antibody dependent enhancement in the context of primary and secondary DENV infections. The Influenza viruses belong to the *Orthomyxoviridae* family and are major pathogens that affect both humans and animals causing severe respiratory illness, including pneumonia. Neutrophils and macrophages play essential roles in the clearance of influenza virus from lungs, before the onset of virus specific immunity but their uncontrolled recruitment and activation contribute to acute lung injury. In this special issue, Rudd et al. study chemokine receptors expression in a murine model of influenza-induced pneumonia and reported a new set of chemokine receptors that modulates several biological functions of neutrophils. On the other hand, Tao et al. performed a comparative analysis of whole-transcriptome RNA expression between two influenza viruses and discuss how differently expressed genes may be involved in host response and evasion mechanisms. The *Herpesviridae* family includes very important human pathogens as the herpes simplex virus (HSV), associated with mucosal lesions and encephalitis. Tognarelli et al. review and update several mechanisms used by HSV that have been described to evade the host antiviral response. The epithelial surfaces of the human body contain complex communities of microorganisms collectively referred to as microbiota. Since the discovery that gut microbiome instruct host immunity, great attention has been directed to this interaction. In their minireview, Domínguez-Díaz et al., provides a general overview of the pro- and antiviral effects of the microbiota to prevent viruses entry into host cells or to help them to evade the host antiviral immunity. Regulation of RNA homeostasis is a central step in eukaryotic gene expression. From transcription to decay, cellular messenger RNAs (mRNAs) associate with specific proteins in order to regulate their entire cycle, including mRNA localization, translation and degradation. The best characterized of such RNA-protein complexes are Stress Granules (SGs) and Processing Bodies (PBs), which are involved in RNA storage and RNA decay/storage, respectively, and are generally associated with repression of gene expression. Gaete-Argel et al. performed an exhaustive update about how viruses have evolved different mechanisms to counteract SGs and PBs assembly or to use them to his own benefit. Moreno-Altamirano et al. explore how viruses mimic, exploit, and/or interfere with host cell metabolic pathways and how, in doing so, they may evade immune responses. Programmed cell death protein (PD-1) and its ligands have received immense attention because of their role in the evasion of tumor cells from antitumor immunity. However, it has been less appreciated that the PD-1/PD-L1 axis also regulates antiviral immune responses and is therefore modulated by a number of viruses. Here, Schönrich and Raftery update and discuss the current literature regarding this important expanding field.

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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ORIGINAL RESEARCH
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Comparative Analysis of Whole-Transcriptome RNA Expression in MDCK Cells Infected With the H3N2 and H5N1 Canine Influenza Viruses

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This study aimed to detect changes in the complete transcriptome of MDCK cells after infection with the H5N1 and H3N2 canine influenza viruses using high-throughput sequencing, search for differentially expressed RNAs in the transcriptome of MDCK cells infected with H5N1 and H3N2 using comparative analysis, and explain the differences in the pathogenicity of H5N1 and H3N2 at the transcriptome level. Based on the results of our comparative analysis, significantly different levels of expression were found for 2,464 mRNAs, 16 miRNAs, 181 lncRNAs, and 262 circRNAs in the H3N2 infection group and 448 mRNAs, 12 miRNAs, 77 lncRNAs, and 189 circRNAs in the H5N1 infection group. Potential functions were predicted by performing Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the target genes of miRNAs, lncRNAs and circRNAs, and the ncRNA-mRNA regulatory network was constructed based on differentially expressed RNAs. A greater number of pathways regulating immune metabolism were altered in the H3N2 infection group than in the H5N1 infection group, which may be one reason why the H3N2 virus is less pathogenic than is the H5N1 virus. This study provides detailed data on the production of ncRNAs during infection of MDCK cells by the canine influenza viruses H3N2 and H5N1, analyzed differences in the total transcriptomes between H3N2- and H5N1-infected MDCK cells, and explained these differences with regard to the pathogenicity of H3N2 and H5N1 at the transcriptional level.

Keywords: canine influenza, H3N2, H5N1, deep sequencing, total transcriptome

INTRODUCTION

Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses that cause influenza in humans or animals, and the viral genome is composed of a single negative strand of segmented RNA. To date, four types of influenza viruses have been identified: influenza A virus (IAV), influenza B virus (IBV), influenza C virus (ICV), and most recently influenza D virus (IDV) (Hause et al., 2013, 2014).

IAV, which causes acute respiratory diseases in many hosts such as birds, humans and pigs, is an important pathogenic microorganism worldwide. Moreover, IAV has a wide host specificity and infects a variety of hosts. Influenza has been divided into avian influenza, swine influenza, human influenza and canine influenza. In the early days, IAVs were believed to be unable to infect dogs under natural conditions, but several subtypes were subsequently isolated from dogs with respiratory symptoms. Currently, two main subtypes of canine influenza virus (CIV) have been identified: equine-origin H3N8 (Crawford et al., 2005; Daly et al., 2008; Kruth et al., 2008; Kirkland et al., 2010) and avian-origin H3N2 (Song et al., 2008; Lee et al., 2009; Li et al., 2010). In addition, various subtypes of IAVs are reported to be able to infect dogs, including influenza A H1N1 pdm09 viruses, H5N1 avian influenza viruses (AIVs), H5N2 subtypes of AIVs, reassortants of wild-type H3N1 IAVs and H9N2 subtypes of AIVs (Songserm et al., 2006; Dundon et al., 2010; Lin et al., 2011; Guang-jian et al., 2012; Song et al., 2012; Sun et al., 2013).

Avian-origin H3N2 CIV, which was initially circulating only in Asian countries (Song et al., 2009; Li et al., 2010; Li G. et al., 2018), has now spread to the United States and the rest of the world (Pulit-Penaloza et al., 2017; Voorhees et al., 2017). H3N2 CIV causes sneezing and clinical symptoms of typical respiratory diseases, such as runny nose, cough and fever, as well as damage to many other organs outside the respiratory tract, in dogs (Luo et al., 2018; Zheng et al., 2018).

The highly pathogenic avian influenza (HPAI) H5N1 virus was first reported in Thailand in October 2004 in dogs with severe pulmonary congestion and edema and a bloody nose (Songserm et al., 2006). An epidemiological survey of 629 village dogs in Thailand found that approximately one-quarter had antibodies against H5N1, indicating that they were infected with the virus or had been infected in the past (Butler, 2006). Our laboratory also isolated an avian influenza H5N1 virus from a dog in 2013. The dogs that were infected with the highly pathogenic AIV H5N1 subtype showed anorexia, dyspnea, cough, conjunctivitis and a brief increase in body temperature within 2 days, but the virus did not spread between dogs (Maas et al., 2007; Giese et al., 2008). The highest viral replication titer of the nose swab was 6.3 $\log_{10}$TCID$_{50}$/mL on average, and lung lesions in the H5N1 infection group were more severe than those in the H3N2 infection group (Zheng et al., 2018).

Why is the H5N1 influenza virus more pathogenic than H3N2, and why does it lead to a more severe inflammatory response? Current studies on H3N2 and H5N1 avian-origin CIV transcriptomes are limited to miRNAs and mRNAs (Fu et al., 2018; Zheng et al., 2018), whereas no studies have examined changes in mRNAs, miRNAs, lncRNAs, and circRNAs or performed a detailed analysis of the correlations between ncRNA and mRNA levels. In the present study, we analyzed the entire transcriptome to obtain an understanding of the mechanism of pathogenicity and differences in inflammation between the viruses and to provide guidance for future treatment.

Dogs are one of the most numerous domesticated animals and often serve as companion animals to humans. Because dogs can be infected with both avian and human influenza viruses, they are likely to be a "mixing vessel" for genetic rearrangement of influenza viruses, making the study of canine influenza important for public and human health (Gonzalez et al., 2014; Zhu et al., 2015).

MATERIALS AND METHODS

Viruses and Cells

The H3N2 (A/canine/Guangdong/B/2013) and H5N1 (A/canine/Guangdong/01/2013) CIVs were isolated in 2013 from dogs with severe respiratory symptoms in Guangdong, China, and preserved in our laboratory. Madin-Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (ATCC) and propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Viruses were propagated in the MDCK cells at 37°C and 5% CO_2 for 48 h. All experiments with live viruses were performed in an enhanced animal biosafety level 3 facility at the South China Agricultural University. The protocol number was SYXK (YUE) 2016-0136.

Sample Collection and RNA Isolation

MDCK cells were cultured with DMEM containing 10% FBS in a 37°C incubator with a 5% CO_2 atmosphere. Upon reaching 90% confluence, MDCK cells were infected with the H3N2 (MOI = 0.1) and H5N1 (MOI = 0.1) influenza viruses, and the viruses used in these infections were purified using a sucrose gradient. Then, the cells were cultured with DMEM containing 2% FBS

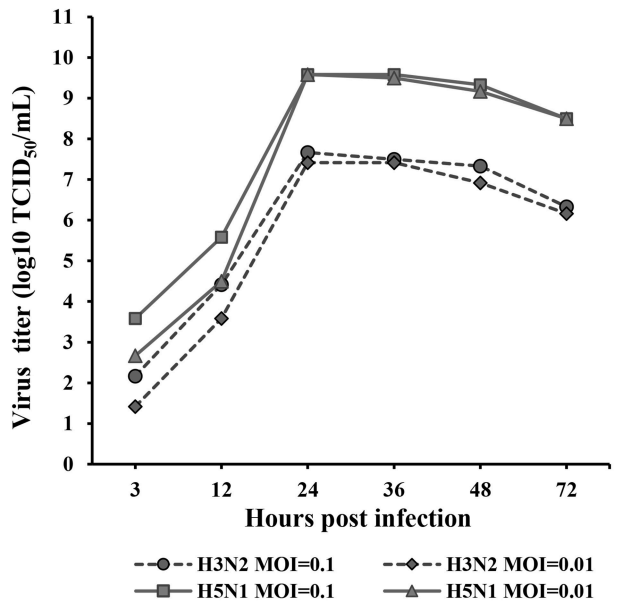

FIGURE 1 | Replication kinetics of H3N2 and H5N1 in MDCK cells. Monolayers of MDCK cells were infected with each virus at MOIs of 0.1 and 0.01. Virus titers were determined using the TCID$_{50}$ assay at 3, 12, 24, 36, 48, and 72 hpi. TPCK-trypsin (0.25 mg/mL) was added to the medium to promote the propagation of the H3N2 influenza A virus. The data were analyzed using one-way ANOVA ($p < 0.05$). Data are presented as the means ± SD of independent triplicate experiments.

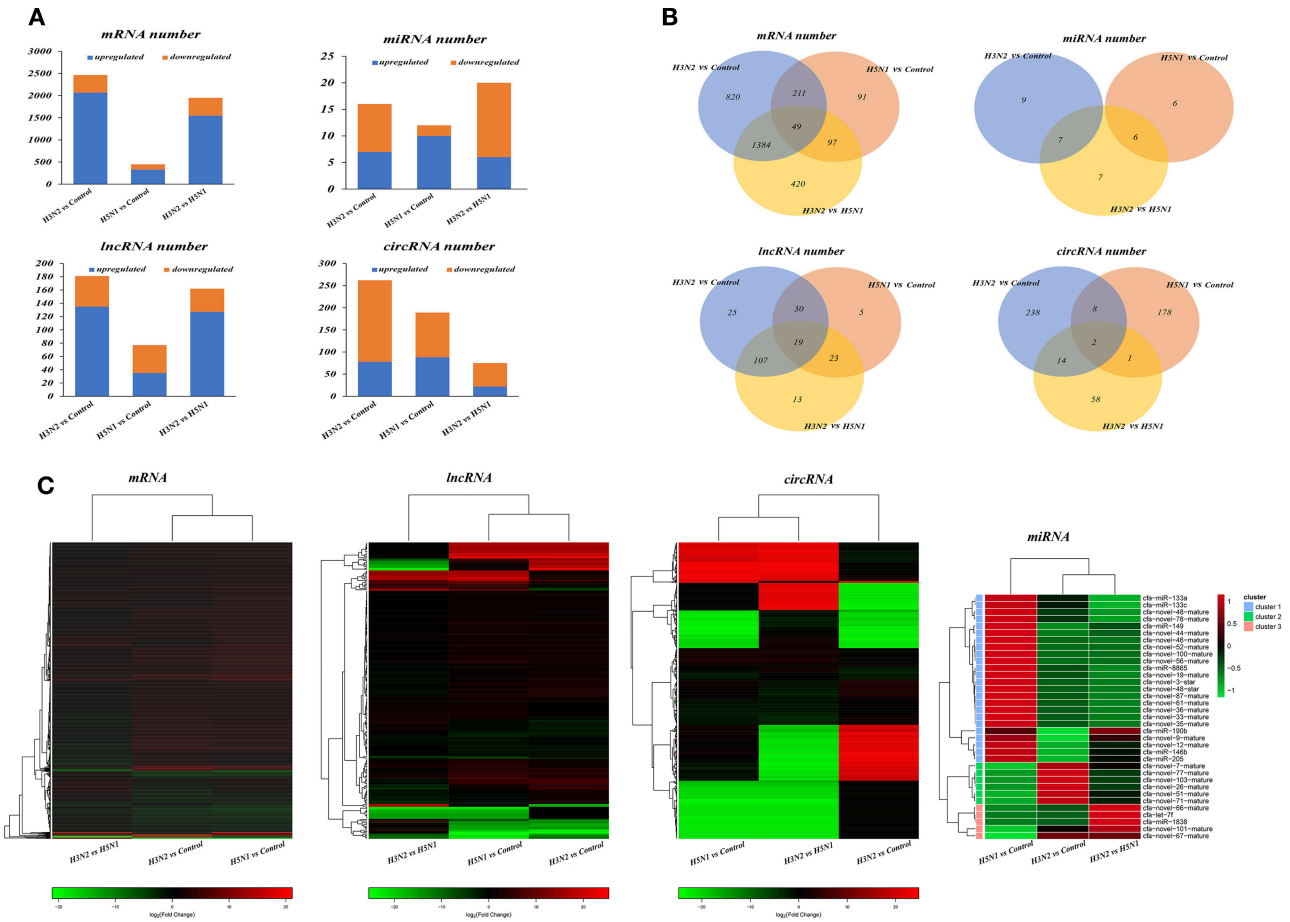

FIGURE 2 | (A) Statistical histogram of the differential expression analysis. The horizontal axis represents the comparison and the vertical axis shows the number of differentially expressed transcripts. Orange represents downregulated transcripts, and blue represents upregulated transcripts. **(B)** Differentially expressed transcripts. In the Venn diagram, different comparison groups are represented by different colors. The numbers in the figure represent specific or common differences in the number of transcripts. The overlapping areas represent the number of differentially expressed transcripts shared by different comparison groups; the non-overlapping areas represent the number of differentially expressed transcripts that were unique to the different comparison groups. **(C)** Heat map of differentially expressed transcripts based on fold changes. Each row represents a transcript and each column represents a comparison group. Red indicates upregulated expression, and green indicates downregulated expression.

in a 37°C incubator with a 5% CO_2 atmosphere for 24 h before being harvested. The control group was cultured without virus under the same conditions (independent triplicate experiments). Total RNA was isolated from MDCK cells using TRIzol (Takara) according to the manufacturer's protocol. RNA concentrations were detected using a Qubit2.0 fluorometer (Invitrogen), and the RNA integrity and genomic DNA contamination were detected by separating the samples on an agarose gel. RNA concentrations and purity were determined by measuring the OD, A260/A280 (>1.8) and A260/A230 (>1.6). RNA samples were stored at −80°C until further use.

RNA Sequencing and Data Analysis

Small RNA library construction: T4 RNA ligase 2 (New England Biolabs) was used to connect the 3′-end connector to the RNA. Reverse transcription primer hybridization: T4 RNA ligase 1 (New England Biolabs) was used to connect the 5'-end connector to the RNA. Reverse transcription reaction: The final library product was obtained by PCR amplification of the reverse transcription product. Construction of a chain-specific library for ribosome removal: Sequencing libraries were generated using RNase R digestion and rRNA-depleted RNAs. The library preparations were sequenced on an Illumina Hiseq™ platform (repeated 3 times). The raw sequencing data were analyzed with FastQC using cutadapt to remove joints and trimmomatic to remove low-quality bases and reads at both ends.

Screen of Differentially Expressed mRNAs, miRNAs, lncRNAs, and circRNAs and Clustering Analysis

The differences in expression among mRNAs, lncRNAs and circRNAs were analyzed using DESeq2, and the differential expression of miRNA was analyzed using edgeR (Anders and

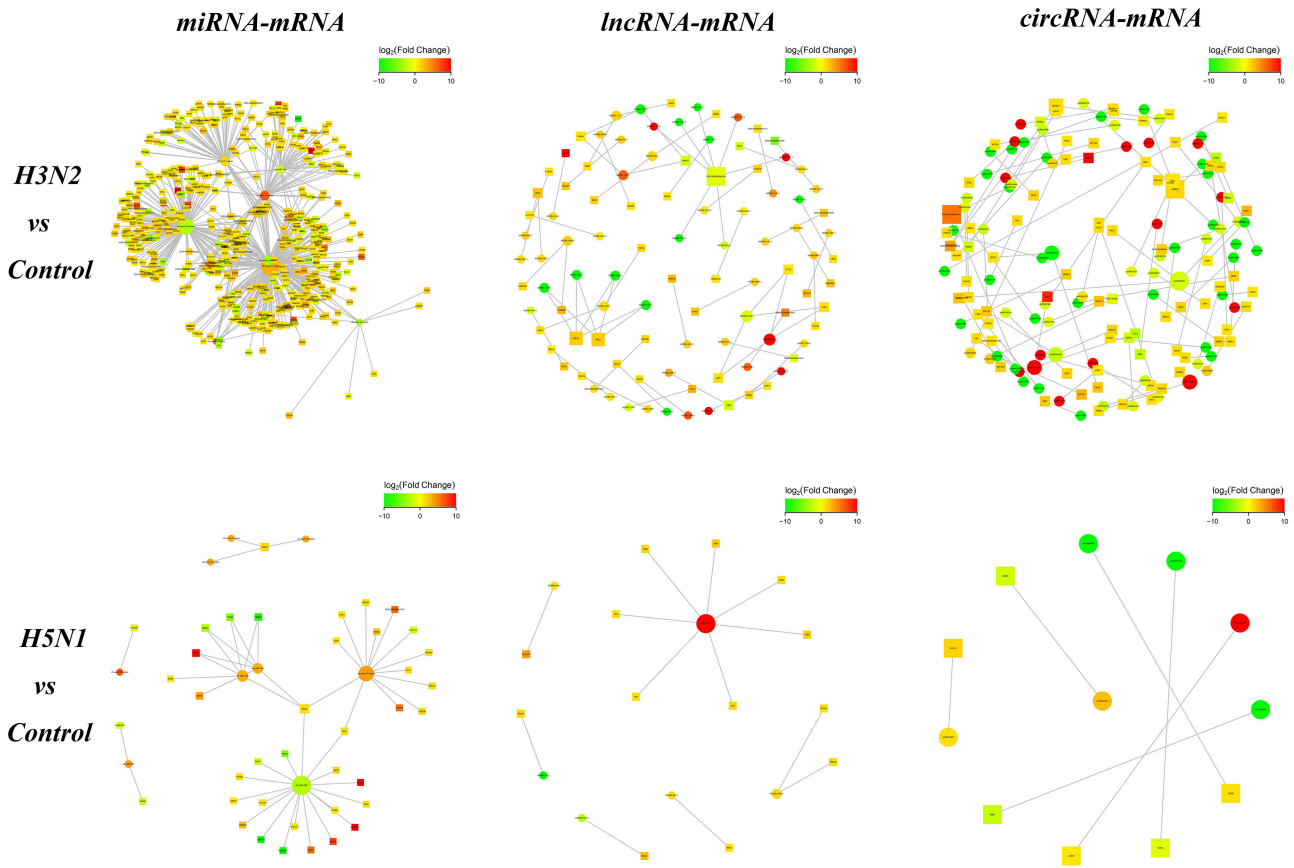

FIGURE 3 | The network diagram of ncRNA and mRNA interactions. Square nodes indicate ncRNAs, round nodes indicate mRNAs, and the edges indicate the interactions between the two genes. The size of the node is proportional to the connectivity (degree) of the node; namely, the more edges are connected to the node, the larger the degree of the node, indicating that the gene is more important in the network. The color of the node represents the difference in gene expression in this group of samples, namely, the logFC value; green represents downregulation, red represents upregulation, and the color depth represents differences in the altered expression.

Huber, 2010). The results of the differential analyses were visualized, with a $p < 0.05$ and multiple differences >2 as screening conditions. A Venn diagram and a heat map were constructed, and a clustering analysis was performed based on the results of the differential expression analysis. A network diagram was drawn based on the correlations between miRNA and mRNA, lncRNA and mRNA, and circRNA and mRNA expression.

Annotation of GO Terms and Analysis of KEGG Signaling Pathways

The differentially expressed miRNA, lncRNA, and circRNA target genes and mRNAs were annotated using GO and analyzed for enriched KEGG signaling pathways.

Analysis of the ceRNA Network (ceRNET)

The circRNA-miRNA-mRNA ceRNET and the lncRNA-miRNA-mRNA ceRNET were constructed after determining the negative regulatory relationship between the differentially expressed miRNAs and their differentially expressed target genes (mRNAs/lncRNAs and mRNAs/circRNAs).

Real-Time qPCR

Significant regulatory pathways were selected from the ceRNET for RT-qPCR verification of differential expression. The cDNA templates were synthesized using PrimeScript^{TM} RT Master Mix (Perfect Real Time) (Takara, Otsu, Japan, Product no: RR036A). RT-qPCR was performed using the SYBR Premix Ex Taq^{TM} (Tli RNaseH Plus) (Takara, Otsu, Japan, Product no: TRR820A) and an LC480 Real-Time PCR System (Roche, Basel, Switzerland) in accordance with the manufacturer's specifications. Small RNA samples were isolated using the E.Z.N.A.^{TM} miRNA Kit (OMEGA BIO-TEK. Product no: R6842-01). The miRcute Plus miRNA First Chain cDNA Synthesis Kit (Tiangen, Beijing, China. Product no: KP211) was used for cDNA synthesis. RT-qPCR was performed using the miRcute Plus miRNA qPCR Detection Kit (Tiangen, Beijing, China, Product no: FP411) and an LC480 instrument (Roche, Basel, Switzerland). GAPDH was used as the endogenous control for mRNAs and lncRNAs, and U6 was used as the endogenous control for miRNAs. We used the $2^{-\Delta\Delta Ct}$ method to analyze the data. All samples were analyzed in triplicate, and the data are presented as means ± standard deviations ($n = 3$).

RESULTS

Replication Kinetics of H3N2 and H5N1 Strains in MDCK Cells

The kinetics of H3N2 and H5N1 CIV replication were measured and analyzed based on the $TCID_{50}$ at 3, 12, 24, 36, 48, and 72 hpi (**Figure 1**) in Reed-Muench. H3N2 and H5N1 CIVs replicated well in MDCK cells. H5N1 replicated significantly more vigorously than H3N2 ($p < 0.01$) at every assessed time point throughout the replication kinetics experiment. Viral replication was distinctly dose dependent for both H3N2 and H5N1 CIVs during the first 24 hpi, whereas the dose dependency was negligible from 24 to 72 hpi. The peak titers of the H5N1 CIV reached up to $10^{9.6}$ of the $TCID_{50}$/mL at 24 hpi, whereas H3N2 CIV replicated significantly less rapidly, with peak virus titers reaching only $10^{7.7}$ of the $TCID_{50}$/mL.

Analysis of Differentially Expressed mRNAs, miRNA, lncRNAs, and circRNAs

Through comparative analysis, we found that 2,464 mRNAs, 16 miRNAs, 181 lncRNAs, and 262 circRNAs were significantly differentially expressed in the H3N2 group compared with the control group and that 448 mRNAs, 12 miRNAs, 77 lncRNAs and 189 circRNAs were differentially expressed in the H5N1 group compared with the control group. Moreover, 1,950 mRNAs, 20 miRNAs, 162 lncRNAs, and 75 circRNAs were differentially expressed in the H3N2 group compared with the H5N1 group (**Table 1**, **Figure 2** and **Supplementary Material**).

Correlation Analysis of mRNAs and ncRNAs

We analyzed the targeting relationship between differentially expressed ncRNAs and mRNAs to further study the correlation between the ncRNA-mRNA regulatory network in MDCK cells infected with CIVs. The analysis identified 706 differentially expressed miRNA-mRNA, 66 lncRNA-mRNA and 86 circRNA-mRNA pairs in the H3N2 group compared with the control group and 50 differentially expressed miRNA-mRNA, 13 lncRNA-mRNA, and 6 circRNA-mRNA pairs in the H5N1 group compared with the control group (**Figure 3**).

TABLE 1 | Differential expression profiles of mRNAs, miRNAs, lncRNAs, and circRNAs.

Group	Type of RNA	Up	Down	Total
H3N2 vs. Control	mRNA	2,068	396	2,464
	miRNA	7	9	16
	lncRNA	135	46	181
	circRNA	78	184	262
H5N1 vs. Control	mRNA	328	120	448
	miRNA	10	2	12
	lncRNA	35	42	77
	circRNA	88	101	189
H3N2 vs. H5N1	mRNA	1,546	404	1,950
	miRNA	6	14	20
	lncRNA	127	35	162
	circRNA	22	53	75

Gene Ontology (GO) Functional Enrichment Analysis of Differentially Expressed Genes

We conducted a GO enrichment analysis and statistical analysis of biological processes to determine the functional classifications of these differentially expressed mRNAs and target genes of ncRNAs. A large number of genes in the H3N2 and H5N1 infection groups are involved in cellular process, single-organism process and metabolic process. However, many genes in biological processes were enriched in the H3N2 infection group, and each enriched functional classification also contained a large number genes. Based on the results of the GO enrichment analysis, miRNAs were more abundant than lncRNAs and circRNAs in terms of the number of target genes and functional classifications (**Figure 4**).

Analysis of the Enrichment of Differentially Expressed Genes in Various Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways

KEGG analysis was also performed for mRNAs and target genes of miRNAs, lncRNAs and circRNAs that were differentially expressed in cells infected with H3N2 and H5N1 to explore canine host resistance mechanisms to H3N2 and H5N1 infection and their differences. Then, we conducted a statistical analysis

TABLE 2 | Primer sequences used for real-time PCR.

Gene name	Primer sequences (5′-3′)
MSTRG.300.2	F: GATCCCGTGGGCGTTTACCCG
	R: GCAAGACACCCAACAGCGGCG
MSTRG.17313.7	F: GGAGTGCTGAGAAGACGGTCGAAC
	R: GCCGCGTTCTCCGTTAATGATCC
MSTRG.7743.1	F: AGGAGCATCTCGGGCTTTTCA
	R: CTTTACCGCTCCATCAACGCA
cfa-novel-52-mature	F: GCCCCCCGGGGGGGCGG
cfa-novel-46-mature	F: GCGGCGGCGGGGAGGGT
cfa-miR-149	F: AGACCGAGGCACAGAAGTGAGGG
U6	F: ACTAAAATTGGAACGATACAGAGA
CISH	F: TTCTTTGCTGGCTGTGGAGCG
	R: GCCTCACTGGCGGTAATGGAA
CREBBP	F: CTTTAAGCCAGAGGAGTTACGC
	R: GGATGTCTTGCGGTTATAGAGC
MCL1	F: ACTGGGGCAGGATTGTGACTCT
	R: GCCAGTCTCGTTTCGTCCTTAC
PERP	F: CCCGAGAGTTCCTTAGCACA
	R: ATGATGTCGAAGGCGATGGC
SND1	F: TAGAGGTGGAGGTAGAGAGCAT
	R: GACAGCAGGGATTTGTAGTAGG
GAPDH	F: AAATGGGGTGATGCTGGTGCT
	R: CATCAGCAGAAGGAGCAGAGA

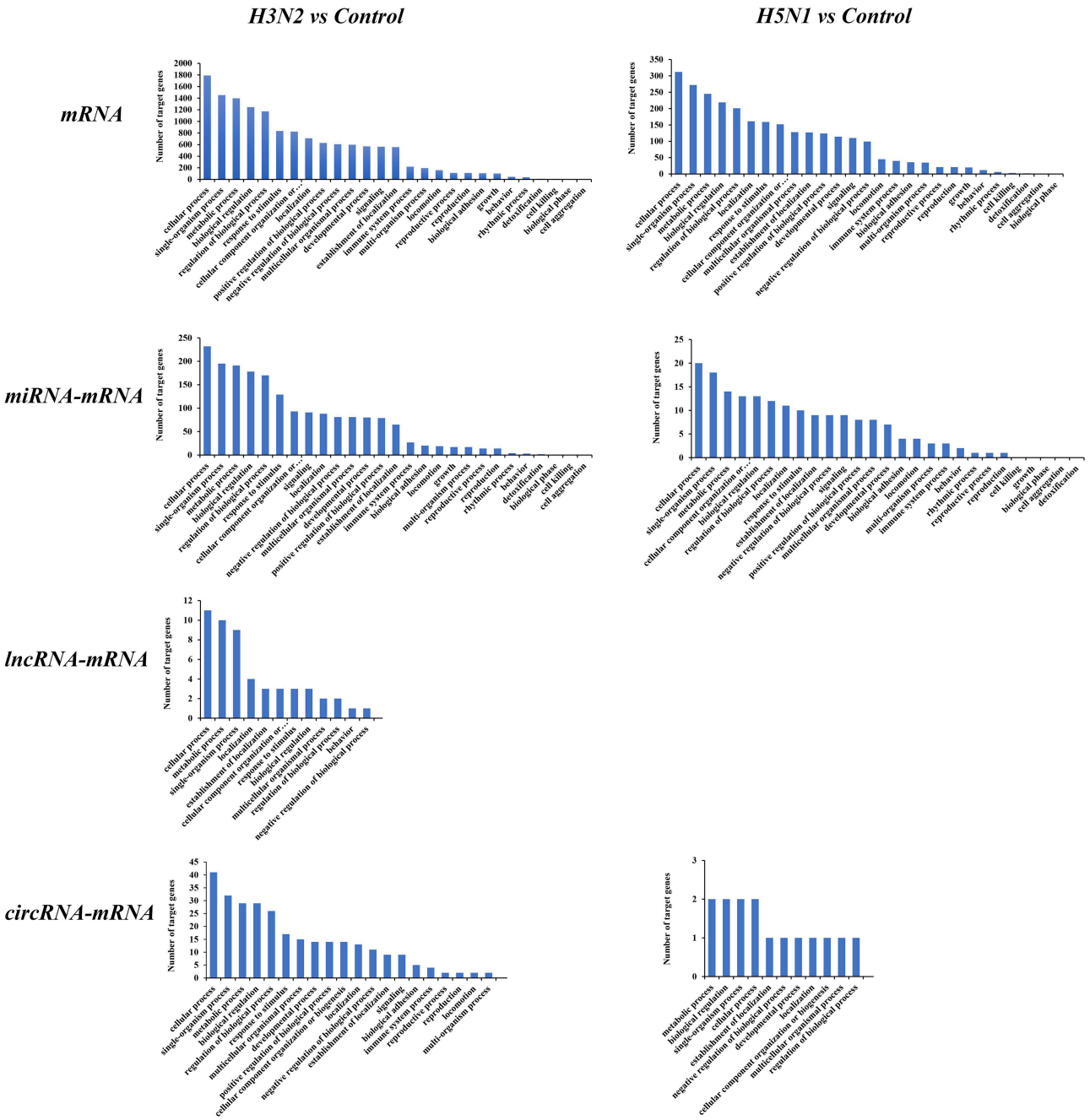

FIGURE 4 | GO pathways of differentially expressed mRNAs and target genes of differentially expressed miRNAs, lncRNAs, and circRNAs. The horizontal axis shows the functional classification, and the vertical axis shows the number of genes in the classification.

of cytokine and pathogen-host interaction pathways and found that a large number regulatory pathways and genes were enriched in the H3N2 infection group, particularly the MAPK signaling pathway, the endocytosis pathway, the p53 signaling pathway and other pathways with more obvious advantages. Notably, miRNAs can target more than one mRNA to regulate gene expression, indicating that miRNA-mediated targeted regulation of mRNA expression is more important than are the regulatory pathways involving lncRNAs and circRNAs (**Figure 5**).

The Competing Endogenous RNA (ceRNA) Regulatory Network

Notably, lncRNAs and circRNAs limit miRNA-mediated regulation of target gene expression and act as miRNA sponges to indirectly regulate gene expression. According to the theory of ceRNAs, we constructed lncRNA-miRNA-mRNA and circRNA-miRNA-mRNA regulatory networks. In the comparison of the H3N2 and control groups, the lncRNA-miRNA-mRNA network contained 15 lncRNAs, 6 miRNAs and 237 mRNAs, and the

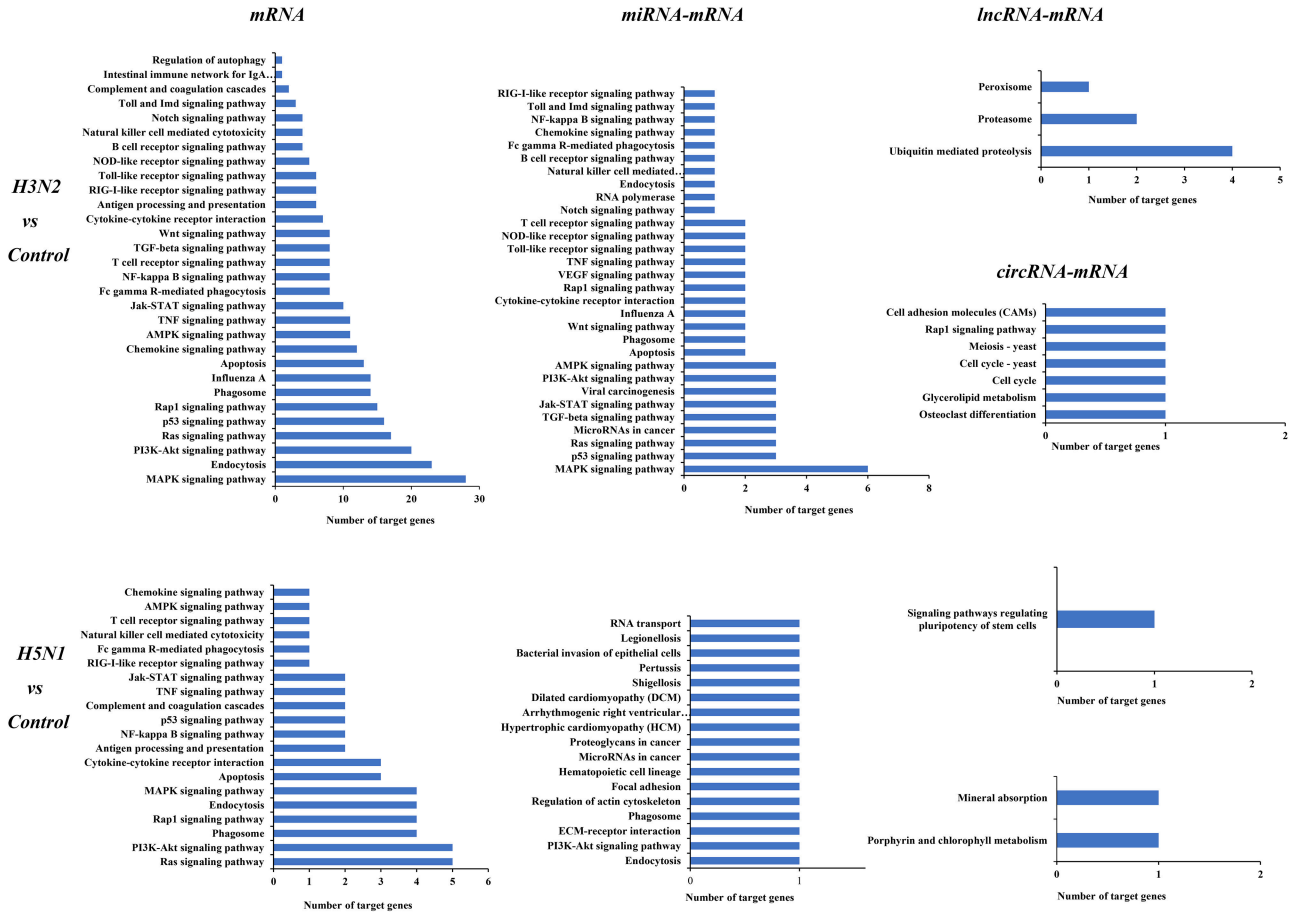

FIGURE 5 | KEGG pathways of differentially expressed mRNAs and target genes of differentially expressed miRNAs, lncRNAs, and circRNAs. The horizontal axis shows the number of genes, and the vertical axis shows each pathway.

circRNA-miRNA-mRNA network contained 3 circRNAs, 1 miRNA and 9 mRNAs. In the comparison of the H5N1 and control groups, the lncRNA-miRNA-mRNA network comprised 6 lncRNAs, 4 miRNAs and 18 mRNAs, and a circRNA-miRNA-mRNA regulatory network was not identified. Enrichment of mRNAs in the regulatory network was assessed via analysis of KEGG pathways (**Figure 6**).

Differential Expression of ncRNAs and mRNAs Confirmed by RT-qPCR

In the ceRNA network, mRNAs in the Jak-STAT signaling pathway, p53 signaling pathway and viral carcinogenesis regulatory network were enriched and verified by RT-qPCR (primer sequences are shown in **Table 2**). The sequencing results were consistent with the trends in the qPCR verification results (**Table 3** and **Figure 7**).

DISCUSSION

In this study, a high-throughput sequencing technique was used to compare the entire transcriptomes of MDCK cells infected with the H5N1 and H3N2 viruses. Although fewer differentially expressed mRNAs were identified in the H5N1 infection group than in the H3N2 infection group, a higher proportion of mRNAs exhibited a more significant differential expression pattern in the H5N1 infection group. In the H5N1 group, 24 (log_2-fold change $>$ 10) of 448 mRNAs were differentially expressed mRNAs, while only 27 (log_2-fold change $>$ 10) of 2,464 mRNAs were differentially expressed in the H3N2 group, 10 of which were shared by the two groups. In the ncRNA-mRNA regulatory network, the H3N2 group had more regulatory pairs than did the H5N1 group. Thus, H3N2-infected MDCK cells may be able to resist the viral infection through more pathways to reach a balanced state. This finding also illustrates why H5N1 is more virulent than H3N2 at the RNA level.

In the circRNA-miRNA-mRNA regulatory network, we identified 3 circRNAs in the H3N2 infection group. One miRNA and 9 mRNAs had a targeted relationship, while the H5N1 infection group did not possess this regulatory network. In the lncRNA-miRNA-mRNA regulatory network, 15 lncRNAs, 6 miRNAs, and 237 mRNAs were identified in the H3N2 infection group. Six lncRNAs, 4 miRNAs, and 18 mRNAs were identified in the H5N1 infection group. Based on this result, H3N2 infection

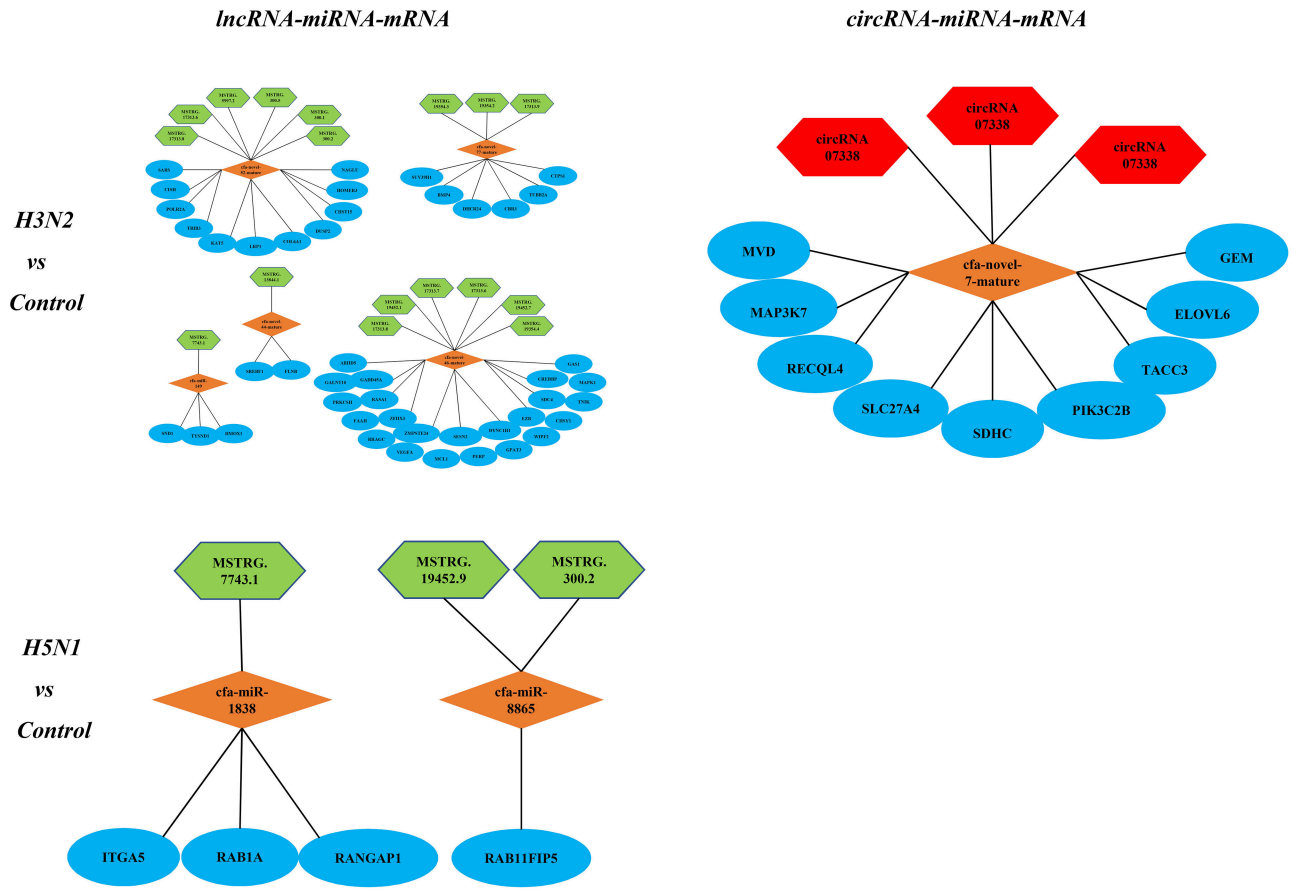

FIGURE 6 | KEGG pathways associated with the lncRNA-miRNA-mRNA and circRNA-miRNA-mRNA regulatory networks; green represents lncRNAs, red represents circRNAs, orange represents miRNAs, and blue represents mRNAs.

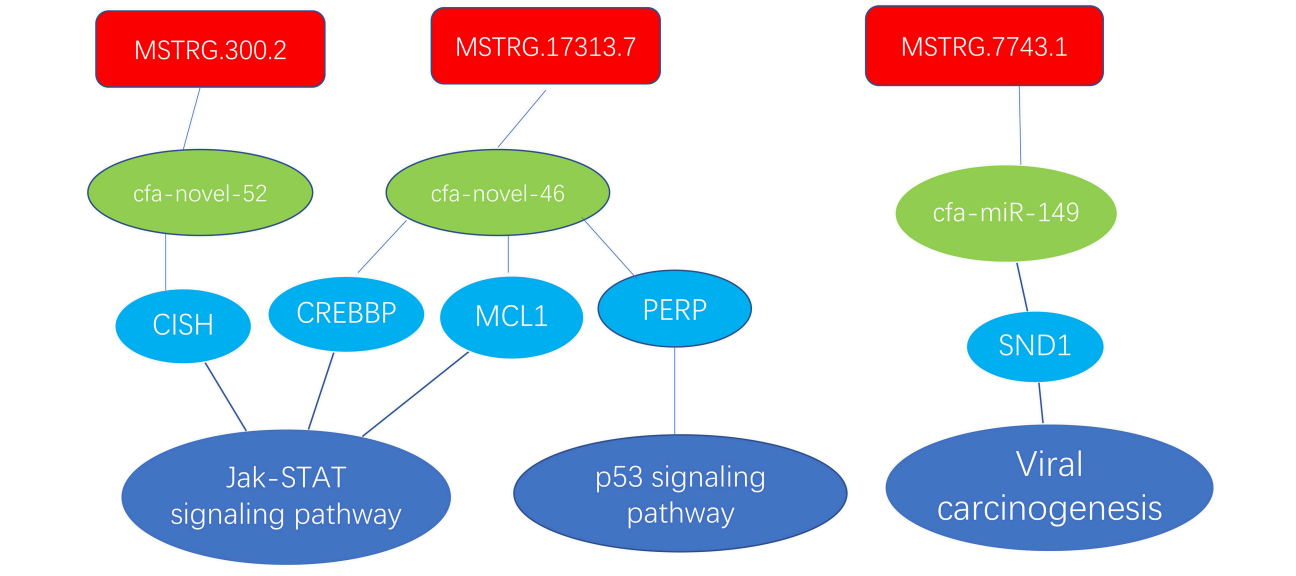

FIGURE 7 | Enrichment of mRNAs in the Jak-STAT signaling pathway, p53 signaling pathway and viral carcinogenesis regulation network; lncRNAs are depicted in red, green represents miRNAs, and blue represents mRNAs.

TABLE 3 | Relative RNA expression of selected differentially expressed genes (DEGs) determined using RNA-seq and quantitative real-time PCR analyses.

lncRNA_name/miR_name/mRNA_name	Accession number	Illumina miRNA-seq (log_2-fold change)	Regulation	Real-time PCR (log_2-fold change)
MSTRG.300.2		5.574059	Up	4.06*
MSTRG.17313.7		24.63398	Up	20.25*
MSTRG.7743.1		1.199492	Up	1.53*
cfa-novel-52-mature		−3.62139	Down	−4.52*
cfa-novel-46-mature		−2.7541	Down	−3.02*
cfa-miR-149		−1.71902	Down	−2.08*
Cytokine inducible SH2 containing protein (CISH)	ENSCAFT00000045397	1.291812	Up	1.06*
CREB binding protein (CREBBP)	ENSCAFT00000044601	1.619728	Up	2.05*
MCL1, BCL2 family apoptosis regulator (MCL1)	ENSCAFT00000019132	2.098362	Up	2.16*
PERP, TP53 apoptosis effector (PERP)	ENSCAFT00000043071	1.268355	Up	1.89*
Staphylococcal nuclease and tudor domain containing 1 (SND1)	ENSCAFT00000002687	1.176868	Up	1.39*

**The statistical significance of differential gene expression with $p < 0.05$ (t-test).*

may induce broad regulation in MDCK cells, and the circRNA-miRNA-mRNA regulatory network is not common in influenza infection. Regarding GO and KEGG enrichment analyses, we also intuitively observed a greater number of GO annotations and enriched KEGG pathways in the H3N2 infection group than in the H5N1 infection group. In conclusion, we postulate that H5N1 is more virulent than H3N2 for several reasons. (1). H5N1 infects cells and causes intracellular cytokine storms, resulting in rapid cell death (Li X. et al., 2018). (2). Cells infected with H3N2 utilize more resistance pathways to eliminate the virus, while H5N1 infection activates relatively few pathways in cells, which showed the some differences in MDCK in antiviral responses between H3N2 and H5N1 at the RNA level of the transcriptome.

Notably, lncRNAs play important roles in many cellular activities, such as the regulation of epigenetics, the cell cycle and cell differentiation, and has become a hot topic in genetic research (Wapinski and Chang, 2011; Kwok and Tay, 2017). MiRNAs and their target genes have a variety of relationships (Perez et al., 2009; Fan and Wang, 2016; Nakamura et al., 2016), and misaligned miRNAs can be used as diagnostic and prognostic biomarkers (Okkenhaug and Vanhaesebroeck, 2003; Hale et al., 2010; Haneklaus et al., 2013). In the present study, we examined the lncRNA-miRNA-mRNA regulatory networks of the Jak-STAT signaling pathway, the p53 signaling pathway and the viral carcinogenesis pathway for RT-qPCR verification. The Jak-STAT signaling pathway, which is closely related to the type I interferon (IFN)-mediated innate immune response, is an important regulator of cell proliferation, differentiation, survival, motility, apoptosis, development and the immune response (Bartunek et al., 1999; Liu et al., 2010). Currently, p53 is the most widely analyzed functional transcription factor. The aggregation and induced activation of p53 are the core cell signaling events in a variety of stress-induced injury responses and have important regulatory roles in inhibiting cell cycle progression and inducing DNA damage repair, cell autophagy and apoptosis (Crighton et al., 2006; Duan et al., 2015; Sui et al., 2015). In the next step, we further explored the mechanisms of these pathways.

In this study, the total transcriptome of the MDCK cell model infected with CIVs was analyzed for the first time. The results further revealed the differences in pathogenicity between H3N2 and H5N1 at the RNA level of the transcriptome. However, this study has only analyzed the differences in the RNA levels at 24 hpi in canine cells and further studies are needed to analyze both earlier time points as well as the differences in the RNA transcriptome in dogs infected with the two types of influenza viruses.

AUTHOR CONTRIBUTIONS

PT, XH, XL, and QZ conducted the experiments. PT and ZN analyzed the data and wrote the paper. SL designed the experiments and revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00076/full#supplementary-material

Supplementary Data Sheet 1 | The original data.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Contribution of Fcγ Receptor-Mediated Immunity to the Pathogenesis Caused by the Human Respiratory Syncytial Virus

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The human Respiratory Syncytial Virus (hRSV) is the leading cause of severe acute lower respiratory tract infections (ALRTIs) in humans at all ages and is the main cause of hospitalization due to pneumonia, asthma, and bronchiolitis in infants. hRSV symptoms mainly develop due to an excessive host immune and inflammatory response in the respiratory tissue. hRSV infection during life is frequent and likely because of non-optimal immunological memory is developed against this virus. Vaccine development against this pathogen has been delayed after the detrimental effects produced in children by vaccination with a formalin-inactivated hRSV preparation (FI-hRSV), which caused enhanced disease upon natural viral infection. Since then, several studies have focused on understanding the mechanisms underlying such disease exacerbation. Along these lines, several studies have suggested that antibodies elicited by immunization with FI-hRSV show low neutralizing capacity and promote the formation of immune complexes containing hRSV (hRSV-ICs), which contribute to hRSV pathogenesis through the engagement of Fc gamma receptors (FcγRs) expressed on the surface of immune cells. Furthermore, a role for FcγRs is supported by studies evaluating the contribution of these molecules to hRSV-induced disease. These studies have shown that FcγRs can modulate viral clearance by the host and the inflammatory response triggered by hRSV infection. In addition, ICs can facilitate viral entry into host cells expressing FcγRs, thus extending hRSV infectivity. In this article, we discuss current knowledge relative to the contribution of hRSV-ICs and FcγRs to the pathogenesis caused by hRSV and their putative role in the exacerbation of the disease caused by this virus after FI-hRSV vaccination. A better understanding FcγRs involvement in the immune response against hRSV will contribute to the development of new prophylactic or therapeutic tools to promote virus clearance with limited inflammatory damage to the airways.

Keywords: hRSV, Fc gamma receptors, re-infection, inflammatory response, lung disease, immune complexes, opsonized virus

INTRODUCTION

The Human Respiratory Syncytial Virus (hRSV) is a single-stranded RNA enveloped virus belonging to the *Pneumoviridae* family (Amarasinghe et al., 2018). The viral particle has a filamentous structure, which consists in a nucleocapsid surrounded by a lipid bilayer envelope obtained from the plasma membrane of the host cell (El Omari et al., 2011). Importantly, infection by hRSV is the most frequent cause of severe acute lower respiratory tract infections (ALRTIs) in children younger than 5 years old (Scheltema et al., 2017) and infection during the first year of life is the main cause of hospitalization in infants (Song et al., 2016). According to epidemiological studies, during the past decade, nearly 33 million cases of new ALRTIs episodes affect children during the first months of life are due to hRSV infection each year (Shi et al., 2017). Therefore, infection by this virus represents a major health and socio-economic burden worldwide (Diez-Domingo et al., 2014; Amand et al., 2018).

Clinical manifestations caused by hRSV infection range from mild symptoms, such as rhinitis, to more severe consequences, which include bronchiolitis, and pneumonia (Pickles and DeVincenzo, 2015). Besides, extra-pulmonary manifestations of hRSV infection have also been reported to occur, such as acute neurological symptoms with seizures and ataxia observed in hRSV-infected children (Eisenhut, 2006; Bohmwald et al., 2015) and long-term behavioral and cognitive impairments in animal models (Espinoza et al., 2013).

Remarkably, it is known that most children become infected with hRSV during the first 2 years of life (Domachowske and Rosenberg, 1999), likely because hRSV can efficiently pass on from one individual to another, but also because of the capacity of this virus to negatively modulate both, T cell and B cell responses upon infection allowing frequent re-infections (PrabhuDas et al., 2011; Cespedes et al., 2014; Zhivaki et al., 2017). These features are thought to be mediated by host and viral factors. For instance, it is known that infants show reduced capacity to produce neutralizing antibodies against hRSV, as compared to adults making the former more susceptible to recurrent infections (Siegrist and Aspinall, 2009). Although maternally-delivered antibodies (matAbs) are reported to delay the onset of primary hRSV infection, their presence in the blood of infants is not associated with the development of less severe disease symptoms (Jans et al., 2017). These observations suggest that antibody-mediated neutralization of hRSV may not be sufficient by itself to limit hRSV infection and disease severity. Furthermore, hRSV encodes several proteins that have the ability to negatively modulate or impair the host antiviral immune response, therefore contributing to re-infections (Mason et al., 2003; Cespedes et al., 2014; Saint et al., 2015; Bohmwald et al., 2016; Gomez et al., 2016; Canedo-Marroquin et al., 2017; Ward et al., 2017). Such knowledge is relevant for designing novel vaccines and therapeutic approaches that can prevent the pathology caused by hRSV. As a result, several clinical trials are currently in progress to assess the safety and effectiveness of different hRSV vaccine candidates (Cautivo et al., 2010; Rey-Jurado and Kalergis, 2017; Rezaee et al., 2017). Among them, we have developed a unique approach to be administered to newborns and young infants. Immunization in the mouse model with a recombinant bacillus of Calmette-Guérin (BCG) that expresses the nucleoprotein (N) of hRSV (rBCG-N-hRSV) induce the production of neutralizing antibodies against hRSV and a T helper 1 (Th1) cellular immunity that protects from hRSV associated-lung pathology by decreasing the infiltration of inflammatory immune cells into the lungs and reduce viral loads in the airways of hRSV-infected mice (Bueno et al., 2008; Cautivo et al., 2010; Leyrat et al., 2014) Furthermore, a single low dose of this vaccine produced using current good manufacturing practices (cGMP), conferred protection against hRSV infection in the mouse model (Cespedes et al., 2017). Given these results, this recombinant-based vaccine arises as a promising candidate to prevent lung damage caused by this virus (Cespedes et al., 2017). In this context, it is possible that a mechanism that contributes to the prevention of hRSV pathology following rBCG-N-hRSV vaccination is the induction of antibodies that recognize the hRSV N protein, which is necessary for viral replication and the inhibition of the immunological synapse (IS) between DCs and T cells that promote T- cell activation (Cespedes et al., 2014). Therefore, if the hRSV N protein becomes neutralized by antibodies during infection it cannot contribute to viral replication, but also will fail in its ability to impair the formation of the IS between DCs and T cells, thus hampering a proper immune response against hRSV. Furthermore, a recent publication from our group shows that immunization with rBCG-N-hRSV can induce the production of antibodies against other hRSV proteins, such as F and G which can serve to neutralize infection, therefore reducing hRSV associated pathology (Soto et al., 2018).

In contrast, vaccine candidates from other groups use the F protein as a target antigen to confer immunity. For example, Novavax Inc. is currently performing a clinical trial based on the use of nanoparticles linked with hRSV F protein to induce the production of neutralizing antibodies against hRSV (Mazur et al., 2018).

Similarly, Janssen is currently testing adenovirus based vector vaccines, encoding pre-fusion forms of the hRSV F protein that also induce the production of anti-hRSV neutralizing antibodies (Mazur et al., 2018).

Finally, other live attenuated vaccines as is the case of rBCG-N-hRSV are based in attenuated hRSV that lack some particular proteins such as M2-2, NS2, or both (Mazur et al., 2018).

Together, these data indicate that it is of vital importance to delineate the mechanisms contributing to hRSV induced pathology in order to prevent or treat infection.

At this latter point, recurrent hRSV re-infection episodes which are common thorough life have encouraged the generation of studies that seek to define the mechanisms responsible for what is considered an impaired or non-optimal immune response elicited against hRSV upon infection to account for re-infection episodes (Openshaw and Chiu, 2013; Cespedes et al., 2014; Shao et al., 2015). Along these lines, a role for the interaction between immune complexes consisting of IgGs and hRSV (ICs) with Fc gamma receptors (FcγRs) could be a process contributing to both, re-infection episodes, and enhancement of hRSV-disease elicited by vaccination with formalin-inactivated

hRSV (FI-hRSV) and later hRSV natural infection (Kim et al., 1969). This hypothesis is supported by the fact that high amounts of antibodies with low neutralizing activity can be induced by immunization with FI-hRSV, which correlates with enhancement of the hRSV-induced disease (Kapikian et al., 1969; Kim et al., 1969). Therefore, it is possible that these low affinity antibodies promote the infection of FcγR-bearing cells through a phenomena called antibody dependent enhancement (ADE), as previously observed for other viruses (Yip et al., 2014; Gu et al., 2015; Flipse et al., 2016). Furthermore, *in vitro* and *in vivo* studies have shown that the blockade or absence of particular FcγRs expressed on the surface of immune cells can modulate the immune response against this virus and the onset of hRSV-induced disease (Osiowy et al., 1994; Kruijsen et al., 2013; Gomez et al., 2016; van Erp et al., 2018). In this article, we review and discuss the current understanding on the contribution of FcγRs to infection and the modulation of the immune response against hRSV both, *in vitro* and *in vivo* and their impact on hRSV-induced pathology.

The Family of Fc Receptors for IgG (FcγRs)

Fc-gamma receptors (FcγRs) bind to immunoglobulin G (IgG) antibodies (Ab), by recognizing the Fc region of the IgG, which promotes receptor clustering on the cell surface and the phosphorylation of tyrosine residues present on signaling motifs within the intracellular region of these receptors. FcγRs engagement ultimately leads to signaling cascades in the cell that can result in the expression of surface molecules and secretion of soluble mediators to modulate the host immune responses (Getahun and Cambier, 2015; Renner et al., 2016); (Soto et al., 2018).

Importantly, these types of receptors are expressed on the surface of immune cells, such as neutrophils, dendritic cells (DCs) and macrophages, among others (Zhang et al., 2004). In general, classic members of this family of proteins were classified according to their immune-modulatory properties, which either promote or inhibit inflammatory responses (Nimmerjahn and Ravetch, 2008; Guilliams et al., 2014). However, FcγRs can also be classified as type-I or type-II, based on their capacity to interact with the two (open or closed) conformational states of the IgG Fc domain (Banegas Banegas et al., 1987). Type-I FcγRs include the classic FcγRs and can only be engaged by the IgG Fc domain in the open conformation state (Banegas Banegas et al., 1987). In contrast, type-II (non-canonical FcγRs), include C-type lectin receptors CD23 and Dendritic Cell-specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin (DC-SIGN), which preferentially bind IgG Fcs in a closed conformation (Banegas Banegas et al., 1987).

In humans, the so-called classic FcγRs are known as: FcγRI (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (CD32c), FcγRIIIa (CD16a), and FcγRIIIb (CD16b) (**Table 1**) (Tripp et al., 2002; Guilliams et al., 2014). Among them, a study performed during 2002 indicates that the expression of FcγRIIIa is increased in Natural Killer cells (NK cells) from patients with severe hRSV associated pathology. Thus, suggesting that this receptor and this particular cell population could be contributing to hRSV disease (**Table 2**, Tripp et al., 2002). Nevertheless, there are two more non-classic human Fc-gamma receptors: neonatal Fc-receptor (FcRn) and cytosolic tripartite motif (TRIM) 21 that bind IgG once internalized into the cells (Guilliams et al., 2014). However, there is no study about it contribution to hRSV induced pathology in hRSV positive patients (**Table 2**). Importantly, all canonical FcγRs with the exception of FcγRIIb are involved in activating functions, such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory cytokines following FcγR-crosslinking by IgG-opsonized complexes (Guilliams et al., 2014). The activation of such processes relies on the Src-family kinase-mediated phosphorylation of an Immunoreceptor Tyrosine-based Activating Motif (ITAM) that is located in the cytoplasmic portion of these activating Fc-receptors (Nimmerjahn and Ravetch, 2008). Subsequently, phosphoinositide 3-kinase (PI3K) is activated, which generates phosphatidylinositol trisphosphates (PIP3s), leading to the recruitment of Bruton's tyrosine kinase (BTK) and the activation of phospholipase Cγ (PLCγ), which promotes the release of calcium (Ca^{2+}) from the endoplasmic reticulum (ER) that in turn activates cell effector functions (Nimmerjahn and Ravetch, 2008).

In contrast, FcγRIIb which is able to terminate the activation cascades associated with the engagement of activating FcγRs (Malbec et al., 1998), is also known as the inhibitory FcγR. During this process FcγRIIb becomes engaged by ICs and it co-aggregates with activating receptors. Following that, different recruited kinases phosphorylate a conserved tyrosine within an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) located in the cytoplasmic tail of FcγRIIb (Malbec et al., 1998). This phosphorylation step leads to the recruitment of tyrosine phosphatases SHP-1 and SHP-2, as well as the inositol phosphatases SHIP-1 and SHIP-2 that suppress the activating signals derived from activating FcγRs (D'Ambrosio et al., 1995; Ono et al., 1996).

In the mouse, there are four different canonical FcγRs expressed on the cell surface: FcγRI, FcγRIIb, FcγRIII, and FcγRIV (**Table 2**) (Nimmerjahn and Ravetch, 2008). Among them, FcγRI, FcγRIII, and FcγRIV are activating, whereas FcγRIIb is the only one that is inhibitory. Of interest, a pro-inflammatory role for the FcγRIII receptor has been reported during hRSV infection in the mouse model (Gomez et al., 2016), whereas the inhibitory FcγRIIb has been shown to hamper inflammatory reactions during allergic-like rhinitis (Malbec et al., 1998), allergic asthma (D'Ambrosio et al., 1995), and hRSV infection (Gomez et al., 2016). Therefore, such receptors appear as attractive targets for novel therapeutic approaches against this kind of diseases.

Contribution of FcγRs to Neutrophil Recruitment, Viral Replication, and Lung Damage During hRSV-Induced Pathology

Based on animal studies, neutrophils have been described to promote inflammation and tissue damage during hRSV infection (Yasui et al., 2005). In addition, other studies in mice that evaluated the role of FcγRs on the lung damage produced by neutrophils in models of acute lung injury (ALI), which

TABLE 1 | Classification of currently described human Fcγ Receptors, and evidences of their role in hRSV-induced pathogenesis.

Type	Receptor	Alternative name/CD	Main function	Evidence after hRSV infection	Suggested role	References indicating a role during hRSV infection
Classical FcγRs (Recognize ICs on the cell surface)	FcγRI	CD64	Activating	—[a]	—	—
	FcγRIIa	CD32a	Activating	—	—	—
	FcγRIIb	CD32b	Inhibitory	—	—	—
	FcγRIIc	CD32c	Activating	—	—	—
	FcγRIIIa	CD16a	Activating	Increased presence of FcγRIIIA^{+} NK cells, and lung damage in patients with severe hRSV infections	The expression of FcγRIIIA on NK cells negatively influences the immune response during hRSV infection	Tripp et al., 2002
	FcγRIIIb	CD16b	Activating	—	—	—
Non-classical FcγRs (C-type lectins that recognize ICs on cell surface or non-classic FcγRs that recognize ICs inside the cell)	CD23	CD23		—	—	—
	DC-SIGN	CD209	Recognition of glycans through a carbohydrate recognition domain (CRD)	*In vitro*: mAb-blockade of DC-SIGN increases human DC maturation markers (CD80, CD86) after hRSV infection.	hRSV-DC interaction through DC-SIGN might impair DC maturation	Johnson et al., 2012
	FcRn	—	Control of endosomal routing	—	—	—
	TRIM 21	—	Elimination of ICs via recruitment of the proteasomal machinery	—	—	—

[a] *No data are available.*

resembles those caused by hRSV infection (Zhang et al., 2016), have suggested that animals lacking activating FcγRs (FcRγ$^{-/-}$ mice) can be protected from ALI triggered by administration of IgG mAbs that recognize self-antigens, such as MHC-I molecules (Looney et al., 2006). Supporting a role for neutrophils and activating FcγRs in this model of lung injury, the same study showed that ALI was observed when *FcRγ*$^{-/-}$ mice were adoptively transferred with wild-type neutrophils followed by the administration anti-MHC-I mAbs (Looney et al., 2006). Taken together, these results suggest that lung disease in this model is dependent on the expression of activating FcγRs by neutrophils.

For the case of hRSV infection, it has been shown that the recruitment of neutrophils to the lungs of infected mice is modulated by the presence of different FcγRs (**Figure 1**) (Gomez et al., 2016). For instance, it was reported that animals lacking the activating FcγRIII (FcγRIII$^{-/-}$) showed decreased neutrophil recruitment and higher viral loads (Gomez et al., 2016), suggesting that FcγRIII could play a pro-inflammatory role during hRSV primary infection and promotes viral clearance. Consistent with the results described above, mice lacking the inhibitory FcγRIIb (FcγRIIb$^{-/-}$) showed increased neutrophil infiltration in lungs due to hRSV infection but decreased viral loads (Gomez et al., 2016), thus suggesting that this receptor can play an anti-inflammatory role during hRSV-induced disease despite it contributes to viral replication (Gomez et al., 2016).

An *in vitro* study using human neutrophils showed that hRSV-ICs, established with hRSV and anti-hRSV autologous serum, but not free hRSV or antibodies alone, could promote the release of reactive oxygen species (ROS) by neutrophils, which could contribute to lung tissue damage (**Figure 1**) (Kaul et al., 1981; Winterbourn et al., 2016). Therefore, it is possible that the activation of neutrophils, mediated by the engagement of FcγRs likely occurs under physiological conditions, when individuals become infected. This notion, is further supported by a study showing increased release of IL-8 by human neutrophils challenged with opsonized hRSV (Arnold et al., 1994). This cytokine is relevant, as it has been described that secreted IL-8 works as a chemotactic signal for neutrophils that induces their activation leading to pro-inflammatory responses (Henkels et al., 2011). This *in vitro* evidence suggests that the engagement of FcγRs can activate neutrophils and therefore contribute to lung inflammation and the progression of hRSV disease (**Figure 1**).

TABLE 2 | Classification of currently described mouse Fcγ Receptors, and evidences of their role in hRSV-induced pathogenesis.

Type	Receptor	Main function	Evidence after hRSV infection	Suggested role	References
Classical FcγRs (Recognize ICs on the cell surface)	FcγRI	Activating	—	—	—
	FcγRIIb	Inhibitory	*In vivo*: FcγRIIb$^{-/-}$ mice display increased lung neutrophil infiltration but decreased viral loads	Anti-inflammatory role	Gomez et al., 2016
			In vitro: WT mice-derived BMDCs loaded with hRSV-ICs were not able to induce the production of IL-2 by CD4^{+} T cells as compared with FcγRIII$^{-/-}$ mice-derived BMDCs	The engagement of FcγRIII by hRSV-ICs impairs DC-mediated T cell activation	Gomez et al., 2016.
			In vitro: FcγRIIb$^{-/-}$mice-derived BMDCs loaded with hRSV-ICs showed unaltered capacity to induce the secretion of IFNγ by CD4^{+} T cells	DC-mediated stimulation of IFN-γ secretion by CD4^{+} T cells does not depend on the presence of the inhibitory FcγRIIb	Kruijsen et al., 2010
	FcγRIII	Activating	*In vivo*: FcγRIII$^{-/-}$ mice display decreased neutrophil recruitment and higher viral loads	Pro-inflammatory role, promotion of viral clearance	Gomez et al., 2016
			In vitro: FcγRIII$^{-/-}$ mice-derived BMDCs loaded with hRSV-ICs showed restored capacity to induce the production of IL-2 by CD4^{+} T cells	The engagement of FcγRIII by hRSV-ICs impairs DC-mediated T cell activation	Gomez et al., 2016
	FcγRIV	Activating	—[b]	—	—
Non-classical FcγRs (FcγRs that recognize ICs inside the cell)	FcRn	IgG recycling	*In vitro: FcγRn*$^{-/-}$ mice-derived BMDCs loaded with hRSV-IC display unaltered capacity to induce IFN-γ production by CD4+ T cells. *In vivo*: FcRn$^{-/-}$ and WT mice display similar CD4^{+} IFN-γ production after hRSV-IC challenge	FcRn does not modulate DC-mediated CD4^{+} T cell activation	Kruijsen et al., 2013
	TRIM 21	Elimination of ICs via recruitment of the proteasomal machinery	—	—	—

[b] *No data are available.*

Modulation of Dendritic Cell Function by FcγRs and ICs Containing hRSV: Implications for T Cell Immunity

Dendritic cells (DCs) can modulate the immune response during viral infections after capturing ICs through either, activating or inhibitory FcγRs (Guilliams et al., 2014). Along these lines, IgG–antigen complexes can trigger activating signals in human DCs (hDCs) after binding to FcγRIII and promote an inflammatory response (Bandukwala et al., 2007). In contrast, binding of ICs to the inhibitory FcγRIIb trigger inhibiting signals that can lead to reduced inflammation (Boruchov et al., 2005). In addition, it has been described that hRSV-ICs containing either, neutralizing or non-neutralizing antibodies can modulate DC function and subsequent T cell responses elicited by the antigen presentation of these cells (Kruijsen et al., 2013; Gomez et al., 2016).

In the context of hRSV infection, it has been reported that DC-mediated T cell activation and IFN-γ production by these cells, is modulated by the presence of activating FcγRs on the DC surface (Kruijsen et al., 2013). In this case, it was observed that DCs derived from WT adult mice were able to induce the production of IFN-γ by CD4^{+} T cells in the presence of anti-hRSV immune serum obtained from mice being challenged with hRSV (Kruijsen et al., 2013). Nevertheless, this observed increase in the IFN-γ response by CD4^{+} T cells was reduced when the DCs were derived from FcRγ$^{-/-}$ mice. Therefore, the expression of all activating FcγRs on the DC surface is required to promote the production of this cytokine by CD4^{+} T cells. Remarkably, unaltered secretion of IFN-γ by CD4^{+} T cells was observed in DCs derived from FcγRIIb$^{-/-}$ mice, when compared to WT mice (Kruijsen et al., 2013), indicating that DC-mediated stimulation of IFN-γ secretion by CD4^{+} T cells does not depend on the presence of the inhibitory FcγRIIb (Kruijsen et al., 2013).

Interestingly, another report indicates that CD4^{+} T cells represent an important source of IFN-γ during neonatal hRSV infection in the murine model, which is required to prevent re-infection and disease severity in adult mice (Lee et al., 2008).

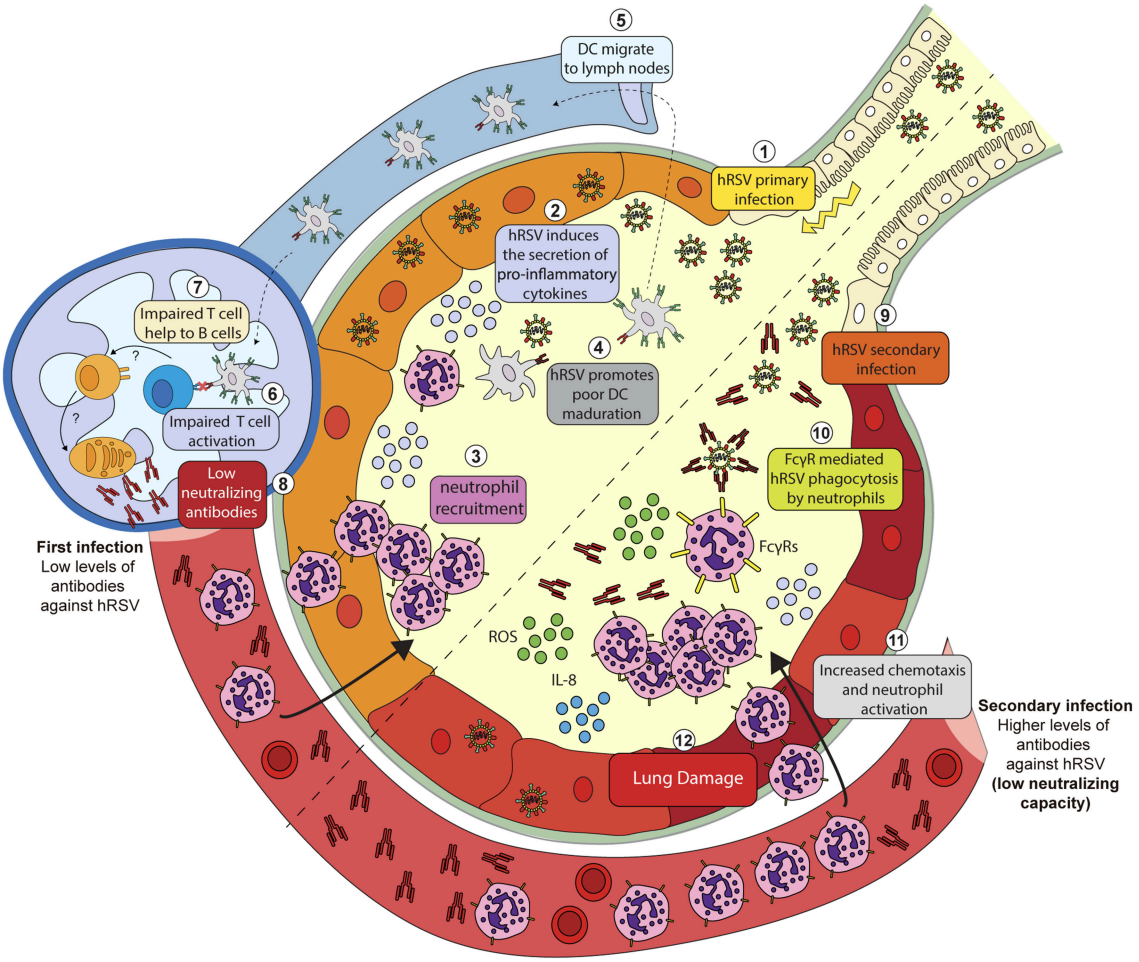

FIGURE 1 | Putative mechanisms explaining hRSV-induced inflammation due to hRSV-IC interaction with Fc-gamma receptors expressed on the surface of neutrophils. During a primary infection (1) hRSV induces the secretion of pro-inflammatory cytokines (2) and chemokines that promote neutrophil recruitment to the lungs and the airways (3). During infection, hRSV is phagocyted by DCs and impair its maturation (4). Infected DCs migrate to lymph nodes (5) but fail to activate T cells (6). By a poorly understood mechanism, T cells fail to help naïve B cells (7) and promote the proliferation of plasma cells that produce anti-hRSV antibodies with a low neutralizing capacity (8). Serum antibodies produced after a primary hRSV infection can opsonize hRSV during secondary infection (9). Opsonized hRSV is then phagocyted by neutrophils through FcγRs (10). The infection of these cells triggers the release of cytokines such as IL-8 that promote the activation and the recruitment of neutrophils (11). Activated neutrophils then release metabolic products, i.e., reactive oxygen species (ROS) that promote lung damage and inflammation (12).

Thus, it is possible that activating FcγRs contribute to prevent re-infection during adulthood, by promoting IFN-γ production by $CD4^+$ T cells through DC-mediated antigen presentation. However, it is necessary to determine whether activating FcγRs on the DC surface also modulate the production of this cytokine by neonatal $CD4^+$ T-cells to prevent re-infection.

Recent studies have shown that another IgG Fc receptor, particularly the neonatal Fc receptor for IgG (FcRn), which is a non-classical Fc receptor that binds IgG at acidic pH (<6,5) (Qiao et al., 2008), does not contribute to the activation of $CD4^+$ T cells when DCs are loaded with hRSV-ICs (Kruijsen et al., 2013). Moreover, Bone Marrow-Derived DCs (BMDCs) from $Fc\gamma Rn^{-/-}$ mice exhibit unaltered capacity to induce the production of IFN-γ by $CD4^+$ T-cells (Kruijsen et al., 2013). These results were validated *in vivo,* as $Fc\gamma Rn^{-/-}$ mice also displayed unaltered IFN-γ production by $CD4^+$ T-cells after being intranasally challenged with hRSV-ICs (Kruijsen et al., 2013).

Results from our group indicate that BMDCs display a reduced capacity to induce IL-2 production by $CD4^+$ T cells after being loaded with hRSV-ICs that had the neutralizing antibody Palivizumab (Synagis^{TM}) (Gomez et al., 2016). In contrast, when the assay was performed with BMDCs derived from either, $Fc\gamma RIII^{-/-}$ or $Fc\gamma RIIb^{-/-}$ mice IL-2 secretion by $CD4^+$ T cells was restored. This results prompts that when present, these receptors impair the capacity of DCs to induce the secretion of IL-2 by $CD4^+$ T cells. It should be noted that, the production of this cytokine is required for the generation of memory regulatory $CD4^+$ T cells (Tregs), which perform anti-inflammatory functions during hRSV infection and protect

against re-infections (Durant et al., 2013). Thus, it is possible that both, FcγRIII and FcγRIIb contribute to hRSV pathogenesis and re-infection by impairing the capacity of DCs to promote the production of IL-2 by $CD4^+$ T cells.

Type II FcγRs Expressed on the Surface of Human DCs Contribute to Immune Responses Against hRSV

In humans, the presence of two types of FcγRs has been recognized (Banegas Banegas et al., 1987). Type-I FcγRs are members of the immunoglobulin superfamily and can be either activating or inhibitory (Nimmerjahn and Ravetch, 2005, 2008). In contrast, Type-II FcγRs are members of the C-type lectin receptor family and comprise two different members: the IgE receptor and the surface protein DC-SIGN (Banegas Banegas et al., 1987; Miettinen, 2004), which is able to recognize the Fc portion of IgG (Kaneko et al., 2006; Svajger et al., 2010), but also the G protein expressed by hRSV (Johnson et al., 2012). Of interest, studies evaluating the role of DC-SIGN in hDCs during hRSV infection, showed that the blockade of this receptor with specific mAbs led to an increase in the expression of maturation markers, such as CD80 and CD86 following hRSV infection (Johnson et al., 2012). This suggests that the interaction between hRSV surface proteins and DC-SIGN can suppress some aspects of DC activation in humans, thus contributing to an impaired protective immunity following hRSV infection. However, further studies are required to study the influence of this receptor during infection *in vivo* and hRSV-induced pathology, as well as its consequences on DC mediated T-cell activation.

Contribution of ADE to hRSV Re-infection Episodes

In a recent study, it was shown that young infants (i.e., <3 months old) generate a highly neutralizing antibody response that is biased from the post-fusion to the pre-fusion form of hRSV F protein. However, as children become older (i.e., from <3 months old to >6 months old), this response is re-directed against post-fusion conformation antigens (Goodwin et al., 2018). Thus, the antibodies generated display a weak neutralizing capacity that fail to prevent hRSV infection. Therefore, it is possible that the generation of a pool of low-neutralizing

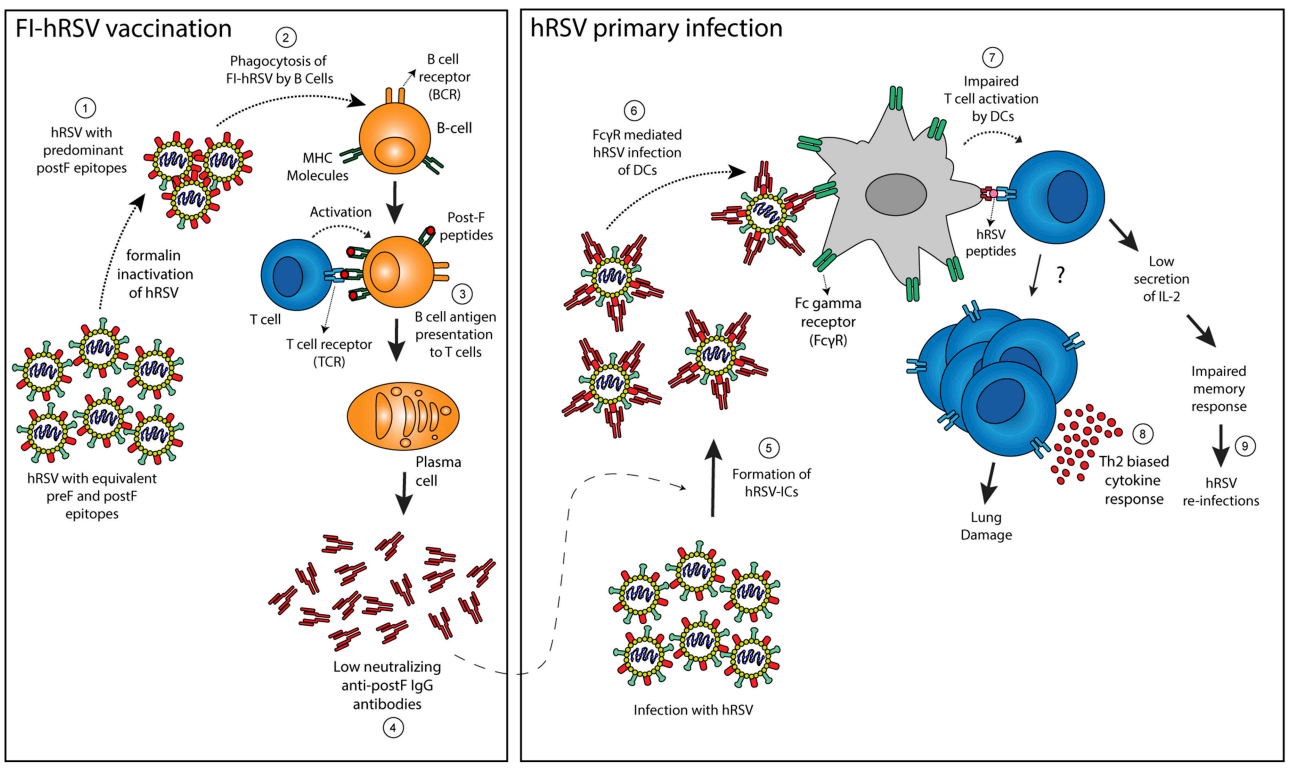

FIGURE 2 | Proposed mechanism to explain enhancement of hRSV-induced disease following FI-hRSV vaccination. Formalin hRSV inactivation produces a non-infectious virus with a high proportion of post-fusion conformation epitopes in the F protein (Post-F) (1). The inactivated virus is then phagocyted by B cells (2) that can present hRSV antigens to T cells in the context of MHC molecules (3). The interaction between B and T cells allows the differentiation of B cells into plasma cells that generate antibodies against the post-fusion conformation of the hRSV F protein (4). Such antibodies failed to neutralize hRSV infection but also may enhance the infection of FcγR bearing cells such as DCs. When infection by hRSV occurs, the low neutralizing antibodies induced by the FI-hRSV vaccine can form immune-complexes (ICs) with hRSV (5) that leads to the activation of Fc-gamma receptors expressed on the surface of DCs (6). Subsequently, an impaired DC-mediated T cell activation (7) can induced $CD4^+$ T cells with a Th2-biased phenotype that promotes lung damage (8). Furthermore, low secretion of IL-2 by $CD4^+$ cells activated by hRSV-IC-loaded DCs can lead to a poor memory response that contributes to hRSV re-infection (9).

antibodies during infancy can facilitate infection of immune cells that express FcγRs, a phenomenon called ADE of infection that has been observed for other viruses such as dengue virus (Flipse et al., 2016), acute respiratory syndrome coronavirus (Yip et al., 2014) and porcine reproductive and respiratory syndrome virus infection (Gu et al., 2015). In this context, antibodies might exert different effector functions through their Fc regions and for hRSV, ADE during infection is an effect that has been reported in *in vitro* studies (Gimenez et al., 1989; Krilov et al., 1989; Osiowy et al., 1994). However, a role of ADE during hRSV pathogenesis *in vivo* has been proposed, but remains to be confirmed during re-infection.

To date, *in vitro* enhancement of infection of monocyte-derived cell lines due by FcγR binding by mAbs and patient sera has been reported (Gimenez et al., 1989; Krilov et al., 1989; Osiowy et al., 1994), demonstrating that non-neutralizing mAbs can enhance the infection of phagocytic cell lines expressing these receptors (Gimenez et al., 1996). Further, when neutralizing antibodies were applied at sub-neutralizing concentrations (i.e., diluted), they induced ADE in phagocytic cells bearing FcγRs. This was also observed using human sera and purified human immunoglobulin (IVIg) (van Erp et al., 2017). Together, these results suggest that the interaction of hRSV-ICs generated with low neutralizing antibodies can promote the infection of immune cells *in vitro,* therefore contributing to hRSV pathogenesis under physiological conditions.

Contribution of ICs Containing hRSV to Enhanced Disease Elicited by Vaccination With Formalin-Inactivated hRSV

The administration of a formalin-inactivated hRSV vaccine to children nearly 50 years ago, which was aimed at preventing severe respiratory disease elicited by hRSV infection was unable to produce protective immunity against hRSV. Contrarily to what was expected, its administration resulted in increased morbidity and mortality in vaccinated infants when they were later infected by the virus (Kim et al., 1969). Although the mechanisms underlying the pathological effects of FI-RSV vaccine have not been totally elucidated, this episode revealed complexities associated to vaccine development, which has been hampered, and raised hypothesis about the pathologic roles of hRSV-ICs (Kim et al., 1969; Polack et al., 2002; Delgado et al., 2009). Enhanced hRSV disease (ERD) after FI-RSV immunization of BALB/c mice has been associated with alveolar deposition of ICs, which was observed 7 dpi of hRSV by means of co-localization of IgG with the complement component 3 (C3 protein). The role of complement fixing ICs in ERD was supported by experiments in $C3^{-/-}$ mice, which showed significantly less airway hyper-responsiveness (AHR) in comparison to WT counterparts, after FI-hRSV vaccination and hRSV challenge, arguing for a role of complement in bronchoconstriction observed in ERD (Polack et al., 2002). These experimental studies were supported by histological analysis of lung sections from two infants that suffered fatal ERD, in which IC-mediated complement activation was observed through extensive peribronchiolar complement component 4d (C4d) deposition in the airway tissue (Polack et al., 2002). Furthermore, a sub-optimal, non-protective antibody response in mice, characterized by high levels of non-neutralizing anti-F and anti-G IgG antibodies, was observed after immunization with FI-hRSV, but not infectious hRSV (Polack et al., 2002). The lack of affinity maturation in Abs elicited by FI-hRSV was associated with enhanced lung histopathology and AHR, whereas the supplementation of Toll-like receptor (TLR) agonists, performed during immunization promoted proper affinity maturation that prevented ERD after hRSV challenge, showing that a deficient TLR stimulation in B cells is likely responsible for the lack of Ab affinity maturation after FI-hRSV vaccination (Delgado et al., 2009). Furthermore, cotton rats vaccinated with FI-RSV elicited high levels of hRSV-specific antibodies, which displayed low neutralizing titers in Vero cells (Piedra et al., 1993). These antibodies were also able to cause ADE in *in vitro* assays. These studies suggest that sub-optimal antibody production and the generation of ICs play a role in ERD development (**Figure 2**). Furthermore, recent studies suggest that $CD4^+$ subsets and a Th2-biased immune response are key for AHR and ERD (Knudson et al., 2015). In this context, TAM (Tyro3, Axl, and Mertk) receptors, which are expressed in various cells and tissues, and their ligand Growth arrest-specific 6 (Gas6) could be involved in the production of a Th2-biased immune responses that reduce the production of type IgG2a subclass antibodies (Shibata and Ato, 2017). These antibodies could have an effective neutralizing capacity against hRSV and therefore prevent hRSV induced disease, but their production is lowered as a consequence of FI-hRSV immunization followed by hRSV infection. Therefore, it is possible that the TAM/Gas6 signaling axis can contribute to the generation of low neutralizing antibodies that failed to neutralize hRSV infection and instead contributes to the pathology caused by hRSV infection through the engagement of FcγRs.

CONCLUDING REMARKS

The hRSV is a leading cause of respiratory illness in infants and a major health burden worldwide. Re-infections with this virus are common and can contribute to additional clinical manifestations, such as asthma and allergies. For this reason, several studies have focused on understanding the mechanisms that can contribute to hRSV induced pathology, but also to elucidate the factors that contribute to re-infection episodes throughout life. In this context, some studies suggested that low number of memory hRSV-specific $CD8^+$ T cells could be associated with re-infection episodes and that the levels of such cells could be regulated by virus-specific antibodies, by modulating the function of antigen presenting cells, such as DCs. Furthermore, recent studies suggest that the generation of regulatory memory T cells could be impaired by the interaction of hRSV-ICs with DCs, pointing out these phenomena as an interesting research topic that deserves analysis. In this review, and based on several studies, we discussed the role of FcγRs during hRSV infection and their immune-modulatory properties that can account for recurrent hRSV infection episodes and the enhancement of the disease caused

by FI-hRSV vaccination. However, further research is needed to understand how hRSV induces the production of antibodies that fail to prevent re-infections. Knowledge of such mechanisms would certainly be appreciated for vaccine and therapy development against hRSV, which represents a major global health problem.

AUTHOR CONTRIBUTIONS

OA and FD are responsible for the writing of this review article. TB, FB, JE-V, JS, and PG reviewed the manuscript. AK is the leading investigator and assisted in the organization and revision of this article. All authors listed approved the version to be published and have made a substantial and intellectual contribution to the work.

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Neutrophils Induce a Novel Chemokine Receptors Repertoire During Influenza Pneumonia

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Exaggerated host innate immune responses have been implicated in severe influenza pneumonia. We have previously demonstrated that excessive neutrophils recruited during influenza infection drive pulmonary pathology through induction of neutrophil extracellular traps (NETs) and release of extracellular histones. Chemokine receptors (CRs) are essential in the recruitment and activation of leukocytes. Although neutrophils have been implicated in influenza pathogenesis, little is known about their phenotypic changes, including expression of CRs occurring in the infected -lung microenvironment. Here, we examined CC and CXC CRs detection in circulating as well as lung-recruited neutrophils during influenza infection in mice using flow cytometry analyses. Our studies revealed that lung-recruited neutrophils displayed induction of CRs, including CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR3, and CXCR4, all of which were marginally induced in circulating neutrophils. CXCR2 was the most predominant CR observed in both circulating and lung-infiltrated neutrophils after infection. The stimulation of these induced CRs modulated neutrophil phagocytic activity, ligand-specific neutrophil migration, bacterial killing, and NETs induction *ex vivo*. These findings indicate that neutrophils induce a novel CR repertoire in the infectious lung microenvironment, which alters their functionality during influenza pneumonia.

Keywords: influenza, neutrophil, acute lung injury, chemokine receptor, mouse model

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INTRODUCTION

Frequent outbreaks of influenza virus infections are causing significant morbidity and mortality in humans, birds, and other animal species (Xu et al., 2006; Traylor et al., 2013; Short et al., 2014; Wang et al., 2016). Neutrophils and macrophages constitute the majority of infiltrated cells in the lungs during influenza, and play essential roles in the clearance of the virus, before the onset of virus-specific immunity (Perrone et al., 2008; Tavares et al., 2017). However, uncontrolled recruitment and activation of these innate immune cells contribute to acute lung injury (ALI), significantly impacting the disease outcome (Crowe et al., 2009; Mauad et al., 2010; Liu et al., 2015). Our earlier studies have demonstrated that in severe influenza, the massive influx of neutrophils into the infected lungs causes collateral damage to the lungs *via* generation of NETs and the release of extracellular histones (Narasaraju et al., 2011; Anandi et al., 2013; Ashar et al., 2018).

The recruitment, extravasation, and activation of neutrophils are largely driven by chemokine ligands *via* binding to their cell-surface receptors called chemokine receptors (CRs) (Moser et al., 2004). CRs belong to a family of seven-transmembrane domain G protein-coupled receptors, divided into four structural groups (C, CC, CXC, and CX_3C) based on the spacing of two conserved cysteine residues. Inflammatory chemokines produced in response to influenza by lung epithelial cells and/or macrophages regulate leukocyte recruitment and activation in infected lungs (Rossi, 2000; Moser and Loetscher, 2001). Neutrophils are generally thought to be limited in expression of CRs, typically consisting predominantly of the CXC group CRs (such as CXCR1, CXCR2); expression of CC chemokine receptors are absent under normal conditions (Sallusto et al., 2000). However, in inflammatory disease conditions such as rheumatoid arthritis, sepsis, and cystic fibrosis, neutrophils have been shown to expand their CR expression repertoire, especially after translocating into various tissues (Speyer et al., 2004; Hartl et al., 2008; Chou et al., 2010; Lebre et al., 2011). Induction of these CC CRs significantly alters neutrophil function, including phagocytosis, respiratory burst, and chemotaxis (Hartl et al., 2008; Chou et al., 2010). Neutrophils isolated from influenza-infected patients display impaired expression of phagocytic receptors such as CD64, CD32, and CD16, indicating that influenza infection modulates neutrophil functionality, which may also contribute to increased susceptibility to bacterial superinfections (Salentin, 2003). Influenza infection also modulates expression and chemotactic responsiveness of CCR1 and CCR2 in monocytes (Pauksens et al., 2008). Following excessive neutrophil influx, their toxic products such as NETs and granule enzymes are associated with pulmonary pathology in influenza pneumonia, although little is known about the phenotypic and functional characteristics of these neutrophils (Narasaraju et al., 2011; Anandi et al., 2013; Rojas-Quintero et al., 2018).

Here, we investigated whether hyper-inflammatory cytokine responses seen during influenza pneumonia alters the phenotypic signature of CR induction in lung-recruited neutrophils. Using Flow cytometry analysis, we have evaluated cell surface receptor expression of CRs (including CC and CXC types) in circulating as well as lung-recruited neutrophils during the course of infection. We evaluated the effects of induced CRs on neutrophil functionality, including phagocytosis, neutrophil migration, bacterial killing, and NETosis. Our results demonstrated induction of various CC and CXC-type CRs in neutrophils after their recruitment into the infected lungs, but not while in circulation. Further, activation of induced CRs with their specific chemokine ligands modulates neutrophil functional activities including phagocytosis, neutrophil migration, and NETosis. These studies suggest that induction of various CRs in lung-recruited neutrophils shape their fate and functional responsiveness in influenza infected-lungs.

MATERIALS AND METHODS

Virus, Animals, and Ethics Approval

Influenza A/Puerto Rico/8/34, H1N1 (PR/8) virus was obtained from the American Type Culture Collection (ATCC, VA). Viral titers were determined by tissue culture infectivity dose ($TCID_{50}$) assay via infection of Madin-Darby canine kidney (MDCK) cells (Ng et al., 2012). Female BALB/c mice (6–8 weeks old) were used in this study. The animals were housed in microisolator cages in a BSL-2 animal facility. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University (protocol number VM-11-43) and were performed in strict accordance with their recommendations.

Animal Infections

For influenza infections, mice were anesthetized with a mixture of xylazine (0.1 mg/kg) and ketamine (7.5 mg/kg). Mice were infected intranasally (IN) with a sub-lethal dose (100 $TCID_{50}$) of PR/8 (H1N1) influenza virus in a 50 μL volume of sterile phosphate-buffered saline. Control mice received equal volumes of PBS.

Collection of Blood, Bronchoalveolar Lavage (BAL) Fluid, and Tissues

For BAL fluid collection, the lungs were lavaged twice using intratracheal administration of 0.5 mL of sterile PBS (Ashar et al., 2018). The recovery of BAL fluid was over 85% for all animals. The BAL fluid samples were centrifuged at 200 x g for 10 min, and BAL cells were resuspended in sterile PBS containing 2% fetal bovine serum for flow cytometry analysis. For differential cell counts, BAL cells were processed onto microscopic slides using a CytoFuge 2 cytocentrifuge (StatSpin, Westwood, MA), subjected to modified Giemsa staining, and cells (more than 200 per animal) were counted at a magnification of 1000x. Whole blood was obtained via terminal procedure of intra-cardiac collection. BAL and blood were collected from control and infected mice at 3, 4, and 5 days post-infection (dpi) for flow cytometry analysis (we found that neutrophils influx peaked from 3 dpi). To exclude that inflammatory responses were due to secondary bacterial infection, 20 μL of each BALF sample was plated onto blood agar and incubated at 37°C for 3 days. In another set of experiments, control and infected animal lungs were fixed with 4% formalin, and subjected to histopathology analysis after hematoxylin and eosin (H&E) staining to evaluate inflammatory and acute lung injury. Histopathologic severity was scored in a blinded fashion on a scale of 1–4 (four being the most severe) based on the following criteria by a board-certified anatomic veterinary pathologist: cellular inflammation, necrotizing bronchiolitis, interstitial pneumonia, alveolitis, hemorrhage, and edema. Total histopathologic scores were evaluated as a sum of all individual scores (Narasaraju et al., 2010).

Flow Cytometry Analyses

The following mouse antibodies were purchased from R&D Systems and used for flow cytometry for detection of chemokine receptors in neutrophils: CCR1 FITC-conjugated antibody (Clone 643854), CCR2 PE-conjugated antibody (Clone 475301), CCR3 PE-conjugated antibody (Clone 83101), CCR5 FITC-conjugated antibody (Clone CTC5), CXCR1/IL-8 RA PE-conjugated antibody (Clone 1122A), CXCR2/IL-8 RB PE-conjugated antibody Clone 242216), CXCR3 PE-conjugated antibody (Clone 220803), CXCR4 fluorescein-conjugated

antibody (Clone 247506), and Ly6G-1A8 PerCP-conjugated antibody (Clone 1A8) were purchased from BioLegend, CA (Hartl et al., 2008).

Phenotypic characterization of neutrophils during the course of infection was performed using whole blood from control mice; blood and BAL cells from influenza-infected mice at 3, 4, and 5 dpi. Control BAL samples were not used as they contained very low numbers of neutrophils to perform flow cytometry. Blood and BAL samples were incubated with RBC lysis buffer (Miltenyi Biotec Inc, CA), followed by incubation with chemokine receptor specific antibodies for 30 min at room temperature. Unstained cells and single-fluorochrome staining controls were used to exclude background and cross-reactivity among different fluorochromes. All samples were then centrifuged and washed thrice with PBS (containing 2% fetal bovine serum) before performing flow cytometry. The latter was performed using BD FACSCalibur flow cytometer, and the data were analyzed using CellPro software. Neutrophils were gated as Ly6G-1A8^{+}SSC$^{med\text{-}hi}$. CD11b analysis was performed by comparing mean fluorescence intensity (MFI) between samples. The Ly6G-1A8 staining on neutrophils was validated by sorting Ly6G-1A8 positive cells on FACSAria flow cytometer, which displayed 99% purity. All flow cytometry experiments were repeated three times, and in each experiment, cells were prepared from a pool of three mice to obtain sufficient numbers of cells.

Neutrophil Isolation

For neutrophil functional analysis, neutrophils were isolated from control blood, infected blood, and BAL samples using a MACS neutrophil isolation kit (Miltenyi Biotec Inc, CA) with Ly6G-1A8 antibody through positive selection (Ashar et al., 2018). Isolated neutrophils were enumerated and used for functional studies. Blood samples were initially incubated with 1x RBC lysis buffer to remove RBCs, prior to isolation of neutrophils.

Phagocytosis Assay

For phagocytosis assays, BAL neutrophils were isolated, and 10^5 cells were stimulated with or without the appropriate CCR1, CCR3, CCR5, CXCR2, CXCR3, and CXCR4 blockers and ligands. One microgram of BX 471 (CCR1 Antagonist; Cayman Chemicals, MI), SB328437 (CCR3 Antagonist; Sigma, MN), anti-CCR5 (Novus, CO), anti-CXCR2 (Cell Applications, CA), CXCR3 (Bio X Cell, NH) blocking antibodies, and AMD3100 (CXCR4 Antagonist (R&D, MN) were added and incubated for 30 min at 37°C. The cells were then stimulated with 10 ng of the appropriate ligands CCL3 (CCR1), CCL11 (CCR3), CCL4 (CCR5), IL-8 (CXCR2), CXCL11 (CXCR3), and CXCL12 (CXCR4). pHrodoTM Red *E. coli* BioParticles (Thermo Fisher, MA) were added to each sample (1 mg/mL), and cells were incubated at 37°C for 90 min. Cells were then stained with Ly6G-1A8 antibody for 30 min at room temperature, washed twice to remove excess bacteria, followed by flow cytometry (Hartl et al., 2008). Results were analyzed by determining MFI. Unstained, single-stained neutrophils and bacteria alone served as controls.

Neutrophil Chemotaxis Assay

Neutrophil chemotaxis assay was performed as described by Szczur et al. (2006). In brief, BAL neutrophils isolated at 4 dpi were purified, resuspended in DMEM containing 1% fetal bovine serum and added to (1×10^5/well) the upper compartment of a Transwell filter system (8.0 μm pore size, 12 mm diameter) in a 24-well culture plate. The chemokine specific ligands including CCL3, CCL4 (R&D), IL-8, and CXCL11 (R&D) (at a concentration of 100 ng/ml) were added to the lower chamber. The plate was incubated for 90 min at 37°C. The culture medium from the lower chamber was centrifuged, and the migrated cells were counted with hemocytometer (Szczur et al., 2006).

In vitro NETs Release

To test the effect of various chemokine receptors on NETosis, neutrophils isolated from influenza-infected lungs at 4 dpi were resuspended in DMEM containing 1% fetal bovine serum and stimulated (2×10^4) with chemokine specific ligands including CCL4, IL-8, and CXCL11 as described above. Phorbol 12-myristate 13-acetate (PMA) at 20 nM concentration was used as a positive control for induction of NETs (Ashar et al., 2018). Released NETs were labeled with SYTOX green staining and visualized under fluorescence microscopy at 400x magnification. Quantification of NETs released was performed as described earlier (Ashar et al., 2018). We evaluated at least 5–10 fields on each slide to quantify the total numbers of positive cells exhibiting NETs release.

Bactericidal Activity

Neutrophils (105) isolated from influenza-infected mice at 4 dpi were incubated with *Streptococcus pneumoniae* at 1:10 ratio for 90 min in the presence or absence of CCL3, CCL4, IL-8, CXCL11 (100 ng/mL). Bacterial killing was measured as a percentage of control bacteria (bacteria incubated without neutrophils) as described previously (Narasaraju et al., 2011). Sample aliquots were plated on chocolate agar to determine the numbers of colony-forming units (CFU).

Statistical Analysis

The data are expressed as the means ± SEM. Statistical analyses were performed using Student's unpaired *t*-test, paired *t*-test or analysis of variance (ANOVA) using GraphPad Prism 7 software. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Lung-Infiltrated Neutrophils Induce Novel Chemokine Receptors During Influenza-Infection

We have evaluated various CC and CXC chemokine receptors in circulating neutrophils as well as lung-recruited neutrophils in influenza-infected mice. Neutrophils were gated based on their FSC/SSC characteristics, followed by detection of Ly6G-1A8 on 10,000 events. Neutrophils, SSC$^{med\text{-}hi}$/Ly6G-1A8^{+} were separated by a cell sorter, resulting in 99% purity based on modified Giemsa staining (**Figure 1A**). We then evaluated

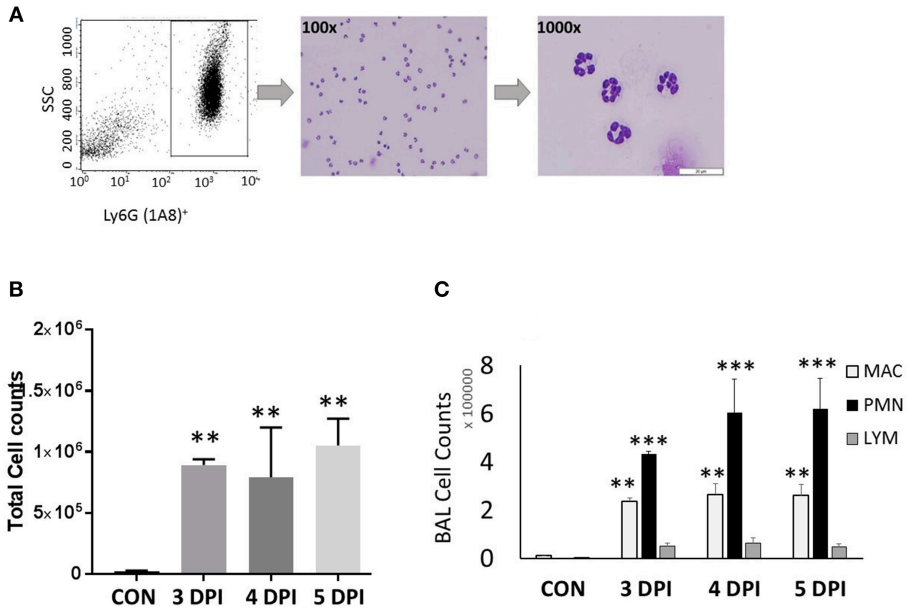

FIGURE 1 | Neutrophil gating and differential counts during influenza infection. BALB/C mice were infected with a sub-lethal dose (100 $TCID_{50}$), intranasally with influenza A/Puerto Rico/8/34 H1N1 virus. Mock-infected mice received equal volumes of PBS. **(A)** Identification of neutrophils and gating. Neutrophils were identified based on their light scatter characteristics (FSC/SSC). The granulocyte region was further differentiated by means of the neutrophil specific marker, Ly6G-1A8. $FSC^{med}SSC^{med\text{-}hi}Ly6G\text{-}1A8^{+}$ cells were further sorted using a FACSAria flow cytometer, which showed that over 99% were neutrophils. Morphologically, neutrophils were identified by modified Giemsa staining. The representing images are showing neutrophils at 100x and 1000x. **(B)** Influenza-infected mice have significantly elevated BAL leukocytes between 3 and 5 dpi. **(C)** Differential cell counts were performed in lung-recruited cells, and revealed neutrophils as the major cell population. Data were expressed as means ± SEM. $n = 3$–5 mice per group; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

induction of a broad range of CRs, including CC (CCR1-3, CCR5) and CXC (CXCR1-4), by flow cytometry analysis using control blood, infected blood, and BAL. Control BAL cells were not included in this study as they contained too few neutrophils to perform flow cytometry. We characterized chemokine receptor profiles in neutrophils between 3 and 5 dpi, which displayed persistent increase of these cells into the infected-lungs (**Figures 1B,C**), with vascular injury evident by protein leakage and total histopathologic changes in the lungs (**Figures 6A–C**).

To evaluate induction of various CRs, neutrophils from blood and BAL samples were labeled with CC (CCR1-CCR3, CCR5) and CXC (CXCR1-4) specific antibodies. Neutrophils were identified by $Ly6G\text{-}1A8^{+}$ staining. Our studies revealed that majority of the CRs do not show significant increase while in circulation in infected-mice. However, the CRs induced in infected-lung microenvironment. Lung-recruited neutrophils induced CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR3, CXCR4, which were absent or marginally induced in peripheral blood neutrophils from influenza-infected mice. Neutrophils from infected-blood showed minimal to absent of CC CRs similar to healthy mice samples. The expression pattern of majority of CC and CXC CRs was consistently elevated from 3 dpi through 5 dpi in lung-recruited neutrophils, but not while in circulation (**Figures 2**–**4**).

Among all CRs, CXCR2 was the most abundant CR detected in control blood, infected blood, and BAL neutrophils (**Figures 2E–H**). Circulating neutrophils from healthy control mice were 85% positive for CXCR2, which increased to 97–100% in infected blood samples (**Figure 2F**). Upon pulmonary infiltration in response to infection, these neutrophils exhibited decline in CXCR2-positive staining, but remained highly induced at over 60% positivity compared to other induced CRs in infected-lungs (**Figures 4A–C**). There was no difference in surface expression levels of CXCR1 in circulating neutrophils, but increased from 3 to 5 dpi with about 30–40% elevation in lung-recruited neutrophils at 4 and 5 dpi (**Figures 2A–D**). The detection of other CXC CRs (including CXCR3 and CXCR4) also displayed a similar trend in their surface expression during the course of infection between 3 and 5 dpi, while there was no difference in circulating neutrophils between control and infected groups (**Figures 2I–P, 4A–C**).

The surface expression of CC CRs was minimal to absent in circulating neutrophils in both control and influenza-infected mice. However, CC CRs including CCR1, CCR2, CCR3, and CCR5 were significantly increased in lung-recruited neutrophils (**Figures 3A–P**). The induction of these chemokine were altered between control and infected blood groups (**Figures 3B,F,J,N**). These CC CRs were present in about 10–15% of BAL cells at 3 dpi, and increased to 20–30% at 4 dpi and to 30–40% at 5 dpi (**Figures 3D,H,L,P**, **4B,C**), suggesting that the inflammatory cytokine environment significantly modulates neutrophil chemokine receptor induction during influenza pneumonia.

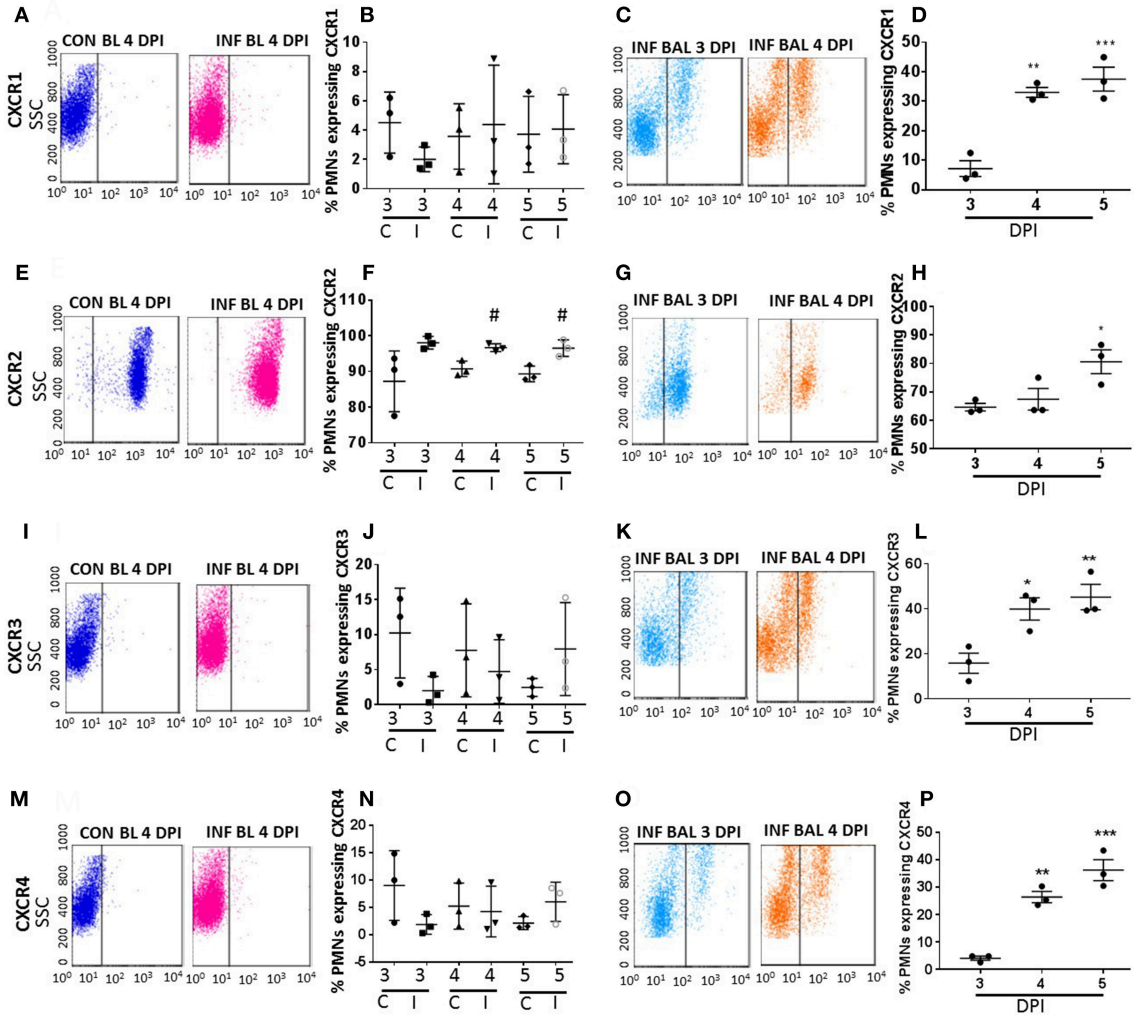

FIGURE 2 | Surface CXC-chemokine receptor detection profiles in blood and lung-recruited neutrophils during influenza infection**.** Blood from control and influenza-infected mice at 3–5 dpi were assessed by flow cytometry. Neutrophils were gated as Ly6G-1A8^{+} cells for subsequent analysis. Induction of receptors was represented as percentage of blood neutrophils showing positive staining for chemokine receptors CXCR1 **(A,B)**, CXCR2 **(E,F)**, CXCR3 **(I,J)**, and CXCR4 (**M,N**). Lung-recruited neutrophils were analyzed at 3,4, and 5 dpi for induction of CXCR1 **(C,D)**, CXCR2 **(G,H)**, CXCR3 **(K,L),** and CXCR4 **(O,P)**. Dot plots represent detection of CXCR1, CXCR2, CXCR3, and CXCR4 in control blood, infected blood (4 dpi) and infected BAL (3 and 4 dpi), and the subsequent graph represents the overall trend in percentage induction of receptor on neutrophils in all samples from 3 through 5 dpi. Data are expressed as mean ± SEM. $n = 3$ replicates and each replicate prepared from a pool of three mice for all receptor expression analysis. $^{\#}p < 0.05$; vs. Con blood. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. 3 dpi BAL. C-Con, I-Infected.

Effect of Induced Chemokine Receptors on Phagocytic Activity in Lung-Recruited Neutrophils Following Influenza Infection

Earlier studies have shown that impaired phagocytic activity during influenza (Ishikawa et al., 2016), and lung-recruited neutrophils fail to kill bacteria *in vivo* (Hashimoto et al., 2007). We found that overall phagocytic activity declined in lung-recruited neutrophils compared to circulating neutrophils (**Figure 5A**). Next, to test if the induced CRs in lung-recruited neutrophils contribute to phagocytic function in infected-lungs, we stimulated upregulated CRs in lung-recruited neutrophils with specific chemokine ligands in the presence or absence of CCR1, CCR3, CCR5, CXCR2, CXCR3, and CXCR4 specific blocking antibodies. Our results demonstrated that antibody blocking of CCR5 and CXCR2 significantly inhibited phagocytic activity. Interestingly, blockade with CCR1 antibody revealed enhanced phagocytic activity (**Figure 5B**). No significant differences in phagocytic activity was observed when CCR3, CXCR3, and CXCR4 were blocked. Based on these findings, we used ligand-specific stimulation for CCR1, CCR5, CXCR2, and CXCR3 to test their effects on neutrophil functional responsiveness including chemotaxis, bacterial killing, and NETosis.

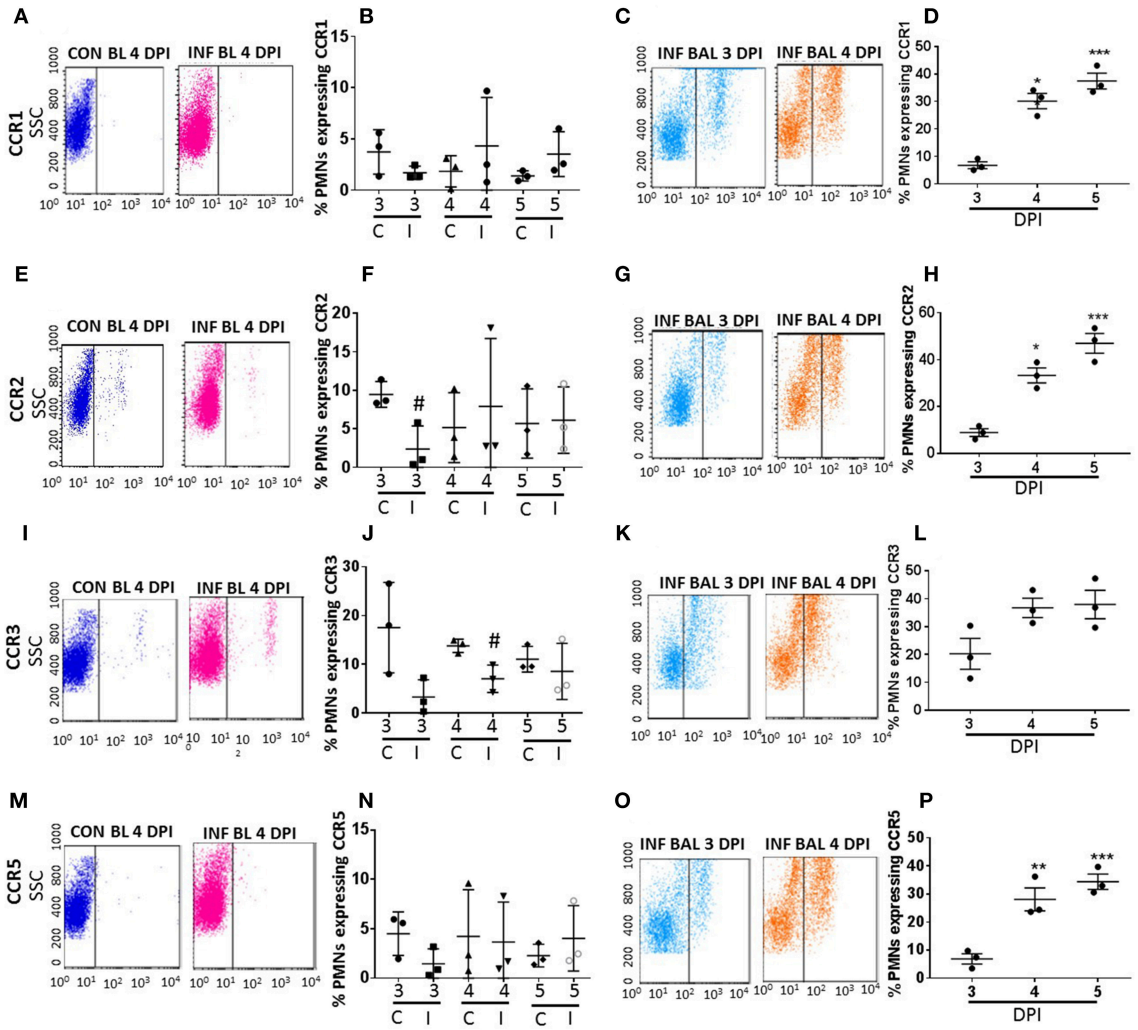

FIGURE 3 | Surface CC-chemokine receptor detection in blood and lung-recruited neutrophils during influenza infection. Blood from control and influenza-infected mice at 3–5 dpi were assessed by flow cytometry. Neutrophils were gated as Ly6G-1A8^{+} cells for subsequent analysis. Detection of receptors was represented as percentage of neutrophils showing positive staining for chemokine receptors CCR1, CCR2, CCR3, and CCR5. Detection of receptors was represented as percentage of blood neutrophils showing positive staining for chemokine receptors CCR1 **(A,B)**, CCR2 **(E,F)**, CCR3 **(I,J),** and CCR5 **(M,N)**. Lung recruited neutrophils were analyzed at 3,4, and 5 dpi for detection of CCR1 **(C,D)**, CCR2 **(G,H)**, CCR3 **(K,L),** and CCR5 **(O,P)** Dot blots represent expression of CCR1, CCR2, CXR3, and CCR5 in control blood, infected blood (4 dpi) and infected BAL (3 and 4 dpi). Data are expressed as mean ± SEM. $n = 3$ replicates and each replicate prepared from a pool of three mice for all receptor expression analysis. $^{\#}p < 0.05$ vs. con blood. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. 3 dpi BAL.

Effect of Induced CRs on Neutrophil Chemotaxis

Neutrophils isolated from influenza-infected mouse lungs were seeded in the upper chamber of the 8.0-μm membrane insert in a Transwell system. CCR1, CCR5, CXCR2, and CXCR3 specific ligands including CCL3, CCL4, IL-8, and CXCL11 (100 ng/mL) were added into the lower chamber and incubated for 90 min. Incubation with IL-8 culminated in a 4-fold increase in neutrophil migration. The addition of ligands including CCL4, and CCL3, but not CXCL11 demonstrated ∼2-fold enhancement in neutrophil migration (**Figure 5C**).

Induced CRs Modulate Release of NETs in Lung-Recruited Neutrophils *in vitro*

Neutrophils isolated from influenza-infected mouse lungs were stimulated with CCR1, CCR5, CXCR2, and CXCR3 specific ligands CCL3, CCL4, IL-8, and CXCL11 (100 ng/mL), respectively, and incubated for 4 h. NETs were stained with SYTOX green (Ashar et al., 2018). Stimulation of neutrophils with IL-8 (CXCR2 ligand) generated pronounced release of NETs. Significantly elevated NETosis was also observed when neutrophils were stimulated with CCL3 and CCL4. However, CXCL11 did not lead to prominent NETosis (**Figure 5D**).

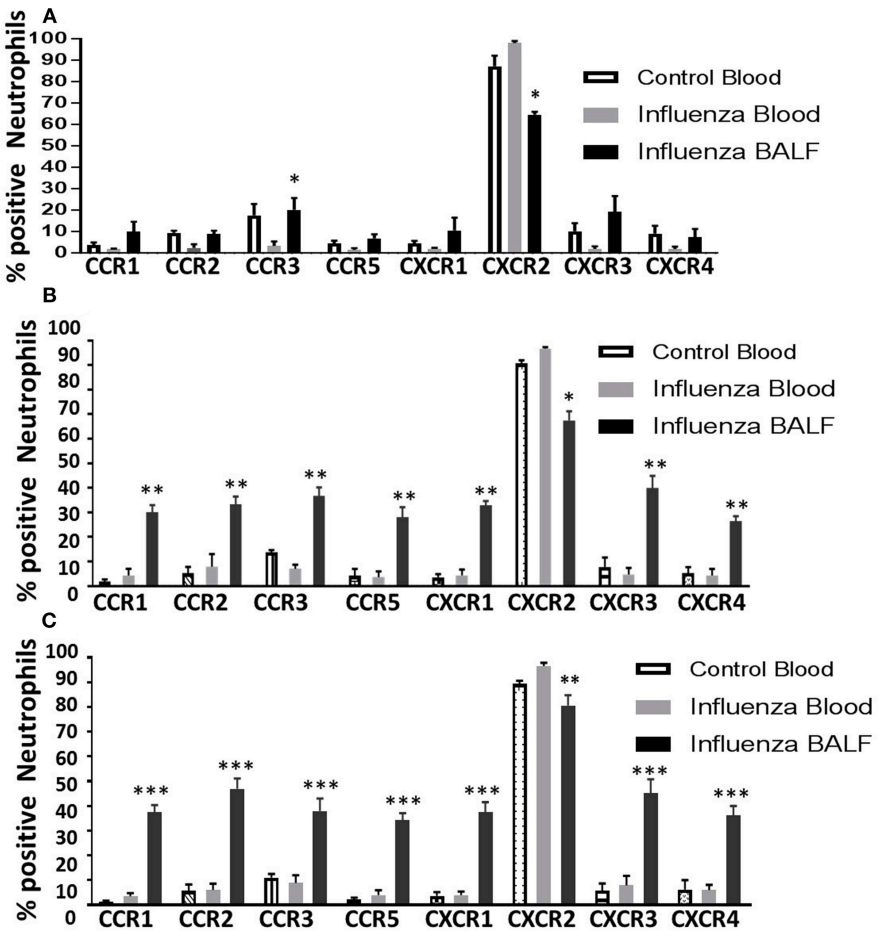

FIGURE 4 | Overall induction of chemokine receptors in neutrophils during the course of infection. The surface detection of CC and CXC CRs were analyzed in a time-dependent fashion and compared with control blood neutrophils. Control blood, infected blood and infected BAL from mice at 3 dpi **(A)**; 4 dpi **(B),** and 5 dpi **(C)**. Samples are represented as percentage expression on neutrophils compared between groups. Data are expressed as mean ± SEM. At each days post infection, was compared with infected (INF) blood samples to visualize the overall surface detection of CRs while in circulation and after recruiting into the infected-lungs. The control BAL samples were not included as they contained very low number of neutrophils to perform flow cytometry. Replicates ($n = 3$); each replicate was prepared from a pool of three mice for all receptor expression analysis. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; vs. Infected blood at each day post infection.

Stimulation of Induced CRs Does Not Enhance Bacterial Killing

Neutrophils isolated from influenza-infected mouse lungs were stimulated with CCR1, CCR5, CXCR2, and CXCR3 specific ligands including CCL3, CCL4, IL-8, and CXCL11 (100 ng/mL) for 20 min, followed by incubation with a 1:10 ratio of *Streptococcus pneumoniae* (serotype 3) for 90 min. No difference in bacterial numbers was observed in cells stimulated with any of these chemokine ligands compared to the bacteria-alone group, thus indicating that activation of these CRs do not interfere with bactericidal activity of neutrophils (data not shown).

Influenza Infection of Lungs Leads to Excessive Neutrophil Influx and Widespread Pulmonary Damage

We performed histopathologic analysis to test for a correlation between neutrophilic inflammation and pathologic lesions between 3 and 5 dpi. Neutrophil-influx was significant and comparable between 3 and 5 dpi, while the changes in neutrophil phenotypic support increase in pathologic lesions with augmented alveolar injury, vascular damage and bronchiolitis (**Figures 6A,B**). Our studies indicate a significant increase in lung pathology. BAL fluid cell counts performed on days 3–5 dpi also displayed an increase in total cell numbers. In support of this, we found significant vascular leakage from 3 to 5 dpi (**Figure 6C**). Further, we did not find any bacterial growth from the BAL samples from influenza-infected mice (data not shown), indicating that neutrophil inflammation or induction of CR are not due to bacterial superinfection.

DISCUSSION

Newly emerging and re-emerging influenza virus infections remain a continuous threat worldwide. Influenza infections trigger hyper-inflammatory cytokine responses together with

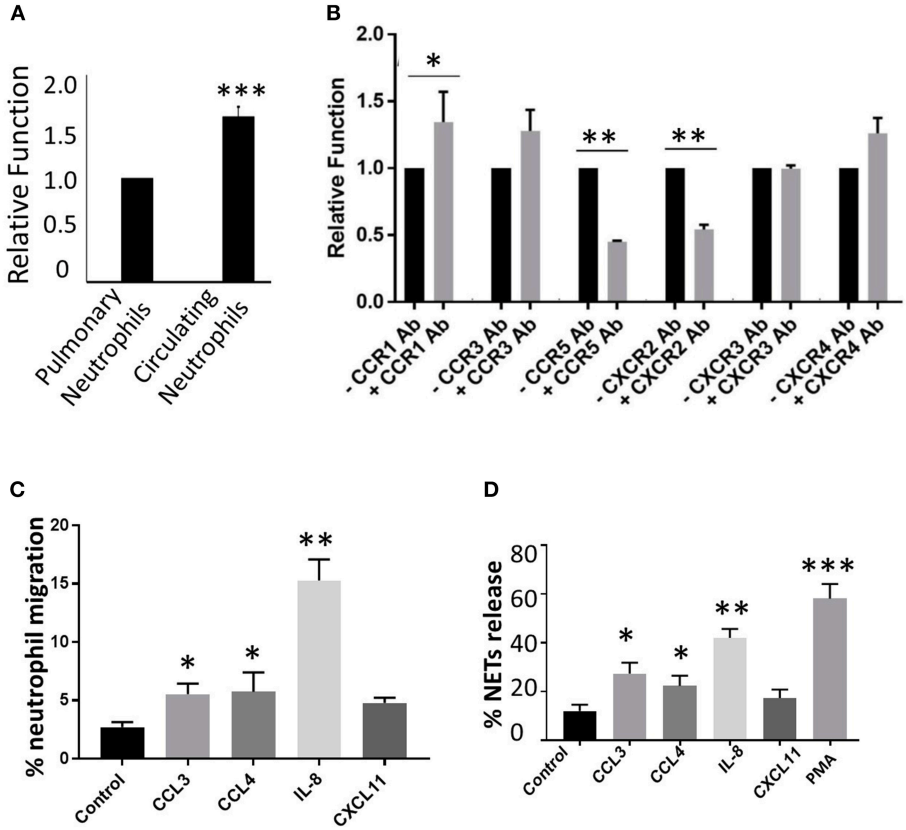

FIGURE 5 | Effect of CR induction on neutrophil phagocytic activity. To test overall phagocytic activity between circulating and lung-recruited neutrophils, we isolated neutrophils from infected blood and BAL, and tested their phagocytic activity. **(A)** Neutrophils isolated from BAL exhibited diminished phagocytic capacity compared with those in circulation. **(B)** Phagocytic activity using CCR1, CCR3, CCR5, CXCR2, CXCR3, and CXCR4 blockers and ligands. BX 471 (CCR1 antagonist), SB328437 (CCR3 antagonist), anti-CCR5, anti-CXCR2, CXCR3 blocking antibodies, and AMD3100 (CXCR4 antagonist) were added and incubated for 30 min at 37°C. The cells were then stimulated with the appropriate ligands CCL3 (CCR1), CCL11 (CCR3), CCL4 (CCR5), IL-8 (CXCR2), CXCL11 (CXCR3), and CXCL12 (CXCR4). Blocking CCR5 and CXCR2 reduced phagocytic capacity of pulmonary infiltrating neutrophils in influenza viral infection, while no change was observed with CXCR3 blockade. In contrast, CCR1 inhibition resulted in enhanced phagocytic activity. **(C)** Neutrophil chemotaxis assay was performed by stimulating neutrophils isolated from infected BAL using CCL3 (CCR1), CCL4 (CCR5), IL-8 (CXCR2), CXCL11 (CXCR3). Data are represented as percentage migration. **(D)** NETosis was evaluated by stimulating neutrophils isolated from infected BAL using CCL3 (CCR1), CCL4 (CCR5), IL-8 (CXCR2), CXCL11 (CXCR3). Data are represented as percentage of NETs formation. Mean ± SEM. $n = 3$ independent experiments, and cells were collected by pooling two mice in each experiment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

rapid, massive cellular influx, predominantly by neutrophils, and macrophages (de Jong et al., 2006; Perrone et al., 2008; Taubenberger and Morens, 2008). We have previously shown that overly exuberant neutrophils produce NETs and extracellular histones which disrupt the alveolar-capillary barrier, resulting in alveolar injury and vascular leakage (Narasaraju et al., 2011; Ashar et al., 2018). Neutrophils are short-lived and terminally differentiated innate immune cells with primary roles in phagocytic clearance of influenza-infected cells. Although exaggerated neutrophil recruitment and their activation are linked to acute lung pathology during influenza, little is known about their phenotypic or functional characteristics (Kobasa et al., 2004; Tumpey et al., 2005; Taubenberger and Morens, 2008; Yokoyama et al., 2010). Here, we provide evidence that lung-recruited neutrophils expand their CR repertoire during influenza infection of lungs. Lung-sequestered neutrophils displayed up-regulation of several CRs (such as CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR3, and CXCR4) that are minimally expressed or absent while in circulation. The surface induction of these CRs increased in a time-dependent manner in pulmonary-recruited neutrophils. Furthermore, induced CRs in lung-recruited neutrophils potentially modulate neutrophil functions, including chemotaxis, phagocytosis, and NETosis. These results indicate that the infected-lung microenvironment significantly affects neutrophil phenotypic signature and their functional responsiveness, and these changes could considerably impact the disease pathogenesis in influenza pneumonia.

Neutrophils conventionally express CXC chemokine receptors, while CC chemokine receptors are generally absent and unresponsive to CC chemokine ligand stimulations. However, studies have shown that neutrophils isolated from lungs or synovial cavities from patients with chronic obstructive pulmonary disease (COPD), rheumatoid arthritis or sepsis (Speyer et al., 2004; Hartl et al., 2008; Chou et al., 2010; Lebre

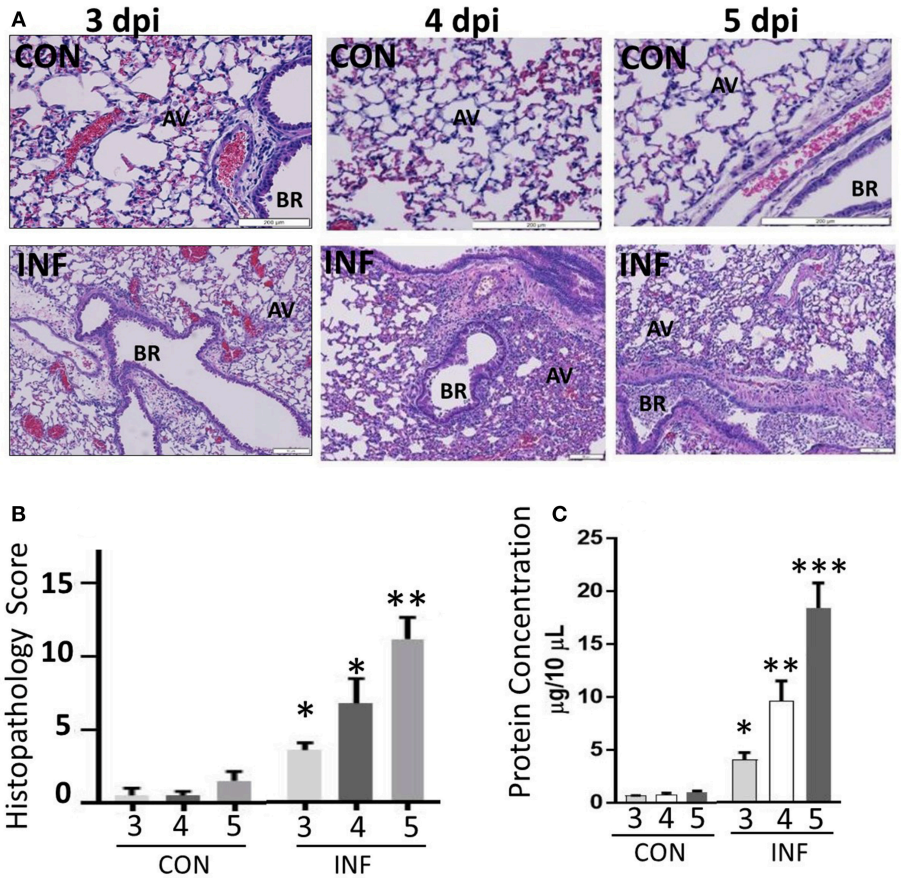

FIGURE 6 | Influenza infection increases inflammation and severe pulmonary pathology. **(A)** Paraffin-embedded lung tissues from 3 to 5 days following challenge with infection or mock infection were stained with hematoxylin and eosin. Infected lungs displayed the highest severity score with notable pulmonary edema, bronchiolitis, alveolitis, hemorrhage, and interstitial disease. **(B)** Total histopathologic scores of infected samples were compared with controls. Data were expressed as means ± SEM. $n = 4$ mice per group. **(C)** Vascular leakage was determined by measuring total proteins present in the BAL fluid samples collected from control and influenza-infected mice at 3, 4, and 5 dpi. AV, alveoli; BR, bronchioles. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

et al., 2011) display induced expression of CC CRs, and that pro-inflammatory cytokines including IFN-γ, TNF-α, and GM-CSF modulate expression of these CRs. Further, the induced CRs alter neutrophil functions, including respiratory burst, degranulation, and chemotaxis thus contributing to inflammation and injury (Hartl et al., 2008). Hyper cytokine responses, also termed as the "cytokine storm" are associated with pulmonary pathology in fatal influenza pneumonia (de Jong et al., 2006; Teijaro et al., 2014; Guo and Thomas, 2017). Influenza primarily infects lung epithelial cells and macrophages, which trigger pro-inflammatory cytokines induction and activation of various toll-like receptors (TLRs) and retinoic acid-inducible receptors (RIG-1)-mediated signaling, leading to persistent elevation in cytokines/chemokines in infected-lungs culminating in immunopathology (Wang et al., 2008; Shirey et al., 2013; Iwasaki and Pillai, 2014; Pulendran and Maddur, 2015; Kandasamy et al., 2016). We found early induction of chemokine ligands such as CCL4, CCL7, CCL2, CCLL, CXCL1, CXCL11, CXCL13 in infected mouse lungs (Ivan et al., 2012). Indeed early induction of pro-inflammatory cytokine response is detrimental in severe influenza pathogenesis (Perrone et al., 2008). Despite evidence demonstrating extensive cytokine induction in severe influenza pneumonia, little is known whether these secreted cytokines regulate induction of CRs in lung-infiltrated neutrophils. It is noteworthy that although neutrophils numbers peaked by 3 dpi, the induction of new CRs appeared up-regulated only from 4 dpi, indicating that induction of these CRs occurs under "cytokine/chemokine stress," which could be critical in shaping the phenotype and functionality of neutrophils in the influenza-infected lung microenvironment.

Among CXC CRs, CXCR2 is the most abundantly expressed in circulating and lung-recruited neutrophils. The surface expression of CXCR2 is regulated by at least two mechanisms, including the receptor internalization/recycling or metalloprotease activity by a disintegrin and metalloproteinase domain-containing protein 17 (ADAM-17) (Mishra et al., 2014). Interestingly, percent neutrophils expressing CXCR2 was decreased in lung-recruited neutrophils at 3 dpi, compared to

circulating neutrophils. However, CXCR2 surface expression increased significantly between 3 and 5 dpi. Similar to these findings, reduction in CXCR2-positive neutrophils is observed in patients with chronic inflammatory conditions (Hartl et al., 2008). The changes in surface expression of CXCR2 in blood and lung-recruited neutrophils may be attributed to receptor internalization and recycling upon increase in ligand-specific interaction in acute influenza infection (Mishra et al., 2014). Persistent increase in neutrophil influx and CXCR2 surface expression indicate that targeting CXCR2 could alleviate excessive neutrophils influx and lung pathology. Indeed, blocking CXCR2 has shown to reduce acute lung injury and inflammation in influenza-infected mice (Tavares et al., 2017) and mice lacking CXCR2 gene have shown decreased inflammation, without affecting viral clearance indicating pathogenic role of neutrophils in severe influenza (Wareing et al., 2007).

The functional significance of the induced CRs was investigated through chemokine-specific ligand activation and/or antibody blocking for neutrophil phagocytosis, chemotaxis, bacterial killing, and NETosis. Our studies revealed highly variable responses to different chemokines that are upregulated during infection. Blocking CCR5 and CXCR2 resulted in reduced phagocytic activity compared to the ligand-mediated stimulation, whereas CCR1 blockade augmented phagocytic activity. Blocking CCR3, CXCR1, CXCR3, and CXCR4 did not modify phagocytic activity. These findings are in partial agreement with previous findings of LPS-injury models, chronic inflammatory diseases in humans, which show that induced CC CRs (such as CCR1, CCR2, CCR3, CCR5) enhance phagocytic activity, and respiratory burst functions (Hartl et al., 2008; Wang et al., 2011). In contrast to the earlier reports, blocking CCR1 enhanced phagocytic activity. However, stimulation of CCR1 enhanced NETosis and chemotaxis, thus indicating that induced CRs exhibit differential functional responsiveness during influenza. Based on the phagocytic functions of different CRs, we tested the effects of induced CCR1, CCR5, CXCR2, and CXCR3 on neutrophil functions including chemotaxis, bacterial killing, and NETosis. Although CXCR2 is a critical CR that regulates neutrophil chemotaxis, and NETosis, induction of CCR5 and CCR1 also significantly impacted these neutrophil functions. Mice deficient of CCR5 gene are more susceptible to influenza infection and exhibit greater neutrophil influx compared to wild-type mice. However, deletion of CXCR3 together with CCR5 ($CCR5^{-/-}$/$CXCR3^{-/-}$) does not alter neutrophil influx. These findings are congruent with our findings that stimulation of CXCR3 does not significantly alter neutrophil migration or NETosis (Fadel et al., 2008).

Interestingly, none of the induced CRs show potential bactericidal effects, when we incubated neutrophils in the presence of *Streptococcus pneumoniae*, which is one of the commonest pathogens causing co-infections during influenza outbreaks (Kash et al., 2011; Moorthy et al., 2016). These results validate our earlier findings that neutrophils from influenza-infected mice lack bactericidal effects. It is noteworthy that close proximity of induced chemokine ligands with enhanced CRs have high probability to augment phagocytic function. However, the lack of *in vivo* bacterial killing suggests that lung-recruited neutrophils may engulf bacteria, but may be defective in bactericidal activity, which was evident from a report demonstrating increased neutrophils containing labeled bacteria (Ishikawa et al., 2016), but fail to kill the pathogen. The lack of bactericidal activity may also be attributed to impaired free radical generation. Influenza infection has also shown to impair NADPH oxidase activity (Sun and Metzger, 2014). A study has shown that seasonal and pandemic influenza viruses differentially regulate neutrophil respiratory burst and phagocytosis (Malachowa et al., 2018). The ability of influenza virus to impair phagocytic function may be due to the inhibition of azurophilic granules with the lysosomes during phagocytosis, thus preventing bacterial killing (Abramson et al., 1982). These findings support our earlier studies showing that massive neutrophil influx during influenza does not reduce bacterial loads. On the other hand, alveolar-capillary injury inflicted by NETs and extracellular histones may facilitate bacterial adhesion and growth, and thus exacerbate pulmonary pathology. Another study has shown that neutrophils also limit pulmonary pathology by suppressing T-cell mediated damage during influenza (Tak et al., 2018). The contribution of neutrophils to protection or injury may ultimately be dependent upon the neutrophil numbers and inflammatory cytokine responses. The virulence of the influenza virus strain may also influence the neutrophil functionality and pathogenesis, which warrants further investigations into the effects of different viral strains of varying pathogenicity on the cytokine storm and neutrophil phenotypic changes. These studies attempted to characterize and compare circulating as well as lung-recruited neutrophils. It would thus be interesting to evaluate neutrophils that are present within the lung parenchyma of infected lungs to determine if induction of CRs is also modified during transmigration of neutrophils from the circulation into the alveolar air space.

In conclusion, this study indicates an induction of CRs occurs upon neutrophil extravasation and activation into the pulmonary environment in a murine model of influenza pneumonia. These induced CRs could serve as potential therapeutic targets for alleviating neutrophil-induced lung pathology. Among all CRs, CXCR2 is most highly induced, and represents a promising target for therapy to reduce neutrophil recruitment to the area of inflammation. The functional properties of these individual chemokine receptors warrant further investigation to further understand how these induced CRs impact deleterious or beneficial effects of neutrophils as well as their roles in the context of influenza-induced acute lung injury.

ETHICS STATEMENT

IACUC, Oklahoma State University. Protocol No: VM-17-32.

AUTHOR CONTRIBUTIONS

JMR, SP, HA, VC, and TN: conception and design of experiments; JMR, SP, HA, JWR, MM, and TN: acquisition and analysis of data; JRM, VC, and TN: critical review; TS: histopathology analysis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Virus Control of Cell Metabolism for Replication and Evasion of Host Immune Responses

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Over the last decade, there has been significant advances in the understanding of the cross-talk between metabolism and immune responses. It is now evident that immune cell effector function strongly depends on the metabolic pathway in which cells are engaged in at a particular point in time, the activation conditions, and the cell microenvironment. It is also clear that some metabolic intermediates have signaling as well as effector properties and, hence, topics such as immunometabolism, metabolic reprograming, and metabolic symbiosis (among others) have emerged. Viruses completely rely on their host's cell energy and molecular machinery to enter, multiply, and exit for a new round of infection. This review explores how viruses mimic, exploit or interfere with host cell metabolic pathways and how, in doing so, they may evade immune responses. It offers a brief outline of key metabolic pathways, mitochondrial function and metabolism-related signaling pathways, followed by examples of the mechanisms by which several viral proteins regulate host cell metabolic activity.

Keywords: viruses, cell metabolism, mitochondria, immune response, viral evasion

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INTRODUCTION

Several recent comprehensive reviews have highlighted the key role of eukaryotic cell metabolism in immunity (Ganeshan and Chawla, 2014; O'Neill and Pearce, 2016; O'Neill et al., 2016). Six main and interconnected metabolic pathways have a role in the immune response: glycolysis; the pentose phosphate pathway (PPP); the tricarboxylic acid cycle (TCA), also known as Krebs cycle; the fatty acid oxidation (FAO), also known as β-oxidation; as well as the fatty acid and amino acid synthesis pathways (**Figure 1**).

Mitochondria take central stage in cellular metabolism since TCA, FAO, oxidative phosphorylation (OXPHOS), calcium buffering, and heme biosynthesis take place within this organelle (Mishra and Chan, 2016).

Energetic and biosynthetic metabolism is fueled by carbon sources, including glucose and glutamine (DeBerardinis and Cheng, 2010), which are taken up by the cells by glucose and glutamine transporters, respectively (Bhutia and Ganapathy, 2016; Navale and Paranjape, 2016).

Once in the cytosol, glucose is converted to pyruvate, via glycolysis, yielding two molecules of ATP and two molecules of NADH (which acts as a cofactor in several enzymatic reactions) per unit of glucose. The glycolysis pathway is also the source of biosynthetic intermediates

that serve the purpose of ribose and nucleotides synthesis (glucose-6-phosphate into ribulose 5-phosphate), amino acids (3-phosphoglycerate enters the serine biosynthetic pathway), and fatty acids (by the sequential conversion of glycolysis-derived pyruvate into the TCA intermediate citrate that may be exported from the mitochondria to the cytosol, where it is converted into acetyl-coA).

Glycolysis-derived pyruvate is either converted to lactate, which is exported out of the cells, or converted into acetyl-CoA that enters the TCA cycle through the aldol condensation with oxaloacetate to form citrate (O'Neill et al., 2016). Citrate is then sequentially converted to isocitrate, α-ketoglutarate, succinyl CoA, succinate, fumarate, malate, and oxaloacetate, which starts a new round of the TCA cycle by its reaction with pyruvate-derived acetyl CoA. Fatty acids can also be converted into acetyl CoA through FAO, linking this metabolic pathway with the TCA cycle. Two major products of both the TCA cycle and FAO are NADH and FADH2, which can transfer electrons to the mitochondrial electron transport chain coupled with OXPHOS and the generation of ATP (O'Neill et al., 2016). In addition, succinate, an intermediate in the TCA cycle, is also an electron donor for the mitochondrial respiratory chain at complex II (succinate dehydrogenase) (Rich and Maréchal, 2010).

The pentose phosphate pathway involves a non-oxidative as well as an oxidative branch; the first allows for the diversion from glycolysis intermediates toward the synthesis of nucleotide and amino acid precursors, while the second generates reducing equivalents of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), which maintain a favorable cellular redox environment and allows fatty acid synthesis (O'Neill et al., 2016).

Fatty acid synthesis uses glycolysis, TCA cycle, and pentose phosphate pathway metabolic intermediates. TCA cycle-derived citrate may be exported from the mitochondria to the cytosol and then ATP citrate lyase converts citrate to acetyl-coA, which in turn may be carboxylated by acetyl-CoA carboxylase (ACC) producing malonyl-CoA. Furthermore, fatty acid synthase and NADPH elongate fatty acid chains (O'Neill et al., 2016).

Glutamine is also a primary source of energy as it is converted to glutamate and then to α-ketoglutarate, which enters the TCA cycle (DeBerardinis and Cheng, 2010).

Immune system cells preferentially follow one or other metabolic pathway, depending on cell type, differentiation status, activation conditions, and microenvironment. Resting T lymphocytes rely mostly on OXPHOS, whereas activated and proliferating T lymphocytes upregulate the expression of the glucose transporter glut-1 and key glycolytic enzymes, relying mostly on glycolysis (Frauwirth et al., 2002; Pearce and Pearce, 2013).

Memory T lymphocytes use OXPHOS (Pearce and Pearce, 2013), "classically activated" macrophages (stimulated with LPS plus IFN-γ)—also referred to as M1 macrophages—engage in glycolysis, whereas alternatively activated macrophages (stimulated with IL-4)—also referred to as M2 macrophages—use OXPHOS and FAO to generate energy (Rodríguez-Prados et al., 2010). Stimulated macrophages and dendritic cells engage in glycolysis after activation through pattern recognition receptors (PRRs) (O'Neill and Pearce, 2016).

Neutrophils rely mostly on glycolysis (Pearce and Pearce, 2013) and the release of neutrophil extracellular traps (NETs) is dependent on the increase in cell membrane glut-1, glucose uptake, and the glycolytic rate (Rodriguez-Espinosa et al., 2015).

Activated B lymphocytes undergo metabolic reprogramming in response to changing energetic and biosynthetic demands, and long-lived plasma cells uptake glucose and glutamine at a higher rate; glucose is used to generate pyruvate for spare respiratory capacity, and glutamine is used as a carbon source for

Abbreviations: ACC, Acetyil-CoA carboxylase; Akt, Akt/Protein kinase B; AMP, Adenosine monophosphate; AMPK, Adenosine monophosphate-activated protein kinase; ATP, Adenosine three phosphate; 2B, 2B protein; 2BC, 2BC protein; ANT3, Adenine nucleotide translocator 3; ATLL, Adult T-cell leukemia/lymphoma; BALF1, BamH1-A left frame transcript; BHRF1, BamH1-Hright reading frame; BZLF1, Zebra protein; cGAS, cyclic guanosin monophosphate-adenosin monophosphate synthase; cGMP, cyclic guanosine monophosphate; CoA, Coenzyme A; CTL, Cytotoxic T lymphocytes; COXIII, Cytochrome c oxidase III; $\Delta\psi m$, Mitochondrial membrane potential; Drp1, Dynamin-related protein; dTTP, Deoxythymidine triphosphate; early Zta, early Zta protein; Env, Envelope; ER, Endoplasmic reticulum; FADH2, reduced Flavin adenin dinucleotide; FAO, Fatty acid oxidation; FHV, Flock house virus; F17, F17 protein; Grb10, Growth factor receptor bound protein 10; HBx, Hepatitis B virus x protein; HIF1α, Hypoxia-induced factor 1α; HPV 18, Human papillomavirus 18; KSHV, Kaposi's sarcoma-associated herpesvirus; HSP60, Heat shock protein 60; IFI6-16, Interferon inducible protein 6-16; IFNs, Interferons; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IKK, IκB kinase; IL-4, Interleukin-4; IP3Rs, Inositol 1,4,5-triphosphate receptors; IRF3, Interferon regulatory factor 3; ISGs, Interferon-stimulator genes; JAK-STAT, Janus kinase-Signal transductor and activator of transcription; Lag-3, Lymphocyte activation gene-3; LANA, Latency-associated nuclear antigen; LMP2A, Latent membrane protein 2A; LPS, Lypopolysacharide; M1, Macrophage type1; M2, Macrophage type 2; MAMs, Mitochondria-associated membranes; MAVS, Mitochondrial antiviral-signaling protein; MCU, Mitochondrial calcium uniporter; MDA-5, Melanoma differentiation-associated gene 5; Mfn1, Mitofusin 1; MHV68γHV68, Murine gammaherpesvirus-68; mTORC1, mechanistic target of rapamycin complex 1; mTORC2, mechanistic target of rapamycin complex 2; mtSSB, Mitochondrial single-stranded DNA binding protein; NADPH, reduced Nicotinamide adenine dinucleotide phosphate; Nef, Nuclear elongation factor; NS, Non-structural Proteins; NETs, Neutrophyl extracellular traps; NFκB, Nuclear factor kappa B; NK, Natural killer; NLR, NOD-like receptor; NOD, Nucleotide-binding and oligomerization domain; NS1, Non-structural protein 1; NS2b3, Non-structural protein 2b3; OMM, outer mitochondrial membrane; OPA1, Optic atrophy protein 1; ORF52, Open reading frame 52; OXPHOS, Oxidative phosphorylation; PB1-F2, PB1-F2 protein; PB1-F2 66S, PB1-F2 protein, serine 66 PB1-F2 66N, PB1-F2 protein, asparagine 66; PB2, PB2 protein; PD-1, Programmed death-1; PFK, Phosphofructokinase; PGC-1a, Peroxisome proliferator-activated receptor-gamma coactivator-1alpha; PI3K, phosphatidylinositol 3-kinase; PLC, Phospholipase C; PMA, Phorbol 12-myristate 13-acetate; PPP, pentose phosphate pathway; PRRs, Pattern recognition receptors; p7, protein 7; p13, protein 13; P70S6K, Ribosomal protein S6 kinase beta-1; RIG-1, Retinoic acid-induced gene 1; RLR, RIG-1-like Receptor; ROS, Reactive Oxygen Species; SPCA 1, Secretory pathway calcium ATPase 1; STING, Stimulator of interferon genes; TBK1, TANK binding kinase 1; TCA, Tricarboxylic acid; Tim-3, T cell immunoglobulin mucin-3; TLRs, Toll like receptors; TTP, thymidine triphosphate; UL 12, full length UL 12 protein; UL 12. 5, N-terminally truncated UL 12 protein; UPR, Unfolded protein response; UTP, Uridine triphosphate; VV, Vaccinia virus; VDAC3, Voltage dependent anion channel 3;VMC1, Viral mitochondrial carrier 1; vIRF1, viral Interferon regulatory factor 1; vMIA, viral mitochondrial-localized inhibitor of apoptosis. Note: Other viruses abbreviations are indicated in **Tables 1–5**.

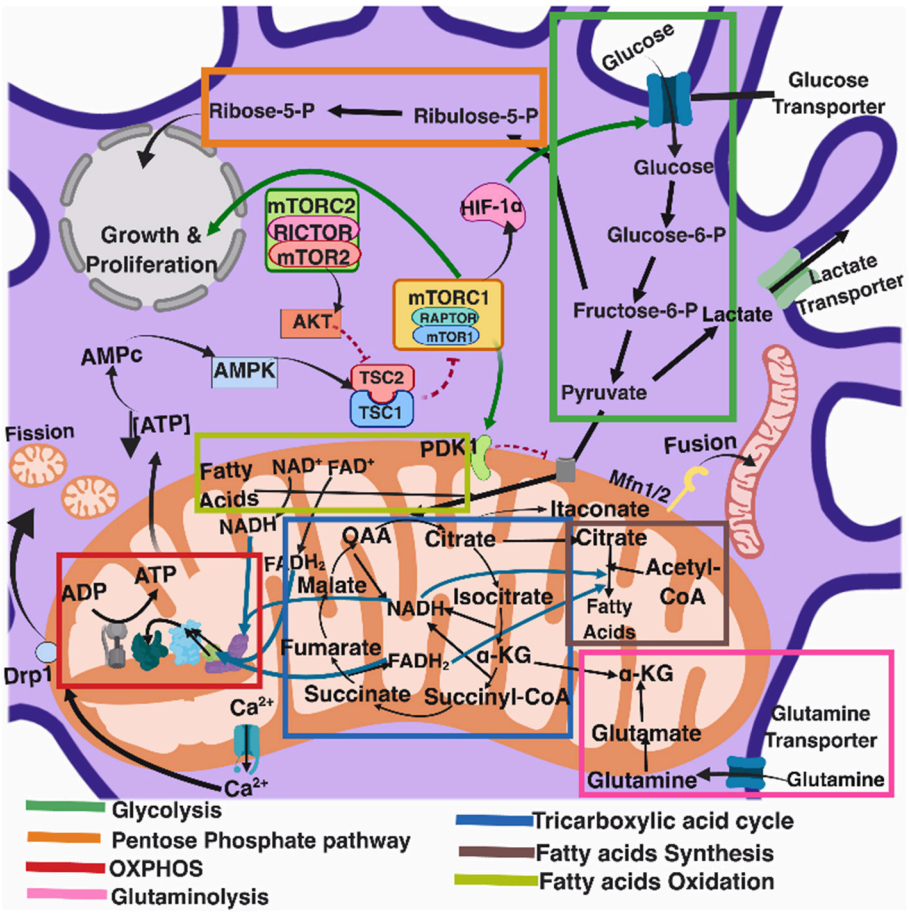

FIGURE 1 | Eukaryotic cell metabolism. Bioenergetic and biosynthetic pathways interconnect glycolysis, glutaminolysis, PPP (pentose phospahate pathway), TCA (tricarboxylic acid cycle), FAO (fatty acid oxidation), fatty acid synthesis, aminoacid synthesis, metabolic sensors such as the AMPK, mTORC1, and mTORC2 pathways, and are also dependent on calcium homeostasis, mitochondrial membrane potential and mitochondrial dynamics. All together they influence cell function and may be the targets of several viruses.

mitochondrial anaplerotic reactions and respiration, promoting cell survival (Jellusova and Rickert, 2016; Lam et al., 2018).

Switching metabolic pathways (metabolic reprograming) leads to changes in cell function (Buck et al., 2017) and the metabolic microenvironment, i.e., tissue O_2 tension, or the concentration of metabolites such as lactate determines cell immune responses (Romero-Garcia et al., 2016).

Interestingly, viral infections such as ocular infection with herpes simplex virus-1 (HSV-1) may change blood glucose levels in the course of infection (Varanasi et al., 2017). Moreover, if glucose utilization is pharmacologically limited *in vivo* in the inflammatory phase, lesions diminish but, if glucose utilization is limited in the acute phase of infection when the replicating virus is still present in the eye, infected mice become susceptible to the lethal effects of HSV-1 infection as the virus spreads to the brain, causing encephalitis (Varanasi et al., 2017). This highlights the fundamental relationship between cell metabolism, immune response, and viral pathogenesis.

ANTI-VIRAL IMMUNE RESPONSES

Among the most effective antiviral immune responses is the production of several type I interferons (**Figure 2**); interferon-α (IFN-α) subtypes and interferon-β (IFN-β), which along with IFN-ε, IFN-τ, IFN-κ, IFN-ω, IFN-δ, and IFN-ζ, are collectively referred to as type I interferons; most cells can produce IFN-α and IFN-β following cell activation through the recognition of viral nucleic acids (McNab et al., 2015).

The RIG-I-MDA5-mitochondrial antiviral-signaling protein (MAVS) axis is the major sensing pathway for RNA viruses, while the axis composed of the cyclic guanosine monophosphate (cGMP)-adenosine monophosphate (AMP) synthase (cGAS) and the stimulator of interferon genes (STING) is the major sensing pathway for DNA viruses (Wu and Chen, 2014). However, there is recent evidence that the cGAS-STING pathway may also restrict the infection by RNA viruses, thus suggesting a connection between the sensing of cytosolic DNA and RNA (Ni et al., 2018).

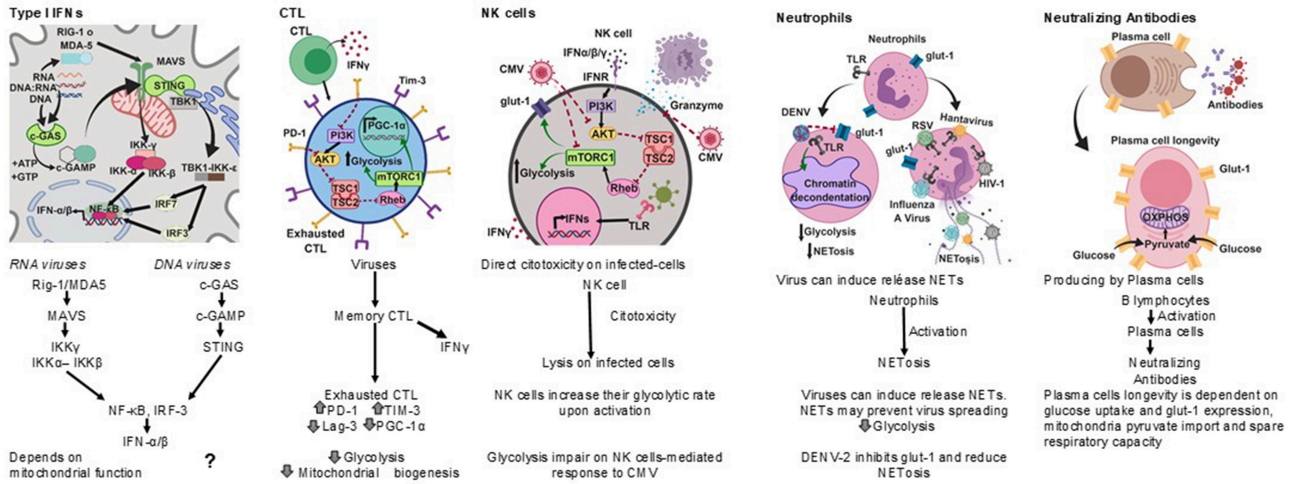

FIGURE 2 | Antiviral immune responses. Type I interferons, cell cytotoxicity, neutrophil extracellular traps and neutralizing antibodies protect against viral infections, each type of response has a metabolic hallmark. Viruses may target specific metabolic pathways for immune evasion.[?], not known.

Both anti-viral pathways converge in the activation of two main transcription factors that regulate the expression of type-I interferons, nuclear factor kappa B (NFκB) and interferon regulatory factor 3 (IRF3). In the case of the RIG-I-MDA5-MAVS pathway, their activation depends on mitochondrial function (Seth et al., 2005; Koshiba, 2013).

Both IFN-α and IFN-β activate the expression of interferon-stimulated genes (ISGs) through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, leading to the inhibition of viral replication and assembly (Darnell et al., 1994; Seth et al., 2005).

Cytotoxic T lymphocytes (CTL) play an important role in the clearance of viral infections (**Figure 2**); memory CTL can be activated by low concentrations of antigen, readily producing cytokines and the lysis of infected cells, thus preventing dissemination (Veiga-Fernandes et al., 2000).

Upon acute viral infection, virus-specific memory CTL quickly produce IFN-γ. However, around 18 h after infection the number of IFN-γ producing CTL drops concomitantly with the upregulation of inhibitory receptors. It has been suggested that the decrease in the synthesis of IFN-γ by CTL is an active regulatory process (Hosking et al., 2013) reminiscent of T cell exhaustion, a process also known to take place during chronic viral infections (Yi et al., 2010; Wherry, 2011).

A hallmark of T cell exhaustion is the upregulation of inhibitory receptors such as programmed death-1 (PD-1), T cell immunoglobulin mucin-3 (Tim-3), and lymphocyte activation gene-3 (Lag-3) (Freeman et al., 2000; Barber et al., 2006). Interestingly, PD-1 negatively regulates glycolysis, represses the transcriptional co-activator peroxisome proliferator-activated receptor-gamma co-activator (PGC)-1alpha (PGC-1α), which plays an important role in the regulation of carbohydrate and lipid metabolism, and impairs CTL responses (Bengsch et al., 2016).

Other anti-viral cell-mediated immune responses include NK cell cytotoxicity (Hammer et al., 2018) and neutrophil extracellular traps (NETs) (Schönrich and Raftery, 2016) (**Figure 2**).

Natural killer (NK) cells have anti-viral activities as they exert direct cytotoxicity on virus infected-cells, and readily produce IFN-γ. NK cells increase their glycolytic rate upon activation (Gardiner and Finlay, 2017), and disruption of glycolysis impairs NK cell-mediated responses to Cytomegalovirus (CMV), for instance (Mah et al., 2017).

Neutrophils are considered a first line of defense against pathogens. However, their role in the control of viral infections is not as clear as for other pathogens (Galani and Andreakos, 2015). It has recently been recognized that viruses can induce the release of neutrophil extracellular traps (NETs), and the mechanisms by which NETs could contribute to anti-viral immunity are emerging (Hammer et al., 2018).

Several viruses, including Hantaan virus (HTNV), H1N1 Influenza A virus (IAV), human immunodeficiency virus (HIV-1), and Respiratory Syncytial virus (RSV), directly stimulate neutrophils to release NETs (Raftery et al., 2014; Delgado-Rizo et al., 2017), and both IFN-α and IFN-γ can prime mature neutrophils to release NETs upon further stimulation (Martinelli et al., 2004; Hammer et al., 2018).

HIV-1 may also prevent the release of NETs by inducing dendritic cells to produce IL-10, which in turn suppresses the reactive oxygen species (ROS)-dependent release of NETs (Saitoh et al., 2012; Hammer et al., 2018).

The Dengue virus serotype-2 (DENV-2) down-modulates the phorbol 12- myristate 13- acetate-(PMA) induced release of NETs, and it has been proposed that one of the mechanisms for this is the interference with the mobilization of the glucose transporter glut-1 to the cell membrane and consequently with the glucose uptake (Moreno-Altamirano et al., 2015).

NETs may prevent virus spreading by being trapped by electrostatic attraction or be inactivated by molecules associated with NETs, such as myeloperoxidase and α-defensins (Saitoh et al., 2012; Hammer et al., 2018).

Antibodies are also important anti-viral effectors (**Figure 2**), and whereas cytotoxic lymphocytes can eliminate infected cells, antibodies are capable of both eliminating infected cells and neutralizing viruses, thereby preventing cell infection. The production of protective antibodies over prolonged periods constitutes a first line of defense against reinfection and, therefore, survival of antibody-producing plasma cells is determinant (Dörner and Radbruch, 2007). It is now known that plasma cell longevity is dependent on enhanced glut-1 expression and glucose uptake, mitochondria pyruvate import and spare respiratory capacity, and that nutrient uptake and catabolism distinguish plasma cell subsets with different lifespans and rates of secreted antibodies (Lam et al., 2016, 2018).

Taken together, it emerges that the activity of immune system cells is dependent on cell metabolism and that viruses could target cell metabolism to evade anti-viral immune responses. The next sections explore some specific mechanisms by which viruses may interfere with cell metabolism.

MITOCHONDRIAL ANTI-VIRAL SIGNALING (MAVS) AND VIRUS SUBVERSION OF MAVS

Mitochondria constitute a metabolic hub, so if a virus is to take control of host metabolism, targeting mitochondria is perhaps the best way.

In 2005 Seth et al. reported the identification of a new protein essential for the activation of the transcription factors NFκB and IRF3 by RNA viruses. They named the protein MAVS and showed that this contains a C-terminal transmembrane domain that targets the mitochondrial outer membrane. Strikingly, they found that this transmembrane domain and the targeting to mitochondria are essential for MAVS signaling, opening a new avenue of research in which mitochondria took center stage in antiviral immunity (Seth et al., 2005).

In non-stimulated cells, NFκB is located in the cytoplasm, associated with its inhibitor IκBα. Upon stimulation with viruses, other pathogens or cytokines, the IκB kinase (IKK) is activated, leading to the phosphorylation of IκBα and its subsequent ubiquitination and proteasomal degradation. NFκB is then released and translocated to the nucleus, where it activates immune and inflammatory genes (Silverman and Maniatis, 2001; Seth et al., 2005).

IRF3 is located in the cytoplasm of non-stimulated cells, and following viral or other pathogen infection it becomes phosphorylated by TANK-binding kinase 1 (TBK1) and IKK kinases, allowing the formation of homodimers that can translocate into the nucleus and activate the synthesis of IFN-β, acting in synergy with NFκB (Yoneyama et al., 2002; Fitzgerald et al., 2003; Hiscott et al., 2003; Seth et al., 2005).

IRF7 can also be phosphorylated by TBK1 and IKK (tenOever et al., 2004), leading to the production of interferon-α (Honda et al., 2005; Seth et al., 2005). NFκB and IRFs are activated by RNA viruses as well as by other pathogens.

The entry of RNA viruses to the cells produces double-stranded RNA intermediates, which can be recognized by host cell pathogen recognition receptors (PRRs) including TLR -3, -7, -8, and -9 (Akira and Takeda, 2004; Seth et al., 2005).

The receptor Retinoic Acid-Induced Gene I (RIG-1) recognizes intracellular dsRNA and the interaction of viral RNA with RIG-1 leads to a change in its conformation, which then activates NFκB and IRF3 (Yoneyama et al., 2002; Sumpter et al., 2005).

The melanoma differentiation-associated gene 5 (MDA5) is a RIG-I-like protein involved in dsRNA signaling and apoptosis (Kovacsovics et al., 2002; Seth et al., 2005).

In 2011, Koshiba (Koshiba, 2013) demonstrated that mitochondrial fusion and mitochondrial membrane potential ($\Delta\psi_m$) are required for MAVS-mediated signaling. They showed that the deletion-targeting of mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), two molecules involved in mitochondrial fusion, prevented cells from producing interferons and pro-inflammatory cytokines in response to viral infection. This resulted in increased viral replication along with a reduced $\Delta\psi_m$, correlating with a reduced antiviral response. Interestingly, the reduction in $\Delta\psi_m$ did not affect the activation of IRF3, which acts downstream of MAVS, suggesting that $\Delta\psi_m$ and MAVS are coupled at the same stage in the RIG-1-like Receptor (RLR) signaling pathway (Koshiba, 2013).

In addition to mitochondria, MAVS are also found in peroxisomes and mitochondrial-associated membranes (MAMs) (Seth et al., 2005; Vazquez and Horner, 2015).

A natural target for the subversion of IFN type I-mediated antiviral response is the MAVS protein (**Table 1**). As an example, the influenza A virus encodes a protein called PB1-F2, which inhibits the induction of type I interferon at the level of the MAVS (Varga et al., 2012). PB1-F2 is an 87–90-amino-acid-long protein with a serine at position 66 (66S), which accounted for the virulence of the Spanish and avian flu pandemic viruses (H1N1 and H5N1, respectively). Interestingly, PB1-F2 66S has a higher affinity for MAVS than PB1-F2 66N, and more efficiently affects the $\Delta\psi_m$ than the wild-type PB1-F2 (Conenello et al., 2007).

TABLE 1 | Viruses that subvert MAVS.

Virus	Viral proteins	Effect	References
Influenza A virus (IAV)	PB1-F2	Inhibition of type I IFN at the level of MAVS	Conenello et al., 2007
Influenza A virus H1N1(1918) and H5N1	PB1-F2 66S, PB1-F2 66N	Disruption of mitochondrial membrane potential and type I IFN response	Conenello et al., 2007
Hepatitis C virus (HCV)	NS3/4A	Inhibition of type I IFN response by cleaving of MAVS	Meylan et al., 2005

Other viruses, such as hepatitis C virus (HCV), induce the cleavage of MAVS from the outer membrane of mitochondria, reducing the interferon-producing response. In this case, the NS3/4A protein cleaves MAVS at cysteine 508 (Meylan et al., 2005; Bender et al., 2015; Vazquez and Horner, 2015).

Another family of pattern recognition receptors contain a nucleotide-binding and oligomerization domain (NOD) and is called the NLR (NOD-like receptor) family. NOD2 facilitates the activation of IRF3 and the synthesis of type I IFN in response to single-stranded RNA. Interestingly, the activation of NOD2 is dependent on MAVS (Sabbah et al., 2009; Moreira and Zamboni, 2012).

Recently, NLRX1 (also known as NOD5, NOD9, or NOD26), a member of the NLR family that localizes to the outer mitochondrial membrane, was shown to mediate MAVS degradation, allowing HCV to evade type I IFN-mediated antiviral response (Qin et al., 2017).

cGAS-STING ANTI-VIRAL PATHWAY AND ITS SUBVERSION BY VIRUSES

The cyclic guanosine monophosphate (cGMP)-adenosine monophosphate (AMP) synthase (cGAS) recognizes viral as well as bacterial double-stranded DNA (dsDNA) (Wu and Chen, 2014; Ni et al., 2018). After binding to dsDNA, cGAS catalyzes the synthesis of the second messenger cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), which then binds to the stimulator of interferon genes (STING) adaptor protein on the endoplasmic reticulum (ER); STING, as a dimer, translocates from the endoplasmic reticulum to the Golgi complex, where it recruits TANK-binding kinase 1 (TBK1) which activates the transcription factors NFκB and IRF3, both of which translocate to the nucleus and induce the synthesis of type I interferons (Barber, 2015; Ni et al., 2018).

While the activation of the RIG-1-MDA5-MAVS antiviral signaling pathway clearly requires mitochondrial activity, in the form of mitochondrial dynamics and $\Delta\psi_m$, a metabolism-related component in the cGAS-STING antiviral signaling pathway has not been explicitly identified. However, several lines of research suggest crosstalk between cGAS-STING and metabolism. Firstly, the ER has been regarded as a separate metabolic compartment on the basis that the ER luminal micro-environment is different from the cytosol, that it contains its own pool of pyridine nucleotides, and that several metabolic pathways related to carbohydrate and steroid metabolism, biotransformation, and protein processing take place in the ER (Csalaa et al., 2006); viral infections may lead to ER stress and to the unfolded protein response (UPR) (Zhang and Wang, 2012); and the mitochondrial function in cells undergoing ER stress is compromised, particularly at the level of mitochondrial membrane potential, oxygen consumption, and ATP production (Wang et al., 2011). The ER stress and UPR synergy with the cGAS-STING antiviral signaling pathway still needs to be fully elucidated (Smith, 2014).

Among the DNA viruses that activate the cGAS-STING pathway are herpes simplex virus 1 (HSV-1), vaccinia virus (VV), and murine gamma herpesvirus 68 (MHV68). Interestingly, RNA viruses such as HIV-1 generate RNA: DNA hybrids as well as dsDNA that may activate the cGAS-STING pathway (Ma and Damania, 2016; Ni et al., 2018).

Of note, dengue virus (DENV)-induced mitochondrial damage leads to mitochondrial DNA release to the cytosol, and the activation of the cGAS-STING pathway (Sun et al., 2007). Since other viruses may cause mitochondrial damage (see below) it is plausible that other RNA viruses may activate cGAS-STING through mitochondrial DNA release.

Several DNA virus-associated proteins are known to interfere with the cGAS-STING pathway, as reviewed by Ni et al. (2018), either by interfering with DNA binding to cGAS, as is the case of Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein Barr virus (EBV), and murine gammaherpesvirus-68 (MHV68,γHV68) tegument protein open reading frame 52 (ORF52), and the KSHV latency-associated nuclear antigen (LANA) protein which interact with cGAS (Wu et al., 2015; Zhang et al., 2016), or by targeting STING, as is the case for the HSV-1-infected cell protein 27 (ICP27) and the UL46 protein, the KSHV viral interferon regulatory factor 1 (vIRF1), the human papillomavirus 18 (HPV18) E7 oncoprotein, the human adenovirus 5 (hAd5) E1A oncoprotein, and the Hepatitis B virus (HBV) polymerase (Lau et al., 2015; Liu et al., 2015; Ma et al., 2015; Christensen et al., 2016; Deschamps and Kalamvoki, 2017).

A more recent development in the field is the finding that some RNA viruses are also capable to interfere with the cGAS-STING pathway, subverting its anti-viral effect (Ni et al., 2018).

Finally, it has been shown that single- or double-stranded DNA may attenuate glucose metabolism, leading to ATP depletion and so constitute a metabolic barrier for viral replication. However, the mechanism seems to be dependent on the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) and the activation of mechanistic target of rapamycin complex 1 (mTORC1) (see below), but independent of the cGAS-STING anti-viral pathway (Zheng et al., 2015).

MITOCHONDRIAL PROTEINS OTHER THAN MAVS AS TARGETS OF VIRAL INFECTION

Some viruses encode mitochondrial proteins, which allow them a direct functional intervention on host cells mitochondria (**Table 2**). In this regard, the Acanthamoeba polyphaga mimivirus (APMV), one of the largest known viruses (400 nm in its capside diameter) (La Scola et al., 2003; Monné et al., 2007), encodes a mitochondrial transport protein called VMC1 (viral mitochondrial carrier), whose function is to transport dATP and other nucleotide triphosphates (dTTP, TTP, UTP, and ADP). VMC1 can support the replication of the APMV genome by acquiring additional nucleotide triphosphates from the mitochondrial pool in exchange for cytosolic ADP (Monné et al., 2007). The APMV genome additionally encodes other five putative mitochondrial proteins (Monné et al., 2007).

TABLE 2 | Viruses that target other mitochondrial proteins.

Virus	Viral proteins	Effect	References
Acantthamoeba polyphaga mimivirus (APMV)	Virus mitochondrial carrier 1 (VMC1)	Increase of viral replication by transporting dATP from the motochondrial pool	Monné et al., 2007
Epstein Barr virus (EBV)	BHRF1, BZLF1, BALF1, early Zta	Increase of viral replication, prevention of B cell apoptosis, blockage of mDNA replication	Cavallari et al., 2018
Hepatitis C virus (HCV)	p7, NS3/4A, NS5A	Disruption of mitochondrial function, cleaveage of MAVS	Cavallari et al., 2018
Hepatitis C virus (HCV)	Core	Mitochondria depolarization, increased production of mitochondrial ROS	Cavallari et al., 2018
Influenza virus (IV)	PB1-F2, PB2, NS1	Modulation of viral replication, viral mRNA synthesis	Cavallari et al., 2018
Herpes simplex virus-1 (HSV-1)	UL 12.5	Degradation of mitochondrial DNA early during infection	Cavallari et al., 2018
Herpes simplex virus-1 (HSV-1)	UL 12	Generation of mature viral genomes	Cavallari et al., 2018

The Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma (ATLL) and encodes an 87-amino acid protein (p13) that helps this virus to establish a persistent infection. This protein primarily accumulates in the inner mitochondrial membrane of host cells and alters mitochondrial morphology toward a more rounded shape, fragments mitochondria (mitochondrial fission), and reduces mitochondrial Ca^{2+} uptake (Biasiotto et al., 2010; Cavallari et al., 2018).

Several proteins encoded by Epstein Barr virus (EBV) target mitochondria, such as BHRF1 (BamHI-H right reading frame), BZLF1 (also known as Zebra protein), BALF1 (BamHI-A left frame transcript), LMP2A (Latent membrane protein), and immediate early Zta protein. BHRF1 accumulates in the outer mitochondrial membrane (OMM) of B lymphocytes, preventing apoptosis and promoting survival of EBV-infected cells, viral persistence, and replication; BHRF presents homology with the transmembrane domains of some eukaryotic Bcl-2 family members (Kvansakul et al., 2017; Cavallari et al., 2018); and BZLF1 has the capacity to interact with mtSSB (mitochondrial single-stranded DNA-binding protein), which is required for the replication of the mitochondrial genome, and partially redirects mtSSB from mitochondria to the nucleus (LaJeunesse et al., 2005; Cavallari et al., 2018). BALF1 also shares homology with Bcl-2 family members and modulates apoptosis and promotes transformation (Hsu et al., 2012; Cavallari et al., 2018). LMP2A induces mitochondrial fission by a Drp1-dependent mechanism (Pal et al., 2014; Cavallari et al., 2018), and finally, the immediate early Zta protein can also bind mtSSB in the cytoplasm, inducing its re-location to the nuclei, blocking mitochondrial DNA replication and facilitating viral replication (Wiedmer et al., 2008).

Many other viruses encode mitochondrial proteins capable of regulating a broad spectrum of mitochondrial activities, as reviewed by Cavallari et al. (2018), including the control of intracellular Ca^{2+}, apoptosis, mitochondrial dynamics, the levels of cytochrome c oxidase III (COXIII) and COX activity, as well as cellular ROS production, and the aggregation of mitochondria near the nucleus. Others promote mitophagy and interfere with the antiviral interferon response (Wu et al., 2007; Wang and Ryu, 2010). Proteins such as KS-Bcl-2 localize in mitochondria (Gallo et al., 2017), and others such as the KSHV-encoded K7 protein localize in mitochondria as well as in the ER and nucleus (Feng et al., 2002).

The non-structural proteins p7 of HCV can modify the mitochondrial function. The p7 protein is determinant for the assembly and later release of infectious virions, it is capable to form membrane-associated hexameric ion channels, induces mitochondrial membrane depolarization, and binds to the interferon inducible protein 6–16 (IFI6-16) (Nieva et al., 2012; Madan and Bartenschlager, 2015; Qi et al., 2017); HepG2 cells that express HCV core protein have increased levels of prohibitin, a protein that regulates mitochondrial function and apoptosis (Peng et al., 2015), by reducing the levels of COX subunits I and II. Therefore, the interaction between the HCV core protein and prohibitin may interfere with the assembly of the respiratory chain, which could lead to increased production of mitochondrial ROS and viral replication (Tsutsumi et al., 2009; Ren et al., 2016). Other molecular partners for viral-encoded mitochondrial proteins are voltage-dependent anion channel 3 (VDAC3) (Rahmani et al., 2000), and heat shock protein 60 (HSP60) (Tanaka et al., 2004).

Three influenza virus proteins are known to localize into mitochondria: PB1-F2, PB2, and NS1 (Chen et al., 2001; Yamada et al., 2004; Carr et al., 2006; Tsai et al., 2017). Although PB1-F2 is dispensable for viral replication, at least in some host cells, its expression accelerates influenza virus-induced apoptosis in human monocytes through mitochondrial ANT3 (adenine nucleotide translocator 3) and VDAC1 (voltage dependent anion channel 1) (Chen et al., 2001; Zamarin et al., 2005). The PB2 protein has a key role in viral mRNA synthesis and localizes in mitochondria, where it can regulate the viability of mitochondria during infection (Carr et al., 2006). The NS1 protein is highly expressed in Influenza A virus-infected cells, and predominantly localizes in the nucleus, although it may also be found in the cytoplasm at later stages of infection (Melén et al., 2007). Although NS1 does not harbor mitochondria-targeting sequences, it has also been

found in mitochondria at early times (1.5 h) post-infection (Tsai et al., 2017).

The UL12 gene of herpes simplex virus type 1 (HSV-1) encodes two distinct but related alkaline DNases through two separately promoted 3' co-terminal mRNAs, producing full-length (UL12) and amino-terminal truncated (UL12.5) proteins. UL12 localizes to the nucleus while UL12.5 is predominantly located in mitochondria, where it degrades mitochondrial DNA early during infection. Whereas nuclear-targeted UL12 produces mature viral genomes from larger genome precursors (Draper et al., 1986; Saffran et al., 2007; Corcoran et al., 2009), the role of UL12.5 is not well-defined since mitochondrial DNA degradation is not required for HSV-1 replication (Duguay et al., 2014).

MITOCHONDRIAL DYNAMICS AND VIRUSES

Mitochondria constantly undergo fusion and fission depending on the cell metabolic requirements, a process that has been dubbed as mitochondrial dynamics (Mishra and Chan, 2016).

Along with being the "powerhouse" of eukaryotic cells, mitochondria are also involved in cellular innate antiviral immunity (Seth et al., 2005). Mitochondrial fusion and fission processes depend on the activity of mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy protein 1 (OPA1)—which promotes fusion—in addition to Dynamin-related protein 1 (Drp1)—which promotes mitochondrial fission (Mishra and Chan, 2016). There is evidence that antiviral immune responses can be regulated by mitochondrial dynamics (Arnoult et al., 2011; West et al., 2011). The close association between mitochondrial dynamics and several mitochondrial and cellular functions may suggest that mitochondrial dynamics could be a target for viruses to interfere with immune responses (**Table 3**). Likewise, the non-structural protein 4A (NS4A) from HCV, either alone or associated with the non-structural protein 3 (NS3), accumulates in mitochondria, altering the mitochondrial dynamics (Nomura-Takigawa et al., 2006). Infection with HIV-1 re-shapes mitochondrial distribution within the cells (Radovanović et al., 1999), while African swine fever virus (ASFV) induces the clustering of mitochondria around virus factories within infected cells, providing the local energy required for the release of virus (Rojo et al., 1998). The DENV NS2b3 protein partially cleaves Mfn1 and Mfn2, attenuating interferon responses (Yu et al., 2015), and induces mitochondrial fusion by inhibiting Drp1 activation and in turn the activation of the interferon response (Chatel-Chaix et al., 2016).

Excessive mitochondrial fission may lead to mitochondrial damage, and this may have a role in hepatitis B virus (HBV)-induced liver disease (Kim et al., 2013).

Hepatitis B virus, through its HBx protein, triggers the translocation of Drp1 to the mitochondria by stimulating the phosphorylation of Drp1 at the Ser616 residue, and on the other hand, contributes to the degradation of Mfn2, favoring mitochondrial fission and mitophagy, attenuating the virus-induced apoptosis in the process (Kim et al., 2013).

TABLE 3 | Viruses that disrupt mitochondrial dynamics.

Virus	Viral proteins	Effect	References
Hepatitis C virus (HCV)	NS4A, NS3	Change of mitochondria distribution	Nomura-Takigawa et al., 2006
Human immunodeficiency virus-1 (HIV-1)		Clustering of mitochondria	Radovanović et al., 1999
African swine fever virus (ASFV)		Cluster of mitochondria around virus factories, providing ATP for virus release	Rojo et al., 1998
Dengue virus (DENV)	NS2b3	Cleavege of Mfn1 and Mfn2, attenuation of IFN responses	Yu et al., 2015
		Mitochondrial fusion by inhibition of Drp1	Chatel-Chaix et al., 2016
Hepatitis B virus (HBV)	HBx	Mitochondrial fission, and mitochondrial injury	Kim et al., 2013

Intracellular calcium concentrations also regulate mitochondrial dynamics since the calcium-dependent phosphatase calcineurin dephosphorylates Drp1 at S637, facilitating the recruitment of Drp1 to the mitochondria and the consequent mitochondrial fission (Cereghetti et al., 2008).

INTRACELLULAR CALCIUM HOMEOSTASIS AND VIRAL INFECTIONS

Intracellular calcium participates in cell signaling, mitochondrial function, and cell death (Duchen, 2000; Contreras et al., 2010), and Ca^{2+} uptake by mitochondria activates Krebs cycle enzymes and oxidative phosphorylation, leading to higher ATP production (Nasr et al., 2003).

Several viruses regulate host cell calcium concentrations in the cytoplasm as well as in mitochondria, allowing viral gene expression, virus replication, and the control of host cell viability (**Table 4**). HSV1 downregulates the uptake of Ca^{2+} by mitochondria along its lytic cycle, modulating virus replication (Lund and Ziola, 1985). Other viruses such as HCV target mitochondria, increasing Ca^{2+} concentration (Li et al., 2007; Campbell et al., 2009). Among the HCV proteins known to interfere with Ca^{2+} homeostasis, are the core protein, the NS5A, and the p7 protein (Gong et al., 2001; Griffin et al., 2004; Kalamvoki and Mavromara, 2004; Dionisio et al., 2009).

HBV induces the mobilization of Ca^{2+} from mitochondria and endoplasmic reticulum to the cytoplasm through the interaction of the HBV protein X with voltage-dependent anion channels (VDAC) (Bouchard et al., 2001; Choi et al.,

TABLE 4 | Viruses that disrupt calcium homeostasis.

Virus	Viral proteins	Effect	References
Human T leukemia virus (HTLV-1)	p13	p13 accumulates in the inner mitochondrial membrane, reduces Dym and mCa^{2+} uptake	Biasiotto et al., 2010
Herpes simplex virus 1 (HSV1)	?	Modulation of viral replication by down-regulation of Ca^{2+} uptake by mitochondria	Lund and Ziola, 1985
Hepatitis C virus (HCV)	NS5A, p7	Increase of Ca^{2+} concentration	Gong et al., 2001; Griffin et al., 2004
Hepatitis B virus (HBV)	HBx	Ca^{2+} release from mitochondria and ER	Bouchard et al., 2001
Human immunodeficiency virus-1 (HIV-1)	Nef	Increase in viral replication by IP3R-dependent increase of cytosolic Ca^{2+}	Foti et al., 1999
Rotavirus	NSP4	virus release by decreasing Ca^{2+} concentration	Tian et al., 1995; Ruiz et al., 2007
Poliovirus	2BC	Increase in viral gene expression and apoptosis by increse in Ca^{2+} concentration	Aldabe et al., 1997
Coxsackievirus B3	2B	Control of apoptosis and virus release by regulation of Ca^{2+} concentration	Campanella et al., 2004
Human cytomegalovirus (HCMV)	pUL37x1	Increased viral replication by mitochondria Ca^{2+} uptake and increased ATP	Sharon-Friling et al., 2006; Bozidis et al., 2010

?, *not known.*

2005). The HIV-1 protein Nef (nuclear elongation factor) interacts with the Inositol 1,4,5-trisphosphate receptor (IP3Rs), increasing cytosolic Ca^{2+} concentration, promoting the transcription of virus-encoded genes and viral replication (Kinoshita et al., 1997; Foti et al., 1999). Rotavirus, through its NSP4 protein, activates phospholipase C (PLC) and the release of Ca^{2+} from the endoplasmic reticulum to the cytosol. However, by the end of its life cycle there is a decrease in cellular Ca^{2+} concentrations enabling rotavirus release (Tian et al., 1995; Ruiz et al., 2007; Díaz et al., 2008).

Poliovirus increases intracellular Ca^{2+} concentrations shortly after infection, increasing viral gene expression (Irurzun et al., 1995; Aldabe et al., 1997). By the end of the virus life cycle Ca^{2+} accumulates within mitochondria at the expense of ER stores in a mitochondrial calcium uniporter (MCU) and voltage-dependent anion channel (VDAC)-dependent process, leading to mitochondrial dysfunction and apoptosis (Brisac et al., 2010).

Enteroviruses control apoptosis through Ca^{2+} regulation; in this way, low levels of cytosolic Ca^{2+} provide the conditions for viral replication while high concentrations of cytosolic Ca^{2+} lead to the formation of vesicles which allow virus release (Campanella et al., 2004; Van Kuppeveld et al., 2005).

Human cytomegalovirus (HCMV) protein pUL37 × 1, also known as viral mitochondrion-localized inhibitor of apoptosis (vMIA) localizes into mitochondria and induces the transfer of ER Ca^{2+} into mitochondria, increasing the production of ATP and virus replication (Sharon-Friling et al., 2006; Bozidis et al., 2010).

The maturation of viral glycoproteins is dependent on both pH and intracellular Ca^{2+} concentrations. Ca^{2+} acts as a cofactor for several enzymes including glycosyl- and sulfo-transferases (Vanoevelen et al., 2007). Measles virus (MV), Dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV), and Chikungunya virus (CHIKV) use the host calcium pump secretory pathway calcium ATPase 1 (SPCA1) for Ca^{2+} loading into the trans Golgi network, which activates glycosyl transferases and proteases allowing viral maturation and spreading (Hoffmann et al., 2017).

mTOR AND AMPK AS METABOLIC HUBS AND VIRAL TARGETS FOR EVASION

The mechanistic target of rapamycin (mTOR) and the adenosine monophosphate-activated protein kinase (AMPK) constitute an integrated metabolic sensor. High levels of ATP (high ATP/AMP ratio) activate mTORC1, resulting in enhanced nutrient-dependent protein synthesis, cell growth and proliferation, whereas low levels of ATP (low ATP/AMP and ATP/ADP ratios), a hallmark of metabolic stress (starvation, hypoxia or viral infection), lead to AMPK-mediated inhibition of mTORC1 and activation of mTORC2, which restores energy homeostasis by switching the ATP-consuming biosynthetic pathways off and the ATP-producing catabolic pathways on (Hardie et al., 2012; Saxton and Sabatini, 2017).

MTOR acts as the catalytic subunit of either of two molecular complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2); mTORC1 is bound to the protein Raptor (Hara et al., 2002; Kim et al., 2002) and mTORC2 is bound to the protein Rictor (Hresko and Mueckler, 2005).

MTORC1 induces metabolic reprograming from OXPHOS to glycolysis by upregulating the transcription factor hypoxia-induced factor 1α (HIF1α) and, as a result, increases the expression of several glycolytic enzymes including phospho-fructo kinase (PFK). On the other hand, mTORC2 regulates cell proliferation and survival by activating the PI3K-Akt pathway (Düvel et al., 2010; Thomanetz et al., 2013; Saxton and Sabatini, 2017). The mTORC1 complex acts downstream of Akt and, as a way of regulation, the mTORC1 substrate p70S6K suppresses mTORC2, and the mTORC1 substrate Grb10 suppresses PI3K signaling (Hsu et al., 2011; Yu et al., 2011; Saxton and Sabatini, 2017), establishing a negative feedback that balances mTORC1 and mTORC2 activities (Meade et al., 2018).

TABLE 5 | Viruses that target mTOR or AMPK.

Virus	Viral proteins	Effect	References
Herpes simplex virus 1 (HSV1)	viral kinase Us3	Enhancement of mTORC1 activity	Martin et al., 2012
Poliovirus (PV)		Inhibition of mTORC1 activity	
Human immunodeficiency virus-1 (HIV-1)	Env	Activation of mTORC1 activity	Le Sage et al., 2016
Sindbis virus (SINV)		Activation of mTORC	Le Sage et al., 2016
Chikungunya virus (CHIKV)	?	Controversial activation/Inhibition of mTOR	Le Sage et al., 2016
Influenza A virus (IAV)	NS1	Differential activation of mTORC1 and mTORC2, supports viral replication	Kuss-Duerkop et al., 2017
Andes virus (ANDV)	glycoprotein Gn	Activation of mTOR, supports viral protein expression and replication	McNulty et al., 2013
Hepatitis C virus (HCV)	NS5A	Activation of mTORC1 supports viral protein expression and replication	Stohr et al., 2016
Poxviruses	F17	Evasion of cytosolic sensing by disruption of the mTORC1-mTORC2 circuit	Meade et al., 2018
Dengue virus (DENV)	?	Viral replication by activation of AMPK and inhibition of mTORC1	Jordan and Randall, 2017
Zika virus (ZIKV)	?	AMPK activation evokes antiviral innate responses and restricts virus replication	Kumar et al., 2018

?, *not known.*

Extracellular growth factors, the cell energy status, and different stressors such as viral infection are integrated into the mTOR pathway. Not surprisingly, viruses can modulate mTOR signaling to their advantage (Le Sage et al., 2016; Saxton and Sabatini, 2017) (**Table 5**). HSV-1 can enhance mTORC1 activity; whereas Poliovirus, HIV-1, Sindbis virus, and CHIKV can inhibit this same complex (Martin et al., 2012).

Activation of mTORC1 supports viral protein expression and replication of Influenza A virus, Andes virus (ANDV), and HCV (McNulty et al., 2013; Stohr et al., 2016; Kuss-Duerkop et al., 2017). On the other hand, poxviruses are capable of evading their cytosolic sensing by means of a conserved structural protein that disrupts the mTORC1-mTORC2 regulatory circuit while maintaining the metabolic benefits of mTOR activity (Meade et al., 2018).

DENV activates AMPK, decreases the activity of mTORC1, and induces lipophagy, a process that is required for the robust DENV replication; the autophagic-mediated mobilization of lipids increases the β-oxidation in DENV-infected cells (Jordan and Randall, 2017) whereas AMPK activation evokes antiviral innate responses and restricts ZIKV replication (Kumar et al., 2018).

CAN VIRUSES REPLICATE WITHIN MITOCHONDRIA?

In addition to the interaction of viral proteins with mitochondria, which modify mitochondrial function, the Alphanodavirus flock house virus (FHV) can infect yeast, insect, plant, and mammalian cells, and replicates its RNA in the mitochondrial outer membrane. Miller et al. showed that the FHV RNA-dependent RNA polymerase, required for FHV RNA replication, localizes to the outer mitochondrial membrane and by electron microscopy these authors identified 40–60 nm membrane-bound spherical structures, similar to other virus-induced membrane structures, within the mitochondrial intermembrane space of infected cells from *Drosophila* (Miller et al., 2001).

CONCLUDING REMARKS

This review explores how viruses may subvert immune responses by controlling host cell metabolism.

Viruses may target MAVS (RIG-I-MDA5-MAVS anti-viral pathway) interfering with RNA virus-induced type 1 interferon responses and target other mitochondrial-associated proteins, disrupting mitochondrial dynamics, mitochondrial membrane potential, and calcium handling—all of which may affect anti-viral immunity. They may also regulate the production of ATP to their advantage by interfering with mitochondrial calcium mobilization, mitochondrial enzymatic activities, and key metabolic sensors such as mTORC1, mTORC2, and AMPK. They may also induce cytotoxic T lymphocyte exhaustion, which implies metabolic reprogramming.

Viruses may also target the cGAS-STING anti-viral pathway, interfering with DNA virus-induced type I IFN responses. Since this anti-viral pathway is not directly connected with host cell metabolism (at least not in the way the RIG-I-MDA5-MAVS is), one key outstanding question is why anti-RNA viruses IFN responses are more "metabolically directed" compared to anti-DNA virus responses. Moreover, why do some RNA viruses induce the release of mitochondrial DNA and in this way recruit the RIG-I-MDA5-MAVS pathway?

In the context of HCV infection, there are at least two mechanisms accounting for the degradation of MAVS, direct cleaving by the HCV-encoded NS3/4A protein, and the NLRX1-induced proteosomal degradation. As both MAVS and NLRX1 localize in the outer mitochondrial membrane, and MAVS signaling is dependent on mitochondrial function, it remains to be determined whether NLRX1 activity is also dependent

on mitochondrial function. However, it is currently known that NLRX1 regulates OXPHOS and cell integrity in a model of ischemia-reperfusion injury, and that loss of NLRX1 increases oxygen consumption and oxidative stress in epithelial cells (Stokman et al., 2017).

The role of glycolysis, β-oxidation, and oxidative phosphorylation on viral infections is continuing to emerge, but there are still outstanding questions on the role and mechanism that some metabolic intermediates may play in viral infection. For instance, dimethyl fumarate enhances the infection of cancer cell lines and human tumor biopsies with several oncolytic viruses (Selman et al., 2018), whereas ZIKV infection upregulates the enzyme cis-aconitate descarboxylase, which converts the TCA intermediate cis-aconitate to itaconate, an endogenous inhibitor of succinate dehydrogenase, inhibiting the conversion of succinate to fumarate and generating a metabolic state that restricts ZIKV replication in neurons (Daniels et al., 2019). These topics require further exploration.

On the other hand, the success of anti-viral antibody responses as well as of antibody-mediated anti-viral vaccine protection depends on plasma cell lifespan, which ultimately relies on plasma cell metabolism; something that differs from B lymphocyte metabolism (Lam et al., 2018). It would therefore be interesting to determine whether there are viruses that specifically target plasma cell metabolism, and in which case whether protecting plasma cell metabolism could be therapeutically useful in helping to support long-lasting anti-viral immune responses.

AUTHOR CONTRIBUTIONS

MM-A and FS-G conceived and designed the review, wrote the paper, edited, and approved the final draft. SK contributed to discussions on the paper, edited, and approved the final draft.

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REVIEW
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Herpes Simplex Virus Evasion of Early Host Antiviral Responses

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Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) have co-evolved with humans for thousands of years and are present at a high prevalence in the population worldwide. HSV infections are responsible for several illnesses including skin and mucosal lesions, blindness and even life-threatening encephalitis in both, immunocompetent and immunocompromised individuals of all ages. Therefore, diseases caused by HSVs represent significant public health burdens. Similar to other herpesviruses, HSV-1 and HSV-2 produce lifelong infections in the host by establishing latency in neurons and sporadically reactivating from these cells, eliciting recurrences that are accompanied by viral shedding in both, symptomatic and asymptomatic individuals. The ability of HSVs to persist and recur in otherwise healthy individuals is likely given by the numerous virulence factors that these viruses have evolved to evade host antiviral responses. Here, we review and discuss molecular mechanisms used by HSVs to evade early innate antiviral responses, which are the first lines of defense against these viruses. A comprehensive understanding of how HSVs evade host early antiviral responses could contribute to the development of novel therapies and vaccines to counteract these viruses.

Keywords: interferon (IFN), inflammasome, toll-like receptors (TLRs), natural killer cells (NK cells), dendritic cells (DCs), cytosolic nucleic acid receptors, innate immunity, apoptosis

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INTRODUCTION

Herpes simplex viruses (HSVs) type 1 (HSV-1 or human herpesvirus 1, HHV-1) and type 2 (HSV-2 or human herpesvirus 2, HHV-2), are members of the *Herpesviridae* family and *Alphaherpesvirinae* subfamily, similar to varicella zoster virus (VZV) (Davison, 2010; Sharma et al., 2016). HSVs are present among humans at a high prevalence (Looker et al., 2008; CDC, 2010; Yawn and Gilden, 2013; Dickson et al., 2014; Suazo et al., 2015b), with two thirds of the global population infected with HSV-1 (Looker et al., 2015a), and ∼11% of the world population infected with HSV-2 (Looker et al., 2015b). HSV-1 and HSV-2 are associated with diverse clinical manifestations, yet disease widely varies from one individual to another, with nearly 40% of those that are infected displaying symptoms during primary infection (Langenberg et al., 1999; Bernstein et al., 2013).

Additionally, during recurrent viral reactivations, most individuals are asymptomatic, with 5–15% of those infected displaying clinical symptoms related to HSV infections (Benedetti et al., 1994; Wald et al., 2000; Sudenga et al., 2012; Suazo et al., 2015b). Although a relatively low proportion of the infected individuals show clinical manifestations, the high percentage of the world population infected with these viruses yields an enormous number of individuals that effectively suffer from HSV-related illnesses.

HSV-1 is mainly associated with orofacial lesions, yet it is also the leading cause of infectious blindness in developed countries and the number one cause of viral encephalitis in adults (Kaye and Choudhary, 2006; Horowitz et al., 2010; Farooq and Shukla, 2012; Bernstein et al., 2013). On the other hand, HSV-2 is mainly associated with genital lesions and neonatal encephalitis (Gupta et al., 2007; Berger and Houff, 2008; Looker et al., 2008; Suazo et al., 2015b), despite the fact that HSV-1 is nowadays more frequently related to primary genital infection worldwide (Buxbaum et al., 2003; Coyle et al., 2003; Xu et al., 2006; Pereira et al., 2012). However, HSV-2 reactivates more frequently from the genital tissue than HSV-1 and hence, despite the finding that the latter is commonly detected during primary infection, HSV-2 is more often isolated from this site than HSV-1 at any time during infection (Lafferty et al., 1987; Kaneko et al., 2008). A similar phenomenon may occur in the orofacial area, with most viral reactivations being attributed to HSV-1. Variable reactivation of HSV-1 and HSV-2 from neurons within the trigeminal or sacral ganglia may be given by differences in gene expression profiles by neurons that innervate these tissues (Kaneko et al., 2008; Flegel et al., 2015; Lopes et al., 2017).

A clinically relevant concern associated with HSV-2 genital infection is that it is associated with a three-fold increase in the likelihood of acquiring the human immunodeficiency virus type 1 (HIV-1), due to synergistic aspects related to the co-infection with both viruses (Wasserheit, 1992; Freeman et al., 2006; Barnabas et al., 2011). For instance, evidence of an indirect interplay between HIV and HSV occurs with HSV-2 infection of macaques and humans eliciting an increase in the amounts of dendritic cells present in the genital tissue, as well as $\alpha_4\beta_7$- and CCR5-expressing $CD4^+$ T cells, both known to be substrates for HIV (Rebbapragada et al., 2007; Martinelli et al., 2011). HSV-2 also elicits lesions in the infected tissue that provide an entry portal for HIV (Bagdades et al., 1992; Suazo et al., 2015b). Additionally, proposed interactions between HSV-2 and HIV would support HSV-2 infections being associated with a relative risk of HIV incidence nearing 50% in the African region (Looker et al., 2017). The association between HSV-2 and HIV suggests that tackling HSV-2 infection could help reduce the HIV pandemics (Rebbapragada et al., 2007; De Jong et al., 2010; Johnson et al., 2011; Martinelli et al., 2011; Sartori et al., 2011; Stefanidou et al., 2013a). Therefore, HSV-2 infection should be considered a major matter of public health concern.

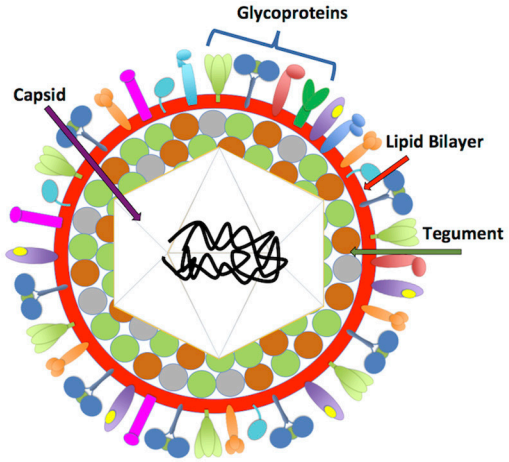
Infections with HSVs remain latent and are characterized by sporadic reactivation episodes accompanied by virus shedding, regardless of the presence of clinical symptoms (Kaneko et al., 2008; Tobian et al., 2013). Lifelong infection in the host by HSVs is achieved thanks to their capacity to infect neurons, mainly those enervating infected tissues and then remain latent within these cells (Margolis et al., 2007; Yao et al., 2014). In the skin, mucosae and eyes, HSVs access neurons by infecting sensorial nerve termini and then traveling in a retrograde manner through the axon of these cells up to the soma. Later, HSVs may reactivate from these cells and exit them through anterograde movements either, to infect other neurons that eventually may innervate the brain or infect cells located nearby the initial site of infection (Linehan et al., 2004; Gonzalez and Sanjuan, 2013).

Importantly, HSVs not only infect epithelial cells and neurons but virtually any cell type in the body, including immune cells thanks to the fact that the main receptors of HSVs are widely distributed in host tissues and cells (Krummenacher et al., 2004). By infecting immune cells, these viruses can modulate and escape diverse antiviral mechanisms evolved by the host to counteract infection and furthermore, establish long-term infection with sporadic recurrences that produce new infectious particles (Retamal-Diaz et al., 2015; Suazo et al., 2015a). Here, we review and discuss recent studies that report the relationship between HSVs and early cellular antiviral responses, both in immune and non-immune cells.

Replication Cycle of HSVs

HSV-1 and HSV-2 share ∼74% identity at the nucleotide level and are structurally very closely related (Baines and Pellett, 2007). Both viruses have a linear, double-stranded DNA (dsDNA) genome with sizes ranging from 150 to 154 kbp, which encode more than 70 open reading frames (ORFs) (Kieff et al., 1971; Dolan et al., 1998; Koelle et al., 2017). The viral genomes are covered by a 125 nm icosahedral capsid (Wu et al., 2016), which is surrounded by a mesh composed by many proteins (>20) called the tegument (**Figure 1**). This protein stratum is in turn enveloped by a lipid bilayer, from which multiple viral glycoproteins protrude and play roles in virus entry and exit, as well as immune-modulation and immune-escape (Roller and Roizman, 1992; Loret et al., 2008; Retamal-Diaz et al., 2015; Suazo et al., 2015a; Suk and Knipe, 2015).

Although HSV-1 and HSV-2 share common aspects during cell entry, they do have some differences. For instance, unlike HSV-1, HSV-2 does not require its glycoprotein C (gC) for attaching to target cells (Shukla and Spear, 2001). On the other hand, both of these HSVs do require the viral glycoprotein B (gB) for the virus to attach to heparan sulfate proteoglycans on the cell surface (Atanasiu et al., 2013). In immune cells such as dendritic cells (DCs) and natural killer cells (NK cells), gB has been reported to bind to an additional cell receptor for viral attachment, namely the paired immunoglobulin like-type 2 receptor (PILR) (Shiratori et al., 2004; Satoh et al., 2008). Once the virus has attached to the cell surface, the viral glycoprotein D (gD) will bind to either, nectin-1 (or nectin-2) expressed on the surface of most anchored cells in the organism, such as epithelial and neuronal cells, or the herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor (TNFR) family that signals intracellularly depending on the orientation of its ligand, either in *cis* or *trans* (Kovacs et al., 2009). The latter HSV receptor is preferentially expressed on the surface of immune cells, such as DCs and T cells (Krummenacher et al., 2004; Jones



Tegument proteins involved in immune evasion

MW (kDa)	Protein	Gene
335.9	VP1-2	UL36
120.6	ICP32	UL37
78.5	ICP0	RL2
78.2	VP11/12	UL46
57.2	UL13	UL13
54.9	vhs	UL41
54.3	VP16	UL48
52.8	US3	US3
32.3	VP22	UL49
26.2	ICP34.5	RL1
17.8	US11	US11

FIGURE 1 | HSV virion structure. HSVs possess linear, double-stranded DNA genomes (152–154 kbp) encoding more than 70 ORFs. The viral genomes are contained within icosahedral capsids of ∼125 nm, which in turn are surrounded by complex meshes of viral proteins known as the tegument. The tegument is enveloped by lipid membranes, which harbor numerous transmembrane glycoproteins. A table with tegument proteins involved in immune evasion is shown on the right, ordered from highest to lowest molecular weight (MW).

et al., 2016). In addition, gD has been described to bind to 3-*O*-sulfated heparan sulfates on the surface of CHO cells, which permitted viral entry when gB, gD, glycoprotein H (gH), and glycoprotein L (gL) were present in the virion (Xia et al., 2002; Tiwari et al., 2004). As a result of gD binding to its ligand, this glycoprotein will undergo a conformational changes that enable this protein to activate the viral gH/gL glycoprotein complex, which in turn triggers the fusion of the virus and cell membranes in a process that is dependent on the activity of gB, which acts as the fusion protein for these viruses (Lazear et al., 2014). Moreover, the glycoprotein complex gH/gL of HSV-1 and HSV-2 participate in a process distinct to the conventional viral entry, since they have been reported to bind αv3, αvβ6 and αvβ8 surface integrins causing dissociation from the heterodimer permitting gH activation to promote virion entry through a mechanism involving acidic endosomes (Gianni et al., 2013, 2015; Cheshenko et al., 2014). Lastly, it has also been observed that HSV-1 can enter cells via a phagocytosis-like uptake mechanism (Clement et al., 2006).

Once the viral and cell membranes have fused, the viral capsid, which is surrounded by tegument proteins, will be released into the cytoplasm. These tegument proteins will have the opportunity to rapidly modulate host antiviral determinants upon entry into the cell, interfering with the detection of viral components, that altogether aim at diminishing the progression of infection (Owen et al., 2015). Within the cytoplasm, the viral capsid will associate to microtubules and travel toward the nuclear membrane to deliver the viral genome into the cell nucleus (Sodeik et al., 1997; Dohner et al., 2002; Radtke et al., 2010). However, it is possible that the capsid reaches the nucleus by simple diffusion within the cytoplasm, as morphological changes take place in HSV-infected cells (Ibanez et al., 2018). Once the capsid reaches the outer nuclear membrane, the tegument viral protein VP1/2, which travels associated to the capsid, will anchor this structure to nuclear pore proteins and favor its docking to the nucleopore for the injection of the viral DNA into the nucleus. This process will allow the initiation of viral gene transcription within the nucleus, and later on, viral genome replication (Abaitua et al., 2012). In parallel, the viral protein VP16, which is present in the tegument, will localize in the nucleus in such a way to promote the transcription of viral genes, acting as a transactivator (Milbradt et al., 2011; Roizman and Zhou, 2015; Suk and Knipe, 2015).

Within infected cells, HSV genes are transcribed sequentially in three main waves; the first set of viral genes that are transcribed are called *immediate early* (or alpha) genes, with many of their functions being related to limiting host immediate antiviral mechanisms. This set of genes also encodes proteins that act as transcription factors that promote the transcription of the second set of viral genes (Silva et al., 2012). After the transcription of alpha genes, *early* (or beta) viral genes are expressed, which are involved among others in promoting the replication of the viral genome (Ibanez et al., 2018). During the replication of the viral genetic material, the genomes of HSVs undergo circularization in a form known as "*rolling circle*," which is regulated by viral factors that ultimately generate linear genomes that are packaged into new viral capsids within the nucleus (Jackson and Deluca, 2003). After the expression of alpha and beta genes, HSV-infected cells transcribe *late* (or gamma) viral genes, which are occasionally separated into *late early* (or gamma-1) and *late* (or gamma-*2*) genes, and are involved among others in providing the structural components that are present in the virion (Chen et al., 1992). During viral transcription, host cells equipped with a zinc-finger antiviral protein (ZAP) that can utilize this restriction factor to inhibit the replication of viruses by promoting the degradation of critical viral mRNAs. Nevertheless, the HSV-1 UL41 protein which is also known as the virus host shutoff protein (VHS), has been reported to rapidly degrade human ZAP mRNA upon

infection, before this host factor can block viral gene expression (Su et al., 2015).

Importantly, infectious HSV particles to be released from the infected cells will require that the viral capsids leaving the nucleus contain the viral genome. For this, HSV capsids are assembled with the viral DNA within the nucleus and then envelope in the inner nuclear membrane (INM) and de-envelope from the outer nuclear membrane (ONM) (Mettenleiter et al., 2013; Funk et al., 2015). At this time, tegument proteins coating the capsid are acquired both, in the nucleus and cytoplasm. Importantly, viral glycoproteins located within the perinuclear space are needed to carry out the capsid budding and fusion processes with these membranes (Bucks et al., 2007; Mou et al., 2009; Ott et al., 2011; Mettenleiter et al., 2013; Owen et al., 2015). Once in the cytoplasm, the capsid which will be covered with tegument proteins will be enveloped into the Golgi apparatus, generating enveloped particles within this compartment that are ready for virion exocytosis (Johnson and Baines, 2011). Notably, it has been reported that viral glycoproteins acquired by the enveloped capsid in the Golgi apparatus are first exported to the cell surface by this organelle and then re-internalized through the Trans Golgi Network before associating to the coated capsids (Wisner and Johnson, 2004; Turcotte et al., 2005). Infected cells will attempt to prevent the release of mature virions using the host antiviral restriction factor tetherin, an interferon (IFN)-inducible membrane protein that has been shown to prevent egress of several enveloped viruses (Perez-Caballero et al., 2009; Kuhl et al., 2011). However, the HSV-1 VHS protein depletes tetherin by degrading its mRNA (Zenner et al., 2013), while, HSV-2 gD has been observed to directly interact with a long disulfide-rich coiled-coil structure (CC) that is found within the extracellular domain of tetherin, thereby targeting the latter to lysosomes for degradation (Liu et al., 2016b). Both effects evidence how HSVs intervene with cell antiviral mechanisms meant to stop virions exit and prevent dissemination.

Aside from the previously described events, HSVs can propagate onto close cells through cell-cell interactions. In these cases, viral proteins are directed to the interface of adjacent cells in a process termed virological synapse, in which cells in close proximity undergo membrane fusion events favoring virus propagation (Johnson et al., 2001). An advantage of this type of infection is that it allows HSVs to propagate onto neighboring cells while avoiding being targeted by immune components, such as complement or neutralizing antibodies (Hook et al., 2006a; Lubinski et al., 2011). This mechanism of infection has not only been reported for epithelial cells, but also for the infection of immune cells, such as T cells by HSV-infected fibroblasts (Aubert et al., 2009).

In sum, HSVs have evolved molecular determinants to effectively bind to and infect various cell types, causing productive infection in multiple tissues and establishing latency in neurons. Alternatively, these viruses are also capable of infecting immune cells and modulate their functions to further interfere with host early antiviral responses.

HSV Modulates Apoptosis Differentially in Non-immune and Immune Cells

For a virus to produce significant amounts of infectious particles from an infected cell, it will need the cell to be viable for as long as possible and to provide the building blocks required for replicating its genetic material and producing its proteins. HSVs have been reported to modulate cellular death in different cell types either, to promote cell viability for the generation of new virions or to promote the death of cells that may be detrimental for their replication and shedding. For instance, the HSV-1 glycoproteins J (gJ) and D (gD) have been described to produce, at least partially the inhibition of Fas-mediated apoptosis in a neuroblastoma cell line and Jurkat cells (Zhou et al., 2000; Jerome et al., 2001). Surprisingly, the expression of gJ alone also induced the production of reactive oxygen species (ROS), which can eventually trigger apoptosis (**Figure 2**) (Fleury et al., 2002; Aubert et al., 2008). HSV-1 has also been reported to reduce cell apoptosis in epithelial cells, despite eliciting processes that involved FLICE-inhibitory protein (c-FLIP) downregulation, which is an inhibitor of caspase-8 that generally results in cell death (Kather et al., 2010). This apparent discrepancy was attributed to the presence of latency-associated transcript (LAT) sequences, which have been described to act as inhibitors of caspase-8-mediated apoptosis, similar to what occurs in infected neuronal cells (Henderson et al., 2002).

Additionally, an intrinsic mechanism of apoptosis consists on the activity of pro-apoptotic Bcl-2 cell death in mouse fibroblasts and monocytes, as well as in human colon carcinoma cells (**Figure 2**) (Sciortino et al., 2006; Papaianni et al., 2015). Importantly, HSV-1 infection promotes increased expression of p53 upregulated modulator of apoptosis (PUMA), a protein that is a host Bcl-2 homology 3 (BH3)-only family member that activates Bax/Bak and produces mitochondrial outer membrane permeabilization (MOMP) to release cytochrome c from the mitochondria and activates caspase-3, ultimately culminating in apoptosis (Papaianni et al., 2015). Furthermore, during HSV infection, caspase-8-interacting domains within the HSV-1 viral protein ICP6 and the HSV-2 viral protein ICP10, both which are R1 large subunits of a ribonucleotide reductase (RR), have been suggested to bind to caspase-8 and cause inhibition of apoptosis induced by TNF-α-induced apoptosis through the TNF receptor TNFR1 (**Figure 2**) (Guo et al., 2015a). However, this inhibition of apoptosis may cause cells to enter necroptosis 12 h post-infection, as an alternative defense mechanism to limit virus replication and spreading (Sridharan and Upton, 2014). Nevertheless, HSV R1 proteins have been reported to bind to host receptor-interacting protein (RIP) 1/3 and inhibit necroptosis in human cells, while necroptosis was observed in mouse cells (Guo et al., 2015b; Huang et al., 2015). RIP3 likely mediates necroptosis in infected fibroblasts cells to limit the dissemination of HSV-1 in the mouse, similar to what has been described for RIP3 with other viruses, such as vaccinia virus and murine cytomegalovirus (MCMV) (Wang et al., 2014; Huang et al., 2015). Effective inhibition of both, apoptosis- and necroptosis-related mechanisms likely allow these viruses to generate high virus yields and

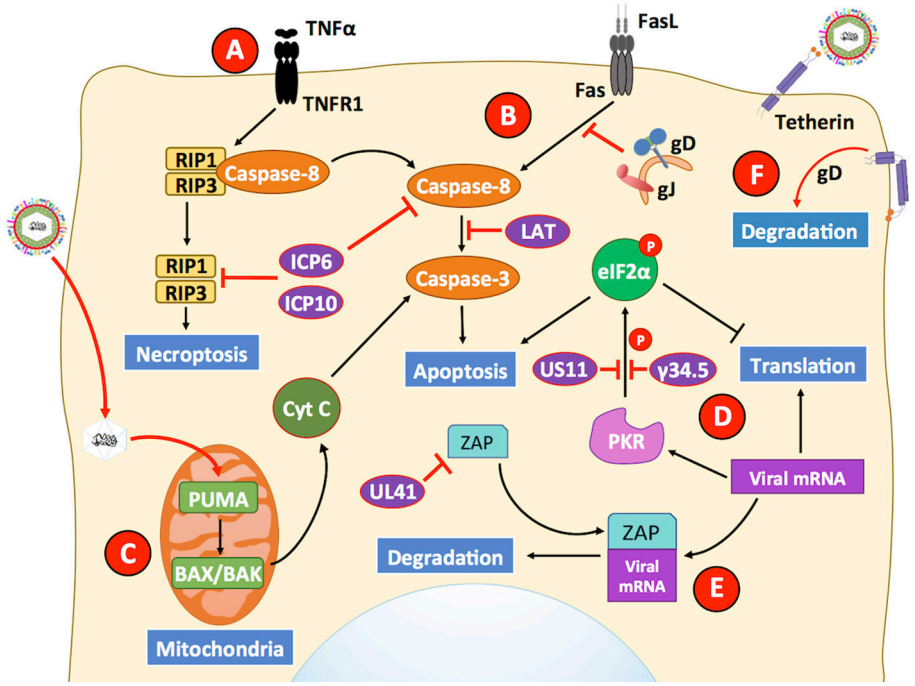


FIGURE 2 | HSVs modulate antiviral mechanisms related to cell death in non-immune cells. HSVs utilize numerous mechanisms to hamper the capacity of host cells to restrict viral infection. **(A)** The engagement of the TNFR receptor leads to the activation of caspase-8 eliciting apoptosis, or eventually RIP1/3 to induce necroptosis. However, HSV proteins ICP6 and ICP10 hamper signaling events related to these pathways, thus prolonging cell survival during infection. **(B)** Engagement of the Fas receptor with Fas ligand (FasL) generally leads to extrinsic apoptosis events mediated by the activation of caspase-8. However, HSV glycoproteins J (gJ) and gD block signaling events by this receptor. Additionally, the LAT transcript also interferes with caspase-8 mediated signaling that usually leads to apoptosis. **(C)** However, HSV infection has been described to upregulate the expression of PUMA in the mitochondria of HSV-infected cells, which leads to BAX/BAK-dependent apoptosis mediated by caspase-3. Thus, HSV may induce the intrinsic apoptotic pathway at later time points of infection after inhibiting apoptosis. **(D)** Another antiviral mechanism hampered by HSV infection is inhibition of cell-induced apoptosis due to translation arrest. Upon detection of viral components, host PKR triggers eIF2α phosphorylation, which inhibits its function and consequently mRNA translation, leading to global protein synthesis arrest and caspase-3 activation. However, the viral proteins US11 and γ34.5 impair eIF2α phosphorylation, allowing viral gene translation to ensue during infection and limiting apoptosis through this pathway. **(E)** The host protein ZAP can act as an antiviral factor that promotes degradation of viral mRNAs. However, its function is inhibited by the HSV protein UL41 (VHS), which promotes ZAP mRNA degradation. **(F)** Finally, infected cells may attempt to prevent the release of mature virion through a membrane protein called tetherin that is capable of binding to enveloped virions. As a countermeasure, the viral glycoprotein gD interacts with tetherin which ultimately provokes degradation of the latter. Black lines show cellular processes. Red lines show processes modulated by HSVs.

sufficient amounts of progeny virions for the dissemination of infection onto adjacent cells and other tissues within the host.

On the other hand, HSVs have been described as capable of inducing apoptosis in immune cells (Jones et al., 2003; Stefanidou et al., 2013a). For instance, HSV-1 induces apoptosis in natural killer cells (NK cells) upon interacting with virus-infected macrophages that expresses Fas/FasL (**Figure 2**) (Iannello et al., 2011), and kills dendritic cells (discussed in the followings sections) (Peretti et al., 2005; Stefanidou et al., 2013b). Although the specific mechanism by which HSVs induce apoptosis in DCs is unclear to date, the process was found to be likely mediated by reduced c-FLIP expression, because it was targeted to degradation in a proteasome-dependent manner (Kather et al., 2010; Stefanidou et al., 2013a). Importantly, an HSV-2 mutant virus lacking the gene that encodes glycoprotein D (*US6*), was shown to be non-lethal for DCs, yet it is unknown if the mutated or deleted viral gene is directly involved in cell death or if its deletion interferes with viral processes that relate to cell death (Petro et al., 2015; Retamal-Diaz A. et al., 2017).

HSVs have also been described to induce the death of T cells either, directly or indirectly. Indeed, a study reported that HSV-2 induced apoptosis in T cells through the activation of caspase-9, -8, and -3 (Vanden Oever and Han, 2010). Although the mechanism by which apoptosis was induced involved intrinsic apoptotic pathways, the addition of inhibitors of apoptosis was unable to completely revert cell death (Pongpanich et al., 2004). Indirectly, HSV-1 has been described to induce T cell "fratricide," a process in which activated T cells infected with HSV-1 increase their surface expression of FasL and induce the apoptosis of neighbor T cells, through FasL signaling through Fas receptor (Raftery et al., 1999). Overall, the findings discussed above indicate that HSVs can differentially modulate apoptosis in immune and non-immune cells, which may favor interference with the host antiviral immune response while allowing viral replication to occur in epithelial cells.

HSVs INTERFERE WITH TOLL-LIKE RECEPTOR SENSING OF VIRAL COMPONENTS

Immune and non-immune cells express numerous molecular sensors that detect virus components or infection-related stimuli that promote the induction of rapid antiviral responses that hamper viral replication and virus propagation (Mogensen, 2009). A type of stimuli that may be encountered or produced during virus infection are pathogen-associated molecular patterns (PAMPs) (Tang et al., 2012), as well as danger signals released due to cellular stress in response to viral replication and known as damage-associated molecular patterns (DAMPs) (Johnson et al., 2013). Host receptors that sense these stimuli include Toll-like receptors (TLRs), which include both cytosolic and nuclear proteins (Mogensen, 2009). Upon the engagement of ligands by such types of receptors, signaling pathways take place which results in the expression of factors with antiviral activity, as well as the production of soluble and membrane-bound molecules that modulate the activity of the infected cell and neighboring cells (Pandey et al., 2014). The early recognition of viral factors by the host, immediately after infection will favor an effective control of the pathogen and hamper its replication and dissemination, altogether likely promoting the establishment of a protective and long-lasting immunity (Mogensen and Paludan, 2001; Tang et al., 2012).

Toll-like receptors, such as TLR2, TLR3, TLR7, and TLR9 have been described to mediate antiviral activities against HSVs during infection (Alexopoulou et al., 2001; Triantafilou et al., 2014). Experimental findings indicate that TLR2 recognizes glycosaccharides within the virion structure, which provides some degree of protection against HSVs. Indeed, it has been reported that TLR2 recognizes the glycoprotein B (gB) of HSV-1, promoting NF-κB activation and the secretion of interleukin (IL)-8 (Cai et al., 2013). Additionally, TLR2 seems to work in concert with the integrin αvβ3, acting as a coreceptor for its activation which leads to type-I IFN production in response to the HSV-1 proteins gH/gL (Gianni and Campadelli-Fiume, 2014). *In vivo* assays showed that in TLR2 knockout mice neuronal CCL2 levels were decreased, in association with reduced macrophage recruitment into the enteric nervous system after intragastric HSV-1 infection (Brun et al., 2018).

On the other hand, the use of agonists of TLR3, a receptor that recognizes pathogen or host double-stranded RNA (dsRNA) that may be produced during viral infections or abnormal cellular processes, has been reported to promote effective antiviral responses (Alexopoulou et al., 2001; Weber et al., 2006). Among HSV-related viral processes that occur during viral transcription, overlaps within (intra-molecular) or between (inter-molecular) viral and host mRNAs may yield dsRNA structures that induce the activation of dsRNA sensors. Additionally, HSVs encode micro RNAs (miRNAs, miR), which are processed from dsRNA intermediates. Some of these miRNAs have been shown to be involved in regulating virus latency. For instance, miR-H2 targets ICP0, which is required for immediate early gene expression and lytic infection, while miR-H3 and miR-H4 encode antisense sequences that counteract the neurovirulent virus lytic factor ICP34.5 (γ34.5). Furthermore, miR-H6 targets ICP4 and promotes LAT transcription (Piedade and Azevedo-Pereira, 2016). Other miRNAs, such as miR-H1, miR-H5, miR-H7, miR-H8, and miR-H11 are also loaded onto the RNA-induced silencing complex (RISC), which may also help trigger dsRNA sensors within infected cells (Flores et al., 2013). Although the precursors of these miRNAs may eventually be involved in the activation of host dsRNA sensors, the precise mechanisms by which these receptors are activated have not been determined and calls for further research in this area. Interestingly, the application of the TLR3 agonist Poly I:C was reported to confer protection against HSV-related disease in the mouse genital infection model (Ashkar et al., 2004). Recently, an HSV vaccine candidate based on sub-unit viral antigens used Poly I:C as a potent adjuvant, which elicited a robust antibody response and induced protection to a lethal vaginal challenge with HSV-2 in the mouse infection model. Importantly, protection was associated with the activation of TLR3 by this formulation (Bardel et al., 2016). On the other hand, it has been suggested that CD8α dendritic cells TLR3 expression contributes to the establishment of an antiviral response that is dependent on NK and $CD8^+$ T cell activation (Swiecki et al., 2013).

Importantly, several findings suggest that the host has set mechanisms dependent on TLR3 function to detect HSV infection in the central neural system (CNS) and restrict viral replication (Zhang et al., 2008; Carty et al., 2014). For instance, experiments with TLR3 knockout mice have shown that the expression of TLR3 in astrocytes favors the control of HSV infection in the CNS, mainly thanks to NF-κB-dependent secretion of IL-6 and TNF-α (Reinert et al., 2012; Liu et al., 2013). On the other hand, induced pluripotent stem cells (iPSCs) obtained from TLR3-deficient patients that were differentiated into various neural populations, displayed increased susceptibility to viral infection and impaired IFN secretion (Lafaille et al., 2012). Accordingly, mutations present in genes of the TLR3 signaling pathway, such as the gene encoding for TANK-binding kinase 1 (TBK1), correlated with the development of herpes simplex encephalitis (HSE) in children and young adults (Herman et al., 2012; Lim et al., 2014). Therefore, positive modulation of the TLR3 pathway may help control HSV infection in infected individuals, yet this remains to be determined.

TLR7, which recognizes exogenous single-stranded RNA (ssRNA) has been reported to induce a response that reduces HSV infection and disease in a genital mouse infection model when engaged with the synthetic agonist Imiquimod (Miller et al., 1999). Furthermore, application of this TLR7 agonist in HIV-1-positive patients suffering from acyclovir-resistant HSV-2 disease has been shown to elicit favorable results against this virus. Thus, artificially engaging TLR7 during HSV-2 infection may eventually prove an effective mechanism to reduce virus-related disease and shedding in these patients (Hirokawa et al., 2011; Deza et al., 2015).

TLR9 is expressed in immune and non-immune cells and can sense bacterial and viral DNA, as well as synthetic CpG-oligodeoxynucleotides (CpG ODNs). Interestingly, intranasal

application of CpG ODNs that are TLR9 agonists in BALB/c mice previous to HSV-1 infection was reported to reduce viral load and the production of pro-inflammatory cytokines IL-6, CCL2, and CCL5 by neurons in the CNS, which increased the survival rate of the infected mice (Boivin et al., 2012). Moreover, local mucosal TLR9 engagement with CpG ODNs prior to infection has been described to promote thickening of the genital epithelium and increase immune cell infiltration into the submucosa in order to control HSV-2 replication, conferring protection in the genital tissue after infection in mice (Ashkar et al., 2003). Although TLR9 knockout mice did not die after CNS infection with HSV-1 in one study, these animals were highly susceptible to HSV infection (Krug et al., 2004; Mancini and Vidal, 2018). In another study, TLR9 expression in the trigeminal ganglia was reported to be required to prevent HSV encephalitis induced by intranasal HSV-1 infection, as more than half of the animals that lacked this receptor died. Interestingly, if the animals lacked both, TLR2 and TLR9 all animals died after infection pointing out the relevance of these receptors in HSV infection (Lima et al., 2010). A similar result has been reported in the HSV genital infection model, as both TLR9 and TLR2 together have been observed to be relevant for resisting intravaginal infection by HSV-1. Indeed, a double TLR2/9 knockout mouse was more susceptible to infection than single knockout animals (Uyangaa et al., 2018). The anti-HSV response in the presence of TLR2/9 involved increased differentiation of TNF-α- and iNOS-producing DCs (Tip-DCs) and the activation of NK cells, which was accompanied by increased recruitment of the latter to the site of infection (Uyangaa et al., 2018). Furthermore, CpG treatment has been shown to induce plasmacytoid DCs (pDCs) to secrete IL-12 and type-I IFNs during HSV-2 infection in TLR4 knockout mice, but not TLR9 knockout animals suggesting that these cells need TLR9 to produce these cytokines (Lund et al., 2003; Boivin et al., 2012). Although IFN-α production during HSV infection *in vivo* is mostly TLR9-independent, CpG also elicited significant IFN-α secretion by splenic pDCs in a TLR9-dependent manner during HSV-1 infection *in vitro* (Hochrein et al., 2004).

Taken together, several TLRs recognize HSV components leading to limited disease, while other TLRs are not stimulated by HSV. However, when these receptors are engaged with activating ligands they also display antiviral activities, which suggests that targeting TLR receptors could be an attractive strategy to treat or limit HSV infection.

HSVs ALSO HAMPER THE SENSING OF VIRAL NUCLEIC ACIDS BY NON-TLR RECEPTORS

Besides TLRs, other host receptors also sense nucleic acids expressed during HSV infection, such as cytosolic retinoic-acid-inducible gene-1 (RIG-1)-like receptors and a broad class of putative DNA sensors (Mogensen, 2009). Importantly, viral nucleic acids can act as strong activators of host signaling pathways that lead to antiviral cellular responses (Iwasaki, 2012). Furthermore, the detection of viral nucleic acids frequently leads to the secretion of pro-inflammatory cytokines, as well as the production of IFNs that hamper viral replication and infection (Diner et al., 2015). Interferon-γ inducible protein 16 (IFI16) is a host sensor of nucleic acids that has been reported to be able to recognize episomal dsDNA, particularly DNA replicating in the nucleus of cells, which results in IFI16 acetylation (Ansari et al., 2015). This process is followed by the translocation of IFI16 to the cytoplasm, which leads to the promotion of IFN-β secretion by the cell and the activation of a host multiprotein complex called the inflammasome, able to initiate an inflammatory response (Unterholzner et al., 2010; Kerur et al., 2011). Importantly, HSV-1 and HSV-2 recognition by IFI16 induces the activation of the transcription factors interferon regulatory factor 3 (IRF3) and NF-κB, which once translocated to the nucleus induce IFN-α/β and IL-6 production in vaginal epithelial cells (Dawson and Trapani, 1995; Conrady et al., 2012; Triantafilou et al., 2014). IFI16 recognition of foreign DNA likely depends on the sensing of naked DNA. During HSV-1 infection, IFI16 may silence viral gene expression in human fibroblasts by adding nucleosomes and heterochromatin marks to the viral DNA, thereby restricting the host transcription machinery from accessing the viral genome (Orzalli et al., 2013). On the other hand, in epithelial cells the HSV-1 ICP0 protein has been reported to partially inhibit IFI16 activation by targeting it to the proteasome for degradation (**Figure 3**) (Johnson et al., 2013). A role for IFI16 in HSV infection has been assessed *in vivo*, with IFI16 knockdown mice unable to produce IFN-α and clear HSV-1 from the cornea after ocular infection (Conrady et al., 2012). Taken together, the studies described above indicate that HSVs readily modulate downstream pathways related to IFI16, as its activation seems to be detrimental to these viruses and their replication. Regretfully, to date only a few studies have assessed the roles of these sensors in immune cells in response to HSV infection.

cGMP-AMP synthase (cGAS) is a cytosolic DNA sensor that triggers cytosolic GMP-AMP (cGAMP) production upon binding to an activating DNA (Cai et al., 2014), cGAMP, in turn, acts as a messenger that signals through the transmembrane adaptor stimulator of interferon genes (STING) and leads to the recruitment and phosphorylation of TBK1, which ultimately activates IRF3-dependent production of IFN-α/β (Sun et al., 2013; Wu et al., 2013). Interestingly, HSV-1 recognition by cGAS leads to IFN-α and IFN-β secretion in fibroblasts, as well as immune cells (Orzalli et al., 2015). Furthermore, it has been shown that cGAS and IFI16 detect HSV cooperatively, with cGAS partially localizing in the nucleus and associating with IFI16 to promote the stabilization of the latter (Orzalli et al., 2015). Nevertheless, HSV-1 has been reported to be able to deregulate the function of these sensors. For example, the HSV-1 UL37 tegument protein has been shown to target cGAS and elicit its inactivation through the deamidation of an asparagine residue that is found both, in the human and mouse versions of this protein (**Figure 3**) (Zhang et al., 2018). In addition, apoptosis was observed following activation of cGAS after HSV-1 infection in human foreskin fibroblasts, which required cyclic dinucleotides and the activation of STING (Diner et al., 2016). On the other hand, protein kinase B (PKB, AKT) activation during HSV-1 infection has been observed to phosphorylate and suppress cGAS activity in epithelial cells, macrophages and fibrosarcoma cells

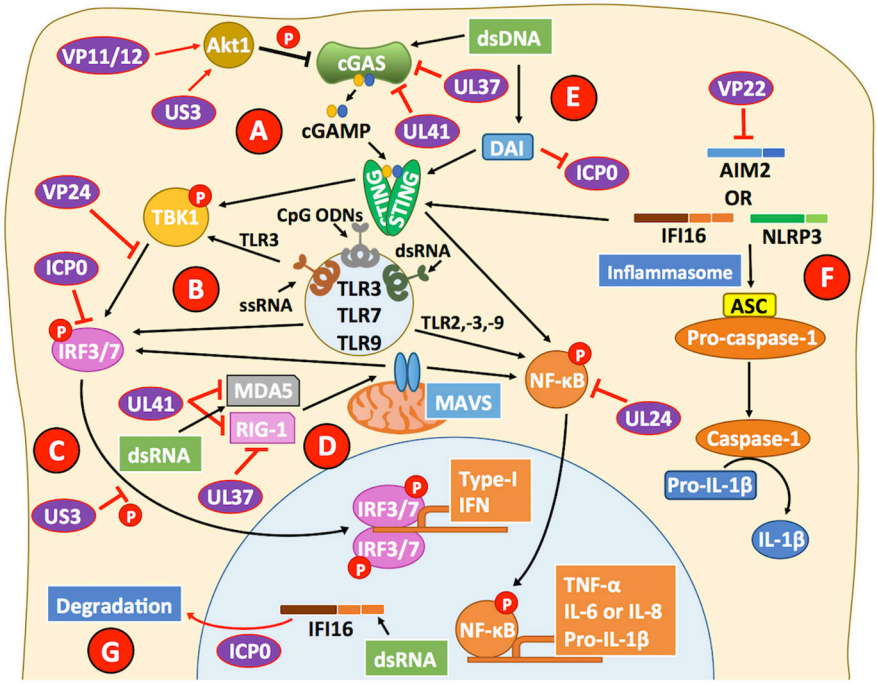


FIGURE 3 | HSVs interfere with viral sensing. **(A)** cGAS is a cytosolic DNA sensor that triggers the activation of STING, which can lead to the phosphorylation of the transcription factor NF-κB and the transcription factor IRF3 through the activity of TBK1. HSV proteins, such as UL37 and UL41 interfere with cGAS activity. VP11/12 and US3 modulate Akt signaling to promote cGAS phosphorylation and suppress its activity, further impairing the capacity of cGAS to mediate STING activation. **(B)** Toll-like receptors (TLRs) are involved in recognizing pathogen and danger signals. Engagement of TLRs with agonists leads to improved antiviral responses due to increased type-I IFN secretion, which is dependent on IRF3/7 and leads to the production of cytokines dependent on NF-κB activation. Importantly, VP24 can target TBK1 to block IRF3 phosphorylation. Downstream of TBK1, ICP0 binds IRF3, and IRF7 to inhibit their activity. **(C)** US3 also blocks IRF3 activation and its translocation to the nucleus reducing type-I IFN production by HSV-infected cells. **(D)** MDA5 and RIG-1 can recognize dsRNA products elicited during viral infection and replication. HSV proteins UL37 and UL41 can impair the function of these cellular sensors, which signal through MAVS to activate NF-κB and promote cytokine production. **(E)** DNA-dependent activator of interferon (DAI) can sense HSV likely through the recognition of HSV dsDNA and inhibit the activity of ICP0, leading to a decrease in viral genome replication. However, after DAI recognition downstream signaling events from STING, through NF-κB are blocked by the viral protein UL24. **(F)** The inflammasome is a multiprotein complex that assembles upon host sensor (e.g., AIM2, IFI16, NLRP3) encounter with viral determinants. The HSV protein VP22 has been reported to block AIM2 sensing of HSV and hence, block pro-caspase-1 activation by adaptor protein apoptosis-associated speck-like protein containing CARD (ASC). By blocking pro-caspase-1 activation, HSV inhibits the production of the pro-inflammatory cytokine IL-1β. Although the host sensor IFI16 has been reported to signal mainly through STING, it can also participate in inflammasome activation. **(G)** The HSV-2 protein ICP0 can direct IFI16 to degradation compartments, thus blocking downstream signaling events by this sensor. Black lines show cellular processes. Red lines show processes modulated by HSVs.

in vitro (**Figure 3**) (Seo et al., 2015). The latter effect is likely due to HSV-1 US3 inhibiting Src family kinases and UL13-dependent VP11/12 tyrosine phosphorylation that leads to the modulation of the phosphatidylinositol-3 kinase (PI3K)/AKT signaling pathway (Eaton et al., 2014). Overall, PI3K/AKT modulation by HSVs would likely provide the virus the ability to interfere with cellular processes related to this pathway, such as cell metabolism, proliferation, gene expression, and cell survival (Liu and Cohen, 2015). Signaling through STING has been shown to be particularly important for conferring protection against ocular HSV-1 infection, as increased disease and virus replication were observed in the corneas and trigeminal ganglia of STING knockout mice, as compared to control animals (Parker et al., 2015). Consistently, treatment with 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a STING agonist prior to infection protected mice from HSV neurological disease, which was associated with reduced viral replication thanks to increased type-I IFN production (Ceron et al., 2019).

Although cGAS is targeted early after infection by HSV, one wonders if it would be possible to detect this host sensor at later time points during cell infection. In this regard, the virion host shutoff protein (VHS, UL41) has been described to target cGAS for degradation even at 20 h post-infection, significantly reducing the chances that this receptor signal for IFN-β production upon HSV infection of epithelial cells and fibroblasts (**Figure 3**) (Su and Zheng, 2017).

Although some level of interference has been described by HSVs over the nucleic acid sensors described above, the DNA sensor termed DNA-dependent activator of interferon (DAI), which is expressed in primary vaginal tissue has been reported to readily detect HSV-2 and lead to IL-6 and IFN-β release upon infection (Triantafilou et al., 2014). This host sensor has been described to interact with the HSV-1 protein ICP0 to hamper viral genome replication, yet independent of the canonical DNA sensing function of this host factor (**Figure 3**) (Pham et al., 2013). Importantly, downstream events

of the cGAS-STING signaling pathway, which are shared with those of DAI-STING, can be blocked by the HSV-1 serine protease UL24 protein that impairs NF-κB activation (Xu et al., 2017). Furthermore, VP24 can target TBK1 and hamper IRF3 phosphorylation, thus blocking alternative downstream signaling pathways associated with STING activation (**Figure 3**) (Zhang et al., 2016). Again, impairing IRF3 activation within infected cells will result in impaired IFN-I production and subsequent inhibition of interferon-stimulated genes (ISGs) in infected and neighbor cells.

RIG-1, as well as melanoma differentiation-associated protein 5 (MDA5), are two host sensors specialized in recognizing dsRNA (Weber et al., 2006). In the context of DNA viruses, such molecules are likely generated as byproducts during the transcription of viral genes and may derive from viral or host products, although this has not been reported for HSVs. Importantly, both receptors have been reported to have their signaling pathways modulated by the HSV protein VHS early after infection (Cotter et al., 2010; Yao and Rosenthal, 2011). This effect has been described to lead to impaired signaling events that otherwise should elicit IRF3 activation and an IFN-β-mediated antiviral response (**Figure 3**) (Yao and Rosenthal, 2011). RIG-1 has been reported to activate the STING pathway through an RNA-DNA sensor crosstalk mechanism aimed at restricting HSV-1 infection in epithelial cells and fibroblasts, as well as *in vivo* (Liu et al., 2016a). Additionally, the HSV-1 UL37 viral protein has been shown to directly block the function of RIG-1, through the deamidation of its helicase domain, which is needed for sensing dsRNA products (**Figure 3**) (Zhao et al., 2016).

In neuronal tissues DAI and RIG-1 work in tandem to detect HSV-1 in the CNS and elicit the production of the inflammatory cytokines TNF-α and IL-6 by murine glial cells, which altogether promote CNS inflammation and increased CNS permeability that allows immune cells to cross the blood-brain barrier, as well as IFN-I type-I to limit viral replication (Crill et al., 2015). Accordingly, RIG-1-mediated recognition of viral nucleic acids in this context depends on host DNA-dependent RNA polymerase III transcription of viral genes into mRNA harboring a 5′ triphosphate CAP structure, which is a substrate for RIG-1 and would allow an antiviral response either, directly or indirectly through DAI or RIG-1, respectively (Crill et al., 2015).

Another viral sensing pathway related to HSV and nucleic acids is the recognition of viral DNA and the activation of the inflammasome early after infection and then, its inhibition later during the virus replication cycle (Johnson et al., 2013). The inflammasome is a multiprotein complex composed by either one of the cytoplasmic sensors NLRP3 or AIM2, combined with IFI16 and has been described to sense HSV in keratinocytes (Chen and Ichinohe, 2015; Gimenez et al., 2016; Strittmatter et al., 2016). Consistent with this finding, a recent study found that IFI16 and NLRP3 are activated in human fibroblasts early after HSV infection (4 h) with consequent IL-1β release (Johnson et al., 2013). However, at later time points (8 h), IFI16 was found to be directed to the proteasome by the viral protein ICP0 and caspase-1, which is a pro-inflammatory effector induced by the inflammasome, and appeared to be trapped within actin clusters instead of being free in the cytosol to enact its catalytic activity (**Figure 3**) (Johnson et al., 2013). Additionally, HSV-1 has been reported to inhibit AIM2-dependent inflammasome signaling events by preventing its oligomerization through the viral protein VP22 (Maruzuru et al., 2018). Thus, HSVs also seem to have evolved molecular mechanisms to block the activation of the inflammasome within infected cells, as a mechanism to hamper the overall function of this sensor and therefore limit its effector capacity of alerting the cells of the presence of the virus.

Finally, virus-infected cells can also detect tertiary RNA structures derived from viral mRNAs thanks to protein kinase R (PKR), a host factor that once activated can help hamper the replication of viruses by inducing NF-κB activation and the expression of cytokines that control virus replication and infection (IFNs) (Kang and Tang, 2012). Furthermore, PKR can control protein synthesis by inducing its shutdown within the cell through the phosphorylation of the host translation initiation factor 2-alpha (eIF2α), which ultimately leads to cell apoptosis (Vattem et al., 2001). Because inhibition of translation within infected cells would be detrimental to the replication cycle of HSVs, these viruses override PKR function by inhibiting the phosphorylation of eIF2α thanks to the viral proteins γ34.5 and US11 (**Figure 1**) (He et al., 1997; Poppers et al., 2000; Carr et al., 2005). Thanks to these viral factors, HSVs can bypass cellular processes elicited after contact of host sensors with viral nucleic acids to enable productive viral infection and virus replication within infected cells.

Taken together, several nucleic acid receptors other than TLRs can sense activating nucleic acids generated during HSV infection. The recognition of such ligands likely helps counteract virus infection and dissemination to other cells and tissues within the host. Importantly, several of these receptors are known to recognize dsRNA structures, yet the origin of these nucleic acids in the context of HSV infection has not been established, and further studies are needed for their identification.

HSVs INTERFERE WITH THE HOST INTERFERON RESPONSE

The activation of pathogen recognition receptors (PRR), can lead to the activation of immune and non-immune cells and trigger antiviral responses that restrict and interfere with virus replication. A significant antiviral response elicited by the sensing of viruses is the IFN response. IFNs are cytokines that once bound to their receptor can potentiate antiviral activities both, in the cell that secretes these molecules and neighbor cells (Schoggins, 2014). IFNs are classified as type-I, -II, or -III. Type-I IFNs are a broad family of molecules that can be secreted by numerous cell types early after infection in response to pathogens such as viruses, with some well-known members being IFN-α, IFN-β, and IFN-ε, and others more recently described IFN-υ, IFN-ω, and IFN-ζ (Hemmi et al., 2002; Al-Khatib et al., 2004; Diebold et al., 2004; Oritani and Tomiyama, 2004; Theofilopoulos et al., 2005; Ma et al., 2018). On the other hand, type-II IFNs have a sole family member, namely IFN-γ which is secreted by specialized subsets of immune cells usually late during infection (Boehm et al., 1997; Bigley, 2014). Finally, type-III IFNs such as

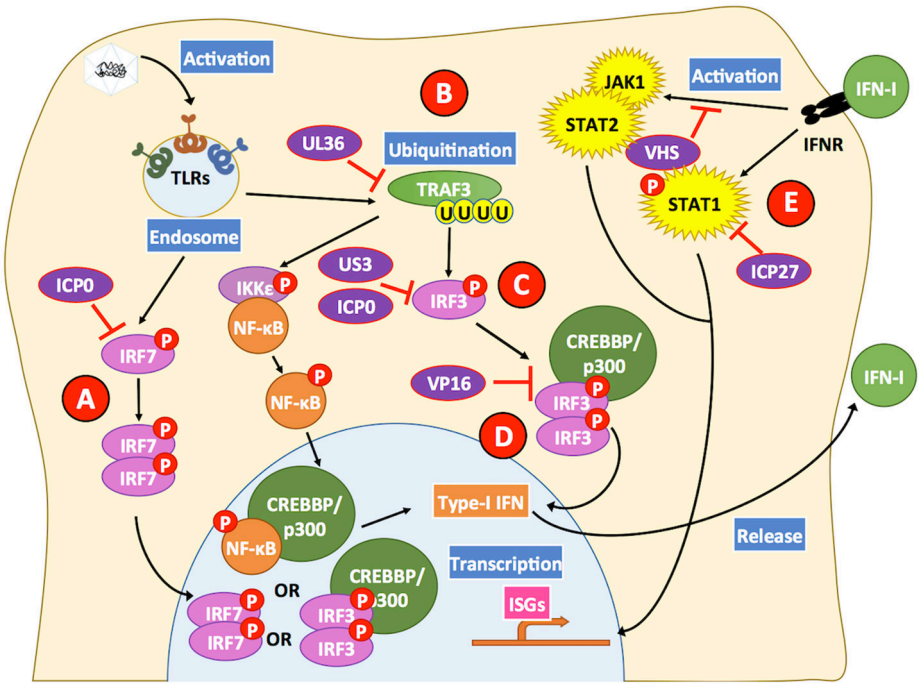


FIGURE 4 | HSV proteins modulate key steps in interferon-related pathways. HSV proteins inhibit interferon-related pathways. Engagement of Toll-like receptors (TLRs) by viral determinants leads to the activation of transcription factors that induce the expression of type-I IFNs. **(A)** The HSV ICP0 protein can block IRF7 activation by hampering its phosphorylation and consequently inhibit its translocation to the nucleus. **(B)** Additionally, UL36 inhibits the ubiquitination of TRAF3 which is required for positive downstream signaling and activation of the transcription factors NF-κB and IRF3. **(C)** HSV proteins US3 and ICP0 can interfere with IRF3 activation at this stage, thus blocking this signaling pathway that otherwise would lead to type-I IFN expression. **(D)** Furthermore, VP16 inhibits the formation of the IRF3-CREBBP/p300 complex hampering signaling events that would lead to IFN-I expression. **(E)** Upon IFN-I engagement, IFNR on the cell surface elicits intracellular signaling cascades mediated by STAT1, STAT2, and JAK1. However, the viral protein ICP27 interferes with STAT1 activation and the viral protein VHS hampers STAT2- and JAK1-related signaling pathways that otherwise would induce the expression of ISGs, which elicit antiviral effects. Black lines show cellular processes. Red lines show processes modulated by HSVs.

IFN-λ1, IFN-λ2, and IFN-λ3 are usually secreted early during infection and have somewhat similar effects than type-I IFNs, although their secretion is limited to epithelial cells (Donnelly and Kotenko, 2010). While type-I and type-III IFNs are related to the induction of multiple antiviral effects in several cell types, type-II IFNs are more related to regulatory roles among immune cells and are accordingly mainly expressed by such types of cells, such as T helper cells (Tau and Rothman, 1999).

Because IFNs have detrimental effects on viruses, HSVs encode an array of molecular factors that negatively modulate the induction of IFN, their production, secretion, and their associated effects by interfering, among others with their intracellular signaling pathways (Peng et al., 2009). For example, the ICP0 proteins of both, HSV-1 and HSV-2 have been described to directly bind and interfere with the activation of IRF3 and IRF7, two transcription factors related to the expression of type-I IFNs (**Figure 4**) (Eidson et al., 2002; Lin et al., 2004; Zhang et al., 2015). Importantly, mice that lack both IRF3 and IRF7 (IRF3/7 double knockout mice) have been described to suffer increased HSV-1 replication and display enhanced dissemination of this virus to several organs after corneal infection (Murphy et al., 2013). Additionally, the HSV-1 US3 protein has been reported to hyperphosphorylate IRF3, which impairs its dimerization and nuclear translocation, thus hampering the transcription of *IFNB* mediated by this transcription factor (Wang et al., 2013b). Furthermore, the HSV-1 tegument protein VP16 has been shown to block IFN-β expression through the inhibition of IRF3 and NF-kB, by impairing the recruitment of the shared coactivator CREB binding protein (CBP) to IFN-I promoters, which is required by these transcription factors to induce IFN-I expression (**Figure 4**) (Xing et al., 2013). Accordingly, the HSV-1 protein UL36, an ubiquitin-specific protease has been shown to de-ubiquitinate TRAF3 (TNF receptor-associated factor-3), thereby hampering stimuli-induced IRF3 dimerization, which is required for IRF3 translocation to the nucleus thus, inhibiting IFN-β transcription (**Figure 4**) (Wang et al., 2013a). Moreover, the advantage for HSVs in interfering with the signaling events associated to type-I IFN secretion has been evidenced *in vivo*, as low IFN-α and IFN-β production is observed in the genital tract of mice after infection (Milligan and Bernstein, 1997; Peng et al., 2009).

When IFNs are released from infected cells, they can act either as paracrine or autocrine ligands by binding to IFN receptors on the cell surface and induce ISG within cells. Antiviral effects of IFNs include restricting the replication of the viral genome, inhibition of protein translation, and impaired

virus egress (Schoggins and Rice, 2011). In order to counteract these outcomes, HSVs interfere with signaling events that occur downstream of the IFN receptors. For instance, the HSV-1 ICP27 protein affects STAT-1 activation, which is a signal transducer for ISG transcription. ICP27 has been reported to interfere with the phosphorylation and nuclear accumulation of STAT-1 in order to impair its activity as a transcription factor (**Figure 4**) (Johnson et al., 2008). Additionally, there is indirect evidence, through an HSV-1 mutant, that suggests that the viral protein VHS could partially be responsible for reducing the activity of signal transducers such as JAK1 and transcription factors like STAT-2, as observed in HSV-infected HeLa cells (**Figure 4**) (Chee and Roizman, 2004). Additionally, the HSV-1 ICP27 protein has been reported to be involved in the secretion of an uncharacterized soluble factor that has antagonizing activity over IFN-I signaling pathways in neighboring uninfected cells (Johnson and Knipe, 2010). *In vivo* studies have assessed the relevance of IFN-I in HSV infection in mice that lack the receptors for type-I IFNs, namely IFNAR1 and IFNAR2c, and shown that inoculation of HSV-1 in the footpads of such animals results in a reduced capacity of the host to control HSV replication, leading to systemic infection, although non-lethal (Luker et al., 2003).

On the other hand, IFN-αβγR$^{-/-}$ mice have been found to be highly susceptible to acute liver failure after HSV-1 corneal infection, with IFN-αβγR expression in both, immune and non-immune cells playing relevant roles in the control of systemic HSV infection (Pasieka et al., 2011; Parker et al., 2016). Moreover, a key role for type-I IFN signaling has been identified in neurons, since immune cell and non-neuronal cell IFN responses do not protect from lethal corneal HSV infection when the these pathways are abrogated in neurons (Rosato and Leib, 2015). In a later study, it was found that IFN-I signaling in neurons was dispensable for the establishment of latency and that cells deficient in IFN-I signaling supported reduced reactivation yet, displayed higher levels of LAT indicating that IFN-I likely regulates LAT expression in neurons (Rosato et al., 2016). Consistent with the relevance of type-I IFNs in HSV infection, topical application of IFN-α was reported to significantly reduce the frequency of recurrences and viral shedding in patients suffering from genital HSV reactivations (Shupack et al., 1992). Although HSVs have mechanisms to impair type-I IFN secretion and their effects, such molecules may eventually reach adjacent cells that are non-infected and elicit signaling events in these cells (Gill et al., 2011; Lee et al., 2017).

IFN-γ induction is associated with positive outcomes during HSV-1 and HSV-2 infections, with reduced viral replication. Furthermore, IFN-γ may be considered a marker related to the potential efficacy of prophylactic formulations (Svensson et al., 2005; Bird et al., 2007; Sato et al., 2014; Khan et al., 2015). Without IFN-γ, T cells are incapable of conferring protection against HSV genital infection (Johnson et al., 2010). However, the relationship between IFN-γ and HSV control is intricate, as the antiviral effects of this cytokine are tissue-dependent and vary depending on whether the virus remains latent in infected cells or is productive in the lytic cycle (Bigley, 2014). Among numerous effects, IFN-γ causes microtubule remodeling in infected cells, which is mediated through the activity of the molecules suppressors of cytokine signaling 1 and 3 (SOCS1 and SOCS3). However, elevated SOCS expression elicits microtubule stabilization and an inhibition feedback on IFN-γ effects, which has been exploited by the HSV-1 ICP0 protein, capable of upregulating SOCS during lytic infection in keratinocytes (Frey et al., 2009). Although IFN-γ acts over promoters of IFN-γ-stimulated genes (ISGs) that have antiviral functions, ISG expression is restricted by epigenetic regulations of histone 3 (H3) in the trigeminal ganglia and is dependent on histone deacetylases (HDACs) to maintain chromatin in a transcriptionally inactive state (Gao et al., 2013). During HSV-1 infection of the trigeminal ganglia, neurons may respond to stress stimuli (e.g., UV light) and inhibit HDACs, which results in SOCS1 and SOCS3 acetylation and the loss of IFN-γ effects. Additionally, chromatin may suffer relaxation processes allowing the viruses to exit latent infection of neurons (Guise et al., 2013). Although a relevant role for IFN-γ has emerged from some studies, paradoxically mice lacking IFN-II receptors IFNGR1 and IFNGR2 showed comparable levels of viral loads as controls when challenged with HSV-1, suggesting that the effects of IFN-γ are somewhat complex in the context of HSV infection (Luker et al., 2003).

Regarding type-III IFNs, relatively few studies have assessed their role during HSV infection. However, one study has reported that the administration of IFN-λ1 (IL-29) prior to HSV-1 infection promoted the expression of numerous antiviral proteins in primary human keratinocyte cultures. One of them, IFN-β helped prevent their infection. This effect was dependent on TLR3 engagement and JAK-STAT signaling events (Zhang et al., 2011). Furthermore, in human neurons HSV-1 infection was shown to be suppressed by IFN-λ1 and IFN-λ2 (IL-28A), particularly through the upregulation of TLR3 and TLR9 expression and subsequent TLR3/9-mediated antiviral responses involving the transcription factor IRF7 (Zhou et al., 2011). Interestingly, type-III IFNs have been reported to be secreted in the vaginal mucosa mainly by DCs, yet if this is the case during HSV infection remains to be determined (Iversen et al., 2010).

Taken together, HSVs have evolved several mechanisms to interfere with the host IFN response at multiple levels. Indeed, HSVs can impair IFN secretion and their related signaling events in infected cells. Collectively, the capacity of HSVs to interfere with IFN responses at various steps highlights the importance of these molecules and pathways in HSV control. Unsurprisingly, potential therapeutic approaches, such Imiquimod induce type-I IFN secretion (Sainathan et al., 2012).

HSVs DOWN-MODULATE THE ANTIVIRAL ACTIVITIES OF THE COMPLEMENT AND INNATE IMMUNE CELLS

If HSV-infected cells are unable to restrict the replication of these viruses or their dissemination, an innate immune response consisting on both acellular and different cell types, will likely interact with the viruses or virus-infected cells in an attempt to impede further infection of nearby cells or other tissues (Halford et al., 2005; Nandakumar et al., 2008; Tegla et al., 2011).

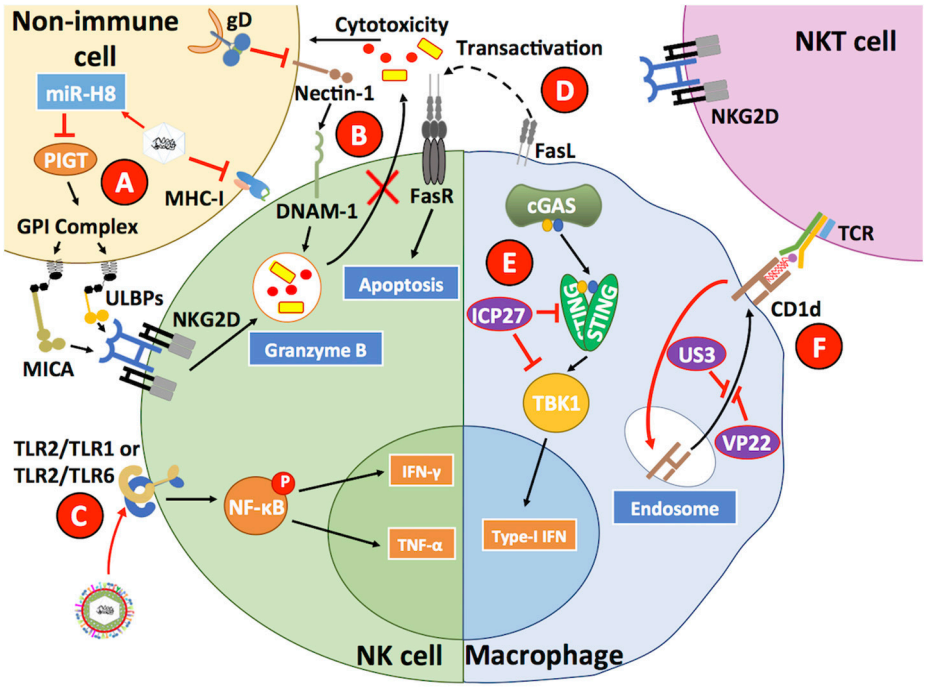


FIGURE 5 | HSVs interfere with antiviral processes in innate immune cells. **(A)** HSV has been described to reduce MHC-I expression on the surface of infected cells. In addition, HSV also reduces MICA and ULBP1-3 expression through the inhibition of PIGT, a member of the GPI anchoring complex by the HSV-1-encoded microRNA H8 (miR-H8). **(B)** The HSV glycoprotein gD reduces nectin-1 expression on the surface of infected cells, hampering DNAM-1 binding to this host factor and diminishing the capacity of NK cells to mediate the lysis of HSV-infected cells, which is normally mediated by granzymes. **(C)** HSV has been reported to directly engage TLR2 on the surface of NK cells, which leads to IFN-γ and TNF-α secretion. **(D)** FasL expressed on the surface of HSV-infected macrophages has been reported to induce Fas-mediated apoptosis in NK cells. **(E)** Within HSV-infected macrophages, the HSV protein ICP27 has been reported to inhibit STING and TBK1 activation, thus interfering with this signaling pathway that generally leads to IRF3-dependent type-I IFN secretion by virus-infected cells. **(F)** HSV infection of macrophages reduces the surface expression of CD1d, which in combination with a glycolipid acts as a receptor for NKT cell TCRs. CD1d is sequestered by the HSV proteins US3 and VP22. Thus, HSV reduces NKT expansion and function by hiding its activating ligand. Black lines show cellular processes. Red lines show processes modulated by HSVs.

However, HSVs are able to inhibit the chain reactions carried out by the host complement which is intended to hamper pathogens by initiating a cascade of protein activations that lead to a membrane attack complex (MAC) (Serna et al., 2016). Indeed, the gC glycoprotein of HSVs can bind to the complement component C3b and block alternative pathways that otherwise lead to the formation of a MAC on the pathogen surface, or the surface of virus-infected cells (Friedman et al., 1984; Mcnearney et al., 1987. Additionally, gC also binds to the complement components C3 and C5, further inhibiting pathways related to the activation of this antiviral mechanism (Lubinski et al., 2002; Hook et al., 2006b).

On the other hand, natural killer (NK) cells are innate immune cells capable of sensing and destroying virus-infected cells that either lack the expression of major histocompatibility complex I (MHC-I) molecules, or express NK-activating molecules on the surface because of abnormal cellular processes betray infection (Mandal and Viswanathan, 2015). HSVs hampers MHC-I expression on the surface of infected cells, which under normal conditions should elicit the activation of NK cells (Orr et al., 2005). However, HSV-1 infection has been shown to reduce the expression of MHC class I polypeptide-related sequence A (MICA) and UL16 binding proteins 1–3 (ULBP1, ULBP2, ULBP3) on the surface of infected cells, which are activators of NK cells that mediate signaling events through the engagement of NKG2D in these cells (**Figure 5**) (Schepis et al., 2009). This inhibition has been reported to be mediated by HSV-1-encoded mir-H8, which downregulates PIGT expression, a member of the GPI anchoring complex that anchors MICA and ULBP1-3 and results in the surface downregulation of these NK ligands (Enk et al., 2016). Therefore, NK cells do not release cytotoxic molecules, such as granzymes onto HSV-1-infected cells, protecting these cells from NK-mediated apoptosis. Nevertheless, NK cells pulsed with HSV-1 and HSV-2 glycoprotein gD antigens and inoculated with TLR2 agonists produced IFN-γ that activated antiviral $CD4^+$ T cells (Kim et al., 2012). Consistent with immune evasion properties by HSVs, the HSV-1 gD glycoprotein has been reported to sequester nectin-1 from the cell surface of infected cells and induce decreased DNAX accessory molecule-1 (DNAM-1) receptor engagement on the surface of NKs by this ligand, thus preventing NK cell-mediated lysis of infected cells (**Figure 5**) (Grauwet et al., 2014). Finally, macrophages infected with HSV-1 have been described to express

FasL and induce apoptosis in NK cells that express Fas receptors (Iannello et al., 2011).

Another innate immune cell type known to participate in antiviral responses is Natural Killer T cells (NKT cells). NKTs recognize antigens in the form of glycolipids presented on CD1d molecules that share structural similarities with MHC-I (Godfrey et al., 2010). Importantly, HSV-1 has been described to negatively affect NKT activation by downregulating CD1d expression on the surface of infected cells (**Figure 5**) (Yuan et al., 2006; Rao et al., 2011). More specifically, HSV-1 was shown to redirect CD1d from the cell surface to intracellular compartments through the phosphorylation of the host factor KIF3A by the viral kinase US3 (Xiong et al., 2015). Furthermore, cellular recycling of CD1d was also inhibited by the viral protein VP22 working along US3 (Liu J. et al., 2016). Importantly, the administration of α-galactosylceramide, an NKT ligand that elicits the recruitment of these cells to the vaginal tissue was reported to decrease the susceptibility of mice upon HSV-2 intravaginal infection (Iversen et al., 2015).

Macrophages are also targets of HSVs. In these cells, HSV-1 has been reported to inhibit downstream events related to the cGAS-STING-TBK1 axis, particularly through the direct interaction of ICP27 with STING and TBK1, which produced a reduction in IFN-I secretion by these cells (**Figure 5**) (Christensen et al., 2016). Interestingly, STAT-1-knockout mice, which are unresponsive to IFN-α and IFN-γ, have been found to be more susceptible to HSV-1 in terms of macrophage infection, as compared to wild-type mice suggesting that these cells utilize a JAK-STAT-1 signaling pathway to restrict HSV replication (Mott et al., 2009). Additionally, HSV-1 has been shown to produce higher levels of pro-inflammatory cytokines in M1 macrophages as compared to M2 macrophages, with M1 characterized as "classically polarized" macrophages vs. M2 macrophages that are "alternatively polarized" (Martinez and Gordon, 2014). The latter observation suggests that pro-inflammatory M1 macrophages infected by HSV-1 promote increased eye inflammation (Lee and Ghiasi, 2017). On the other hand, in the same study when macrophages were stimulated to induce their differentiation toward an M2 phenotype, these cells produced anti-inflammatory cytokines (e.g., IL-10), which was associated with less eye pathology.

Regarding other innate immune cell types, such as neutrophils or mast cells, these cells have been described to participate at the onset of immune cell infiltration into skin and corneas during HSV infection (Royer et al., 2015; Hor et al., 2017). However aside from contributing to exacerbated inflammation, a protective role has not been attributed to neutrophil activity in these tissues in mice models, yet mast cells seem to be necessary for assisting innate immunity in the cornea of mice (Royer et al., 2015; He et al., 2017).

Altogether these results suggest that HSVs target NK and NKT cells, as well as macrophages because these cells likely play a crucial role in controlling HSV infection. Thus, potentiating the activation and functions of these cells during HSV exposure and infection could elicit improved responses against these viruses.

HSV INFECTION MODULATES DENDRITIC CELL MATURATION, ANTIVIRAL ACTIVITY AND MIGRATION

Dendritic cells (DCs) are key immune cells that promote and regulate immune responses by modulating the activity of innate and adaptive immune cells (Gonzalez et al., 2008; Cespedes et al., 2013). DCs are strategically located throughout the body acting as sentinels that probe the environment surrounding mucosae, skin, as well as internal organs. Ultimately, DCs sense and capture foreign and self-antigens for their processing (Soloff and Barratt-Boyes, 2010). DCs degrade protein-derived antigens and present them to T cells as small peptides loaded on MHC-I and -II molecules (pMHC) that can be recognized by T cell receptors (TCR) on the surface of $CD8^+$ and $CD4^+$ T cells, respectively (Galvez et al., 2016). DC antigen presentation to T cells can lead to a process termed the immunological synapse, which involves close DC-T cell interactions that can result either in T cell activation or its inactivation (Gonzalez et al., 2007; Murphy et al., 2012; Retamal-Diaz et al., 2015; Retamal-Diaz A. et al., 2017). Importantly, the interaction between DCs and antigen-specific T cells will determine the phenotype of T cells which will depend on the expression of membrane-bound and soluble molecules presented at the cell-cell interphase (Zheng et al., 2004). As a result of DC-T cell activation, T cells can become among other cell types, cytotoxic or regulatory by secreting soluble factors that kill infected cells, modulate immune, and non-immune cells, or promote tolerance to antigens, eventually ignoring cognate antigens (Gonzalez et al., 2007).

Because of the role of DCs in defining the phenotype of T cells, which in turn can affect the overall immune response against a viral pathogen such as HSV, the interaction between DCs and these viruses has gained increasing attention in the last decade. Importantly, DCs are permissive to HSV infection, although virus yields are somewhat limited as compared to other cellular substrates, such as epithelial cells (Pollara et al., 2003; De Jong et al., 2008; Grosche et al., 2017; Retamal-Diaz A. et al., 2017). Once infected with HSVs, DCs display reduced antigen presentation on MHC-I molecules, which is mediated by the viral protein ICP47 that acts over transporters associated with antigen processing (TAP) at the endoplasmic reticulum and impedes antigen translocation to this organelle for the loading of viral antigenic peptides onto MHC-I molecules (**Figure 6**); yet, this phenomenon has been reported to occur at a lower extent in murine cells, as compared to human cells (Hill et al., 1995; Tomazin et al., 1998; Elboim et al., 2013; Oldham et al., 2016a). Interestingly, ICP47 has been reported to adopt a helical hairpin structure that blocks TAP function and peptide translocation, as it precludes substrates from binding to the transporter and prevents the two cytoplasmic nucleotide-binding domains (NBD) of TAP from hydrolyzing ATP, which is required for their activity (Oldham et al., 2016b). Despite the existence of several variants of TAP-1 and TAP-2 in humans, ICP47 does not seem to have a particular preference over one or other polymorphism (Praest et al., 2018).

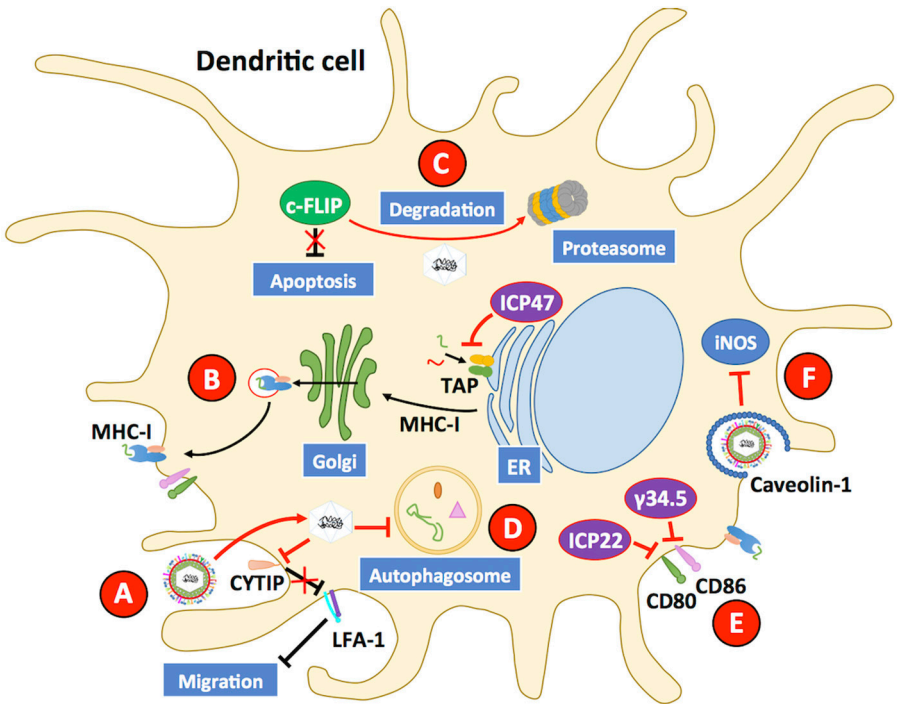


FIGURE 6 | HSVs interfere with dendritic cell function. Dendritic cells (DCs) are susceptible to HSV-1 and HSV-2 infection. **(A)** Upon infection with HSV, the host protein CYTIP is degraded, which causes the upregulation of LFA-1 and reduces the capacity of DCs to migrate to draining lymph nodes and activate T cells. **(B)** HSV infection hampers the capacity of DCs to present virus-derived antigens to T cells on MHC-I molecules by interfering with the activity of transporters associated with antigen presentation (TAP proteins). Inhibition of TAPs is mediated by the viral protein ICP47. **(C)** HSVs elicit apoptosis in DCs through the downregulation of c-FLIP, a potent anti-apoptotic protein, which is directed to the proteasome during infection of these cells. **(D)** HSV infection hampers the activity of the autophagosome, which has been reported to reduce antigen presentation to $CD8^+$ T cells. **(E)** CD80 and CD86 are co-stimulatory molecules that are commonly upregulated during infection, and along with MHC-peptide complexes enable DCs to activate T cells. HSV inhibits the expression of CD80 and CD86 on the DC surface thanks to the viral proteins γ34.5. The viral protein ICP22 also inhibits the expression of CD80 on the cell surface. **(F)** HSV infection inhibits inducible nitric oxide synthase (iNOS) in DCs through the downregulation of caveolin-1, which will reduce the antiviral capacity of these cells. Black lines show cellular processes. Red lines show processes modulated by HSVs.

HSV-1 and HSV-2 can also reduce the capacity of DCs to activate T cells by decreasing the expression of the co-stimulatory molecules CD80 and CD86 on the cell surface, which has been suggested to occur through the downregulation of IFNα/β levels by the viral protein γ34.5 (**Figure 6**) (Jin et al., 2009; Suazo et al., 2015a). Consistently, an HSV-1 with a mutation in γ34.5 is capable of inducing the maturation of DCs through TBK-1-dependent phosphorylation of IRF3 (Ma et al., 2017). However, a later study suggests that inhibition on IRF3 activation by γ34.5 is also mediated by mechanisms other than TBK-1, as the deletion of the TBK-1 binding domain (TBD) of γ34.5 did not restore IRF3 activation, although this finding remains to be confirmed in DCs as the study was performed in human foreskin fibroblasts cells (Manivanh et al., 2017). On the other hand, the HSV-1 protein ICP22 has been reported to be capable of binding to the CD80 promoter in DCs circulating through HSV-infected cornea, inhibiting the expression of this important co-stimulatory molecule for T cells (Matundan and Ghiasi, 2018).

Moreover, both HSV-1 and HSV-2 have been reported to inhibit autophagosome formation in DCs, by interfering with cellular degradation processes and affecting antigen presentation to $CD8^+$ T cells (Suazo et al., 2015a; Budida et al., 2017). Because DCs utilize autophagy as a means to limit viral replication within these cells, inhibition of this process likely contributes to HSV subversion of DCs (**Figure 6**) (Rasmussen et al., 2011). HSV-1 has been described to interfere with nitric oxide synthase within lung DCs via downregulation of caveolin-1 (Cav-1), further hampering the antiviral capacities of HSV-infected DCs (**Figure 6**) (Wu et al., 2015).

Additionally, HSV-1 and HSV-2 have been reported to hamper DC migration from the infected tissue to the corresponding lymph nodes (LNs), thereby likely reducing the efficacy of DCs at activating $CD4^+$ and $CD8^+$ T cells at this site (Prechtel et al., 2005; Bedoui and Greyer, 2014; Retamal-Diaz A. et al., 2017). Indeed, HSV-1 has been shown to promote the degradation of cytohesin-interacting protein (CYTIP) in mature DCs, which regulates DC motility by downregulating integrin expression and causes the upregulation of lymphocyte function-associated antigen-1 (LFA-1), a β2-integrin protein; therefore enhanced adhesion of DCs occurs in the infected tissue reducing their migration to the LNs (**Figure 6**) (Theodoridis et al., 2011). Additionally, HSV-1- and HSV-2-infected Langerhans cells (LCs) have been reported to undergo apoptosis after infection with HSVs and to be unable to downregulate E-cadherin, which

needs to be reduced at the cell surface to promote the migration of these cells to the LNs (Puttur et al., 2010). In this context, HSV-infected LCs have been described to act as a source of HSV antigen for dermal DCs (dDCs) within the infected skin, which would result in the phagocytosis of apoptotic HSV-infected LCs by dDCs (Kim et al., 2015). The interaction between LCs and HSV, and then by HSV-infected and apoptotic LCs with dDCs would likely result in the priming of HSV-specific T cells *in vivo,* which would be difficult to assess *in vitro* with HSV-infected bone marrow-derived DCs (BMDCs) and monocyte-derived DCs. Importantly, the effects of HSV infection over the capacity of DCs to activate T cells seems to more pronounced *in vitro* than *in vivo* (Bedoui et al., 2009; Kim et al., 2015; Whitney et al., 2018).

However, contrarily to the negative effects described above for HSV over DCs, another study found that upon exposure to HSV-1, a human $CD8\alpha^+$ plasmacytoid DC subset increased the expression of markers associated with the migration of these cells to lymph nodes, and that these DCs were able to promote the activity and functions of T cells, B cells and NK cells, which were recruited to the infection site (Schuster et al., 2015).

Overall, most of the findings described above support the notion that HSVs have evolved different mechanisms and strategies to hamper DC function impacting virus control by these cells and likely negatively affecting adaptive immune responses in the host.

Despite numerous studies describing approaches that elicit protective immunity against HSVs, identifying a correlate of protection for HSV infection has remained elusive. Interestingly, recent studies suggest that the outcome of the DC-HSV interaction may relate to the establishment of protective immunity, as specific HSV mutants that are attenuated in DCs confer particularly protective and robust immunity against HSV infection *in vivo* (Retamal-Diaz A. et al., 2017; Retamal-Diaz A. R. et al., 2017). One of these studies reported that anti-HSV antibodies mediated the protection conferred by the HSV-inoculated DCs, which likely results from the help of B cell-supportive anti-HSV helper T cells (Long et al., 2014). On the other hand, vaginal DCs primed with estradiol have been described to promote $CD4^+$ T cells with a Th17 profile that enabled these cells to efficiently respond against an HSV-2 challenge (Anipindi et al., 2016). An IL-1β-related signaling pathway mediated this favorable response. The relevance for DCs in eliciting protective anti-HSV responses has been further emphasized by studies that assess their contribution at re-stimulating tissue-resident memory T cells (T_{RM}) (Iijima et al., 2008). After HSV-2 infection, T_{RM} $CD8^+$ are recruited to the genital tissue by chemokines such as CXCL-9 and CXCL-10, which are expressed by the infected epithelium (Nakanishi et al., 2009; Iijima and Iwasaki, 2014). Importantly, this recruitment was found to be mediated, at least partially by IFN-γ produced by DCs which came into contact with HSV antigen-specific Th1 helper $CD4^+$ cells and stimulated them to establish T_{RM} $CD8^+$ cells (Smith et al., 2004; Nakanishi et al., 2009). In line with this notion, a "prime and pull" immunization approach was recently described with which protective immunity was achieved against HSV-2 genital infection upon inoculation of an attenuated HSV virus which induced vaginal tissue memory T cells that could be recalled to this tissue in a CXCL10-dependent manner (Shin and Iwasaki, 2012). dDC populations present in the skin within the $CD301b^+$ subset were found to be present at the site of infection after applying "prime and pull" strategy mentioned above and were held responsible for re-stimulating HSV antigen-specific memory $CD8^+$ T cells (Shin et al., 2016).

HSVs have also been reported to induce the synthesis and release of pro-inflammatory cytokines by DCs that promote their infection with HIV and the replication of this virus from previously-infected cells, likely increasing the dissemination of the latter virus during co-infections (Stefanidou et al., 2013a). HSV-2-infected DCs secrete TNF-α, which through signaling processes mediated by TNFR1 and TNFR2 has been reported to induce increased expression of CCR5 in DCs, enabling subsequent infection of these cells with HIV-1 (Marsden et al., 2015; Herbring et al., 2016).

Recent studies support the notion that DCs may promote neuron infection with HSV, thus contributing to virus latency within the host. In addition, it has been observed that animals depleted of DCs display up to fivefold less latently infected neurons in the trigeminal ganglia, as compared to wild-type mice suggesting that DCs participate in processes related to neuron infection (Mott and Ghiasi, 2008). Accordingly, the depletion of the $CD11c^+CD8\alpha^+$ DC subset reduced the amounts of latent HSV-1 in neurons after ocular infection (Mott et al., 2009). Furthermore, Flt3L treatment, which increases the numbers of DCs in tissues, produced increased neuronal infection upon a similar infection (Mott et al., 2008). Taken together these studies suggest HSV may use DCs as Trojan horses to reach neurons, which may occur by virus attachment to the cell surface or virus replication within these cells. Despite these findings, another study found that depleting DCs with diphtheria toxin targeting CD11c-expressing cells was associated with increased viral loads in neurons after HSV infection in the footpads (Kassim et al., 2006). Another study found that mice lacking $CD8\alpha^+$ DCs had increased amounts of latent HSV-1 and more recurrences (Mott et al., 2014).

Overall, HSVs have evolved multiple mechanisms to negatively modulate the function of DCs, which likely results in a reduced capacity of the host to control HSV infection and mount and effective antiviral response. Identifying strategies that improve the interaction between HSVs and DCs should likely ameliorate the overall host response to these viruses either, immediately upon infection or during the establishment of long-term protection.

CONCLUDING REMARKS

Herpes simplex viruses elicit a diverse array of diseases in humans, both in individuals that have immune-related complications, as well as otherwise healthy persons. The capacity of HSVs to elicit disease during primary infections, as well as recurrences after establishing lifelong infection relates, among others to their ability to evade and neutralize host antiviral mechanisms that act in immune and non-immune cells. Importantly, HSVs interfere with early antiviral steps, such as the capacity of the host to sense viral determinants,

signaling pathways that lead to cellular antiviral effects and the function of innate immune cells that act early after infection against these viruses. Evasion of these processes gives HSVs the chance to infect host cells and reach neurons favoring viral latency and lifelong infections, altogether dampening antiviral activities that could help immune cells establish effective and protective immunity against these viruses. The fact that the host somewhat fails at initiating an effective early antiviral response may provide grounds for the establishment of ineffective adaptive immunity, mainly through the interference of DC function, which is crucial for linking innate and adaptive immunity. Thus, improving the outcome of the early host antiviral responses against HSVs could help both, the generation of better anti-HSV therapies, as well as the design of prophylactic strategies intended at preventing infection with these viruses.

AUTHOR CONTRIBUTIONS

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

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Respiratory Syncytial Virus Exacerbates Kidney Damages in IgA Nephropathy Mice via the C5a-C5aR1 Axis Orchestrating Th17 Cell Responses

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Respiratory viral infections can directly lead to kidney damage such as IgA nephropathy (IgAN), partly due to mucosal immune system dysfunction. Although the activated C5a-C5aR1 axis results in increased Th1 and Th17 frequencies but reduced Treg frequencies in Respiratory syncytial virus (RSV) infection, how this axis affects Th cell disorders in RSV-induced IgAN exacerbation remains unknown. Here, we used a mouse model to dissect the activation of C5a-C5aR1 by RSV and the consequences on the regulation of Th1, Th17, and Treg immune responses in IgA nephropathy. RSV fusion protein was clearly deposited not only in the pulmonary interstitium but also in the glomerulus in RSV-IgAN mice, and RSV infection led to more severe pathological changes in the kidneys in IgAN mice. Blocking the C5a-C5aR1 axis resulted in a decrease in the albumin-to-creatinine ratio, and the attenuation of kidney damage in IgAN and RSV-IgAN mice might be partly attributed to the inhibition of Th cell and cytokine dysfunction. Th1, Th17 and Treg immune responses and their corelative cytokines were disrupted by RSV infection and rescued by C5aR1 inhibition. Moreover, we constructed a coculture system of human mesangial cells and $CD4^+$ T cells and found that RSV infection might lead to $CD4^+$ T cell production via human mesangial cells-enhanced $CD4^+$ T cell proliferation, consequently increasing IL-17 levels. These pathological behaviors were augmented by C5a stimulation and decreased by C5aR1 inhibition. Thus, C5aR1 inhibition alters both kidney damage and Th1, Th17, and Treg cell dysfunction in RSV-induced IgAN exacerbation and locally regulates HMC antigen presentation function in the kidney. Taken together, our data offer profound evidence that blocking the C5a-C5aR1 axis might be a potential therapy for RSV-induced IgAN.

Keywords: RSV, C5a-C5aR1 axis, IgA nephropathy exacerbation, $CD4^+$ T cells, human mesangial cells

INTRODUCTION

Immunoglobulin A nephropathy (IgAN), a common disease characterized by prominent IgA deposits in the renal mesangium, is the most prevalent primary chronic glomerulonephritis, particularly in the Asia-Pacific region (Lv et al., 2017). Mucosal immune system dysfunction is chiefly involved in the pathogenesis of IgAN (Fellström et al., 2017). The onset and exacerbation of IgAN are often related to respiratory and gastrointestinal syndromes caused by viral infections (Amore et al., 2004; Wyatt and Julian, 2013). Previous research has focused on the correlation between renal injury and injection with viruses such as coxsackie virus, cytomegalovirus, herpes simplex viruses, Epstein-Barr virus, and adenovirus by detecting viral antigens, DNA or RNA in kidney tissues of IgAN patients (Kawasaki, 2011). Although an IgAN mouse model can be constructed by immunization with infectious or inactivated *Sendai virus* (Amore et al., 2004; Zhang et al., 2017), chronic inflammatory diseases of the respiratory mucosa, whether or not they result in IgAN development, remain uncharacterized (Floege and Feehally, 2016). Respiratory syncytial virus (RSV), a common pathogen of respiratory tract infection, is involved in the mechanism by which minimal change disease causes nephrotic syndrome onset and exacerbation through cytokine dysfunction and direct kidney injury (Liu et al., 2007; Zhai et al., 2016). However, the potential pathogenic mechanism of RSV infection in the IgAN process should be explored.

Our research group demonstrated that $CD4^+$ T lymphocytes, a crucial component of the mucosal immune system that can defend against pathogens, play a key role in IgAN development (Meng et al., 2014; Xiao et al., 2016; Gan et al., 2018b). Increased frequencies of Th17 cells and Th22 cells and decreased Treg frequencies in blood and kidney were observed in IgAN mice compared to normal mice (Meng et al., 2014; Gan et al., 2018b). Moreover, the imbalances in Th17 and Treg cells were further disturbed in mice with IgA nephropathy by hemolytic streptococcus infection (Meng et al., 2014) and tonsillitis (Gan et al., 2018b), respectively. In addition, we found that RSV infection led to $CD4^+$ T cell disorders in normal mice, while the activated C5a-C5aR1 axis could exacerbate the above imbalance (Hu et al., 2017). Furthermore, Bera et al. reported that RSV infection resulted in Th17 relevant cytokine production and lung inflammation in wild-type mice and that C3aR deficiency reversed these reactions (Bera et al., 2011).

The C5a-C5aR1 axis functions as a modulator and effector of immune responses. Liu et al. proposed that C5a and C5aR expression in the urinary tract and kidney was significantly associated with the activity and severity of kidney injury in IgAN patients (Liu et al., 2014). C5aR deficiency reduces proteinuria and attenuates histologic injury in an IgAN mouse model, perhaps partly contributing to the inhibition of kidney cytokine and chemokine expression (Zhang et al., 2017). Notably, blocking C5aR can inhibit cultured human mesangial cells (HMCs) proliferation and cytokine and chemokine secretion (Zhang et al., 2017). In addition, we found that RSV infection apparently enhanced the frequencies of Th1, Th2, and Th17 cells but decreased the Treg cells frequencies by stimulating C5a and C5aR1 production, and the above changes were alleviated by a C5aR antagonist (C5aRA) in an asthma mouse model (Hu et al., 2017). Although the C5aR1-mediated regulation of $CD4^+$ T cells in RSV infection is understood in detail and the C5a-C5aR1 axis can function in IgAN pathogenicity, the mechanisms of RSV-mediated IgAN exacerbation, whether via activating the C5a-C5aR1 axis or orchestrating Th17 cell immune responses, remain unknown.

The main focuses of this project were as follows: (1) to ascertain how RSV infection exacerbates kidney damage in IgAN mice, perhaps through C5a-C5aR1 axis-mediated regulation of Th17 cell responses; and (2) to clarify the capabilities of HMCs to function as antigen-presenting cells to induce Th17 cell proliferation during RSV infection.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from the Experimental Animal Center of Central South University (Changsha, Hunan, China). All animals were fed and housed under desired temperature and humidity conditions in a specific pathogen-free environment. All studies were conducted in accordance with Institutional Animal Care guidelines. This project was approved by the Animal Experimental Ethics Committee of Hunan Province.

Animal Model

Thirty-six BALB/C mice were randomly assigned to six groups (age: 6–8 weeks, weight: 20 ± 2 g, $n = 6$ per group): control group (Control), RSV-infected group (RSV), IgAN group (IgAN), RSV-infected IgAN group (RSV-IgAN), C5aRA-treated IgAN group (C5aRA-IgAN), and C5aRA-treated RSV-IgAN group (C5aRA-RSV-IgAN).

The RSV infection mouse model was mainly developed as described previously (Hu et al., 2017). Mice were inoculated under isoflurane anesthesia by intranasal instillation and intraperitoneal (i.p.) injection with $\sim 10^6$ PFU of purified RSV (A2 strain, 50 μl) in endotoxin-free PBS from days 0 to 2. The control group received an equal amount of PBS. Mice were sacrificed on day 7 **(Supplementary Figure 1A)**.

The IgA nephropathy mouse model was constructed as previously described (Meng et al., 2014; Xiao et al., 2017). This model was generated by intragastric gavage of mice with BSA (Roche) in acidified water (800 mg/kg body weight) every other day, subcutaneous injection of CCL4 and castor oil (mixed at the ratio of 1 to 5; 0.1 ml) once a week and i.p. injection (0.08 ml) biweekly, and intravenous injection of LPS (Sigma) (50 μg) twice in weeks 6 and 8. For RSV-IgAN mice, RSV was inoculated as described above daily for up to 3 days in the 10th and 11th weeks. For the C5aRA-treated groups, IgA and RSV-IgAN mice were treated with C5aRA (W54011, Abcam) by caudal vein injection 24 h before RSV infection. The control mice received an equal amount of PBS. All mice were killed in the 12th week for sample harvest **(Supplementary Figure 1B)**.

Cell Culture

HMCs were purchased from ScienCell™ Research Laboratories, and $CD4^+$ T cells were isolated from healthy people by human CD4 microbeads (130-045-101) bought from Miltenyi Biotec. For the coculture of HMCs with $CD4^+$ T cells, the two different cell types were cultured in mesangial cell medium (4201, Scien Cell) in an incubator at 37°C in 5% CO_2. Purified $CD4^+$ T cells were cultured with HMCs at a ratio of 1–5 for 48 h in the absence or presence of RSV.

Functional Studies

Before sample harvest, all mice were housed in metabolic cages for 24 h to collect urine samples. The albumin-to-creatinine ratio (ACR) was determined by standard laboratory methods.

Histological Analyses

The upper left kidney and right lung were fixed in 4% neutral-buffered formalin, dissected, embedded in paraffin, and cut into 2- and 3-μm-thick sections. Sections were stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) and then examined by light microscopy.

The mouse renal tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Three hours later, specimens were placed in 2% O_sO_4 for 2 h, hydrated in a decreasing series of ethanol solutions and embedded in Epon-Araldite. The specimens were cut into ultrathin sections (70 nm) and stained with uranyl acetate and lead citrate. The specimens were examined by transmission electron microscopy.

Paraffin-embedded sections were subjected to immunohistochemistry (C5aR1 and CD4 protein) and immunofluorescence (RSV F protein, IgA and IgM). Serial 2-μm-thick sections of kidney tissues and serial 3-μm-thick sections of lung tissues were dewaxed by xylene, rehydrated in different gradient alcohol and washed by PBS. Then antigen retrieval was performed with citrate (pH 6.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 20 min. After blocking nonspecific binding with diluted normal rabbit serum for 60 min, the sections were incubated for 16–8 h at 4°C with anti-C5aR1 antibody (ab117579, Abcam) or CD4 antibody (44038, SAB). The slides were developed using an SP goat IgG kit (ZSGB-Bio). Chromogenic reactions were performed with DAB liquid (ZSGB-Bio), and counterstaining was performed with Mayer's hematoxylin (ZSGB-Bio). For immunofluorescence, dewaxing, rehydration, and antigen retrieval were performed as described for immunohistochemistry. The sections were incubated for 16 h at 4°C with RSV F protein (SC-57998, Santa Cruz), anti-mouse IgA (ab97234, Abcam), and anti-mouse IgM (ab190369, Abcam). Normal rabbit and normal rat sera were used in the control group for immunohistochemistry and immunofluorescence.

The integrated density and area of each immunofluorescence or immunohistochemistry section was measured by Image J program according to regular instruction. Then mean density is calculated by the ratio of integrated density to the area. The mean density of RSV F, IgA, C5aR1, and CD4 protein was used for statistical analysis in every group.

Cell Isolation From Blood and Kidney Tissue

Blood samples of different mice (100 μl) were collected before sample harvest, and then red blood cell lysis buffer (C3702, Beyotime Biotochnology) was used to remove red cells. The washed cells were used for flow analysis.

Kidney tissues were excised completely, minced in serum-free RPMI 1640 medium under aseptic conditions and then incubated with 0.4 mg/ml collagenase IV (LS004186, Worthington) for 1 h at 37°C. Cell suspensions were filtered through a series of nylon meshes and washed with PBS. Lymphocyte-enriched cell suspensions were acquired by Percoll density gradient (70 and 30%, GE Healthcare) centrifugation. Cells were stained for flow cytometric analyses.

Flow Cytometry

For Th1 and Th17 cell detection, isolated cells were suspended in RPMI 1640 (Gibco) with 10% FCS and activated by phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma) and ionomycin (1 mg/ml; Sigma) in an incubator (37°C, 5% CO_2) for 5 h. After 30 min of incubation, Brefeldin A (3 mg/ml, eBioscience) was added to the cell suspensions. The postintervention cells were divided equally into tubes, stained with normal mouse serum (Sigma) to block nonspecific staining, incubated with antibodies against the surface markers CD3 (APC, eBioscience) and CD4 (FITC, eBioscience) for 30 min in the dark at 4°C, and then permeabilized with Cytofix/Cytoperm (eBioscience) at 4°C for 30 min. Intracellular cytokines were stained with anti-mouse IFN-γ (PE, eBioscience) and anti-mouse IL-17A antibodies (PE, eBioscience). Foxp3 staining was performed according to the manufacturer's instructions. Isolated lymphocytes were incubated with anti-mouse CD4 (FITC, eBioscience) and CD25 (APC, eBioscience) in the dark at 4°C for 30 min, rinsed in Fix/Perm buffer (eBioscience), and stained with anti-mouse Foxp3 antibody (PE, eBioscience) for 45 min. For $Ki67^+CD4^+$ T cells detection, the treated cells were incubated with anti-human CD4 (564419, BD Biosciences) antibody in the dark at 4°C for 30 min, rinsed in Fix/Perm buffer (eBioscience), and stained with anti-human Ki67 antibody (350535, Biolegend) for 45 min. Finally, the cells were analyzed with a Becton Dickinson FACS Calibur system using Cell Quest software.

Assessment of Cytokines in Serum and Kidney

Serum was diluted with PBS, while the same weight kidney samples in all different mice were prepared by homogenization in PBS containing protease inhibitors (Roche Diagnostics). Serum and kidney IFN-γ, IL-10, and IL-17A (eBioscience) levels and C5a levels (RayBiotech) were tested by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols.

RNA Preparation and Real-Time PCR

Real-time PCR analysis of total RNA extracted from cells using RNeasy Mini Kits (Qiagen, Valencia, CA) was performed according to the manufacturer's guidelines. RNA was reverse transcribed into cDNA using SuperScript III Reverse

Transcriptase (Invitrogen). Real-time PCR was conducted in an ABI Prism 7000 sequence detector (Applied Biosystems, CA) as previously described (Bin et al., 2018).

The CD80 (Hs01045161_m1), CD86 (Hs01567026_m1), and IL-17A (Hs00936345_m1) primers used for real-time PCR were purchased from Applied Biosystems. Quantities of all target genes in test samples were normalized to the corresponding HPRT1 and 18S quantities.

Statistical Analysis

Data is appeared as mean ± sem. Statistical analyses for every group were assessed with one-way analysis of variance (ANOVA), and between-group comparisons were evaluated by the least significant difference (LSD) *t*-test (Prism software; Graphpad). Significance was assumed at $P < 0.05$.

RESULTS

The ACR in IgAN Mice Is Enhanced by RSV and Reduced by C5aRA

To explore the impact of RSV infection and C5aRA on kidney dysfunction in IgAN mice, urine samples were obtained from all mice to detect the ACR before sample harvest. As shown in **Figure 1**, the ACR was significantly increased in IgAN mice compared with control mice, and RSV infection further increased the ACR in RSV-IgAN mice. This finding implied that we successfully generated an IgAN mouse model and verified that RSV infection indeed exacerbates kidney dysfunction. However, C5aRA treatment of IgAN and RSV-IgAN mice reversed the above phenotype, as evidenced by an obvious decrease in the ACR. Overall, our data suggest that RSV infection can clearly increase the ACR, which is significantly reduced by C5aRA in IgAN mice. ACR evaluation indicated that C5aRA can effectively rescue the adverse effect of RSV infection on kidney dysfunction.

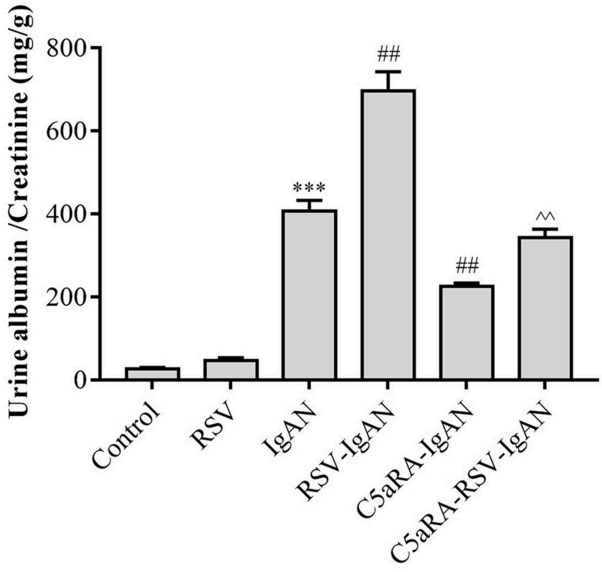

FIGURE 1 | The ACR in IgAN mice is increased by RSV and decreased by C5aRA. Urine samples were collected for 24 h before sample harvest to assessed ACR. Data are expressed as mean ± sem of experiments performed in duplicate in $n = 6$ mice per group, *t*-test. ***P < 0.001 vs. control *group.* ##$P < 0.01$ vs. IgAN group. ^^$P < 0.01$ vs. RSV-IgAN group.

Kidney Damage in IgAN Mice Is Exacerbated by RSV but Alleviated by C5aRA

To further assess pathological damage in the kidney, PAS-stained sections of kidneys from all experimental mice were examined. As shown in **Figure 2A**, there was more proliferation of the mesangium in IgAN mice than control mice. Moreover, RSV infection exacerbated this proliferation in RSV-IgAN mice, but it was ameliorated in C5aRA-treated mice. Furthermore, immunofluorescence staining with a specific IgA antibody (**Figure 2B** and **Supplementary Figure 2**) uncovered IgA deposition in IgAN mice that was exacerbated by RSV exposure. However, C5aRA-treated IgAN and RSV-IgAN mice showed markedly fewer IgAN deposits.

In addition, the changes in electron-dense deposits detected by electron microscopy were related to histology changes and IgA deposition. Specifically, many electron-dense deposits in the glomerular mesangial region, minor mesangial proliferation, and segmental fusion of podocyte foot processes were observed in IgAN mice. Notably, the above changes were more obvious in RSV-IgAN mice, while the opposite trends were observed in the C5aRA treatment group (**Figure 2C**). In accordance with the ACR changes, morphological changes described above indicate that kidney damage is aggravated by RSV and alleviated by C5aRA in IgAN mice.

C5aRA Lessens the RSV Deposition in Kidney and Lung Tissues of RSV-Infected Mice

To further investigate the inhibitory effect of C5aRA on RSV deposition in RSV-IgAN mice, immunofluorescence staining of RSV F protein was detected. Representative images show that RSV F protein was principally deposited in the kidney glomeruli (**Figures 3A,C**) and lung interstitial areas (**Figures 3B,D**) in RSV-infected mice. Nevertheless, C5aRA treatment reduced RSV deposition in RSV-IgAN mice. Taken together, the data indicate that C5aRA might antagonize the detrimental effect of RSV infection on kidney function by decreasing RSV deposition.

C5a and C5aR1 Expression Is Further Upregulated by RSV Infection in IgAN Mice

The aforementioned results suggest that C5aRA can antagonize the negative effects of RSV infection, but the influence of RSV infection on the C5a-C5aR1 axis during IgAN development is still unknown. As shown by the immunohistochemical results, C5aR1 expression was localized in the kidney glomeruli of RSV-infected mice, with lower expression in control mice. Moreover, C5aR1 expression was increased in kidney tissues in IgA mice and was further increased by RSV infection in RSV-IgAN mice. C5aR1 expression was obviously lower in RSV-infected mice, IgAN mice and RSV-IgAN mice treated with C5aRA. In addition, ELISA was used to assess serum and kidney C5a levels in the different groups, and the trends in C5a levels were similar to those in

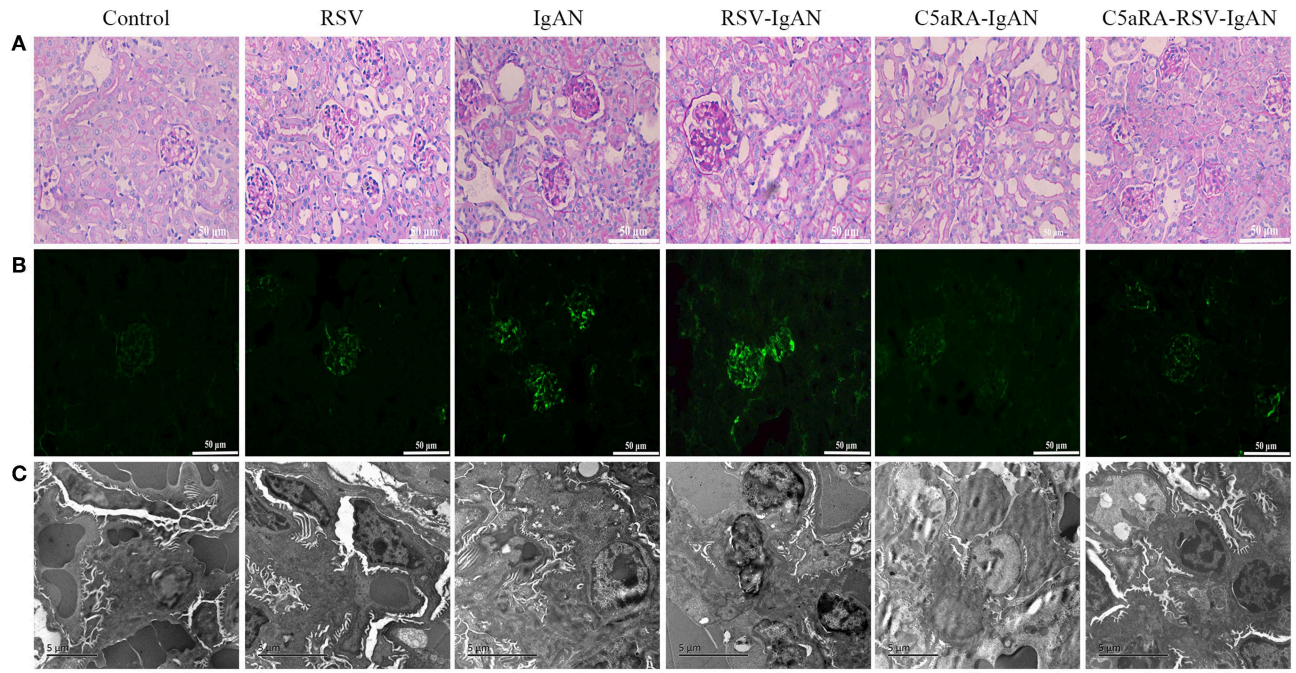

FIGURE 2 | Kidney damage in IgAN mice is exacerbated by RSV but alleviated via C5aRA. **(A)**Representative images of pathological changes of kidney of PAS staining in different mice (400×). **(B)** IgA deposition in local kidney area were detected by immunofluorescence staining (200×). **(C)** Ultrathin kidney sections (70 nm) were stained with uranyl acetate and lead citrate, and then examined by transmission electron micrographs. $N = 6$ per group.

C5aR1 expression. All the data described above imply that RSV might exacerbate IgAN development by excessively activating the C5aR-C5aR1 axis (**Figure 4**).

Lung Damage and IgA Deposition in IgAN Mice Are Aggravated by RSV Infection but Reduced by C5aRA Treatment

RSV infection can lead to lung damage, but we were interested in determining whether the lung damage caused by RSV infection in IgAN mice could be cured by C5aRA. Compared with the control and IgAN mice, RSV-IgAN mice exhibited significant inflammatory cell infiltration around blood vessels and bronchi in lung tissues assessed via CD4 immunostaining (**Figures 5A,C,E**). Moreover, IgA deposition was found in the lung tissues of IgAN mice, and RSV infection might have further increased IgA deposition in IgAN mice (**Figures 5B,D**). This lung damage and potential increase in IgA deposition were reduced by C5aRA treatment. However, there was no IgM deposition in the lung tissues of IgAN mice (data not shown). In short, these findings proclaim that respiratory mucosal infection is related to IgAN onset and development.

The Balance of Th17 Cell Responses and Correlative Cytokines Is Perturbed by RSV but Normalized by C5aRA

To further expound the potential regulatory relationship between C5a-C5aR1 axis activation and Th17 cell responses in RSV-induced IgAN mice, the frequencies of Th1, Th17, and Treg cells in the blood and kidney were examined. The proportions of Th1 and Th17 cells in the blood and kidney were both remarkably augmented, while the Treg proportions were reduced in RSV-infected mice and IgAN mice compared with control mice. Additionally, these changes in Th1, Th17, and Treg cells were further increased in RSV-infected IgAN mice. Interestingly, C5aRA treatment decreased the Th1 and Th17 cell frequencies but increased the Treg frequency in IgAN and RSV-IgAN mice (**Figure 6**).

Additionally, correlative cytokines in serum and kidney tissues were evaluated by ELISA. Serum and kidney IFN-γ, IL-17A, and IL-10 levels presented similar trends as those described above for Th1, Th17, and Treg cells in all the groups (**Figure 7**). In summary, C5aRA might remedy kidney immune dysfunction caused by RSV infection through regulating Th1, Th17, and Treg cell frequencies and related cytokine expression.

Antigen Presentation to $CD4^+$ T Cells by HMCs *in vitro* Stimulates $CD4^+$ T Cell Proliferation and Increases IL-17A Levels in Response to RSV Infection

RSV infection may exacerbate IgAN in mice by inducing the production of more $CD4^+$ T cells, but little is known about the function of HMCs in this pathogenic mechanism. To elucidate the effect of HMCs on $CD4^+$ T cell proliferation induced by RSV infection, we first stimulated normal HMCs with RSV. The data revealed that CD80 and CD86 expression by HMCs was increased by RSV infection (**Figure 8A**), which suggested

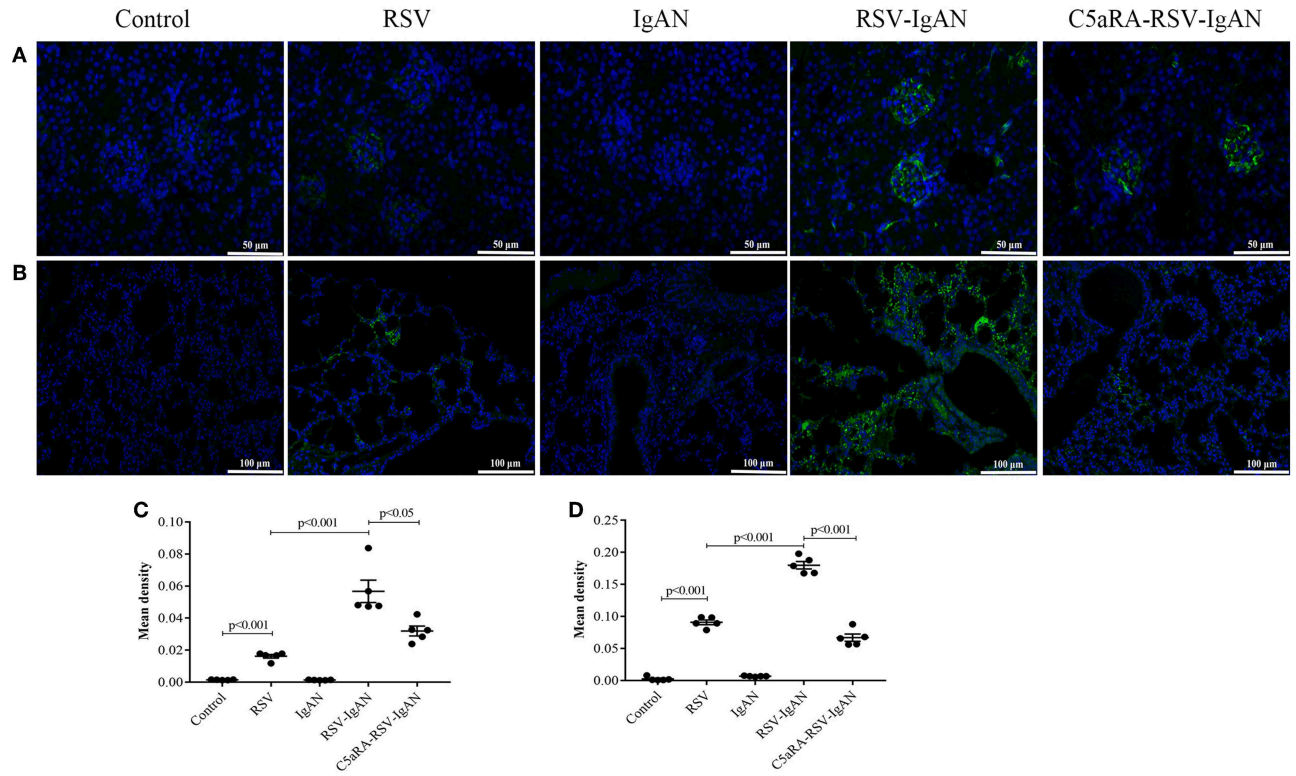

FIGURE 3 | C5aRA decreases RSV F protein deposition in kidney and lung tissues of RSV-infected mice. Representative images of immunofluorescence staining for RSV F protein in kidney **(A,** 400×**)** and lung tissues **(B,** 200×**)**. Light green, RSV F protein deposition, blue, nuclear counterstain. The mean density of RSV F protein deposition in kidney tissues **(C)** and lung tissues **(D)** was calculated through Image J program. Data are calculated as mean ± sem of experiments in triplicate in $n = 5$ per group, *t*-test.

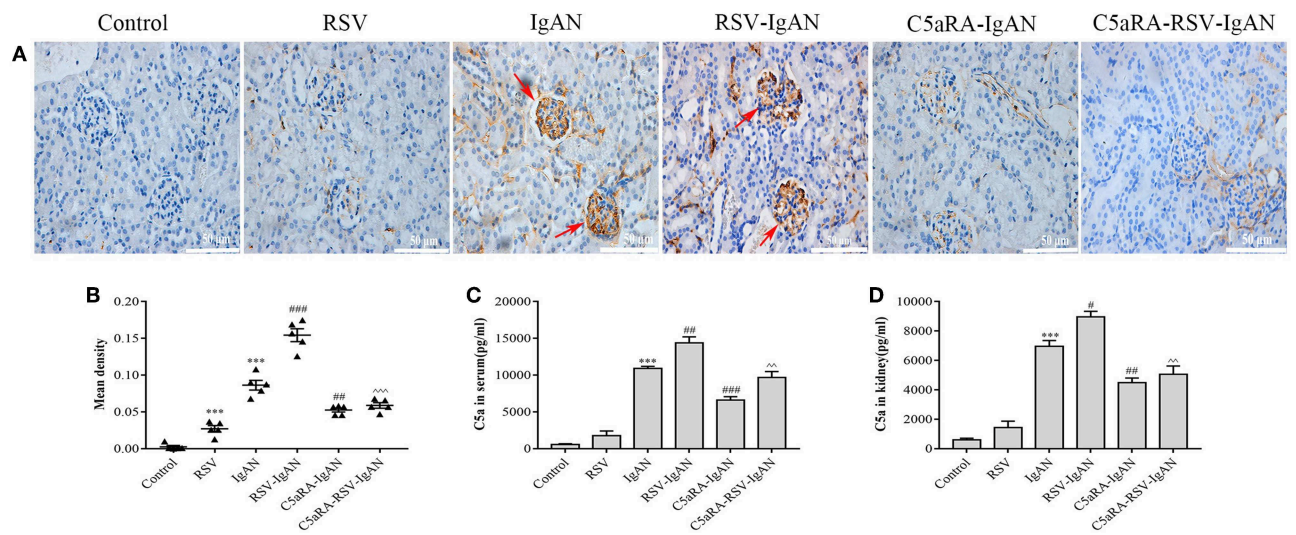

FIGURE 4 | C5a levels and C5aR1 expression in different mice. **(A)** Representative images of C5aR1 expression in kidney tissue was assessed by immunohistochemistry (400×). Red arrowheads, C5aR1 positive expression area in glomerular. **(B)** The mean density of C5aR1 expression in kidney tissues evaluated by Image J software. **(C,D)** Serum C5a levels **(C)** and kidney C5a levels **(D)** tested by ELISA in different groups. Results are assessed as mean ± sem of repeated experiments in triplicate, $n = 5$ per group, *t*-test. ***P < 0.001 vs. control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. IgAN group. $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P <$ 0.001 vs. RSV-IgAN group.

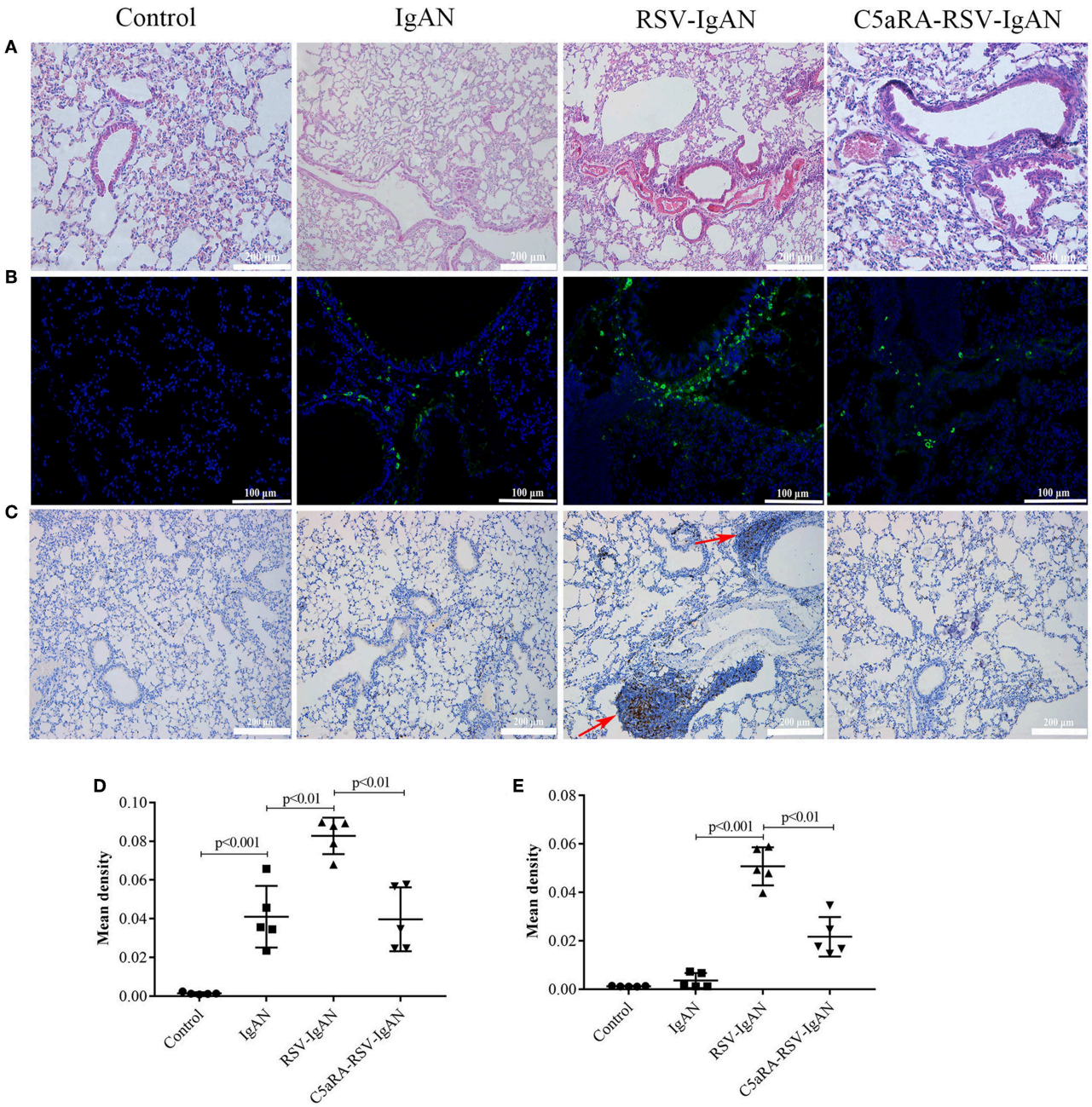

FIGURE 5 | RSV exacerbates and C5aRA reduces lung damage and IgA deposition in IgAN mice. **(A)** Representative images of HE staining in lung tissues (200×). **(B)** Specific IgA deposition in lung tissues detected by immunofluorescence staining (200×). Light green, IgA deposition, blue, nuclear counterstain. **(C)** CD4 protein expression **(**200×**)** of lung tissues were assessed by immunohistochemistry. Red arrowheads, CD4 positive expression. The mean density of IgA deposition **(D)** and CD4 immunostaining **(E)** in lung tissues was calculated by Image J program. Data are expressed as mean ± sem of experiments in triplicate, $n = 5$ per group, *t*-test.

that HMCs antigen presentation function could be enhanced by RSV infection.

Based on our above results in IgAN mice, RSV infection could activate the C5a-C5aR1 axis, lead to kidney damages and Th cells disorder, cocultures of HMCs with $CD4^+$ T cells were constructed and maintained in the absence or presence of RSV, C5a, C5aRA, and a costimulatory molecule inhibitor to further verify the specific interaction between HMCs and $CD4^+$ T cells. As shown in **Figures 8B,D**, we found that RSV infection alone increased $Ki67^+CD4^+$ T cell proliferation and IL-17A levels, but these increases were even more obvious in coculture conditions. It is speculated that HMCs might play a part in $CD4^+$ T cell dysfunction caused by RSV infection. Moreover, C5a stimulation resulted in more significant enhancements of $Ki67^+CD4^+$ T cell

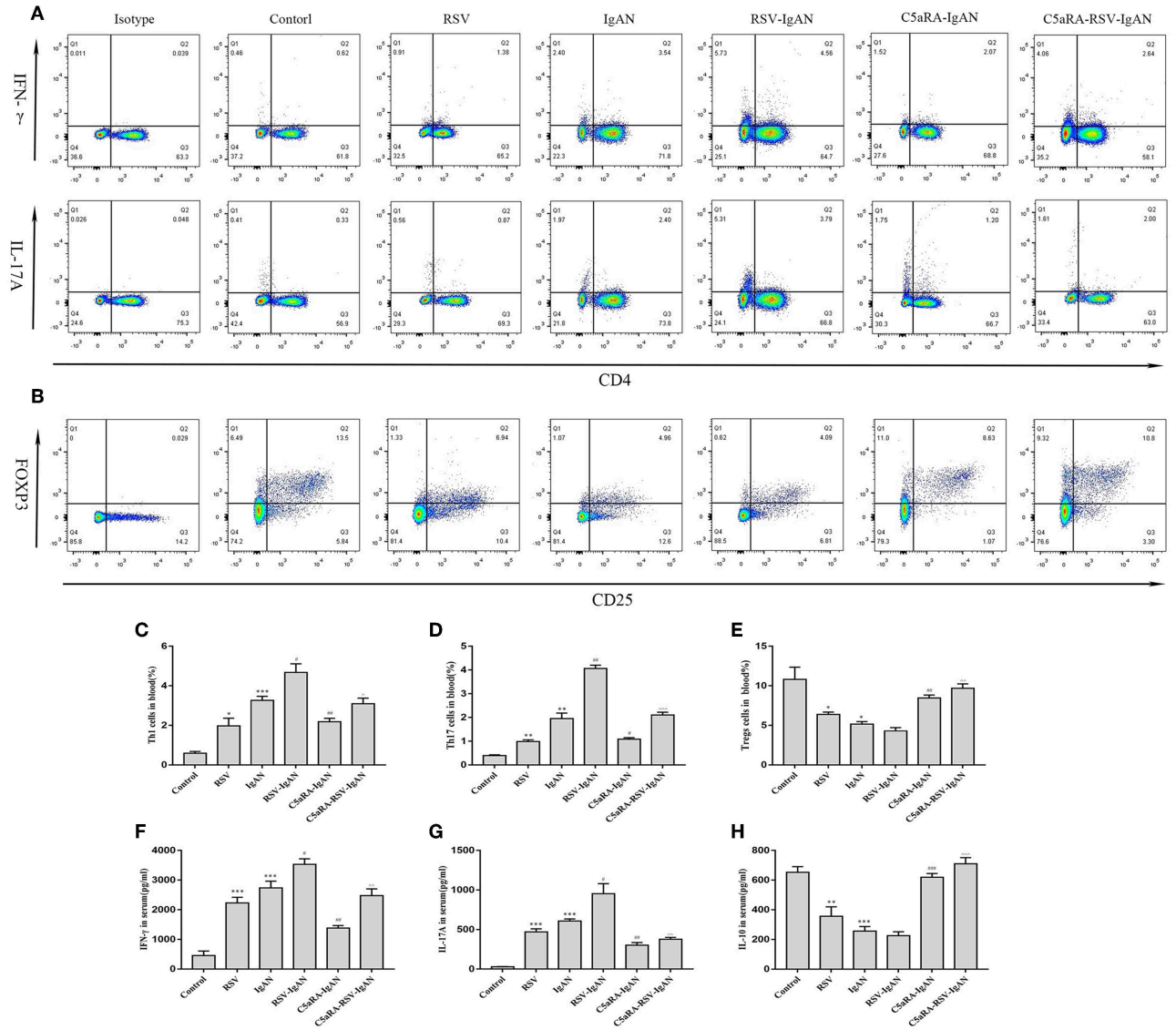

FIGURE 6 | Percentages of Th1, Th17, and Treg cells in blood and serum levels of IFN-γ, IL-17A, and IL-10. Blood samples were collected before sample harvest, and then red blood cell lysis buffer was use to remove red cells. Anti-mouse CD3, CD4, IFN-γ, and IL-17A antibody were stained as method described above and then tested by flow cytometry to evaluate Th1 and Th17 percentages, respectively. Anti-mouse CD4, CD25, and Foxp3 antibody were stained to show Tregs proportions. **(A)** Representative flow chart of Th1 and Th17 cells in blood as percentages of $CD3^+CD4^+$IFN-γ+ cells $CD3^+CD4^+$IL-$17A^+$cells. **(B)** Representative flow chart of Treg cells in blood as percentages of $CD4^+CD25^+$Foxp3+cells. **(C–E)** Percentages of Th1 **(C)**, Th17 **(D)**, and Treg **(E)** cells in the blood of all different groups. **(F–H)** Serum IFN-γ **(F)**, IL-17A **(G)**, and IL-10 **(H)** levels assessed by ELISA in different groups. Data are shown as mean ± sem of experiments in triplicate in $n = 3$–5 mice per group, *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. IgAN group. $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ vs. RSV-IgAN group.

proliferation and IL-17A levels in coculture conditions with RSV infection (**Figures 8B,D**).

However, $Ki67^+CD4^+$ T cell proliferation and IL-17A levels were reduced by treatment with C5aRA, anti-CD80 mAb, anti-CD86 mAb, a combination of anti-CD80 and anti-CD86 mAbs, CTLA-4Ig, and control Ig, which decrease the effectiveness of HMC antigen presentation (**Figures 8C,E**). Taken together, the results show that RSV infection and C5a stimulation might lead to $CD4^+$ T cell production via HMC-enhanced $CD4^+$ T cell proliferation, thereby increasing IL-17 levels.

DISCUSSION

Accumulating evidence suggests that abnormalities in the IgA mucosal immune system could be key elements in the pathogenesis of IgAN, and a characteristic clinical presentation of IgAN is episodic visible hematuria coinciding with mucosal infection, most commonly of the upper respiratory tract (Floege and Feehally, 2016). Moreover, IgAN exacerbation is often associated with viral infections of the upper respiratory tract (Amore et al., 2004).

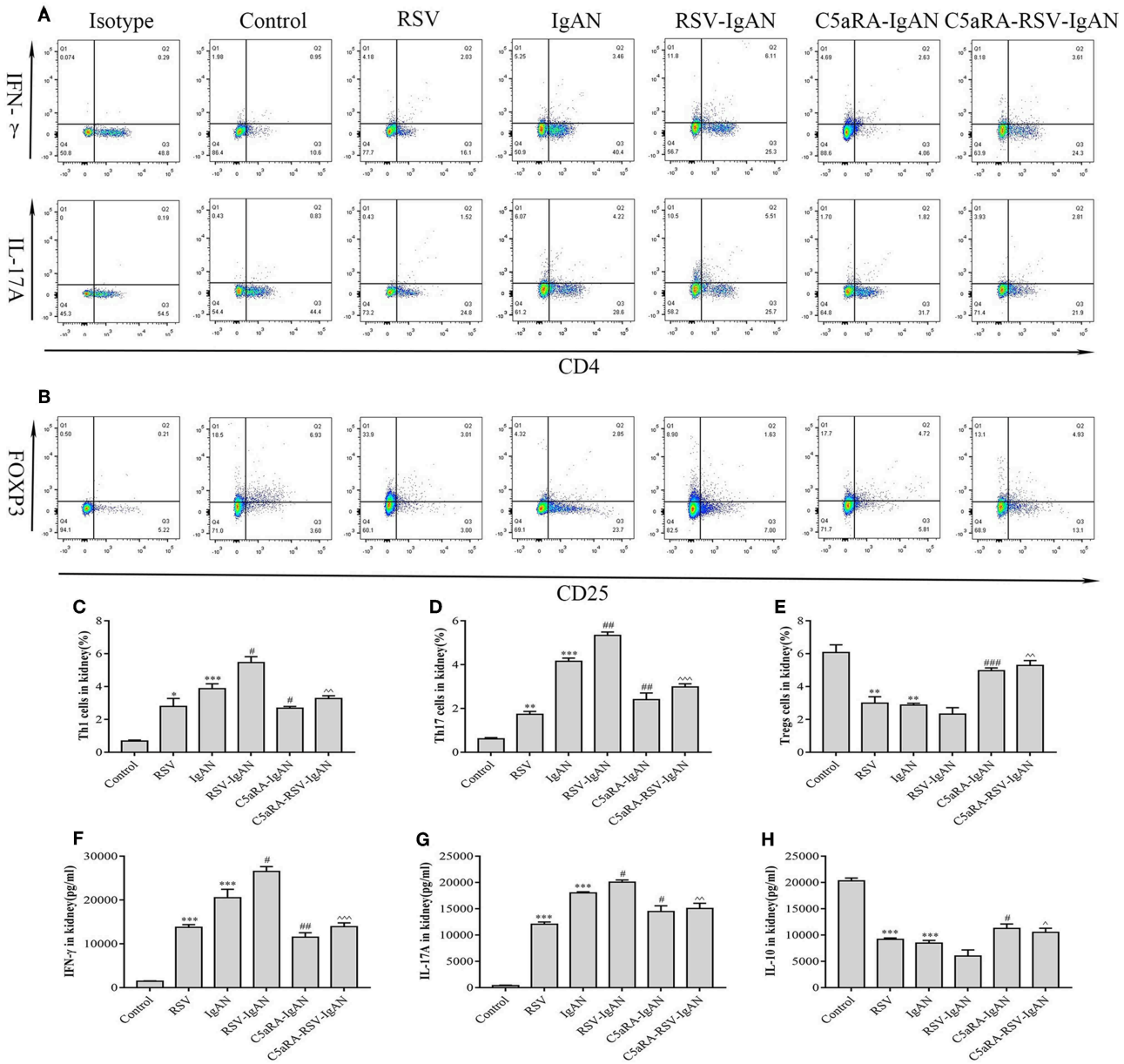

FIGURE 7 | Percentages of Th1, Th17 and Treg cells and levels of IFN-γ, IL-17A, and IL-10 in the kidney. Kidney samples were collected and isolated lymphocyte via Percoll density gradient (70% and 30%) centrifugation. Anti-mouse CD3, CD4, IFN-γ, and IL-17A antibody were stained and then examined by flow cytometry to evaluate Th1 and Th17 percentages, respectively. Anti-mouse CD4, CD25, and Foxp3 antibody were stained to show Tregs proportions. **(A)** Representative flow chart of Th1 and Th17 cells among kidney lymphocytes as percentages of $CD3^+CD4^+IFN\text{-}\gamma^+$ cells $CD3^+CD4^+IL\text{-}17A^+$ cells. **(B)** Representative flow chart of Treg cells in the kidney as a percentage of $CD4^+CD25^+Foxp3^+$ cells. (**C**–**E**) Percentages of Th1 **(C)**, Th17 **(D)**, and Treg **(E)** cells in the kidneys of different groups. **(F–H)** Levels of IFN-γ **(F)**, IL-17A **(G)**, and IL-10 **(H)** in the kidneys were measured by ELISA of different groups. Data are expressed as mean ± sem of experiments in triplicate in n = 3–5 mice per group, t-test. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. IgAN group. ^P < 0.05, ^^P < 0.01, ^^^P < 0.001 vs. RSV-IgAN group.

In the present study, we investigated the effect of RSV infection on IgAN mice and tried to clarify the underlying pathogenic mechanism. Inconsistent with a previous study that showed less RSV F protein deposition and mRNA levels in the glomerulus and renal tubules of RSV-infected mice at day 14 (Liu et al., 2007; Zhai et al., 2016), we did not find RSV F protein deposition in the kidney in RSV-infected normal mice. There are two possible reasons to explain the above discrepancy: (1) female BALB/c mice were used in our study, while male Sprague-Dawley rats were used in the previous study; and (2) because two different model construction methods were used, the RSV incubation time was different. However, we observed that RSV F protein was clearly deposited not only in the pulmonary interstitium but also in the glomerulus in RSV-IgAN mice, and RSV infection led to

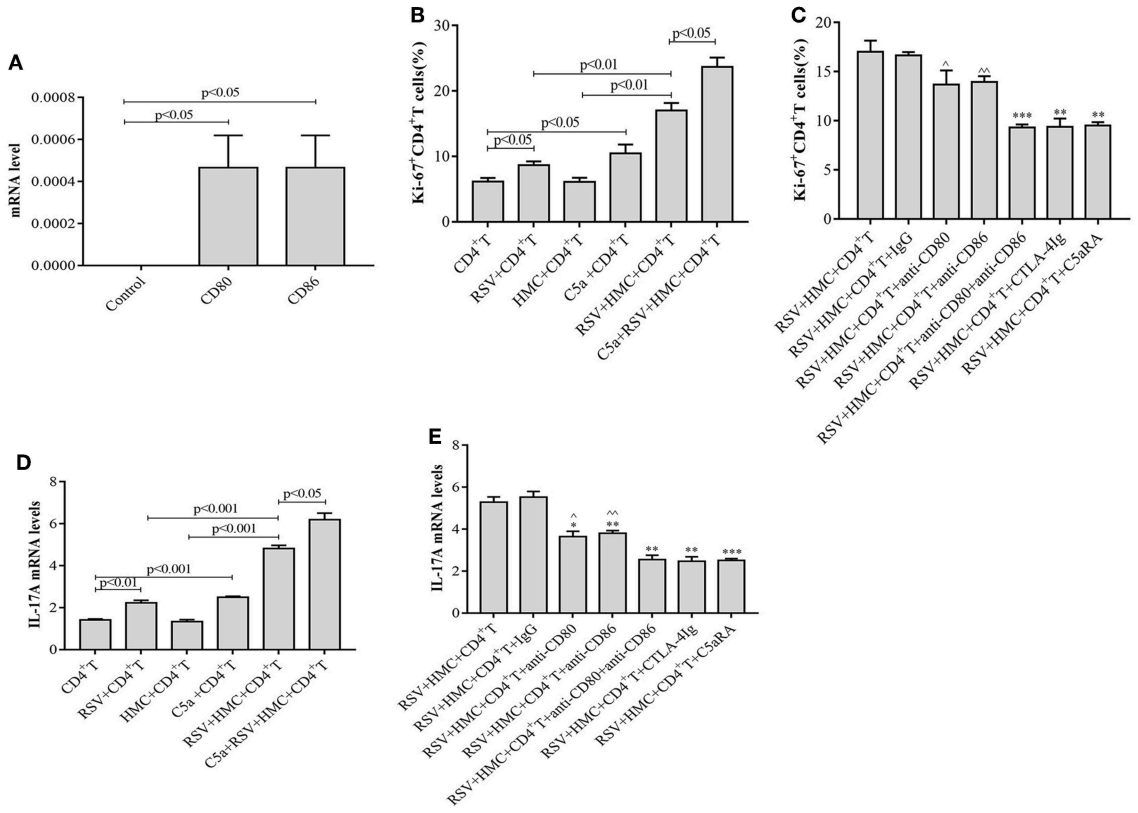

FIGURE 8 | $CD4^+$ T cell proliferation and IL-17A levels are increased by RSV and C5a but reduced by C5aRA via human mesangial cell antigen presentation. **(A)** Effect of RSV infection on HMCs antigen presentation function. CD80 and CD86 expression in RSV-infected HMCs assessed by real-time PCR. **(B)** Effect of RSV infection and C5a stimulation on $CD4^+$ T cell proliferation. **(C)** C5aRA and costimulatory antibodies decrease $Ki67^+CD4^+$ T cell proportions in the coculture system of HMCs and $CD4^+$ T cells in response to RSV infection. $Ki67^+CD4^+$ T cell percentages were detected by flow cytometry **(B,C)**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RSV+HMCs+$CD4^+$T group. $^\wedge P < 0.05$, $^{\wedge\wedge}P < 0.01$ vs. RSV+HMCs+$CD4^+$T+anti-CD80+anti-CD86 group. **(D)** Effect of RSV and C5a stimulation on IL-17A expression. **(E)** IL-17A levels are downregulated by C5aRA and co-stimulatory inhibitor treatment in the coculture system of HMCs and $CD4^+$ T cells in response to RSV infection. Total RNA extracted from $CD4^+$T cells were collected and then IL-17A levels were assessed by real-time PCR **(D,E)**. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RSV+HMCs+$CD4^+$T group. $^\wedge P < 0.05$, $^{\wedge\wedge}P < 0.01$ vs. RSV+HMCs+$CD4^+$T+anti-CD80+anti-CD86 group. Data are expressed as mean ± sem and each experiment was performed in triplicate repeated in cells, $n = 3$, *t*-test.

more severe pathological changes in the kidney in IgAN mice. Moreover, marked infiltration of inflammatory cells surrounding the airway and IgA deposition in the kidney and lung were detected in RSV-IgAN mice, which supports the notion that immune responses induced by RSV infection could cause the progression of the immune-mediated kidney damage of IgAN.

Th cells play multifaceted roles in RSV infection and IgAN. The immunomodulatory mechanisms of RSV infection are highly effective at inhibiting long-term protection by disrupting type I interferon signaling, antigen presentation, and the quality and durability of T cells, B cells and antibodies; chemokine-induced inflammation is another possible contributor (Ascough et al., 2018). As shown in our previous study, RSV infection increases Th1 and Th17 cell frequencies but decreases Treg cell populations in normal mice (Hu et al., 2017). In addition, we found that Th17 cells, viewed as vital T cells, might affect the pathology or disease outcome of streptococcus-associated IgAN (Meng et al., 2014). In agreement with the results of previous studies, our present results verify that Th1 and Th17 frequencies were higher in IgAN mice than in controls, while the results for Tregs were the opposite. Notably, higher Th1 and Th17 frequencies and lower Treg frequencies were detected in IgAN mice infected with RSV. Based on the above findings, $CD4^+$ T cell response might be a pivotal part of RSV-induced IgAN exacerbation, with Th1 and Th17 cells functioning as proinflammatory cells and Treg behaving as protective cells. Therefore, decreasing Th1 and Th17 cells and promoting Treg cells will be potential beneficial aspects of RSV-induced IgAN treatment.

Complement activation is recognized to play a prominent role in the pathogenesis of IgAN, as confirmed by the renal deposition of complement components of the alternative and mannose-binding lectin (MBL) pathways (Zhai et al., 2016). Although C5aR expression has been observed in RSV infection (Hu et al., 2017) and in IgAN patients (Liu et al., 2014) and mice (Zhang et al., 2017), how it affects RSV-induced IgAN exacerbation has not been defined. Consistent with previous research (Zhang et al., 2017), we found that C5a levels and C5aR1 expression were elevated in IgAN mice compared to normal mice, but the novel finding that C5a levels and C5aR1 expression were

further increased in RSV-IgAN mice attracted our attention. Furthermore, blocking the C5a-C5aR1 axis partially reversed the aforementioned phenomena and alleviated kidney lesions. Our results provide conclusive evidence that RSV infection might exacerbate IgAN by strengthening C5a-C5aR1 axis activation and represent a foundation supporting the future use of latent therapies targeting C5aR to remedy RSV-induced IgAN.

It is likely that genetic absence or pharmacological inhibition of C5aR1 reduces the generation of myeloperoxidase-ANCA, with an attenuated Th1 response and an increased number of $Foxp3^+$ regulatory T cells (Dick et al., 2018). Moreover, there was research proposed that C5aR-deficient mice have fewer Th17 cells and therefore are less likely to develop lupus nephritis than wild-type mice (Pawaria et al., 2014). Our previous study found that inhibition of C5aR1 could decrease Th1 and Th17 cell responses but augment Treg responses in RSV-infected mice (Hu et al., 2017). To date, studies investigating the role of the C5a-C5aR1 axis in regulating Th1, Th17, and Treg cell immune responses in IgAN and RSV-IgAN mice have not been performed. In our study, to further address the above focuses, IgAN and RSV-IgAN mice were treated with C5aRA, and the properties of Th1, Th17, and Treg cells were detected. Interestingly, C5aRA not only reversed kidney damage in IgAN and RSV-IgAN mice but also reduced the Th1 and Th17 frequencies while increasing the Treg frequency. According to our data, the C5a-C5aR1 axis participates in the IgAN pathogenic process by amplifying the proinflammatory functions of Th1 and Th17 cells but weakening the protective effects of Treg cells, and these functions could be further strengthened during RSV infection in IgAN mice.

Based on our murine experiments, RSV infection could activate C5a-C5aR1 axis and further augment their proinflammatory through increase Th1 and Th17 proportions, meanwhile which kidney inherent cells or immune cells involved in the process catch our attention. HMCs represent approximately one third of glomerular cells. Substantial mesangial cell proliferation in response to injury occurs in IgA nephropathy. Resident renal cells including mesangial cell should no longer be viewed as passive targets in renal inflammation, but as active participants in this process. HMCs can express major histocompatibility complex class II molecules (MHCII), suggesting that it can act as Ag presenting cells and directly regulate the nephritogenic immune response (Timoshanko and Tipping, 2005). Gan et al. proposed that Th22 lymphocytosis can be induced by HMCs through CD80 and CD86 antigen presentation pathway (Gan et al., 2018a), and CD80 and CD86 expression in antigen presentation cells is related to renal function (Wu et al., 2004). Therefore, we constructed the coculture system of HMCs and $CD4^+$ T cells to explore the effect of HMCs in $CD4^+$ T proliferation *in vitro*. In addition, Zhang et al. confirmed that C5aR inhibition can block cytokine and chemokine secretion and cell proliferation of cultured HMCs (Zhang et al., 2017). It is curious whether HMCs induce $CD4^+$ T cell populations during RSV infection and C5a stimulation *in vitro*. As the data show, RSV infection augmented the antigen presentation function of HMCs, as assessed by CD80 and CD86 expression. $Ki67^+CD4^+$ T cells and IL-17A levels showed a small increase in response to RSV infection and C5a stimulation alone but were significantly augmented upon coculture with HMCs. Of note, when the HMC antigen presentation function was suppressed by CD80 antibody, CD86 antibody and C5aRA, $Ki67^+CD4^+$ T cells, and IL-17A levels were lessened. Combined with our previous results, our current data indicate that RSV infection might promote HMC antigen presentation function and C5a secretion, and further lead to $CD4^+$ T cell proliferation and increased IL-17A levels. Therefore, it is speculated that RSV infection exacerbates IgAN onset via $CD4^+$ T cell imbalance, partly due to heightened HMC antigen presentation function in the local area of the kidney. In addition, therapeutics targeting the C5a-C5aR1 axis may be sufficient to affect the HMC pathogenic process.

In conclusion, this work builds on previous studies and extends the role of the C5a-C5aR1 axis in RSV-induced IgAN exacerbation. We demonstrate that RSV infection can exacerbate IgAN pathogenic development. This pathogenic process may be attributed partly to C5a-C5aR1 axis activation, increased Th1 and Th17 proinflammatory function and reduced Treg cell-mediated protective effects. Of note, we also confirmed that HMCs, as antigen-presenting cells, might promote $CD4^+$ T cell proliferation and upregulate IL-17A levels, but these effects could be inhibited by C5aRA. Our data provide profound evidence indicating that blocking the C5a-C5aR1 axis might be a potential therapy for RSV-induced IgAN patients.

ETHICS STATEMENT

All studies were conducted in accordance with Institutional Animal Care guidelines. This project was approved by the Animal Experimental Ethics Committee of Hunan Province (No. 201603376). Additionally, this project was authorized by the Medical Ethics Committee of the Xiangya Hospital of Central South University for Human Studies (No. 201703582).

AUTHOR CONTRIBUTIONS

XL conceived and designed the study, and finalized the manuscript. XH conducted the experiments and edited the manuscript. LL, WT, and SD conducted the experiments. JF, QZ, TM, and YZ analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00151/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MicroRNA and Nonsense Transcripts as Putative Viral Evasion Mechanisms

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Viral proteins encode numerous antiviral activities to modify the host immunity. In this article, we hypothesize that viral genomes and gene transcripts interfere with host gene expression using passive mechanisms to deregulate host microRNA (miRNA) activity. We postulate that various RNA viruses mimic or block binding between a host miRNA and its target transcript, a phenomenon mediated by the miRNA seed site at the 5′ end of miRNA. Virus-encoded miRNA seed sponges (vSSs) can potentially bind to host miRNA seed sites and prevent interaction with their native targets thereby relieving native miRNA suppression. In contrast, virus-encoded miRNA seed mimics (vSMs) may mediate considerable downregulation of host miRNA activity. We analyzed genomes from diverse RNA viruses for vSS and vSM signatures and found an abundance of these motifs indicating that this may be a mechanism of deceiving host immunity. Employing respiratory syncytial virus and measles virus as models, we reveal that regions surrounding vSS or vSM motifs have features characteristics of pre-miRNA templates and show that RSV viral transcripts are processed into small RNAs that may behave as vSS or vSM effectors. These data suggest that complex molecular interactions likely occur at the host-virus interface. Identifying the mechanisms in the network of interactions between the host and viral transcripts can help uncover ways to improve vaccine efficacy, therapeutics, and potentially mitigate the adverse events that may be associated with some vaccines.

Keywords: RNA viruses, microRNA, miRNA, miR, seed sequence, mimics, sponges

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INTRODUCTION

Regulation of gene expression is a complex occurrence involving transcriptional and post-transcriptional mechanisms (Burgess, 2017). Translation of host mRNAs is regulated by small evolutionarily conserved small non-coding RNAs or miRNAs (Chen et al., 2017; Morales et al., 2017; Murashov, 2017) principally by sequence complementarity. Sequence complementarity is also fundamental to pathways such as clustered regularly interspaced short palindromic repeats (CRISPR) and RNA interference (RNAi) pathways (Panek et al., 2016; Kaikkonen and Adelman, 2018; Damas et al., 2019; Hussain et al., 2019) of which miRNAs have an integral function. Host miRNA genes can be intronic, intergenic, or independent transcription units typically processed by RNA polymerase II. Initial transcription produces primary miRNA transcripts (pri-miRNA) which are processed by a nuclear microprocessor complex to produce pre-miRNAs (Bartel, 2004; Finnegan and Pasquinelli, 2013; Ha and Kim, 2014). Pre-miRNAs are exported out into the

cytoplasm and further processed into a 18–25nt long dsRNA by class III RNAse enzyme Dicer to form the mature mRNA duplex. One strand of the duplex is thermodynamically stable and referred to as the guide strand while the other strand is the passenger strand. The mature guide strand at the 5' end encodes a 6 nt 'miRNA seed' that is complementary to a miRNA recognition element (MRE) in target transcripts (Bartel, 2004; Finnegan and Pasquinelli, 2013; Ha and Kim, 2014). Sequence dependent pairing of the seed site with MRE can lead to either mRNA decay or blocked translation (Fabian et al., 2010). Each miRNA can target several genes although the stoichiometry of this interaction is inadequately defined (Weill et al., 2015). Non-seed-mediated miRNA regulation has also been recognized (Fabian et al., 2010; Ghosal et al., 2014; Li et al., 2014; Kumari et al., 2016), is an alternate mechanism of miRNA function (Cloonan, 2015), and multiple miRNAs may regulate a single gene (Bartel, 2009; Friedman et al., 2009; Fabian et al., 2010). There are several features that may contribute to function as different motifs may be able to interact among viral and host transcripts given each position can have one of the four nucleotides (A/U/G/C) and the six nt in the seed site can be promiscuous and bind to many targets (Friedman et al., 2009). Host gene miRNA regulation is well established and has been demonstrated to modulate ∼60% of the human transcriptome (Friedman et al., 2009; Dong and Lou, 2012; Hashimoto et al., 2013; Jia et al., 2014). It is well known that miRNAs regulate cell physiology during normal homeostasis as well as during disease states (Karnati et al., 2015; Alipoor et al., 2016; Vishnoi and Rani, 2017; Olejniczak et al., 2018). Importantly, differential expression of miRNAs has been used as biomarkers for diagnosis, treatment and prognosis, and miRNA expression is modified in response to viral infection (Sullivan and Ganem, 2005; Piedade and Azevedo-Pereira, 2016; Auvinen, 2017; Trobaugh and Klimstra, 2017).

DNA viruses typically replicate with a high fidelity and encode their own miRNAs (Klinke et al., 2014; Flor and Blom, 2016; Albanese et al., 2017; Qin et al., 2017) to regulate virus and host gene expression. In contrast, RNA viruses lack replicative fidelity and arise in the host following infection as swarms of quasispecies (Steinhauer et al., 1992). The quasispecies generally have poor replicative fitness. RNA virus replication and gene transcription are catalyzed by RNA-dependent RNA polymerase (RdRP) which is error-prone (Perez-Rodriguez et al., 2016). It is not understood what proportion of quasispecies are translated during infection, though non-canonical start codons can be used during RNA viral protein synthesis (Firth and Brierley, 2012). Thus, quasispecies can contribute to antigenic diversity via non-canonical translation of alternative viral proteins, and inhibition of these processes can improve viral yield during vaccine production via molecular breeding, increase vaccine safety and stability and reduce potential adverse events (Perez-Rodriguez et al., 2016). While a lack of exonuclease proof reading activity occurs in the RdRP, a feature explaining how low fidelity arises, it remains unclear why RNA viruses have and maintain low fidelity RdRP. It is possible that low RdRP fidelity facilitates virus replication (Hopfield, 1974; Back et al., 1996) by allowing RNA viruses to escape situations where unfit mutations predominate fitness leading to species collapse and attenuation (Lauring and Andino, 2010).

TABLE 1 | Binding energy calculations for vSSs per RNA duplex.

Virus	vSS	miRNA	MFE (Kcal/mol)
RSV A2	NS1-2278	miR-2278	−32.4
	G-4280	miR-4280	−25.2
	G-1273f	miR-1273f	−29.7
RSV B1	NS1-2278	miR-2278	−31.9
	M/IGR4-7161-3p	miR-7161-3p	−34.0
	G-2113	miR-2113	−29.0

Stability of vSS miRNA was determined using RNAhybrid. Mean free energy (MFE) denotes stability of interaction. NS1, Non-structural gene-1; G, RSV G gene; M, Matrix gene. Number indicate the potential miRNA target.

In this article, we speculate that RNA viruses interfere with the host RNAi machinery that regulates both foreign and endogenous gene expression by miRNAs. We postulate that RNA viral genomes and gene transcripts encode motifs that can either mimic or block native miRNA activity through sequence homology or complementarity. We analyzed the genomes of several RNA virus families (**Supplementary Tables 1, 2**) and identified vSMs that potentially may mimic miRNA seed activity and thus increase native miRNA repression of host antiviral pathways. We also identified several viral seed sponges that can potentially block host miRNAs and relieve native miRNA suppression of pro-viral host genes. We focused our analysis on the *Paramyxoviridae* family of RNA viruses owing to their impact on human and animal health. Paramyxoviruses have negative sense, non-segmented, single-stranded RNA genomes that are transcribed in a gradient leading to a differential abundance of viral transcripts with all steps in the viral life cycle occurring in the cytosol where host miRNAs also regulate gene expression. Paramyxoviruses are classified into two subfamilies *Paramyxovirinae* (e.g., Avulavirus, Henipavirus, Morbilivirus, Respirovirus, and Rubulavirus genera) and *Pneumovirinae* (e.g., Pneumovirus and Metapneumovirus genera) (Aguilar and Lee, 2011; Amarasinghe et al., 2017; Rima et al., 2017). Paramyxovirinae members causing morbidity and mortality include measles (MV), Mumps (MuV), Hendravirus (HV), Nipah virus (NiV), and the Pneumoviruses, i.e., respiratory syncytial virus (RSV) and human metapneumovirus (hMPV).

In this article, we propose that quasispecies enable RNA viruses to modulate host gene expression by regulating miRNA function via sequence complementarity or identity with the miRNA seed sites. We also suppose that vSM function to increase native miRNA-based suppression, while vSS inhibit native miRNA activity and increase host gene expression to the advantage of the virus. Preliminary analysis has identified a number of vSM or vSS in several Paramyxovirus genomes. For example, for RSV the regions that neighbor potential vSS or vSM are predicted to form stable stem loop structures that are typically substrates for nuclear and cytosolic RNAses of the RNAi pathway (Cai et al., 2004; Ritchie et al., 2007; Shu et al., 2007; Kurihara and Watanabe, 2010). These findings suggest that these regions

in the viral genome can be templates for cytosolic Dicer activity. We have confirmed that during RSV infection gene transcripts are processed into sncRNAs and have identified viral transcripts that harbor vSS or vSM using next generation sequencing (NGS). Genomic analyses show that these motifs are more abundant in genes that have known or predicted immunomodulatory function or are involved in viral replication/transcription. These data suggest that interactions with host miRNAs may be part of a mechanism to modulate miRNA-mediated host regulatory pathways and regulate viral gene expression. These findings have important implications for better understanding of host-virus interaction as well as rational vaccine design strategies.

MATERIALS AND METHODS

Viruses and Cell Culture

Mycoplasma-free virus stocks of wild type RSV strain A2 were expanded in Vero cells (ATCC CCL81) and maintained in DMEM (Hyclone, Salt Lake City, Utah USA) supplemented with 5% heat-inactivated fetal bovine serum (Hyclone, USA) as previously described (Oshansky et al., 2009). A549 cells (ATCC-CCL185) grown in DMEM supplemented with 5% serum as above were used for all infections. A549 cells were infected at a multiplicity of infection (MOI) of 1.0 as previously described (Oshansky et al., 2009). SHSY5Y cells were maintained in DMEM with 10% heat inactivated FBS.

Nucleotide Sequence Analysis

Complete genome sequences for RSV, MV, HMPV, MuPV, NDV, HV, NiV, MuV were from the National Center for Biotechnology Information (NCBI). Accession numbers for all sequences analyzed are given in **Supplementary Table 1**. A local database of human mature miRNA sequences version 21.0 was constructed locally in BioEdit. Viral sequences were analyzed using BLASTN (Altschul et al., 1990; Altschul and Gish, 1996; Altschul and Pop, 2017) against this local database using the parameters Expect value (E) =10, matrix (M) = BLOSUM62,

TABLE 2 | vSMs in *Paramyxovirus* genomes.

Genera	vSMs
RSV A	miR-4719 > miR-556-3p > miR-4801 > miR-8074 > miR-3613-3p > miR-1253 > miR-3618 > miR-182-5p > miR-8485 > miR-4770
RSV B	miR-4311 > miR-6780a-5p > **let-7b-3p** > miR-499a-3p
HMPV A	**miR-4662a-5p > miR-20b-5p >** let-7f-5p > miR-5194 > miR-548u > **miR-1267** > **miR-3925-5p** > miR-3148 > miR-4799-5p > **miR-6884-5p** > miR-335-5p
HMPV B	**miR-6884-5p** > miR-592 > **miR-1267** > **miR-20b-5p**
NDV	miR-6876-5p > miR-1306-5p > miR-1264 > miR-4280 > miR-6888-3p > miR-6855-3p > miR-338-3p > miR-548aq-5p > miR-6875-5p > miR-8069 > miR-3925-3p > miR-1249-5p > miR-892b > miR-4656 > miR-4679
HV	miR-4661-3p > miR-4651 > miR-30e-5p > miR-6734-5p
NiV	**miR-3925-5p >** miR-4698 > miR-4693-3p
MV	miR-374a-3p > miR-5571-5p > miR-3942-3p > **miR-4662a-5p >** miR-5688 > miR-6861-3p > miR-4275 > miR-3617-3p > miR-4273 > miR-569 > miR-3202 > miR-1297 > miR-26b-5p > miR-3934-3p > miR-1255b-5p > miR-6755-3p > miR-1469 > miR-324-3p > miR-548ah-5p > miR-593-3p > miR-4518 > miR-6803-3p > miR-4690-5p > miR-4466 > miR-210-3p > miR-2909 > miR-3909 > miR-4505 > **let-7b-3p** > miR-2276-5p > miR-4420 > miR-4498 > miR-6075 > miR-2276-3p > miR-4486 > miR-6793-3p
MuV	miR-6861-5p > miR-6877-3p > miR-143-5p > miR-4433a-5p > miR-1236-3p > miR-4781-3p > miR-219a-2-3p > miR-1233-5p > miR-4645-5p > miR-3131 > miR-6881-5p > miR-4269 > miR-7152-3p > miR-4286 > miR-4323

vSMs in bold are conserved across two or more viruses. vSM hits are listed in order of their decreasing abundance among viral genomes. RSV, respiratory syncytial virus; hMPV, human metapneumovirus; NDV, New Castle disease virus; HV, Hendravirus; NiV, Nipah virus; MV, Measles virus; MuV, Mumps virus.

TABLE 3 | vSSs in Paramyxovirus genomes.

Genera	vSSs
RSV A	**miR-2278** > miR-4280 > miR-1273f > miR-4753-3p > miR-592 > miR-4639-3p
RSV B	**miR-2278 > miR-7161-3p** > miR-2113 > miR-7151-5p > miR-8068 > miR-369-3p
HMPV A	**miR-8063 > miR-323b-5p > miR-6831-5p > miR-337-5p >** miR-5584-3p > **miR-494-5p > miR-3678-3p**
HMPV B	**miR-891a-3p > miR-6831-5p > miR-323b-5p > miR-337-5p > miR-8063 > miR-3678-3p** > miR-6730-3p
NDV	miR-219a-1-3p > miR-3165 > miR-1257 > miR-758-5p > **miR-7161-3p** > miR-7844-5p > miR-6868-3p
HV	miR-374a-3p > miR-1250-5p > miR-6815-3p > miR-29b-3p > miR-29c-3p > miR-26a-5p > miR-6740-3p
NiV	miR-6817-5p > miR-6846-3p > miR-3173-5p > miR-1205 > miR-2054
MV	miR-6770-3p > miR-4455 > miR-514b-3p > miR-6506-5p > miR-1183 > miR-6841-3p > miR-4714-5p
MuV	miR-6861-5p > miR-6877-3p > miR-143-5p > miR-4433a-5p > miR-1236-3p > miR-4781-3p > miR-219a-2-3p

vSSs in bold are conserved across two or more viruses. vSSs identified Paramyxovirus genomes. vSMs in bold are conserved across two or more viruses. vSS hits are listed in order of their decreasing abundance amongst viral genomes. RSV, respiratory syncytial virus; hMPV, human metapneumovirus; NDV, New Castle disease virus; HV, hendravirus; NiV, nipah virus; MV, measles virus; MuV, mumps virus.

Low complexity Repeat masking = OFF and output = Tabular. CSV files were imported into Microsoft Excel 2010 and filtered to identify hits where miRNA start or miRNA end was ≤3. Hits in the same orientation as the miRNA (5′-3′) were designated vSMs while vSSs were in anti-sense orientation.

Structure Prediction of RSV vSMs (mimics) and vSSs (sponges)

Nucleotide sequences (100 nt) flanking each predicted vSM or vSS for RSV were extracted from parental genomic sequence and analyzed by miRNAfold (Sullivan and Ganem, 2005) or RNA structure (Xu and Mathews, 2016). Structures were visualized with VARNA GUI (Hashimoto et al., 2013). Pre-miRNA sequences were used as controls in prediction. Hybridization stability was calculated using RNA hybrid (Jia et al., 2014).

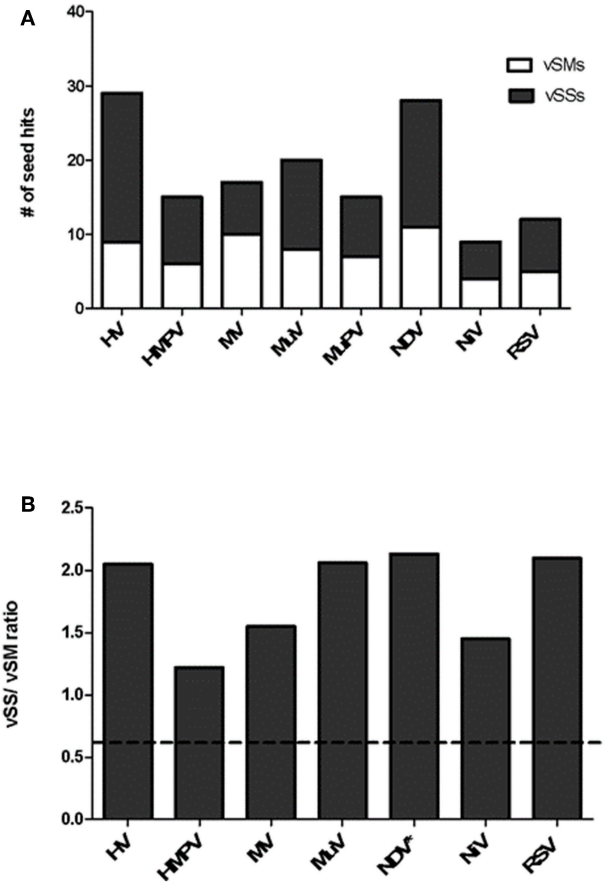

FIGURE 1 | Distribution of vSMs and vSSs across genera of the Paramyxoviridae family. **(A)** Viral sequences identical to host miRNA seed site were designated vSMs (open bars) while those complementary to miRNA seed sites were designated as vSSs (filled bars). **(B)** Ratio of vSS/vSM across genera is shown. Dotted line represents ratio equal to 1.0. HV, hendravirus; HMPV, human metapneumovirus; MV, measles virus; MuV, mumps virus; NDV, New Castle's disease virus; NiV, nipah virus; RSV, respiratory syncytial virus.

Analysis of Small RNA Processing

Total RNA from mock-treated or RSV A2-infected (MOI = 1.0) Vero cells was isolated and fractionated using RNAzol RT according to the manufacturer protocol (MRCgene, Ohio). Fractionated small RNA was polyadenylated and then reverse transcribed with Protoscript II (New England Biolabs, MA) RSV G- and L-specific reverse oligomers followed by PCR using gene specific forward and reverse oligomers in a reaction containing 10 uM final primer concentration as per manufacturer's recommendations. PCR amplicons were resolved on a denaturing 12% PAGE gel in 1X TBE buffer and stained with SYBR Gold. Sequences for RSV G and L oligomers are provided in **Supplemental Methods**.

Next Generation Sequencing (NGS)

Type II respiratory epithelial (A549) cells or neuronal (SHSY5Y) cells were mock-treated or infected with RSV A2 (MOI = 1.0) for 24 h. Total RNA was isolated using RNAzol RT (MRCgene, Ohio) per the manufacturer protocol and size fractionated. Small RNA was quantified using Qubit broad range RNA kit (Invitrogen, USA). Size fractionated small RNA was ligated to proprietary Illumina adaptors using T4 RNA Ligase deletion mutant (Epicenter, USA). Adaptor ligated RNA was reverse transcribed and amplified for limited number of cycles during which index barcodes were attached to each cDNA pool. cDNA libraries for each sample at the end of the incubation temperature were again analyzed on a Tapestation 2200, quantified and then pooled at equimolar ratios as recommended by manufacturer. Pooled cDNAs were denatured using freshly prepared 0.2 N NaOH at room temperature for 3 min and then mixed with hybridization buffer. Diluted libraries at 20 pM concentration were loaded onto Illumina MiSeq and run to generate fastq files as per Illumina MiSeq protocol. Sequencing reads obtained had quality scores >30, were trimmed of adaptors, and were then analyzed by BLAST against RSV A2 genome to determine virus specific transcripts and determine gene location for these transcripts.

RESULTS

Paramyxovirus Transcripts Mimic or Bind to Host miRNAs by Seed Sequence

Paramyxovirus genome replication, gene transcription, and translation occur in the cytoplasm, which is the primary site of host post-transcriptional gene regulation by miRNAs. To identify the sequence motifs in *Paramyxovirus* genomes that could mimic or inhibit host miRNA function based on seed sequence homology/complementarity, RSV, HMPV, MuPV, NDV, HV, NiV, MV, and MuV genomes were analyzed by basic local alignment search tool (BLAST) (Altschul et al., 1990) against a database of either human mature miRNAs or cognate species using criteria optimized for finding short matches (matrix = BLOSUM 62, Expect value = 10). Hits were filtered to identify matches in the miRNA seed site that were either in the sense (+/+) or antisense (±) orientation relative to the miRNA. Sense orientation hits were designated as vSMs (**Table 2**), while antisense hits were designated vSSs

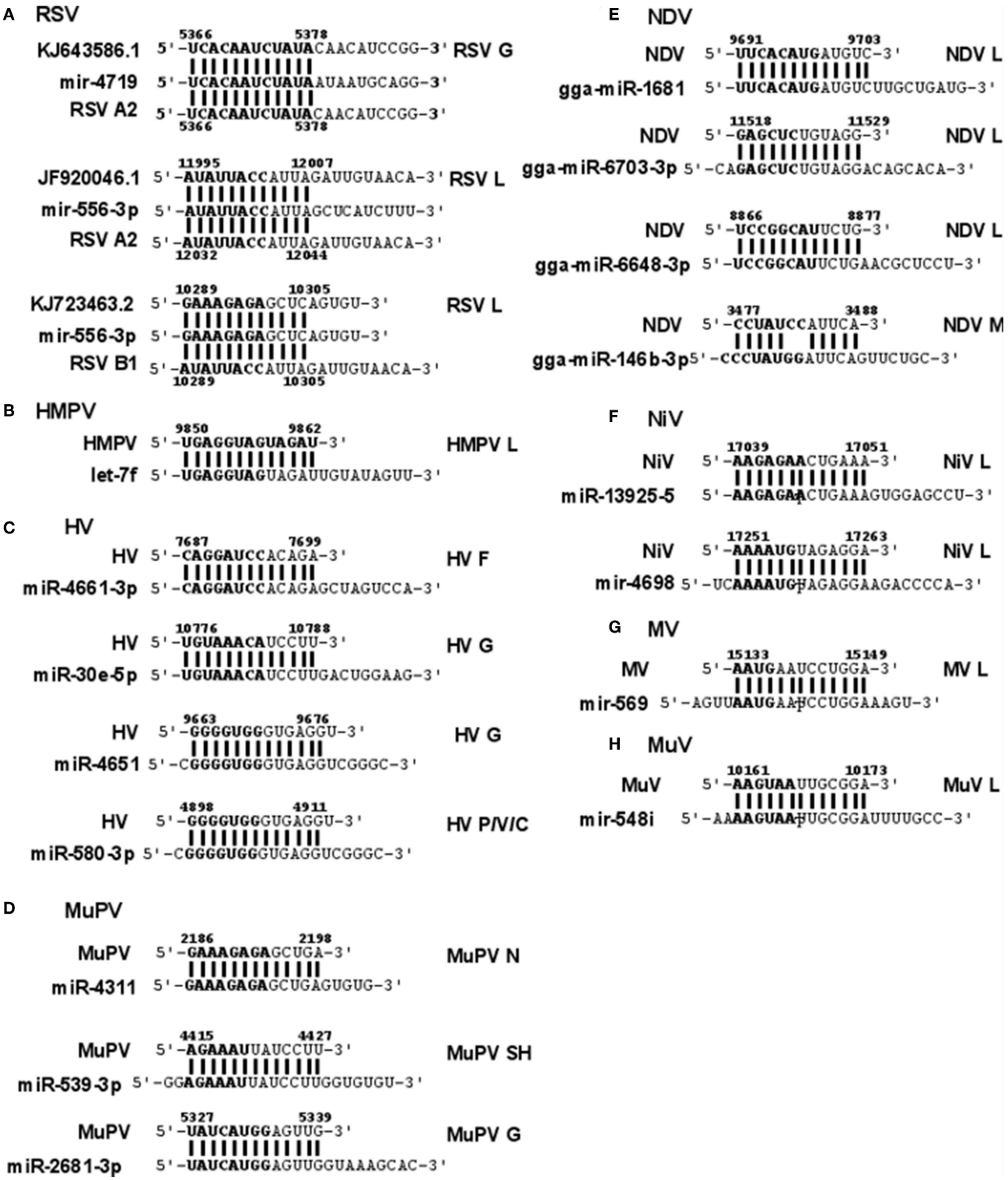

FIGURE 2 | Sequence alignments of miRNA vSMs encoded by different paramyxoviruses. Alignments show identity between host miRNA seed sites and the corresponding vSM across *Paramyxoviruses*. Nucleotide numbers indicated viral genome coordinates while labels on the right indicate encoding viral gene. miRNA seed sequence is indicated in bold. Straight lines indicate perfect identity.

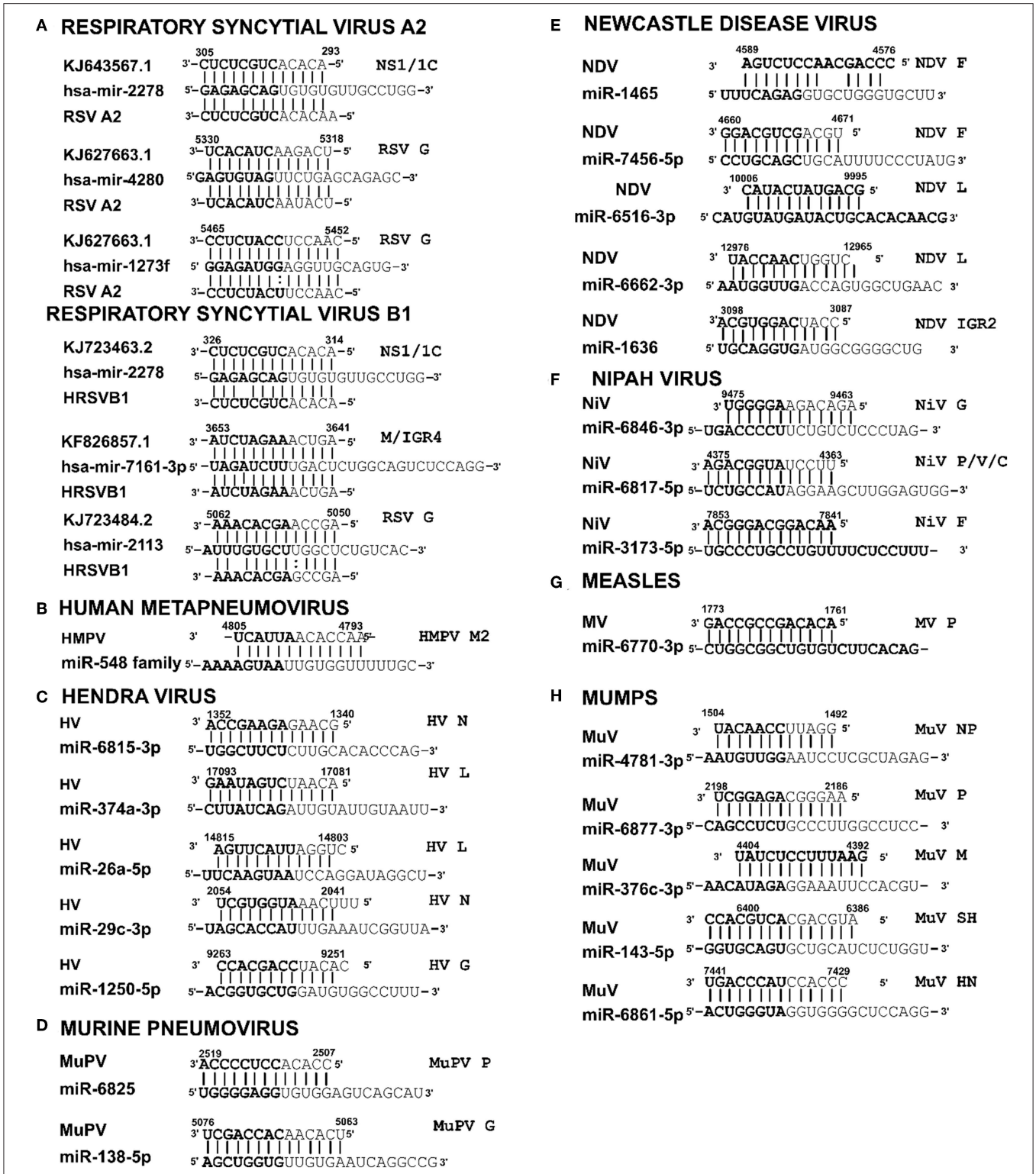

FIGURE 3 | Sequence alignments of miRNA vSSs encoded by different paramyxoviruses. Alignments show complementarity between host miRNA seed site and the corresponding vSS across *Paramyxoviruses*. Nucleotide numbers indicated viral genome coordinates while labels on the right indicate encoding viral gene. miRNA seed sequence is indicated in bold. Straight lines indicate Watson-Crick base pairing (AU/GC) while colon indicates a non-Watson-Crick base pair (GU).

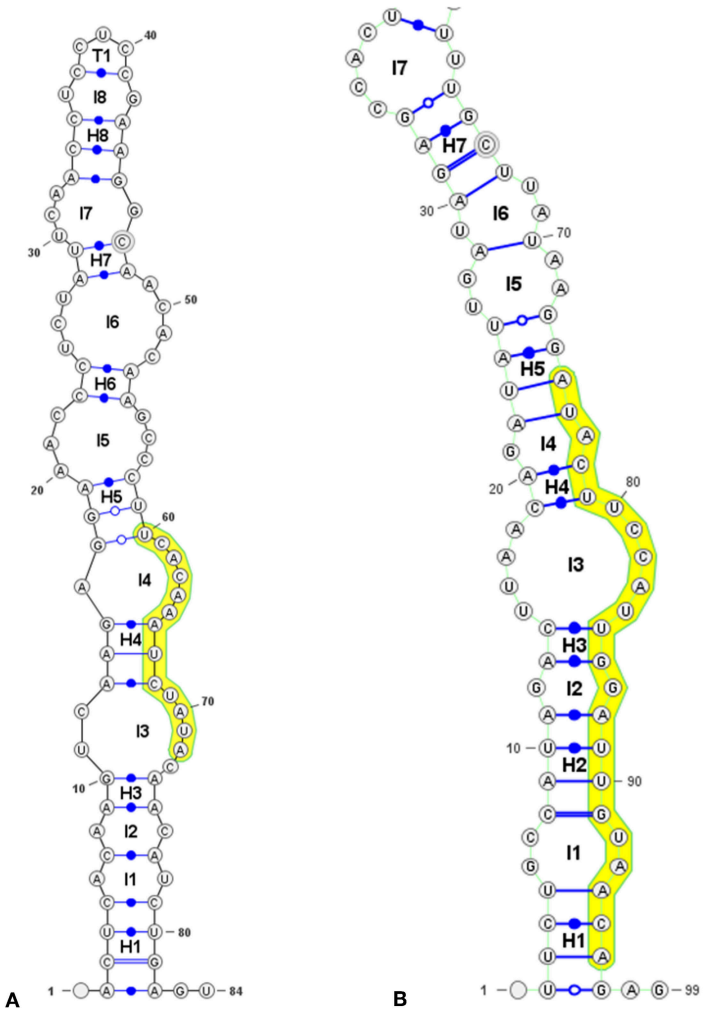

FIGURE 4 | Viral genomic regions form characteristic stem- loop folds in vSM and vSS encoding regions and are processed into smaller transcripts. Secondary structures of a 100 nt sequence flanking vSM-4719 **(A)** and−556-3p **(B)** were predicted using RNAfold and drawn using VARNA. Highlighted region corresponds to the vSM. Internal and terminal loops are prefixed with L and T, respectively. Helices are prefixed with H and the 5′ and 3′ ends of the molecule are as indicated. **(C)** Size-fractionated small RNA from mock or RSV A2 infected (24 h or 48 h pi) Vero cells was reverse transcribed using RSV G- and L-specific reverse primers. Amplicons obtained using a G/L specific primer pair were electrophoresed on a 12% PAGE gel alongside a molecular ladder and stained with SYBR gold.

(**Table 3**). Across genera, we identified 85 vSSs compared to 60 vSMs for the type strains (**Figure 1A**). The ratio of the number of vSSs to vSMs was typically in the 1.5–2.0 + range for most genera except HMPV where they were in approximately equal numbers (**Figure 1B**). The ratio of vSS/vSM was determined to determine if vSMs vs. vSSs were selected for or against during evolution. As noted in the Introduction, vSMs are expected to enhance native miRNA mediated suppression while vSSs are expected to bind to miRNAs and relieve their repressor effect. A high vSM/vSS ratio would indicate that mimicking host miRNA activity could be a predominant mechanisms of modulating host miRNA activity. Alternatively, a low vSM/vSS ratio might indicate that inhibiting miRNA activity would be a more conserved mode of regulating miRNA function. Most vSMs or vSSs were located in the L gene across the *Paramyxovirus* genera followed by F protein and G protein genes. Details of vSMs and vSSs in are discussed below.

Respiratory Syncytial Virus

RSV is grouped as A and B strains based on the diversity in the G protein (Johnson and Graham, 2004; Papenburg and Boivin, 2010; Pangesti et al., 2018). For strain A2 (GenBank accession number M74568), we did not identify any vSM or vSS while for strain B1 (GenBank accession number AF013254), one vSM was identified in the L gene. Analysis of clade A (326 sequences identified) and clade B (60 sequence strains) (accession numbers in **Supplementary Table 1**) identified two vSMs, one mapping to miR-4719 in the G gene and the other mapping to miR-556-3p in L gene (**Figure 2A**). Strains that encoded vSM-4719

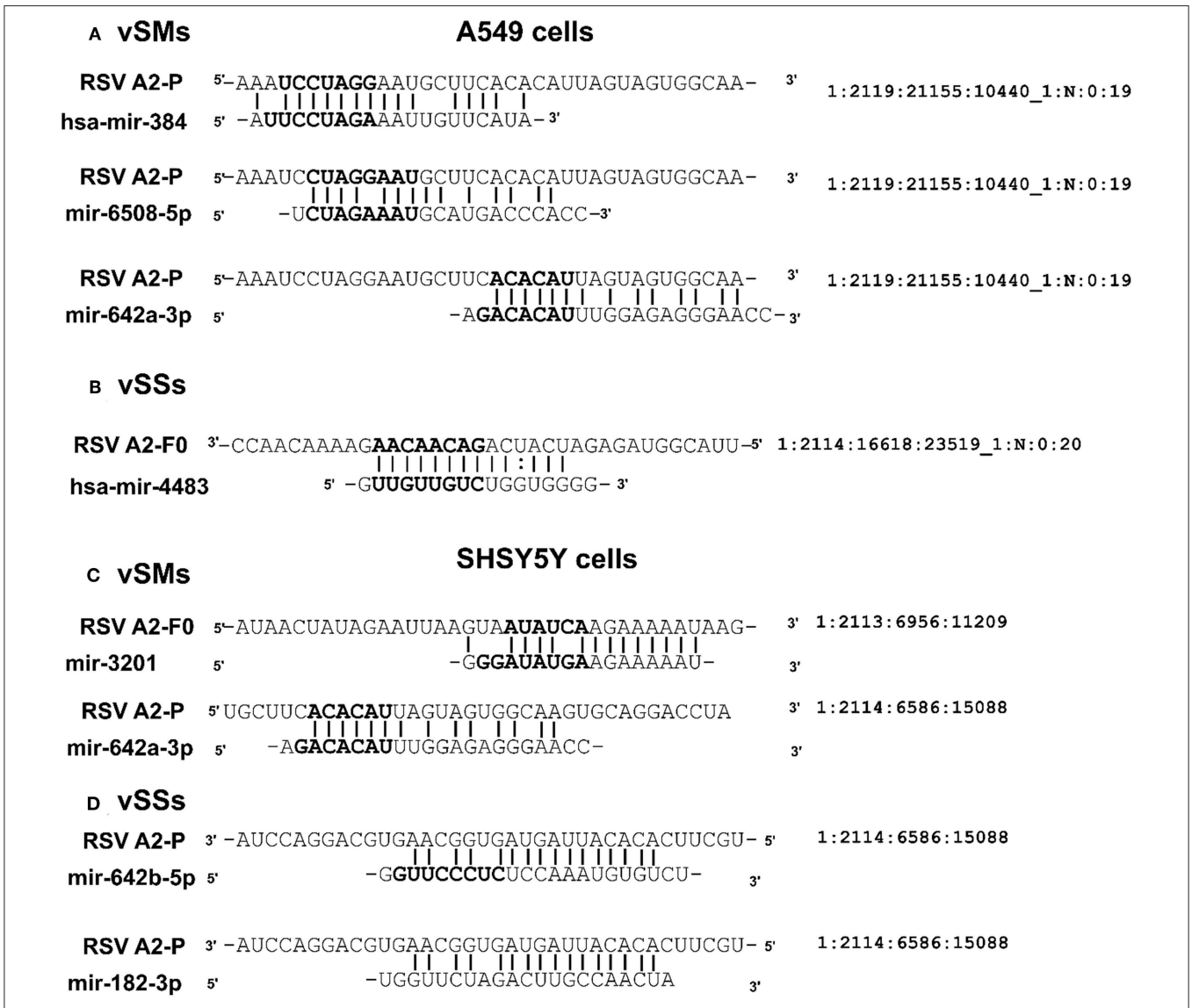

FIGURE 5 | Small RNA from RSV-infected respiratory epithelial (A549) **(A,B)** or neuronal (SHSY5Y) **(C,D)** cells were sequenced on an Illumina MiSeq. Alignments show RSV vSMs or vSSs in bold.

did not encode vSM-556-3p and vice-versa. For B1, the single vSM corresponding to miR-4311 mapped to the L gene. The viral gene segments that encoded vSSs included miR-2278 in the NS1/1c gene and miR-4280 and miR-1273f in the G gene (**Figure 3A**).

Human Metapneumovirus

HMPV, like RSV, is grouped as A and B strains based on G gene diversity (Kahn, 2006; Papenburg and Boivin, 2010; Schuster and Williams, 2014). A single vSM for miRNA let-7f was located in the L gene (**Figure 2B**), and a single vSS complementary to multiple members of the miR-548 family was found in the M2 gene (**Figure 3B**).

Hendravirus

Analysis of the HV genome identified three major vSMs for miR-4661-3p, miR-30e-5p, and miR-580-3p in F, G, and P genes (**Figure 2C**). vSSs for miR-6815-3p and miR-29c-3p in the N gene, miR-374a-5p, and miR-26a-5p in the L gene, and miR-1250-5p in the G gene were identified (**Figure 3C**).

Murine Pneumovirus

Analysis of MuPV genome identified several conserved vSMs containing seed sequence of miR-4311 in the N gene, miR-539-5p in the SH gene, and miR-2681-3p in the G gene (**Figure 2D**), as well as vSSs complementary to miR-6825 and miR-138-5p in the P and G genes, respectively (**Figure 3D**).

New Castle Disease Virus

Avian Paramyxovirus-1 (APMV-1) is the etiological agent of NDV. Analysis of the APMV-1 genome identified four highly conserved vSMs for miR-1681, miR-6703-3p, and miR-6648-3p in the L gene and miR-146b-3p in the M gene (**Figure 2E**). The genome also encoded two vSSs each for miR-1465, miR-7456-5p in the F gene, and miR-6516-3p and miR-6662-3p in the L gene, and miR-1636 in the intergenic region (**Figure 3E**). APMC-1 encoded the highest number of vSMs and vSSs among all paramyxoviruses.

Nipah Virus

Analysis of NiV genomes identified two vSMs for miR-3925-5p and miR-4698 in the L gene (**Figure 2F**). The genomes also

TABLE 4 | miRs targeted by vSMs and vSSs for HMPV or RSV strain A or B were analyzed by DIANA miRPath server.

	# of vSMs			# of vSSs		
Pathways deregulated by virus infection	**RSV A**	**RSV B**	**Total**	**RSV A**	**RSV B**	**Total**
Prostate cancer	1	1	2	1	1	2
Axon guidance	1	1	2		1	1
PI3K-Akt signaling pathway	1	1	2	1	1	2
Chronic myeloid leukemia	1	1	2		1	1
Non-small cell lung cancer	1		1	1	1	2
Transcriptional mis-regulation in cancer	1	1	2	1		1
Endometrial cancer	1		1	1	1	2
T cell receptor signaling pathway	1	1	2		1	1
Focal adhesion	1	1	2			0
Neurotrophin signaling pathway	1		1		1	1
Vasopressin-regulated water reabsorption	1		1		2	2
ErbB signaling pathway	1		1		1	1
Regulation of actin cytoskeleton	1		1			0
Prion diseases	1	1	2			0
Glioma	1		1	1		1
Endocytosis	1	1	2			0
Wnt signaling pathway	1	1	2			0
Colorectal cancer	1	1	2			0
Ubiquitin mediated proteolysis	1	1	2			0
Dopaminergic synapse	1	1	2			0
MAPK signaling pathway	1	1	2			0
Renal cell carcinoma	1		1		1	1
Melanogenesis	1		1		1	1
TGF-beta signaling pathway	1		1		1	1
B cell receptor signaling pathway	1		1		1	1
mRNA surveillance pathway	1	1	2			0

Significance of association based on p value ≤ 0.05 and False Discovery Rate (FDR) correction. Pathways with p-values < 0.05 are shown which are significant. Numbers indicate number of vSMs or vSSs.

encoded vSSs against miR6846-3p in the G gene, miR-6817-5p in the P gene and miR-3173-5p in the F gene (**Figure 3F**).

Measles Virus

The MV genome had a vSM for miR-569 in the L gene (**Figure 2G**), and a vSS for miR-6770-3p in the P gene (**Figure 3G**).

Mumps Virus

MuV genome has a vSM in the L gene for mir-548i (**Figure 2H**). The genome also encodes five vSSs against miR-4781-3p in the NP gene, miR-6877-3p in the P gene, miR-376c-3p in the M gene, miR-143-5p in the SH gene, and miR-6861-5p in the HN gene (**Figure 3H**).

These comparative analyses of *Paramyxoviruses* revealed an enrichment of vSSs relative to vSMs and localization of vSM sequences in L or G genes. It is not clear if these miRNA sequences evolved as an adaptation to a host response or are an outcome of low polymerase (L gene) fidelity. The conservation of these sequences in the circulating viral strains of multiple paramyxoviruses suggests that they may facilitate virus replication possible by contributing to immune evasion.

Viral RNA Is Processed to Smaller Transcripts During Infection

Stem-loop structures are a feature of miRNA templates and are recognized by both nuclear and cytosolic class III RNAses (Fabian et al., 2010; Weill et al., 2015). Several studies suggested RSV genome/antigenome might not fold to form secondary structures due to N protein encapsidation (Ghosal et al., 2014; Kumari et al., 2016). However, recent data has shown that RNA probes can bind to RSV genomic RNA (Bartel, 2009; Li et al., 2014; Cloonan, 2015) support the hypothesis that the RSV genome/antigenome is exposed during infection and may be able to form secondary structural motifs that can act as templates for Dicer, a class III cytosolic RNAse (Friedman et al., 2009). There is no published data to indicate that viral transcripts are protein encapsidated in the cytosol during infection. We analyzed genomic sequences near predicted vSMs and vSSs, and RNA secondary structure predictions indicated the potential of these regions to fold into stable stem loop structures similar to pre-miRNA transcripts (**Figures 4A,B**). Randomization of the sequence disrupted the structure. Stability of interaction between miRNAs and vSSs were computationally determined with RNAhybrid, a tool for finding the minimum free energy hybridization of a long and a short RNA to predict miRNA targets (Jia et al., 2014). Interactions

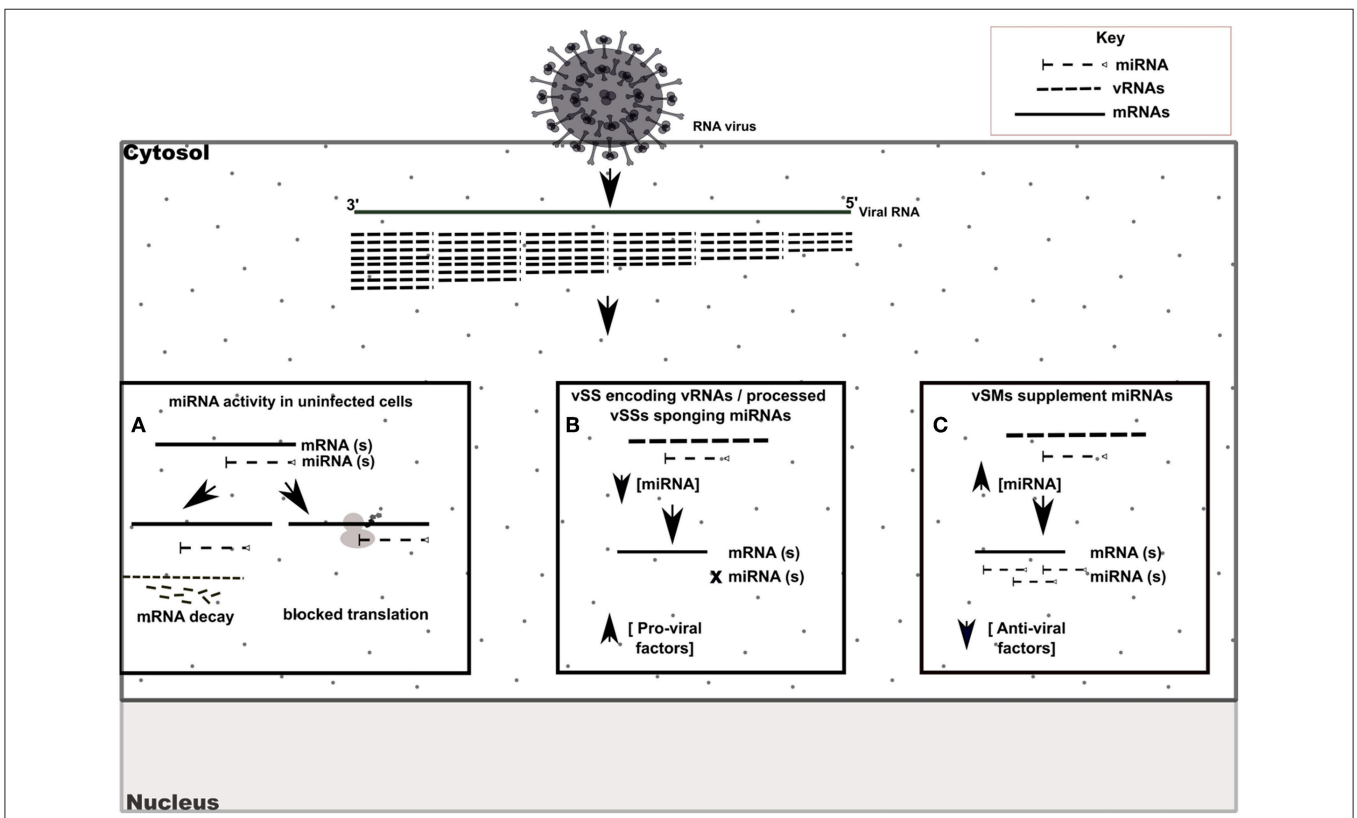

FIGURE 6 | Model of vSM / vSS modulation of host responses during infection. Host mRNA are depicted as solid lines, microRNAs are depicted as dashed lines with end caps while viral transcripts are depicted as dashed lines. **(A)** Normal post-transcriptional miRNA regulation of gene expression. Degree of miRNA complementarity determines if gene knockdown proceeds via mRNA decay/translation following miRNA binding to target transcript in the RISC complex. **(B)** Viral RNAs encoding vSSs/processed vSSs bind to host miRNAs and prevent native suppression of pro-viral factors. **(C)** Viral RNAs encoding vSMs can supplement host miRNA activity and suppress anti-viral responses.

between the predicted vSS and complementary miRNA are very stable as evident from the low mean free energy of the hybrid (**Table 1**). To establish if the RSV transcripts are processed into small RNAs during infection, Vero cells were infected with RSV A2 (MOI = 5.0) for 24 or 48 h, and size-fractionated small RNA was reverse transcribed using G- or L- specific oligonucleotides and amplified using G- or L-specific forward and reverse oligomers. MOI of 1.0 was used to infect A549 or SHY5Y cells with RSV or MV followed by small RNA isolation at 24hpi and NGS sequencing. MOI of 5.0 was used to infect Vero cells for RT-PCR analysis of small RNA products. The different MOIs were used because of the different sensitivities of the assay methods. With NGS, we expected to observe even small differences owing to the extremely high sensitivity of the method. In contrast, for Vero cells, RSV infection produces little to no cytopathic effect at 24–48 hpi, thus necessitating higher MOI infection. Electropherograms clearly showed bands of ∼40, 50, 70, and 80 bp for the G gene, and 40 and 80 bp for the L gene along with several bands in the ∼120 bp size range that are similar to host pre-miRNA sizes (**Figure 4C**). These data support the hypothesis that vSM- and vSS-encoding regions of the genome are folded and processed into smaller transcripts potentially via cellular RNAi machinery. Experimental validation of predicted structures is out of the scope of this hypothesis article.

Small Viral RNA Transcripts Encode vSMs and vSSs

The results show that RSV infection of Vero cells, which lack a functional IFN α/β locus (Karnati et al., 2015; Alipoor et al., 2016), produces small RNAs from the G and L genes (**Figure 4C**). We also analyzed small RNAs transcriptomes of mock-treated, RSV-infected A549 cells, or RSV-infected neuronal SHSY5Y cells by NGS to identify if these transcripts encoded vSMs and vSSs. Transcripts mapping to viral genome over their entire length (36 nts) were also compared to mature human miRNAs to identify vSMs and vSSs. In RSV-infected A549 cells, we identified viral transcripts that encoded three overlapping full-length vSMs (miR-384, miR-6508-5p, and miR-642a-3p) and others that were perfectly complementary to miR-4483 (**Figure 5A**). Viral transcripts from RSV infected SHSY5Y cells encoded a near full length miR-3201 or miR-642a-3p transcript and vSSs against miR-642b-5p and miR-182-3p (**Figure 5C**). Many of transcripts encoded only vSMs or vSSs in contrast to the transcripts noted above which encoded nearly full-length host miRNAs (**Figures 5B,D**). These findings support our hypothesis that *Paramyxovirus* gene transcripts may modulate host miRNA activity via seed match or complementarity. While host encoded competing endogenous RNA are known, this is the first such analysis done for *Paramyxovirus* genomes thereby opening new areas of investigation.

DISCUSSION

The low replicative fidelity of RdRp of *Paramyxoviruses* appears to be an evolutionary strategy since it allows for quasispecies generation, favors emergence of variants that can escape immune pressure, and may allow the virus to modulate host responses and gene expression via regulation of miRNA function. Tempering host gene expression is important for viral replication, and viruses have evolved several means to counter host responses to virus infection using mechanisms including shutdown of host transcription, translation, and modification of sncRNA and miRNA expression (Vishnoi and Rani, 2017; Olejniczak et al., 2018).

RIG-I and MDA-5 are prototypical pattern recognition receptors that detect and respond to presence of 5′ triphosphorylated and double-stranded RNA, respectively, during RNA virus infection (Piedade and Azevedo-Pereira, 2016; Auvinen, 2017). These receptors trigger a signaling cascade that culminates in establishment of an antiviral state in the infected and neighboring cells (Piedade and Azevedo-Pereira, 2016). Viruses avoid host responses to replicate and moderate these responses to facilitate replication. In addition to modifying the antiviral response, we propose that quasispecies generation during RNA virus replication helps regulate host gene expression by modulation of host miRNA function and activity. We speculate that RNA viral quasispecies produce a cloud of molecular signatures that mimic or inhibit host miRNA activity via sequence complementarity and alter the expression and function of several host pathways during infection. Our data suggest that vSMs and vSSs encoded by *Paramyxovirus* genomes and transcripts aid quasispecies generation by modulating various host pathways (**Table 4**) (**Figure 6**). It is likely that the generation of quasispecies is dynamic and adapts to host cell pressure. Thus, viral transcripts can mutate to produce vSMs or vSSs that modulate different miRNAs in different cell types as well as carry one or more frameshift mutations to produce alternative proteins/peptides that provoke modified or altered responses as these data suggest where viral transcripts can potentially bind to or mimic host miRNAs perturbing their expression and diverting host resources.

AUTHOR CONTRIBUTIONS

AB and AM collected and analyzed the data and wrote the manuscript with RT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00152/full#supplementary-material

Supplementary Table 1 | Lists of all raw data.

Supplementary Table 2 | Lists analysis of other RNA viruses that also show vSMs and vSSs.

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Molecular Simulations Reveal the Role of Antibody Fine Specificity and Viral Maturation State on Antibody-Dependent Enhancement of Infection in Dengue Virus

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Recent clinical studies have revealed that severe symptoms of dengue fever are associated with low pre-existing antibody levels. These findings provide direct clinical evidence for the theory of antibody-dependent enhancement of infection (ADE), which postulates that sub-neutralizing levels of antibodies facilitate the invasion of host cells by the dengue virus. Here, we carried out molecular simulations guided by previous *in vitro* experiments and structural studies to explore the role of antibody fine-specificity, viral conformation, and maturation state—key aspects of dengue virology that are difficult to manipulate experimentally—on ADE in the context of primary and secondary infections. Our simulation results reproduced *in vitro* studies of ADE, providing a molecular basis for how sub-neutralizing antibody concentrations can enhance infection. We found that antibody fine specificity, or the relative antibody response to different epitopes on the surface of the dengue virus, plays a major role in determining the degree of ADE observed at low antibody concentrations. Specifically, we found that the higher the relative antibody response to certain cross-reactive epitopes, such as the fusion loop or prM, the greater was the range of antibody concentrations where ADE occurred, providing a basis for why low antibody concentrations are associated with severe dengue disease in secondary infections. Furthermore, we found that partially mature viral states, in particular, are associated with the greatest degree of ADE.

Keywords: antibody-virus interactions, dengue virus, antibody dependent enhancement, antibody neutralization, molecular simulations

INTRODUCTION

Dengue virus (DENV), a major human pathogen transmitted by *Aedes aegypti* mosquitoes, causes an estimated 390 million infections each year (Bhatt et al., 2013). Four DENV serotypes (DENV1–DENV4), which are found across tropical and sub-tropical regions, vary in prevalence depending on the time and region. Whereas primary dengue infection is typically asymptomatic or results in a mild, uncomplicated fever, secondary infection with a heterotypic serotype is associated with severe disease manifestations, such as dengue hemorrhagic fever, and occasionally, death

(Halstead, 1970; Sangkawibha et al., 1984; Guzman and Harris, 2015). This pattern of outcomes has led to the hypothesis that pre-existing immunity to DENV is responsible for enhanced secondary infections.

Recently, two clinical studies that assessed the longitudinal risk of severe dengue disease following primary and secondary infection found that low pre-existing serum concentrations of antibodies (Abs) to dengue virus were associated with the highest risk of severe symptoms. In a study of 3,451 children in Thailand, Salje et al. (2018) found that individuals developed a stable set-point titer within 1 year of a primary infection, and that individuals with pre-existing titers of <1:40 developed hemorrhagic fever at 7.4 times the rate of naïve individuals, compared with 0.0 times for those with titers >1:40. Likewise, in a study of children in Nicaragua, Katzelnick et al. (2017) found that individuals with pre-existing DENV Ab titers within a narrow intermediate range had the highest risk of severe symptoms, compared to those with high DENV Ab titers and those that were seronegative for DENV infection.

The exact mechanism by which pre-existing immunity leads to severe dengue symptoms is unknown. However, *in vitro* studies of dengue infection suggest that Ab-dependent enhancement of infection (ADE) plays a role. In ADE, sub-neutralizing Ab concentrations facilitate viral invasion of host cells via an Fcγ-receptor (FcγR)-mediated mechanism. Specifically, Abs bound to the virus surface engage Fc receptors, resulting in FcγR-mediated endocytosis. Subsequent acidification of the phagocytic vesicles triggers viral membrane fusion and invasion of the host cell. Although *in vitro* studies using both monoclonal and polyclonal Abs have shown that ADE occurs under various conditions for a range of FcγR-bearing cells, major questions remain regarding its physiological role in dengue disease severity.

First, lower set-point titers are associated with severe dengue disease during secondary infection, but not primary infection, suggesting that serotype specificity, in addition to antibody concentration, plays a role in ADE. Second, it is unclear how the same infecting viral titer that is largely asymptomatic in naïve individuals is pathogenic in exposed individuals. In this study, we sought to address these questions by extending a molecular simulation approach to model the roles of antibody concentration, serotype-specificity, and viral heterogeneity in ADE.

DENV infection triggers a broad immune response, which in part involves the production of hundreds to thousands of distinct monoclonal Abs (mAbs) which bind to a range of epitopes on the surface of the virus. Previous *in vitro* studies of flavivirus infection suggest that a stoichiometric threshold of 20–50 Abs bound to the virion is sufficient for neutralization (Pierson et al., 2007). MAbs to DENV are typically classified as type-specific (TS) Abs that bind to and/or neutralize only one serotype, and cross-reactive (CR) Abs that bind to and/or neutralize all four serotypes. An important study by Beltramello et al. (2010) found that activation of immunological memory years after a DENV infection leads to the production of large amounts of broadly CR Abs. However, most of these Abs are incapable of neutralizing infection even at very high concentrations, and only a small quantity of them may exhibit TS or potent neutralizing activity.

A key feature of these poorly neutralizing CR Abs is that they target immunodominant epitopes, such as the fusion loop (FL) of the envelope (E) protein or the *pr* fragment of the prM protein, which have low accessibility or availability. *In vitro* studies have shown that, Abs which bind to these epitopes are highly prone to ADE (Halstead and O'Rourke, 1977; Beltramello et al., 2010; Dejnirattisai et al., 2010; Yeo et al., 2015). Fully mature DENV viral particles do not contain *pr* fragments or present the FL epitope on their surface (Perera and Kuhn, 2008; Zhang et al., 2013). However, infected host cells produce a wide spectrum of viral particles in different maturation states with varying ratios of prM and E, which are manifest in cryo-electron microscope images as spiky patches and heterogeneous morphologies. Recent studies of *in vitro* models of DENV infection have shown that, mAbs which target these epitopes are typically highly cross-reactive, poorly neutralizing, and highly prone to ADE, even when they are produced by infections due to other flaviviruses, such as the Zika virus (ZIKV) (Barba-Spaeth et al., 2016; Stettler et al., 2016).

We previously presented a method (Ripoll et al., 2016) for estimating the stoichiometry of Ab-flavivirus complex formation and modeling antibody-dependent neutralization of dengue virus, using a molecular simulation approach based on the theory of multiple equilibria in proteins (Tanford and Kirkwood, 1957; Beroza et al., 1991). We used a coarse-grained structural representation of the Ab-flavivirus complex based on high-resolution cryo-EM and X-ray crystallography structures that allowed us to capture important structural characteristics, such as the spatial distribution of the epitope around the virion. Here we extended this approach to consider (1) mixtures of CR and TS mAbs that recognize distinct epitopes, and (2) heterogeneous viral populations that include partially mature states in various structural configurations.

MATERIALS AND METHODS

We modeled the interaction of a mixture of CR and TS Abs with the envelope of flaviviruses, using an extension of the structure-based Monte Carlo (MC) approach described previously (Ripoll et al., 2016). Here, we highlight the changes we introduced to the procedure to model the binding of an Ab mixture to a virion. First, we assumed that the total Ab concentration, $[Ab_{tot}]$, is given by Equation (1), where $[Ab_{TS}]$ and $[Ab_{CR}]$ are the partial concentrations of TS and CR Abs, respectively.

$$[\boldsymbol{Ab_{tot}}] = [\boldsymbol{Ab_{TS}}] + [\boldsymbol{Ab_{CR}}] \quad (1)$$

We assumed that binding occurs as a Brownian-like process where Abs randomly collide with a virus envelope. The virion concentration, $[V]$, was considered infinitely dilute (virion-virion interactions are negligible, i.e., $[Ab_{tot}] >> [V]$). Each set of CR or TS Abs was represented by a single mAb with the highest affinity of the respective group, and the chosen representative Abs corresponded to well-studied mAbs whose epitopes were mapped to E or prM, preferably through Cryo-EM or X-ray crystallography experiments.

Coarse-Grained Structural Model of DENV-Ab Complexes

We used coarse-grained representations of both the Abs and the viral envelope to capture the relevant geometrical features of the complexes. As described above, we generated three-dimensional models of the viral particles by combining homology modeling techniques with existing cryo-EM and X-ray structure data. For the four DENV serotypes, we constructed all-atom models for whole virus envelopes in their immature "spiky," immature "smooth," and mature conformations (Zhang et al., 2003, 2013; Perera and Kuhn, 2008; Yu et al., 2008; Kostyuchenko et al., 2013). Using the fully mature or immature models, we produced partially mature viral particles with varying ratios of immature to mature content, $\mathbf{f}$, and then converted these into coarse-grained models.

We represented the partially mature state as a sphere of radius r [$= (r_{Imm} + r_{Mat})/2$], where r_{Imm} and r_{Mat} correspond to the radii of the immature and mature viral envelopes, respectively, derived from all-atom homology models of the viral envelopes in the particular maturation state (i.e., mature, immature *spiky*, and immature *smooth*) (Zhang et al., 2003, 2013; Perera and Kuhn, 2008; Yu et al., 2008; Kostyuchenko et al., 2013). We used a tessellation procedure to partition the surface of the sphere into elements of equal size (Tegmark, 1996), each of which determined a pixel on the sphere, and whose total number determined the resolution of the spherical grid. We derived a simplified representation of an epitope, ξ, on the tessellated sphere from the collection of surface elements intersected by the radial projections of the actual epitope atoms in the three-dimensional model of the partially mature envelope.

At the start of each MC run, the fraction of prM content, f, was randomly selected based on a normal distribution centered around $\mathbf{f}$, an input parameter defining the mean prM content, and a composite viral particle consisting of E-prM and E subunits (in a ratio corresponding to f). The distribution of E-prM and E subunits was either arranged randomly across the whole viral surface (for the "smooth" conformation) or as a contiguous surface patches (for the "rough" conformation). The types of epitope distributions were consistent with available experimental data indicating the existence of partially mature flavivirus particles containing varying amounts of uncleaved prM (Junjhon et al., 2008; Nelson et al., 2008; Dowd and Pierson, 2011), and cryo-EM data showing viral particles where a portion of the surface remains in the immature "spiky" state (Perera and Kuhn, 2008; Junjhon et al., 2010). Finally, CR and TS epitopes were mapped to the corresponding E-prM and E subunits on the viral surface.

Abs were represented as circular "soft disks" that could interact with other Abs through steric interactions and bind to their epitopes. This simplified "footprint" representation removes the need to account for changes in the Ab orientation relative to the virus surface. An Ab was considered *bound* when it landed on a given surface element and occluded a center of one of its epitopes. The radius of the soft disk, r_{Fab}, reflects the overall excluded volume of the Ab. We previously found that a r_{Fab} of 27.8 Å was sufficient to reproduce the Ab binding stoichiometry for most Ab-flavivirus complexes with available cryo-EM structures (Ripoll et al., 2016).

Ab-Virus and Ab-Ab Interactions

We used the theory of multiple equilibria in proteins to model Ab binding (Edsall and Wyman, 1958; Steinhardt and Reynolds, 1969; Bisswanger, 2008), where Abs represented the ligands, and the virus envelope represented the macromolecule whose binding sites corresponded to the epitopes of the Abs. We modeled the behavior of a mixture of two types of Abs by adapting a methodology used to study pH titration in proteins (Beroza et al., 1991). During the simulation process, we assumed that Abs stochastically bind to and unbind from binding sites on the virus surface in an epitope-specific manner, through the use of a free energy function that derives the free energy of the virus binding state based on its binding configuration and the binding affinity of each mAb in the system (see **Supplemental Materials**: section A. Modeling Ab-virus and Ab-Ab interactions). Previously, we used this approach to model the binding of mAbs to DENV (Ripoll et al., 2016), here, we extended this approach to simulate the binding of *mixtures* of two mAbs, a CR and TS mAb, to DENV. The Abs in the system, in addition to binding to the virus surface, were also assumed to sterically interact with each other, potentially occluding neighboring binding sites in the described above.

Simulating Ab Binding and Neutralization

We carried out simulations for combinations of $[Ab_{TS}]$, and $[Ab_{CR}]$, with each partial concentration ranging from 10^{-1} to 10^{-14} M. For each pair of partial concentrations, we carried out 500 binding simulation runs ($\sim 10^8$ M steps each), collecting statistics from 25,000 independent configurations of Abs bound to the viral capsid. From the simulation data, we computed the observed number of bound Abs $< N_{bound} >$ to generate Ab occupancy curves. Similarly, we computed the mean number of bound CR Abs, $< N_{CR} >$, and the mean number of bound TS Abs, $< N_{TS} >$.

We generated theoretical curves of infectivity, r_{infc}, or neutralization, r_{neut} [$= (1–r_{infc})$], using a structure-based model of neutralization introduced in our previous work (Ripoll et al., 2016). This model represents a variation of the "multiple hit" model (Parren and Burton, 2001), which assumes that docking of multiple Abs to a single virion is required for neutralization. In previous work we showed that this model shows ~50% neutralization for an average $< N_{bound} >$ of ~30, which is close to the neutralization threshold postulated by the "coating theory" for flaviviruses.

Simulating ADE

We defined a quantitative model of ADE that assumes that the enhancement, $\mathbf{E_{ADE}}$, produced by a given virion is determined by two variables: the infectivity of the particle, $\hat{r}_{infc}$, and the rate of phagocytosis, $\hat{\mathrm{r}}_{\mathbf{phg}}$.

$$E_{ADE} = \hat{\mathrm{r}}_{infc} \cdot \hat{\mathrm{r}}_{phg} \tag{2}$$

We assumed the rate of phagocytosis to be a function of the number of successful encounters of the virion with the receptors

on the surface of the invaded cell. Additionally, we assumed all cells to have the same number of receptors. A successful encounter required the presentation of an Ab by the virus to the host cell, hence, the greater the $< N_{bound} >$ on the virion, the more likely the encounter was successful. With these considerations, we assumed that $\hat{r}_{phg} = C\, N_{bound}$, where N_{bound} is the number of Abs bound to the particular configuration of the virus, and C is a constant.

Monte Carlo Simulations

To produce a stoichiometric curve for a given dual Ab mixture and virus complex, we need to determine the average $< N_{bound} >$ at different concentration values of the Ab mixture. To this end, we performed importance sampling using MC methods (Beroza et al., 1991) to simulate the Ab-virus binding process. After first selecting a pair of partial concentration values for the free Abs, $[Ab_{TS}]$ and $[Ab_{CR}]$, we carried out 500 independent simulation runs. Each run started with the initialization of the system, in which the number of CR epitopes for an Ab-free viral envelope was randomly defined using a normal distribution with a mean corresponding to the immature content $\mathbf{f}$ (specified by an input value). During the course of the MC simulation, surface elements were randomly selected along with a type of action (a "trial" move): binding or release of an Ab. For binding moves, the type of Ab was chosen randomly, and a binding attempt was made only when the surface element was associated with an epitope of the correct type. For release moves, the procedure first checked whether an Ab was bound to the surface element under consideration, and upon confirming a bound Ab, attempted to unbind it. Trial moves were accepted and rejected based on the Metropolis criteria.

During a run, which typically ended after 10^8 MC steps, we collected statistics every 2×10^6 steps. This sampling frequency was determined based on the correlation time between approximately independent measurements as computed using the methodology of Beroza et al. (1991). To produce averages for a given concentration, we used all samples collected from the 500 independent runs.

Code Availability

The program code and processing scripts are available upon request.

RESULTS

Simulating Ab Binding in Flaviviruses

We carried out molecular simulations of polyclonal Abs binding to DENV, West Nile Virus (WNV), and ZIKV virions to explore the contributions of Ab concentration, epitope fine specificity, and virus maturation state on infectivity and ADE. We represented polyclonal sera as a mixture of representative TS and CR mAbs and assigned binding affinities that reflect homotypic and heterotypic specificities. Representative TS and CR Abs were selected from published data where serotype specificity and epitope information were available. In particular, we focused on mAbs where cryo-EM, X-ray crystallography, or shotgun mutagenesis methods were used for epitope mapping (**Table 1**).

We carried out MC-based simulations using coarse-grained representations of the flavivirus envelope and mAbs. Specifically, we started from a high-resolution structural model of the virion, mapped the appropriate mAb epitopes onto the virion surface, and then reduced the virion representation to that of a tessellated sphere, with the epitope residues defined as points. Each Ab was modeled as soft disk that represents its binding "footprint," and Abs could interact with one another through steric interactions (Ripoll et al., 2016). We modeled viral structural heterogeneity by varying the degree of maturation and by modeling "smooth" (**Figures 1A–C**) and "rough" (**Figures 1D–F**) conformational states. A given virion in the model assumed a range of maturation states, each defined by the fraction of prM-E heterodimers present in the model, from fully immature (100%) to fully mature (0%).

During simulations, each run started from a coarse-grained model of the partially mature virion. Representative models are shown in **Figures 1C,F** for the smooth and rough partially mature states, respectively. In the smooth form of the virion, prM-E heterodimers were distributed randomly over the virus envelope. We used the smooth form of the WNV envelope, together with WNV mAb E16, to explore the effects of epitope exposure. The smooth form of a DENV virion was also used in simulations that assumed simultaneous binding of CR and TS Abs. In the latter case, we chose DENV mAb 2D22, which binds to E protein dimers (Fibriansah et al., 2015), as a representative of TS mAb, and DENV mAb 5G22, which binds to an epitope on prM, as a representative CR mAb.

For the rough form of the virion, we considered mosaic structures from cryo EM experiments which show partially mature particles containing E-prM heterodimers in the immature spiky state (Junjhon et al., 2010). These heterodimers aggregate over the virus surface to form a single immature patch (**Figure 1D**). For the rough virion, we used the mAb 2H2, which binds a pr epitope, as a representative CR mAb, along with 2D22 as a representative TS mAb. **Figures 1B,E** show the atomic representations of TS and CR epitopes in the rough form of the virus. Simulations of the rough form were also used to investigate the effect of partial maturation on the binding of a CR mAb of the EDE2 family, namely mAb 747(4) A11, which targets E dimer epitopes in DENV and ZIKV (Dejnirattisai et al., 2015, 2016; Barba-Spaeth et al., 2016).

Ab Binding Stoichiometry in Partially Mature Virions

We first carried out simulations in which we varied the concentration of a single type of mAb that binds to partially mature virions either in the smooth or rough state, to observe the impact of reduced epitope exposure on infection, neutralization, and ADE. We considered a wide range of maturation states (prM content from 0 to 100%) and ran the simulations with TS mAb concentrations ranging from 10^{-1} to 10^{-14} M, assuming high binding affinity for the virion ($K_A = 10^{-9}$ M). At each mAb concentration, we carried out 500 independent simulations,

TABLE 1 | DENV-specific mAbs used in simulations.

mAb	Specificity	Virus	Method	PDB ID	Epitope	References
2D22	TS	DENV2	Cryo-EM	4UIF, 4UIH, 5A1Z	E dimer	De Alwis et al., 2012; Fibriansah et al., 2015
2H2	CR	DENV1-4	Cryo-EM	3J42	prM	Henchal et al., 1982; Wang et al., 2013
5G22	CR	DENV1-4	Shotgun mutagenesis	–	prM	Smith et al., 2012, 2016
EDE2 A11	CR	DENV1-4, ZIKV	X-ray diffraction	4UTB, 5LCV	EDE2[a]	Dejnirattisai et al., 2015; Rouvinski et al., 2015; Barba-Spaeth et al., 2016
E16	TS	WNV	X-ray diffraction	3IYW, 1ZTX	E–DIII	Nybakken et al., 2005; Kaufmann et al., 2010

[a] *EDE2 stands for "E dimer-dependent epitope" with sensitivity to disruptions in N-linked glycosylation sites at positions 153 and 155 of the DENV E protein.*

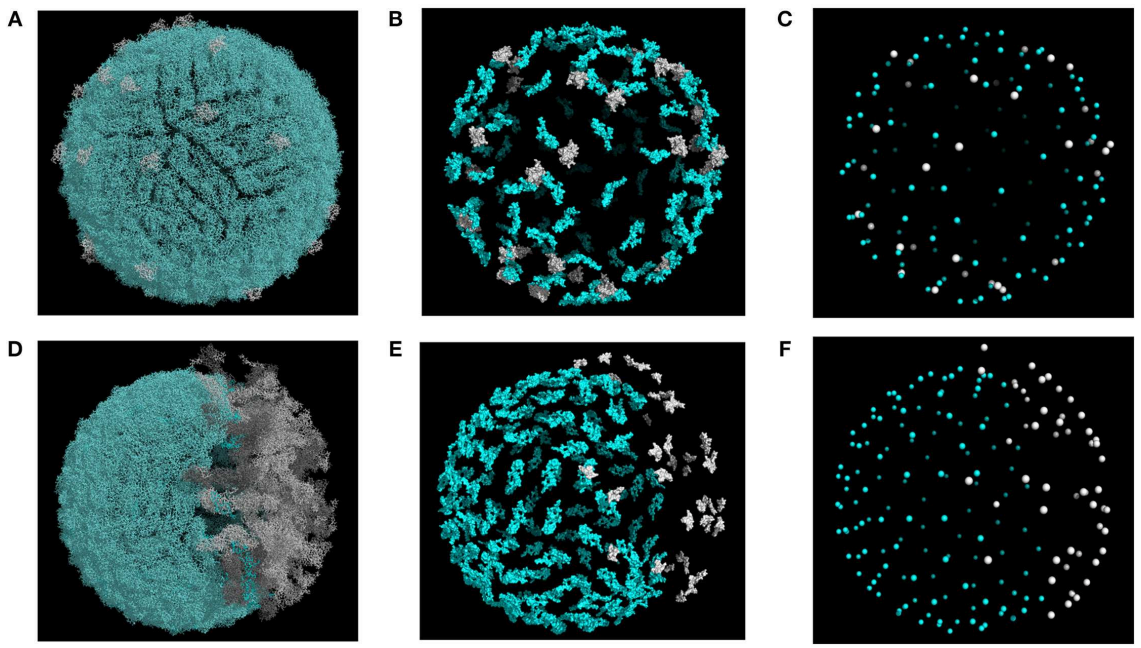

FIGURE 1 | Structural models of DENV envelope used for simulations. Representative structures are shown for smooth **(A–C)** and rough **(D–F)** conformations. Epitopes and epitope centers for TS (blue) and CR (white) mAbs are highlighted in **(B,C,E,F)**. Structures were generated with maturation corresponding to a prM content of 20%.

each 10^8 steps long, and evaluated the mean number of bound Abs. From the simulation trajectories, we computed the average infectivity and average enhancement.

Pierson et al. (2007) carried out *in vitro* experiments on the effects of epitope exposure in the related flavivirus WNV. In one experiment, they investigated Ab occupancy requirements for virus infectivity by controlling the number of epitopes of a type-specific Ab (mAb E24) displayed by recombinant WNV particles. They achieved this by mixing wild-type WNV E protein, which contains the E24 epitope, with mutant WNV E-prM heterodimers, which include a point mutation that abrogates E24 binding. To simulate this experiment, we used published structural information from mAb E16 whose epitopes closely overlap with those of mAb E24 (Pierson et al., 2007). For simulation purposes, we assumed that these mixed particles were in the smooth state (**Figure 1A**), containing wild-type E molecules in the mature state and mutant E-prM in the immature state. We found good agreement between our simulated results and the experimental data with respect to ADE and relative infectivity at varying levels of maturation. Increasing epitope exposure (by increasing the percentage of wild-type E in the particles) led to a reduction in the overall infection rate, as found in the experiments (**Figures S1A,B**). The range of concentrations where ADE occurred broadened and shifted toward higher concentrations as epitope exposure decreased (**Figures 2A,B**).

DENV glycoproteins organized on the envelope surface co-exist in two forms, mature dimers and immature trimers (Junjhon et al., 2008, 2010; Plevka et al., 2011), which form mosaic "rough" viral particles that are often released by infected host cells. We carried out simulations using this rough conformation (**Figure 1B**) for a range of maturation states. We first explored the binding of CR Abs to immature DENV using epitope information for the anti-prM mAb 2H2 (Wang et al., 2013) and compared it with the experimental results of Dejnirattisai et al. (2010), which showed that anti-prM Abs are prone to elicit ADE even at very high Ab concentrations (see

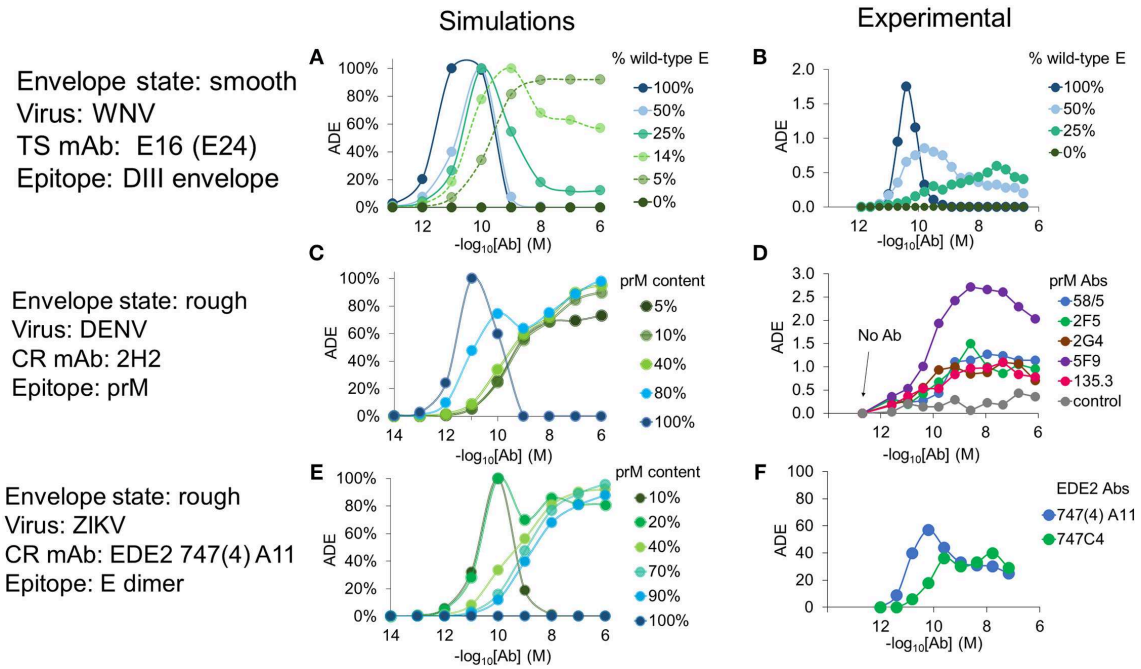

FIGURE 2 | Effects of epitope exposure on Ab activity—experiments vs. simulations. Plots of ADE as a function of Ab concentration for viral particles displaying varying numbers of epitopes. **(A)** Simulated ADE using virions in the smooth state, at various ratios of wild-type to mutated E proteins forming the virus envelope. **(B)** Experimental ADE data for WNV mAb E24, obtained from Pierson et al. (2007). **(C)** ADE predicted by simulations of anti-prM Ab 2H2 binding to epitopes exposed on mosaic particles at different degrees of maturation. **(D)** Enhancement of DENV2 infection of primary monocytes in the presence of human anti-prM antibodies; data from Dejnirattisai et al. (2010). **(E)** ADE predicted by simulations of mAb EDE2 747(4)-A11 binding to mosaic particles of ZIKV at different degrees of maturation. **(F)** Enhancement of infection in human myeloid cell U937 by ZIKV strain HD78788 in the presence of variable concentrations of EDE2 mAbs 747(4)-A11 and 747C4; data from Dejnirattisai et al. (2016). Simulated ADE values were normalized by the maximum ADE observed in that condition.

Figure 2D). Our simulations showed similar results: unlike with an anti-E mAb, such as E24, 2H2 exhibited ADE across a broad range of Ab concentrations and maintained high ADE even at very high concentrations (**Figure 2C**).

Finally, we examined a recently studied class of CR mAbs that target the EDE2 epitope (Dejnirattisai et al., 2015). EDE2 Abs can strongly neutralize DENV, but some members of the family enhance infection of ZIKV (Dejnirattisai et al., 2016). The epitope of EDE2 Abs is a conformational one that forms when the virus matures and exposes E dimers on the envelope surface. We used structural information for the mAb EDE2 747(4)A11 bound to DENV and ZIKV to define the epitopes (Rouvinski et al., 2015; Barba-Spaeth et al., 2016). Our simulations for EDE2 mAb 747(4)A11 in complex with the rough form of ZIKV showed peak ADE activity for highly mature particles (prM content $<$ 10%) and monotonically increasing ADE at lower Ab concentrations at a wide range of maturation states (prM content $>$10% and $<$90%) (**Figure 2E**). Both of these ADE characteristics are reproduced qualitatively as in experimental observations (**Figure 2F**).

Neutralization and ADE in Primary DENV Infections

To explore the role of ADE in secondary heterotypic DENV infections, we used a semi-quantitative approach to describe differences in host immune status between primary and secondary infections. We assumed that changes in the neutralization properties of blood sera between primary and secondary infections are mainly related to variations in the binding affinity of the Abs. In our model, variations in binding affinity are specified through changes in the dissociation constants of Abs, K^{TS} and K^{CR}. To model blood sera conditions that follow a primary or secondary homotypic infection, we first produced stoichiometric plots for dual mixtures of CR and TS Abs assuming that the affinity of the TS Ab is comparable to that of the CR counterpart ($K^{TS} \approx K^{CR}$). Conditions associated with a secondary heterotypic infection, on the other hand, were modeled assuming a substantial loss in affinity of TS Abs with respect to CR Abs ($K^{TS} \gg K^{CR}$).

We carried out binding simulations at a range of TS and CR Ab concentrations (10^{-2} to 10^{-14} M) and immature (prM) contents ranging from 5 to 40%, using the smooth conformation of the virus. **Figure 3A** shows the Ab occupancy as a function of Ab concentration and maturation state. For highly mature virions (5–10% prM content), CR epitopes, which are found on prM, contributed little to the total occupancy even at high CR Ab concentrations where the epitopes are saturated, and overall Ab occupancy was primarily driven by TS Ab concentration. At lower maturation levels (30 and 40% prM content), CR Abs played a greater role in overall Ab occupancy, although TS Ab

concentration continued to be the primary contributing factor. This is because, TS epitopes greatly outnumbered CR epitopes, even at higher maturation states.

Figure 3B shows infectivity as a function of Ab concentration and maturation state. At high maturation states, it was determined almost entirely by the TS Ab concentration, as the number of CR epitopes to drive neutralization was insufficient. At lower maturation states (20–40% prM content), CR Abs and TS Abs contributed comparably to neutralization, and infectivity was only seen in conditions of low CR and TS Ab concentrations.

Figure 3C shows ADE as a function of Ab concentration and virus maturation state. We calculated ADE as a function of both Ab occupancy and Ab neutralization, with the peak ADE at an Ab occupancy just under the neutralization threshold. For highly mature virions (5% prM content), ADE occurred in conditions corresponding to low TS Ab concentration and high CR Ab concentration. Under these Ab concentration conditions, ADE peaked for a prM content of around 10% and became almost negligible for a prM content above 20%. At low maturation states (prM content $\geq$30%), ADE only occurred in a narrow range of Ab concentrations corresponding to the transition between high occupancy and no occupancy.

Overall, for primary infections, Ab occupancy and neutralization was primarily driven by TS Ab concentration, and ADE occurred only at a low TS Ab concentration and a moderate to high CR Ab concentration in highly mature virions. In virions of low to moderate maturation states, ADE rarely occurred, and was restricted to a very narrow range of Ab concentrations whenever it did.

ADE in Heterotypic Secondary DENV Infections

We extended our model to capture secondary heterotypic DENV infection. We assumed that TS Abs produced in a primary infection had low binding affinity against a heterotypic virion, while the binding affinity of CR Abs was similar between homotypic and heterotypic infections. To reproduce these conditions, we chose dissociation constants of $K^{TS} = 1E^{-5}M$ for TS Abs and $K^{CR} = 1E^{-9}M$ for CR Abs in heterotypic infections.

We carried out binding simulations under the same conditions as in the case of the primary infection. **Figure 4A** shows Ab occupancy as a function of the partial concentrations of TS and CR Abs and the maturation state of the virus. For highly mature virions (5% prM content), occupancy was determined primarily by TS Ab concentration because of the low numbers of CR epitopes. Unlike in the primary infection, however, appreciable Ab occupancy occurred only at high TS Ab concentrations, due to the poor binding affinity of TS Abs to a heterotypic serotype. At moderate to low levels of viral maturation (prM content $>$ 10%), CR Ab concentration plays a significant role in determining Ab occupancy, owing to the high binding affinity for its epitope.

Figure 4B shows infectivity as a function of partial TS and CR Ab concentrations and viral maturation state. For highly mature virions (5% prM content), neutralization was only observed at high concentrations of TS Ab, owing to its poor binding affinity for the heterotypic serotype. Under conditions of low TS and high CR concentrations, only virions with a low maturation state (prM content $\geq$ 30%) exposed enough CR epitopes to become fully neutralized. On the other hand, virions with moderate to high maturation (prM content $\leq$10%) were only partially neutralized.

Figure 4C shows ADE as a function of partial TS and CR Ab concentration and viral maturation state. For the highly mature virion, ADE occurred at a wide range of TS and CR Ab concentrations. This is because the low affinity of TS Abs and the low epitope availability of CR Abs led to sub-neutralizing Ab occupancy at a wide range of concentrations. As in the case of a primary infection, under conditions of low TS and high CR concentrations, ADE peaked at a prM content of around 10% and became negligible at a prM content above 20%. For moderate to highly immature virions, ADE occurred at a narrow range of concentrations, limited to conditions where Ab occupancy was $>$0 but sub-neutralizing.

Overall, whereas ADE occurred mainly during conditions of very low TS Ab concentration in primary or homotypic infection, it occurred under a wide range of TS Ab concentrations in heterotypic secondary infection.

Role of Rough Viral Conformation in ADE

To explore how different types of partially mature virus particles affect infectivity and ADE, we next carried out a series of simulations in which a fraction of the viral envelope was in the immature rough or spiky state during a heterotypic infection (see **Figure 1D**). Unlike previous simulations of the smooth form of the virus where CR Abs targeted pr epitopes randomly distributed on the virion surface, here we used simulations of the rough form of the virus in which CR Abs targeted exposed pr epitopes forming a single patch on the surface of the virion.

Compared to the simulations of heterotypic infection using the smooth form of the virion, we found several differences. Most notably, occupancy and neutralization were driven almost entirely by the partial concentration of TS Abs, and CR Abs had virtually no neutralization capacity, even at high concentrations (**Figures 5A,B**). Furthermore, the degree of maturation had little impact on the neutralization capacity of CR Abs within the range of maturation states considered (prM content of 5–40%). In contrast to heterotypic infection in the smooth virion, the neutralization capacity of CR Abs increased as the prM content increased, eventually providing a level of neutralization comparable to TS Abs at high levels of virus immaturity (**Figure 5B**).

Finally, whereas ADE occurred only in highly mature virions in the case of the smooth virion, it occurred at all levels of viral maturation in the rough virion (**Figure 5C**). Together, these findings suggest that the rough viral conformation may be particularly prone to ADE under a wide range of conditions during a secondary heterotypic infection.

Simulating Longitudinal Risk of ADE

Recent long-term pediatric cohort studies (Katzelnick et al., 2017; Salje et al., 2018) based on large groups of individuals have shown that the risk of severe dengue disease is correlated with low anti-DENV antibody titers in the blood, with risk

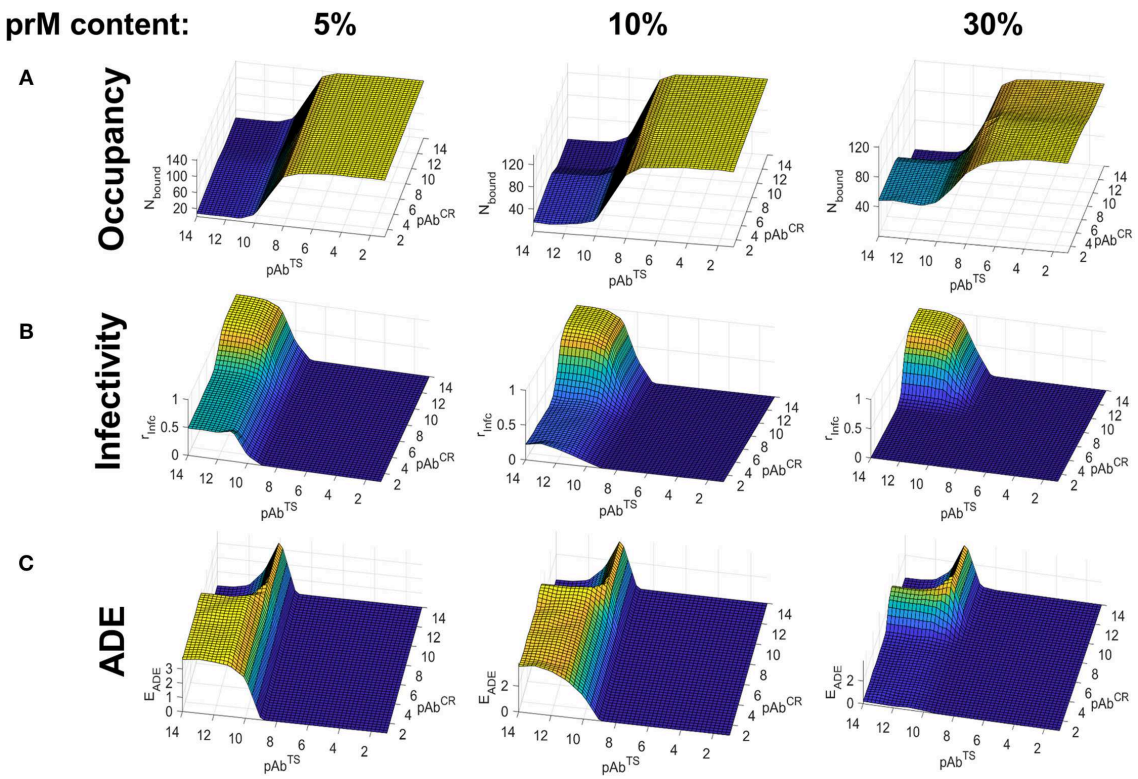

FIGURE 3 | Simulated Ab binding occupancy, neutralization, and ADE in primary DENV infection using the smooth virus conformation. Estimated Ab binding occupancy **(A)**, infectivity **(B)**, and ADE **(C)** at a range of TS and CR Ab concentrations for highly mature (5% prM content), moderately mature (10% prM content), and low-maturity (30% prM content) virions during a primary or homotypic infection.

being significantly lower for children having high antibody titers, and, surprisingly, for seronegative individuals. We sought to use our simulation results to explore alternative scenarios and conditions under which an individual might be prone to enhancement of dengue disease. In particular, we investigated how time-dependent changes of Ab concentrations and other variables determine DENV infection and disease enhancement as the outcome.

In a pediatric dengue cohort study, Katzelnick et al. (2017) showed that serum titers over time could vary substantially from one individual to another. In a separate study, Salje et al. (2018) found that the time-dependent behavior of TS and CR Ab concentrations in an individual during primary and post-primary infections could be modeled as a sharp increase in titers followed by an exponential decay. They found that, after the first year, titers tend to stabilize to a set-point titer.

To our best knowledge, more detailed data on time-dependent changes in the concentration of CR and TS Abs against DENV from individuals are not publicly available. As such, we used information from the above-mentioned studies to produce hypothetical curves of Ab concentrations as functions of time that capture relevant aspects of the observed experimental behavior. **Figure 6A** shows hypothetical curves of total Ab concentration and TS Ab fraction as a function of time. In these hypothetical curves, total Ab concentrations are highest at the convalescent phase of a primary infection (t_0), followed by an exponential decay leveling off to a range of set-point titers, months to years later (t_2). We modeled the risk of ADE as a function of the fine specificity of serum Ab (the fraction of the total Ab concentration occupied by TS Abs), and the total Ab concentration from the convalescent phase of a primary infection until set-point titers are achieved (t_0 to t_2) (**Figure 6B**).

For homotypic infections, the risk of ADE was minimal, even at low Ab concentrations, across all ranges of fine specificity (**Figure 6B**; top) and time points. As such, the risk of ADE during homotypic secondary infection was minimal regardless of the rate of decay of Ab concentration or the endpoint titer, even for individuals with low set-point titers (top panel of **Figure 6B**, showing projections of different Ab concentration time courses onto ADE risk).

For secondary heterotypic infections, however, there was a substantial risk of ADE under a wide range of Ab concentrations and fine specificities (**Figure 6B**; bottom). The risk was particularly pronounced under conditions where the fine specificity of the Ab response is biased toward CR Abs over TS Abs (TS fraction < 0.50), and at intermediate concentrations of total Ab. During the first year after infection (from t_0 to t_1), the risk of ADE was low in all scenarios, as the total Ab concentration was higher than the intermediate concentrations associated with ADE. Thereafter, the outcome varied by scenario. Individuals

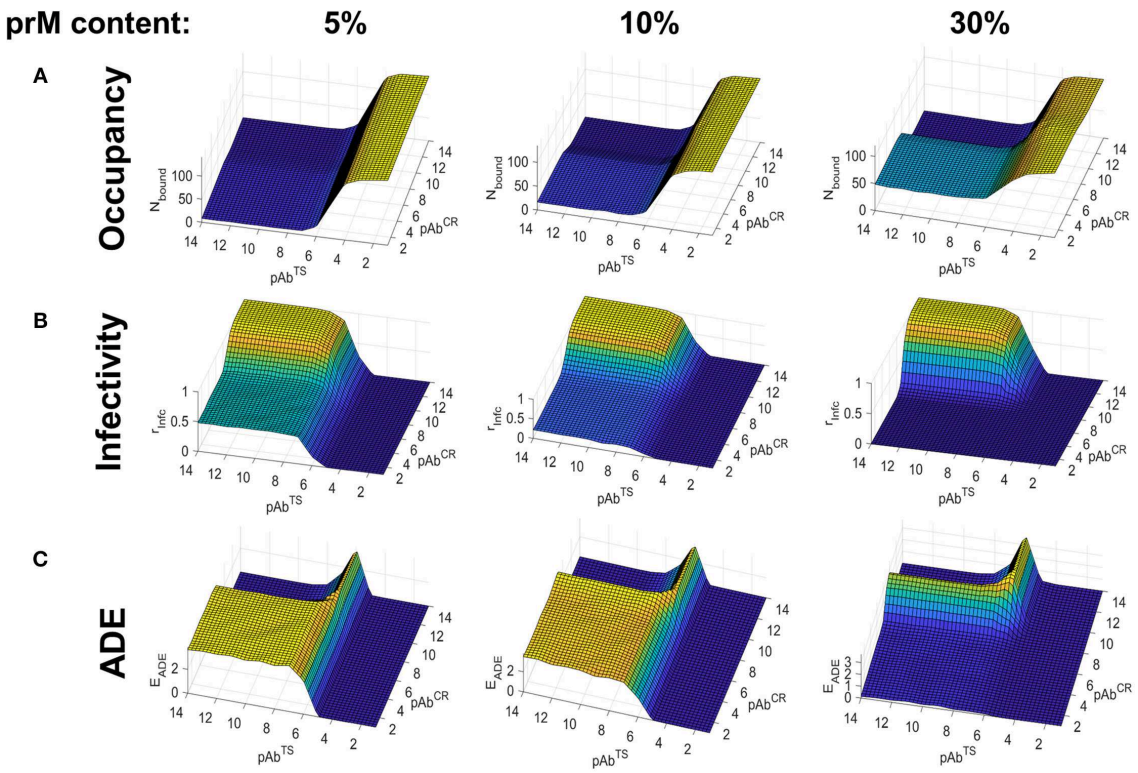

FIGURE 4 | Simulated Ab binding occupancy, neutralization, and ADE in heterotypic secondary DENV infection using the smooth virus conformation. Estimated Ab binding occupancy **(A)**, infectivity **(B)**, and ADE **(C)** at a range of TS and CR Ab concentrations for highly mature (5% prM content), moderately mature (10% prM content), and low-maturity (30% prM content) virions during a heterotypic infection.

with very low set-point titers (purple in **Figure 6A**) were most susceptible to ADE within a specific time window (between 12 and 24 months post-infection), as their serum Ab concentration passed through the high-risk intermediate range. By contrast, for individuals with higher set-point titers (magenta and red in **Figure 6A**), long-term risk of ADE remained high after the first year, as their Ab titers stabilized within the high-risk intermediate range. Finally, individuals with very high set-point titers (pink and brown in **Figure 6A**) were almost entirely free of risk. Finally, these trends were robust for both smooth and rough virions differing widely in prM content.

DISCUSSION

Here, building on previous work, we developed a model of Ab binding to the flavivirus surface in order to determine the molecular and structural basis for ADE. We validated this model with experimental *in vitro* data on antibody occupancy and relative infection from a range of studies (Ripoll et al., 2016). We used smooth conformation of the flavivirus virion to model infection and ADE as a function of Ab concentration and epitope accessibility. Our simulations show infection curves similar to those obtained by Pierson et al. (2007) finding that virions with low epitope exposure are able to avoid neutralization entirely, while virions with epitope exposures >30% showed a typical neutralization curve. Our simulations reproduced general features observed in experimental studies of ADE, such as the bell-shaped curve for high degrees of mature content (Pierson et al., 2007) and the displacement of maximum ADE toward high concentrations with diminishing epitope exposure. Our model predicted ADE to occur over a wide range of high Ab concentrations at an epitope accessibility below 30%. At high Ab concentrations, it predicted some degree of ADE at an epitope accessibility below 25%, and predicted maximum ADE to occur around an epitope accessibility of 5–10%.

In addition, we explored the role of partial maturation on infectivity by modeling viral particles in their "rough" state (i.e., mosaic particles). Comparison of our computational results with the limited experimental data on the neutralizing capacity of CR anti-prM and EDE Abs revealed that our simulations captured the relevant features of infectivity and ADE observed in experiments. Our simulations also linked observed patterns of infection, neutralization, and ADE to specific conditions that determine epitope accessibility: Ab affinity, Ab concentration, and mature content of the virus. The simulations predicted that rough and smooth viral particles produce different patterns of infectivity and ADE for similar levels of maturation. For the smooth particle, anti-EDIII Abs were fully neutralizing at high Ab concentrations ([Ab] >> K_D) and induced ADE only at sub-neutralizing concentrations ([Ab] ~ K_D). By contrast, for the

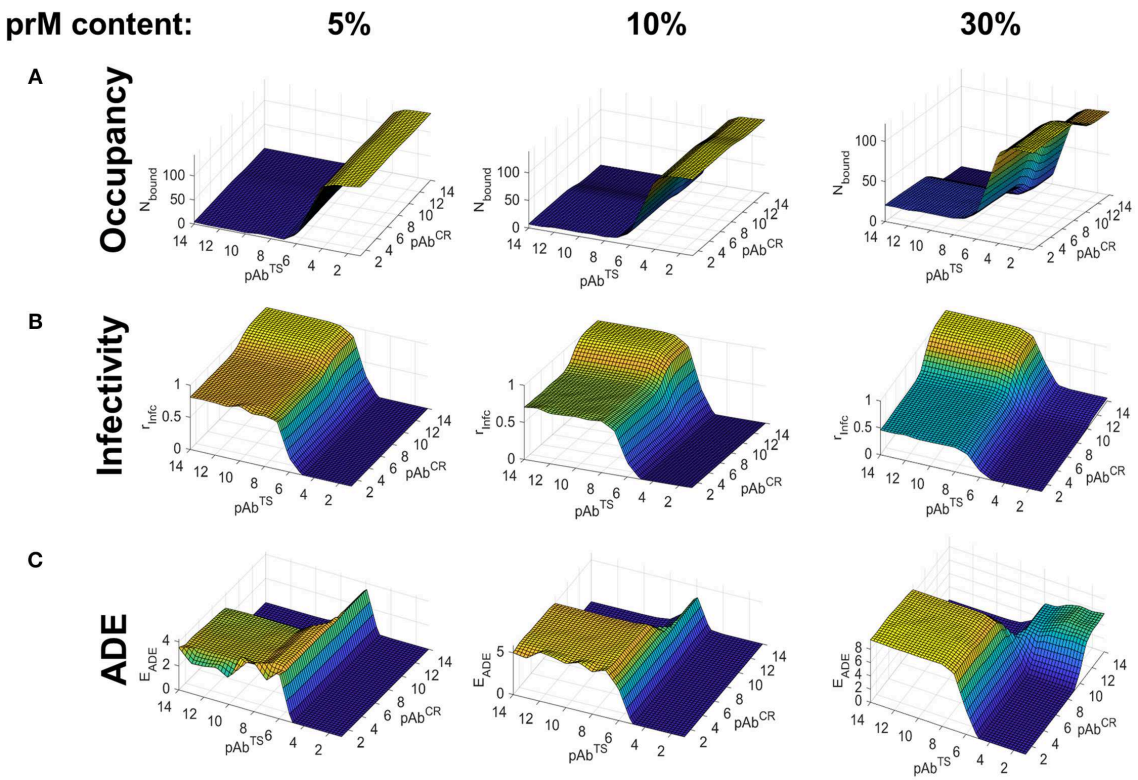

FIGURE 5 | Simulated Ab binding occupancy, neutralization, and ADE in heterotypic secondary DENV infection using the rough virus conformation. Estimated Ab binding occupancy **(A)**, infectivity **(B)**, and ADE **(C)** at a range of TS and CR Ab concentrations for highly mature (5% rough conformation), moderately mature (10% rough conformation), and low-maturity (30% rough conformation) DENV virions during heterotypic infection.

rough particle, both EDE2 and anti-prM Abs induced ADE even at high Ab concentrations for a wide range of maturation states. These trends were robust to changes in maturation state (prM content of 25–90%).

We modeled the polyclonal Ab response as a combination of CR and TS Abs and modeled heterotypic infection by reducing the binding affinity of the TS Ab while maintaining that of the CR Ab. Neutralization and ADE were significantly affected by conditions associated with heterotypic infection. In homotypic infection, neutralization is driven primarily by TS Abs and ADE is limited to conditions of very low TS Ab concentration. In heterotypic infection, both TS and CR Abs generally contributed to neutralization, but in a suboptimal fashion: CR Abs were limited by epitope accessibility while TS Abs were limited by poor binding affinity. During heterotypic infection involving virions in the smooth conformation, ADE became increasingly pronounced across a wide range of Ab concentrations as the prM content decreased below 20%.

The exact maturation state of DENV *in vivo* is unclear, but *in vitro* studies show that the virus can be produced in a wide range of maturation states. In our simulations, maturation state played a prominent role in neutralization and ADE. At lower levels of maturation (higher prM content), CR Abs specific to pr epitopes increasingly played a role in neutralization. Furthermore, in both homotypic and heterotypic infections, ADE was maximal at a maturation state corresponding to a prM content of 5–10% for smooth virions, and over 30% for rough virions. Previous studies have suggested that unlike some flaviviruses, DENV may have evolved to have suboptimal prM cleavage (Rouvinski et al., 2017). This feature of DENV could contribute to ADE in secondary infections.

We found that the immature rough form of the virus also had interesting characteristics with respect to neutralization and infection. It was poorly neutralized by CR Abs, and unlike the smooth form, where ADE is highest at relatively high levels of maturation (prM content of 5–10%), it was prone to ADE at a wide range of maturation states. In our model, CR Abs are poorly neutralizing in the rough form (relative to the smooth form) because the FL epitopes are tightly clustered in trimeric spikes that prevent full occupancy of all three epitopes for steric reasons. Thus, for a prM content of 40%, the most CR epitopes that a virion can display is 32. Our simulations estimate that the average number of bound CR Abs is generally below the neutralization threshold postulated by the "coating theory" (∼30 Abs), even at the highest CR Ab concentrations tested. In the smooth form of the virus, these epitopes are distributed homogenously across the viral surface, with negligible interference between neighboring bound CR Abs. Collectively, our results suggest that the rough form of the virus may be particularly pathogenic in cases of

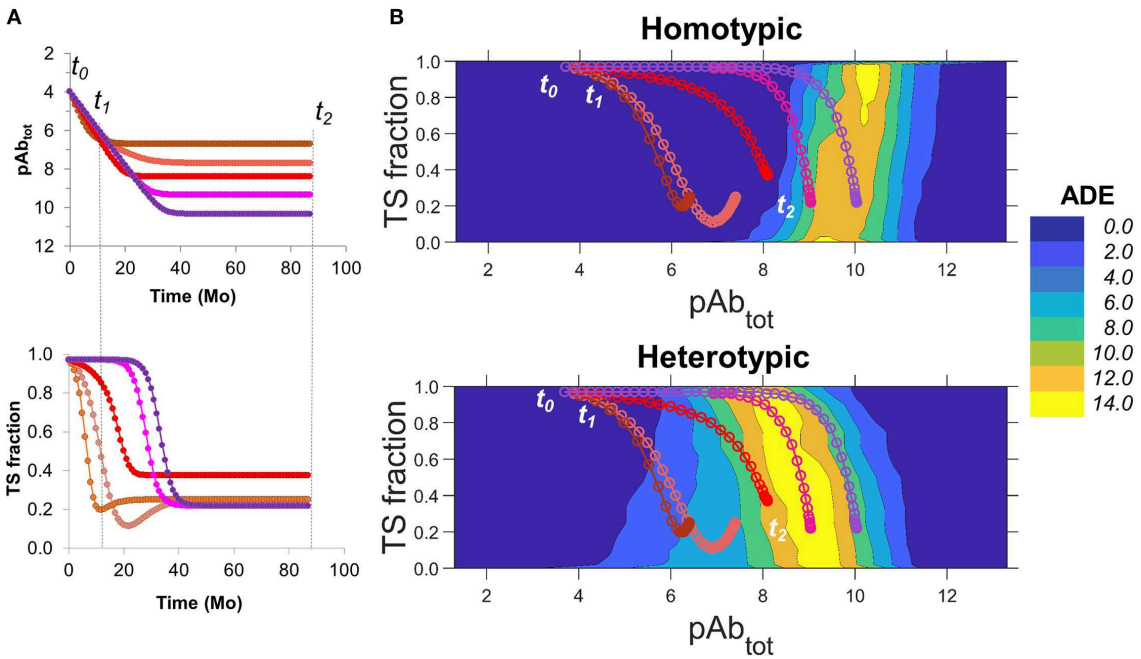

FIGURE 6 | Estimating the longitudinal risk of ADE from simulation data. **(A)** Hypothetical scenarios of changes in DENV-specific serum Ab concentration over time (top) from the convalescent phase of a primary infection (t_0) to a time point where set-point titers are achieved (t_2), where t_1 indicates 1 year post infection. Scenarios with increasingly higher set-point titers are shown from brown to purple. The bottom panel shows the corresponding TS Ab fractions for each scenario. **(B)** Contour plots for ADE as a function of fine specificity, expressed as TS fraction, and total Ab concentration. Scenarios are plotted using corresponding colors in **(A)** for homotypic (top) and heterotypic (bottom) conditions. Plots are derived from simulations of smooth virions with a prM content of 20%.

heterotypic infection, where neutralization is driven primarily by CR Abs.

Our finding that the viral maturation state plays a significant role in ADE has implications for *in vitro* and *in vivo* models of ADE. Two recent studies showed discordant results between a *in vivo* and *in vitro* model of ADE where they found that high concentrations of CR mAbs 4G2 and 6B6C-1 show high mortality in the AG129 mouse model, but do not exhibit ADE at high concentrations in an *in vitro* model using THP-1 cells (Watanabe et al., 2015; Ramasamy et al., 2018). They also found that TS mAb 3H5, which shows similar neutralization and ADE characteristics as 4G2 and 6B6C-1 *in vitro*, was highly protective in the AG129 mouse model. One possible explanation for these discrepancies is that the maturation state of virus produced in the *in vivo* model is different from virus produced in the *in vitro* model. In our model, if the prM content of the virion produced *in vitro* is >20%, then a standard bell-shaped ADE curve is to be expected. By contrast, if the prM content of the virion produced in the *in vivo* model is <20%, then ADE would be expected even at high Ab concentrations. Furthermore, this difference in maturation state would be expected to have a greater impact on CR Abs than TS abs, in an epitope-specific manner. Thus, lower immature contents in the virions produced by AG129 mice could explain the high mortality of mice at high Ab concentrations and the discordance between *in vitro* and *in vivo* results. Differences in the conditions inside host cells have been shown to affect the maturation state of newly produced virions (Nelson et al., 2008). Thus, it is conceivable that *in vivo* and *in vitro* conditions alter the virus maturation state, shifting the neutralization and enhancement capacities of CR Abs. Such mechanism could also explain why ADE can overcome the protective efficacy of Abs in a tissue-dependent manner (Watanabe et al., 2015).

ADE and Severe Dengue Disease

Our ADE model informs a number of recent clinical findings on severe dengue disease in natural infection studies. First, prior studies have shown that low pre-existing antibody levels are associated with an increased likelihood of severe dengue disease only during secondary heterotypic infections (Katzelnick et al., 2017). Our work shows why low pre-existing antibody levels may not enhance secondary *homotypic* infection—namely that highly neutralizing TS Abs generated during primary infection can neutralize a secondary homotypic infection even at low Ab concentrations, precluding the occurrence of ADE.

Second, other studies have shown that after a primary infection, individuals acquire temporary immunity to all four serotypes—an immunity that has been suggested to last anywhere from 6 months to several years (Sabin, 1952; Halstead, 2007; Montoya et al., 2013; Sharp et al., 2014). Our work suggests that this is because serum Ab concentrations are sufficiently high following a primary infection, such that even low affinity (poorly cross-reactive) TS Abs can neutralize a secondary heterotypic infection. As the Ab concentration decays toward a set-point titer, the combination of high-affinity CR Abs that target

poorly accessible epitopes, and low-affinity TS Abs that show poor binding occupancy, results in sub-neutralizing binding stoichiometry and a high propensity for ADE. In short, serum conditions conducive to ADE emerge months to years after a primary infection, depending on the subsequent Ab decay rate.

Finally, previous studies have shown that different individuals achieve different set-point titers ∼24–36 months after a primary infection (Salje et al., 2018). Our simulations suggest that the risk of ADE for individuals with very low set-point titers temporarily increases as the serum Ab concentration passes through the high-risk range and then falls below that level, whereas the risk for those with higher set-point titers may remain consistently high. This suggests that measuring set-point titers may be sufficient to predict an individual's relative risk of severe dengue disease.

Implications for Vaccine Research

Recent studies of Dengvaxia, a tetravalent vaccine developed by Sanofi-Pasteur, have revealed that for vaccine recipients with no prior exposure to DENV, there is a modest *increase* in the risk of severe dengue disease (Aguiar et al., 2016). Other studies have shown that poorly immunogenic dengue vaccines, or tetravalent dengue vaccines in which the subject fails to seroconvert in all four serotypes, can result in the induction of CR Abs over TS Abs (Kanesa-Thasan et al., 2001; Gromowski et al., 2018). Our findings suggest that low Ab titers with mostly CR Abs that target only a few epitopes per virion are prime conditions for ADE, supporting the theory that poorly immunogenic dengue vaccines act as a surrogate for post-primary dengue infection. Our results demonstrate that Ab concentration and specificity are critical host determinants of ADE, underscoring the importance of measuring not only antibody titer but also fine specificity when assessing future dengue vaccine candidates. In this respect, our simulations support vaccine designs such as the one recently reported using tetravalent virus-like particles displaying the domain III of E (Ramasamy et al., 2018), in which the antigen contains well-characterized serotype-specific epitopes that are present in large quantities on the virion surface. Finally, our simulations point to the degree of viral maturation as another important determinant of ADE. The fraction of extracellular DENV particles that exist as mosaic particles can vary substantially depending on various factors, such as the specific DENV strain or the host cell in which the virus was produced (Van Der Schaar et al., 2008; Junjhon et al., 2010; Plevka et al., 2011). We suggest that the maturation state of any live-attenuated strain of DENV used as a dengue vaccine may be critical to its ability to induce protective Ab responses without creating serum conditions that increase the risk of severe dengue disease.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

DR developed the stochastic model for antibody binding and carried out the simulations. DR, AW, and SC designed the computational experiments, analyzed the resulting data, and prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00200/full#supplementary-material

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REVIEW
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The PD-1/PD-L1 Axis and Virus Infections: A Delicate Balance

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Programmed cell death protein (PD-1) and its ligands play a fundamental role in the evasion of tumor cells from antitumor immunity. Less well appreciated is the fact that the PD-1/PD-L1 axis also regulates antiviral immune responses and is therefore modulated by a number of viruses. Upregulation of PD-1 and its ligands PD-L1 and PD-L2 is observed during acute virus infection and after infection with persistent viruses including important human pathogens such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV). Experimental evidence suggests that insufficient signaling through the PD-1 pathway promotes immunopathology during acute infection by exaggerating primary T cell responses. If chronic infection is established, however, high levels of PD-1 expression can have unfavorable immunological consequences. Exhaustion and suppression of antiviral immune responses can result in viral immune evasion. The role of the PD-1/PD-L1 axis during viral infections is further complicated by evidence that PD-L1 also mediates inflammatory effects in the acute phase of an immune response. In this review, we discuss the intricate interplay between viruses and the PD-1/PD-L1 axis.

Keywords: PD-1, PD-L1, PD-L2, antiviral immune responses, viral immune evasion, virus-induced immunopathogenesis, viruses

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INTRODUCTION

Programmed cell death 1 (PD-1, also known as CD279) was discovered by Tasuku Honjo et al. at Kyoto University from a screen of genes involved in programmed cell death (Ishida et al., 1992). PD-1 expression is rapidly induced after signaling through the T cell receptor (TCR) and modulated by cytokines (Agata et al., 1996; Yamazaki et al., 2002; Wherry et al., 2007; Chikuma et al., 2009; Terawaki et al., 2011; Ahn et al., 2018). Other types of immune cells such as B cells, natural killer (NK) cells, NKT cells, dendritic cells (DCs), and monocytes also express PD-1 (Sharpe et al., 2007; Keir et al., 2008).

There is ample evidence that PD-1, a member of the immunoglobulin superfamily, regulates the magnitude and quality of T cell responses. It plays a pivotal role in the induction and maintenance of central as well as peripheral tolerance (Nishimura et al., 1999, 2001; Wang et al., 2005; Okazaki and Honjo, 2006; Francisco et al., 2010; Fife and Pauken, 2011). For example, antigen presentation by resting DCs induces peripheral CD8+ T cell tolerance by signaling through PD-1 on CD8+ T cells (Probst et al., 2005). In fact, PD-1 has been called a 'rheostat' that calibrates threshold, strength, and duration of T cell responses (Okazaki et al., 2013; Honda et al., 2014). PD-1 belongs to a group of structurally different surface molecules that function as co-inhibitory receptors during immune responses against pathogens and cancer (Attanasio and Wherry, 2016; Hashimoto et al., 2018; Sharpe and Pauken, 2018). These molecules counterbalance co-stimulatory

receptors on T cells such as CD28, which bind to CD80 and CD86 on professional APCs and facilitate T cell activation (Esensten et al., 2016).

Clinical studies have shown that blocking the PD-1 pathway is effective against several types of cancer including melanoma, lymphoma, lung, and renal cancer (Sanmamed and Chen, 2018). This type of treatment is referred to as immune checkpoint therapy and the blocking reagents are called immune checkpoint-inhibitors (ICIs). Together with James P. Allison, who worked on another co-inhibitory receptor called cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4), Tasuku Honjo was awarded the Nobel Prize in Physiology or Medicine 2018 for the discovery of cancer therapy by inhibition of negative immune regulation (Wolchok, 2018).

PD-1 interacts with the ligands PD-L1 (CD274; also called B7-H1) and PD-L2 (CD273; also called B7-DC), which show distinct expression patterns. *In vitro*, PD-1 inhibits T cell activation by recruiting Src homology region 2-containing *protein tyrosine phosphatase* 2 (SHP2) after interaction with its ligands on APCs (Chen and Flies, 2013; Okazaki et al., 2013; Sharpe and Pauken, 2018). This is associated with dephopshorylation of crucial tyrosine residues within the CD3 complex and CD28. In virus-infected mice lacking SHP2 in T cells, however, PD-1 signaling is not impaired, suggesting the existence of redundant inhibitory pathways downstream of PD-1 (Rota et al., 2018).

PD-L1 is expressed not only by all hematopoietic cells but also by many non-hematopoietic cell types such as endothelial cells and epithelial cells (Sharpe and Pauken, 2018). In contrast, PD-L2 expression is more restricted and can be induced on hematopoietic cells such as DCs, B cells, and monocytes/macrophages. Besides PD-1, there are other known interacting partners for PD-L1 and PD-L2. PD-L1 also binds to CD80 whereas PD-L2 uses RGM domain family member B (RGMB) as an alternative binding partner. Both types of interaction also inhibit immune responses (Butte et al., 2007; Xiao et al., 2014).

Viruses have to overcome strong barriers to replicate in the hostile environment of their hosts (Virgin et al., 2009). An arsenal of weapons helps viruses to subvert antiviral immunity. This includes the exploitation of host inhibitory receptor signaling pathways (Ong et al., 2016). The impact of the PD-1/PD-L1 axis in chronic virus infections is well described whereas its role during the acute phase of viral infections is less clear (Brown et al., 2010; Attanasio and Wherry, 2016). However, whether virus-induced upregulation of PD-1 ligands represents a viral immune evasion strategy or an adaption of the host defense to minimize immunopathology is a moot point. In this review, we highlight the diverse roles of PD-1 and its ligands during virus infections and their implications for host-pathogen interaction.

THE ROLE OF THE PD-1 PATHWAY IN ACUTE VIRUS INFECTIONS

In mice acutely infected with lymphocytic choriomeningitis virus (LCMV) strain Armstrong (LCMV Arm) PD-1 is rapidly upregulated on naïve virus-specific CD8+ T cells before they clonally expand (Ahn et al., 2018). In this model of acute LCMV infection, CD4+ T cells are not required for virus clearance, which occurs within 1–2 weeks after infection (Matloubian et al., 1994). Blockade of the PD-1 pathway at this stage further increases effector functions of CD8+ T cells by enhancing granzyme B expression and mechanistic Target of Rapamycin (mTOR) signaling. Consequently, virus elimination is accelerated although the total number of virus-specific CD8+ T cells does not change (Ahn et al., 2018). Similarly, the PD-1/PD-L axis inhibits the differentiation of CD8+ T lymphocytes into polyfunctional cytotoxic T cells during acute infection of mice with murine retrovirus (David et al., 2019). This implies that PD-1 negatively regulates the terminal differentiation of naïve CD8+ T cells into effector CD8+ T lymphocytes during acute virus infection.

After virus clearance, PD-1 expression on virus-specific T cells returns to normal levels (Barber et al., 2006; Blattman et al., 2009). The expanded pool of virus-specific effector T lymphocytes contracts due to increased cell death and memory T cells arise from a subset of fate-permissive effector T cells (Akondy et al., 2017; Omilusik and Goldrath, 2017; Youngblood et al., 2017). There are at least three major memory T cell subsets: central memory T cells (Tcm cells), effector memory T cells (Tem cells), and recently defined tissue-resident memory T cells (Trm cells). Tcm cells lack effector functions but express lymph node homing molecules and circulate through the blood and the secondary lymphoid organs (Sallusto et al., 1999). After stimulation, Tcm cells differentiate into Tem cells that lack lymph node homing molecules and continuously recirculate between blood, lymph and non-lymphoid tissues. Tem cells are bestowed with various effector functions (Sallusto et al., 1999). In contrast, Trm cells do not recirculate (Wakim et al., 2008; Gebhardt et al., 2009; Masopust et al., 2010) and express core phenotypic markers including co-inhibitory receptors such as PD-1 (Hombrink et al., 2016; Kumar et al., 2017; Pallett et al., 2017). Functionally, Trm cells participate in the first line of defense to viruses by establishing an antiviral state and recruiting circulating memory T cells to sites of viral infection (Schenkel et al., 2013, 2014; Ariotti et al., 2014; Carbone and Gebhardt, 2014). Located in multiple anatomical sites including barrier tissue such as lung, skin and gut, Trm cells are indispensable for antiviral immunity and immunosurveillance (Shin, 2018; Wu et al., 2018; Szabo et al., 2019). The functional role of the PD-1/PD-L1 axis for CD8+ Trm cells is unclear at the moment but it may prevent uncontrolled Trm activation and inflammation in virus-infected tissues and other inflammatory conditions. In accordance, blockade of PD-1 on Trm cells increases the severity of eczema in a mouse model of allergic contact dermatitis (Gamradt et al., 2019).

Intriguingly, the number of memory precursor T cells increases if PD-1 is blocked by antibodies during acute LCMV infection, possibly due to faster virus elimination (Ahn et al., 2018). Virus-specific memory CD8+ T cells that develop after the elimination of LCMV persist without antigen and are capable of self-renewal due to homeostatic proliferation in response to IL-7 and IL-15 (Wherry et al., 2004; Surh and Sprent, 2008; Abdelsamed et al., 2017). Although the blockade of the PD-1/PD-L1 axis in mice infected with LCMV Arm increases

effector CD8+ T cell function, no excessive tissue damage is observed (Ahn et al., 2018). Similar to the LCMV strain WE (LCMV WE), LCMV Arm does not disseminate but instead is eliminated from infected laboratory mice after acute infection. In contrast, derivatives of LCMV Arm and LCMV WE ("clone 13" and "docile," respectively) replicate more vigorously and persist (Matloubian et al., 1990; Welsh and Seedhom, 2008). These LCMV strains cause lethal immunopathology in mice deficient of the PD-1/PD-L1 axis (PD-L1 KO mice, PD-1 KO mice) during the acute phase of infection (Barber et al., 2006; Mueller et al., 2010; Frebel et al., 2012; Zinselmeyer et al., 2013; Shaabani et al., 2016). This is due to the killing of LCMV-infected vascular endothelium by CD8+ T cells resulting in vascular leakage with pulmonary edema and severe hypotension (Frebel et al., 2012). In a mouse model of acute viral hepatitis, the absence of PD-1 is associated not only with more rapid virus clearance but also with more severe hepatitis (Iwai et al., 2003). These results imply that the stimulation of the PD-1/PD-L1 axis during the acute phase of virus infection helps to adjust the strength and quality of the cytotoxic CD8+ T cell attack so that the good (virus elimination) and the bad (tissue damage) is balanced, preventing excessive tissue damage.

VIRUS-DRIVEN PD-L1/2 EXPRESSION

Many viruses increase PD-L1/2 expression on hematopoietic cells (**Table 1**) and non-hematopoietic cells (**Table 2**). PD-L1/2 expression is regulated by proinflammatory and anti-inflammatory signals (Sun et al., 2018). The promotor regions of PD-L1 and PD-L2, which are paralog genes, are differentially regulated although they show similarly arranged binding sites for transcription factors (Garcia-Diaz et al., 2017).

Type I and type III interferons (IFNs) are important antiviral cytokines. They are induced early in virus-infected barrier tissue such as lung/gut epithelial cells and serve as the first line of antiviral defense (Okabayashi et al., 2011; Wack et al., 2015; Andreakos et al., 2017; Galani et al., 2017; Zanoni et al., 2017; Good et al., 2019; Lazear et al., 2019). Type I IFNs, which in humans include several IFN-α subtypes and IFN-β, increase PD-L1 expression but to a lesser extent than PD-L2 expression (Garcia-Diaz et al., 2017). PD-L2 responds equally well to IFN-γ (type II IFN) and IFN-β (Garcia-Diaz et al., 2017). IL-4 may be an even more potent inducer of PD-L2 (Loke and Allison, 2003) thus accounting for the presence of PD-L2 on monocyte-derived DCs generated *in vitro*. Blockade or absence of type I IFN signaling during chronic LCMV infection results in reduced PD-L1 expression despite enhanced viral replication (Teijaro et al., 2013; Wilson et al., 2013; Shaabani et al., 2016). Although type I IFNs moderately upregulate PD-L1 (Sun et al., 2018) they increase NK cytotoxicity and allow clonal expansion and memory formation of antiviral cytotoxic CD8+ T cells (Biron et al., 2002; Kolumam et al., 2005; Aichele et al., 2006). Type III IFNs signal through a unique heterodimeric receptor and induce the expression of antiviral IFN-stimulated genes (ISGs) similar to type I IFNs (Davidson et al., 2015). Intriguingly, type III IFNs do not upregulate PD-L1 (Raftery et al., 2018). Accordingly, in this early phase of acute infection the PD-1/PD-L1 axis does not inhibit antiviral immune cells.

Recognition of viruses by pattern recognition receptors (PRRs) also upregulates PD-L1. TLR3 signaling in particular strongly increases PD-L1 levels on DCs (Pulko et al., 2009; Boes and Meyer-Wentrup, 2015; Raftery et al., 2018) whereas RIG-I signaling alone has no significant effect (Raftery et al., 2018). Triggering of TLR3, which transmits downstream signals through the TIR-domain-containing adapter-inducing IFN-β (TRIF), also enhances PD-L1 on other cell types including endothelial cells (Cole et al., 2011) and epithelial cells (Tsuda et al., 2005). In accordance, virus-induced PD-L1 upregulation on neuronal cells is severely impaired in TLR3-deficient mice (Lafon et al., 2008). Recently, viral proteins inducing PD-L1/PD-L2 expression have been identified. For example, HIV Tat protein increases PD-L1 expression on DCs through TNF-α and TLR4 signaling (Planes et al., 2014). The HCV core protein *in vitro* induces strong PD-L1 upregulation on primary human Kupffer cells and monocytes in a TLR2- and PI3K-dependent manner (Tu et al., 2010; Zhai et al., 2017). In accordance, the PD-L1 levels on monocytes from HCV-infected patients were significantly higher than on monocytes from healthy individuals (Zhai et al., 2017). A recent study has shown that extracellular vesicles (EVs) produced by HBV-infected hepatocytes are endocytosed by circulating monocytes resulting in PD-L1 upregulation (Huang et al., 2017; Kakizaki et al., 2018). Moreover, PD-L1 and PD-L2 are upregulated by hantaviral N protein most likely via hantavirus-induced TLR3 signaling (Raftery et al., 2018). In addition, the latency-associated transcripts (LATs) of herpes simplex virus type 1 (HSV-1) upregulate PD-L1 on mouse neuroblastoma cells by an unknown mechanism (Chentoufi et al., 2011). Remarkably, as of yet no viral immunoevasin has been discovered that directly interacts with the molecules of the PD-1/PD-L1 axis to exploit its immunosuppressive function.

Viral replication can also result in the production of anti-inflammatory cytokines such as IL-10 (Brooks et al., 2006b; Ejrnaes et al., 2006). Cellular IL-10 has been shown to upregulate the expression of PD-1 and PD-L1 in a STAT-3 dependent manner in DCs and monocytes (Curiel et al., 2003; Selenko-Gebauer et al., 2003; Sun et al., 2015; Lamichhane et al., 2017). Accordingly, the absence of cellular IL-10 in LCMV infected mice results in enhanced effector T cell responses, rapid virus elimination, and generation of antiviral memory T cells (Brooks et al., 2006b; Ejrnaes et al., 2006). Intriguingly, during coevolution with their hosts members of the virus family *Herpesviridae* have acquired numerous genes from their hosts including those that mimic cellular IL-10 (Raftery et al., 2000; Ouyang et al., 2014; Schonrich et al., 2017). These viral IL-10 (vIL-10) molecules act as immunosuppressive cytokines that also paralyze co-stimulatory B7 molecules (Muller et al., 1998; Raftery et al., 2004). It is possible that vIL-10 molecules also increase signaling through the PD-1/PD-L1 axis similar to their cellular counterparts thereby contributing to viral persistence. However, combined blockade of both, IL-10 and PD-L1, during chronic LCMV infection enhances T-cell function more efficiently than a single blockade (Brooks et al., 2008). Thus, IL-10 is pleiotropic and has immunosuppressive functions

TABLE 1 | Virus-induced upregulation of PD-1 ligands on hematopoietic cells.

Virus	Findings	References
LCMV Arm and clone13	Increased PD-L1 expression on myeloid DCs and marginal zone macrophages; decreased T cell motility in the marginal zone of the spleen due to PD-L1	Zinselmeyer et al., 2013
LCMV	High PD-L1 expression on Kupffer cells in the liver	Shaabani et al., 2016
IAV	Type I IFN induced PD-L1 expression on virus-infected professional APCs in the airways	Erickson et al., 2012; Valero-Pacheco et al., 2013; Rutigliano et al., 2014; Staples et al., 2015; McKendry et al., 2016
JEV	PD-L1 upregulation on virus-infected DCs *in vitro* and decreased expansion of Treg cells by virus-infected DCs after PD-L1 blockade	Gupta et al., 2014
EOBV	Increased numbers of PD-L1 transcripts during EOBV infection of monocytes derived from macaques	Menicucci et al., 2017
HV	PD-L1/2 upregulation on DCs; high amounts of soluble PD-1 and PD-L2 in the circulation of HV-infected patients	Raftery et al., 2018
FV	PD-L1 expression on erythroid precursor cells and CD4+ T lymphocytes	Akhmetzyanova et al., 2015
HIV	PD-L1/2 upregulation on monocytes, DCs and macrophages; Correlation between level of PD-L1 expression and disease progression	Boasso et al., 2008; Meier et al., 2008; Wang et al., 2008; Rodriguez-Garcia et al., 2011
SIV	Upregulation of PD-L1 on DCs; correlation between level of PD-L1 expression and disease progression; improved function of antiviral T cells function after PD-L1 blockade	Xu et al., 2010
HSV-1	Increased PD-L1 expression on DCs in the draining lymph nodes after virus inoculation into foot pads of mice	Channappanavar et al., 2012
VZV	PD-L1/2 upregulation on human monocytes, B cells, NK cells, and NKT cells	Jones et al., 2019
KSHV	Increased PD-L1 expression on monocytes	Host et al., 2017

Ad, adenovirus; EOBV, Ebola virus; FV, Friend retrovirus; HIV, human immunodeficiency virus; HSV-1, herpes simplex virus type 1; HV, hantavirus; IAV, Influenza A virus; JEV, Japanese encephalitis virus; KSHV, Kaposi's sarcoma-associated herpesvirus; LCMV, lymphocytic choriomeningitis virus; LCMV Arm, LCMV strain Armstrong; LCMV clone13, LCMV strain clone13; RSV, respiratory syncytial virus; Treg cells, regulatory T cells; VZV, varicella zoster virus.

TABLE 2 | Virus-induced PD-L1 upregulation on non-hematopoietic cells.

Viruses	Findings	References
LCMV	PD-L1 upregulation on fibroblastic reticular cells	Zinselmeyer et al., 2013
Ad	Increased PD-L1 expression on primary human hepatocytes	Grakoui et al., 2006; Muhlbauer et al., 2006
HBV	Upregulated PD-L1 expression on hepatocytes derived from a transgenic mouse model of BV infection	Maier et al., 2007
IAV, MHPV, PIV-3, RSV	Increased levels of PD-L1 on alveolar and bronchiolar epithelial cells after virus infection *in vitro* and in patients with viral acute lower tract infections	Stanciu et al., 2006; Telcian et al., 2011; Erickson et al., 2012; McNally et al., 2013
RABV	Type I IFN-dependent PD-L1 upregulation on virus-infected mouse and human neuronal cells *in vitro* and on neuronal cells in virus-infected mice	Lafon et al., 2008
HSV-1	PD-L1 upregulation on mouse neuroblastoma cells	Chentoufi et al., 2011
HSV-1	PD-L1 upregulation on virus-infected neurons in ganglia	Jeon et al., 2013
HSV-1	PD-L1 upregulation on epithelial cells in the virus-infected cornea	Jeon et al., 2018

Ad, adenovirus; EOBV, Ebola virus; HMPV, human metapneumovirus; HBV, hepatitis B virus; HSV-1, herpes simplex virus type 1; HV, hantavirus; IAV, Influenza A virus; JEV, Japanese encephalitis virus; LCMV, lymphocytic choriomeningitis virus; LCMV Arm, LCMV strain Armstrong; LCMV WE, LCMV strain WE; PIV-3, parainfluenza virus type 3; RABV, rabies virus; RSV, respiratory syncytial virus; Treg cells, regulatory T cells; VHF, viral hemorrhagic fever.

independent of the PD-1/PD-L1 axis during persisting virus infections (Ouyang et al., 2011).

In the late phase of acute virus infection, type II IFN and several other cytokines including TNF-α and IL-10 are released by immune cells such as CD8+ T cell cells (Zhang and Bevan, 2011). IFN-γ strongly upregulates PD-L1 (Garcia-Diaz et al., 2017; Raftery et al., 2018; Sun et al., 2018). In addition, plasmacytoid DCs (pDCs) migrate into virus-infected tissue and secrete large amounts of type I IFNs (Siegal et al., 1999). These cytokines not only induce antiviral ISGs but also drive inflammatory responses such as secretion of TNF-α, IL-1β, or IL-6 (Davidson et al., 2015), which can further increase PD-L1 on various cell types including endothelial cells and at the same time promote non-lytic virus elimination (Sun et al., 2018). Thus, in the late phase of acute viral infection, PD-L1 is strongly upregulated thereby downregulating terminal differentiation of CD8+ T cells and preventing excessive tissue damage due to uncontrolled cytotoxic attack.

FUNCTION OF PD-L1 DURING ACUTE VIRUS INFECTIONS

PD-L1 expressed on hematopoietic or non-hematopoietic cells has different functions (Keir et al., 2006; Mueller et al., 2010). For example, PD-L1 expression on parenchymal cells of the pancreas rather than hematopoietic cells prevent autoimmune diabetes (Keir et al., 2006). In accordance, during LCMV infection of mice PD-L1 expression on non-hematopoietic cells reduces viral clearance and immunopathology (Keir et al., 2008). Thus, upregulation of PD-L1 expression may protect virus-infected cells from being eliminated by cytotoxic CD8+ T cells. On the other hand, selective absence of PD-L1 on hematopoietic cells results in lethal immunopathology (Mueller et al., 2010). This is best explained by an increase in number and function of cytotoxic CD8+ T lymphocytes, which may overwhelm the PD-L1-conferred protection in non-hematopoietic target cells (Frebel et al., 2012).

Virus-induced PD-L1 on professional APCs may help to focus the antiviral CD8+ T cell response on a few strongly stimulatory, i.e., immunodominant, virus-derived epitopes by increasing the threshold of CD8+ T cell activation. In this way, the majority of weakly immunogenic viral peptides fail to activate CD8+ T cells. The adjustment of the "rheostat" on professional APCs may be necessary to prevent autoimmune disease and maintain peripheral tolerance in the face of a highly inflammatory milieu. Indeed, a recent study has shown that the PD-L1/PD-1 axis regulates T cell responses at the activation stage (Sugiura et al., 2019). CD80, which binds to CD28 and CTLA-4 on T cells, also interacts with PD-L1 (Butte et al., 2007, 2008). Importantly, this interaction occurs only in *cis* (Chaudhri et al., 2018) and prevents PD-L1 on DCs from co-inhibitory signaling to T cells via PD-1 (Chaudhri et al., 2018; Sugiura et al., 2019). In contrast, the functions of CD28 (co-stimulatory) and CTLA-4 (co-inhibitory) are not impaired by *cis*-PD-L1/CD80 interactions on DCs (Sugiura et al., 2019). Many viruses upregulate PD-L1 on professional APCs such as DCs (**Table 1**) either directly or through IFN release. Low PD-L1 levels on uninfected DCs have only a weak impact on T cell activation (Brown et al., 2003) due to *cis*-PD-L1/CD80 interactions (**Figure 1**, upper scheme). It is likely, that the high PD-L1 levels on DCs in the context of viral infection will overwhelm the *cis*-binding capacity of CD80 resulting in increased co-inhibitory signaling via PD-1 (**Figure 1**, lower scheme). PD-L1 on professional APCs also promotes the induction and maintenance of regulatory T cells (Treg cells; Francisco et al., 2009). Treg cells help to confine the antiviral defense and to prevent immunopathology during virus infections (Veiga-Parga et al., 2013). Taken together, viruses can reprogram DC function in antiviral immune responses by tipping the balance between co-inhibitory and co-stimulatory signals as shown for murine cytomegalovirus (Loewendorf et al., 2004; Benedict et al., 2008) and vaccinia virus (Kleinpeter et al., 2019).

Strong stimulation of the PD-1/PD-L1 does not prevent immunopathology during viral hemorrhagic fever (VHF). VHF is a designation for distinct but pathogenically similar zoonotic diseases that are caused by several enveloped RNA viruses including Ebola virus (EBOV), hantavirus, and dengue virus (DENV) (Paessler and Walker, 2013). VHF viruses target endothelial cells thereby causing vascular leakage (Zampieri et al., 2007; Schonrich et al., 2008; Basler, 2017). In fact, type III IFN and TNF-α, which upregulate PD-L1 on endothelial cells, also mediate dysfunction of the endothelial barrier (Brett et al., 1989; Koh et al., 2004). Virus-specific CD8+ T cells show high levels of PD-1 on the surface during acute infection with EBOV (McElroy et al., 2015). Moreover, fatal EBOV infection is characterized by a high percentage of T cells expressing PD-1 and other co-inhibitory receptors such as CTLA-4 (Ruibal et al., 2016). Monocytes are susceptible to EBOV infection and upregulate production of PD-L1 transcripts in response to EBOV replication (Menicucci et al., 2017), whereas DENV-infected DCs express higher levels of PD-L2 but reduced PD-L1 (Nightingale et al., 2008). In patients with acute hantavirus infection large amounts of soluble PD-L1/PD-L2 are found in the sera indicating that these molecules are strongly upregulated in hantavirus-infected cells *in vivo* (Raftery et al., 2018). In accordance, strongly increased PD-L1 levels are detected after hantavirus infection of immature DCs *in vitro* and in hantavirus-infected mice with a humanized immune system (Raftery et al., 2018). In striking contrast, CD8+ T cells do not upregulate PD-1 during acute hantavirus infection (Lindgren et al., 2011).

Taken together, in the acute phase of viral infection virus-specific T cells rapidly upregulate the co-inhibitory receptor PD-1 upon recognition of antigen. Simultaneously, viruses upregulate PD-L1 on hematopoietic and non-hematopoietic cells directly through PRR signaling or indirectly by inducing the release of IFNs and other inflammatory cytokines. Ideally, a tailor-made antiviral CD8+ T cell response eliminates viral pathogens with minimal immunopathology (**Figure 2**). The antiviral immune response during VHF, however, eliminates viruses at the cost of vascular leakage. The dysregulation of the immune responses could be due to variations in PD-L1 expression (e.g., timing, cell type, or strength), imbalance between co-stimulatory vs. co-inhibitory receptors (failure of "checks and balances"), or altered usage of PD-L1 interaction partners (PD-1, CD80, and possibly additional unknown partners). On the other hand viruses can also manipulate the "checks and balances" of the immune system in such a way that an effective antiviral immune response is prevented helping the pathogen to persist in the organism.

THE PD-1/PD-L1 AXIS DURING PERSISTING VIRUS INFECTIONS

Chronic Virus Infection

Chronic infections with viruses such as hepatitis B virus, hepatitis C virus (HCV), and human immunodeficiency virus (HIV) represent major causes of chronic disease and death worldwide (Ott et al., 2012; Schweitzer et al., 2015; GBD 2015 HIV Collaborators, 2016; Stanaway et al., 2016). During chronic infection virus particles are continuously released from virus-infected cells and maintain a network of immunosuppressive mechanisms that interfere with virus elimination (Ng et al., 2013). Therefore, T cells enter a state called T cell exhaustion.

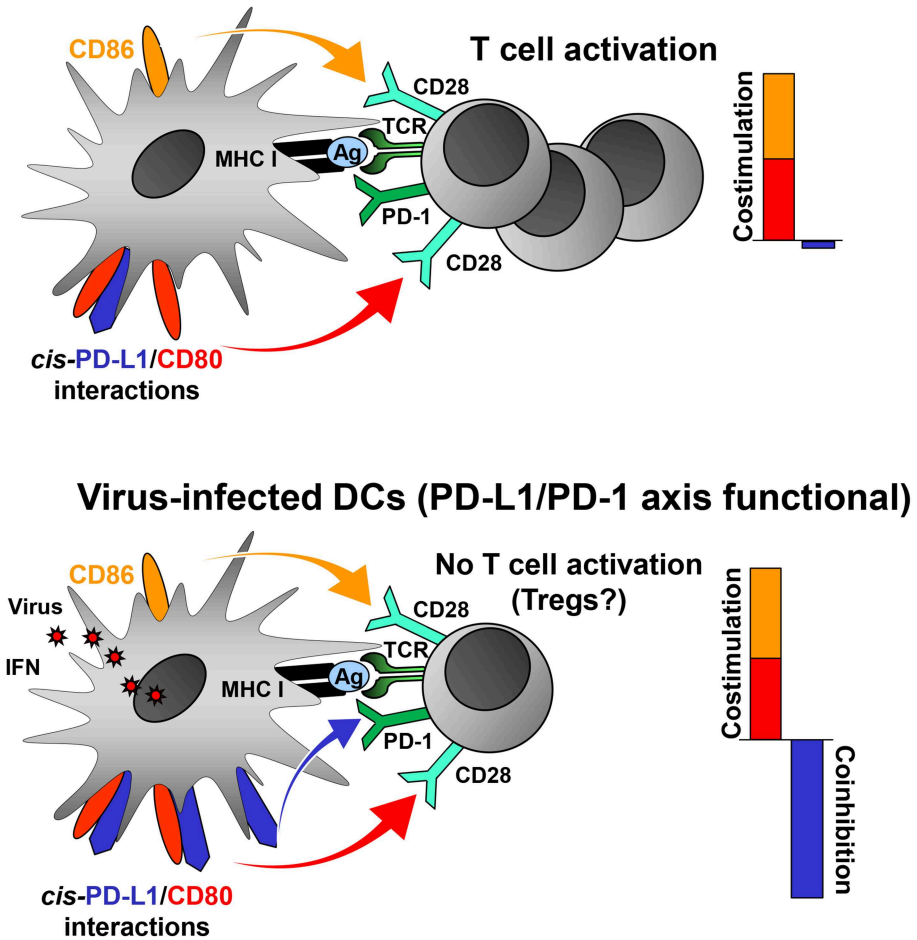

FIGURE 1 | PD-L1 mediated viral regulation of T cell activation. Upper graph: In the absence of viral infection mature dendritic cells (DCs) express relatively low levels of PD-L1. Recognition of cognate antigen (Ag) bound to MHC class I molecules by T cell receptor (TCR) results in upregulation of PD-1 on T cells. DCs express co-stimulatory molecules CD80 and CD86 allowing efficient co-stimulation of T cells via CD28. The PD-1/PD-L1 axis is not co-inhibitory due to restriction by *cis*-PD-L1/CD80 interactions, and thus T cells are activated. Lower graph: In the context of viral infection DCs upregulate PD-L1 due to exposure to viral PAMPs and high levels of type I IFN. The restricting *cis*-PD-L1/CD80 interactions are most likely overwhelmed by virus-induced PD-L1 resulting in PD-1 signaling and prevention of T cell activation. The consequences of this for the generation of Tregs is as of yet unknown.

T Cell Exhaustion and Partial Restoration of T Cell Function by Blockade of the PD-1/PD-L1 Axis

The first evidence for T cell exhaustion was gathered in paradigmatic experiments using LCMV-infected mice (Zehn and Wherry, 2015; Kahan and Zajac, 2019). Derivatives of LCMV Arm and LCMV WE (LCMV clone13 and LCMV docile, respectively) vigorously replicate and disseminate in mice thereby persisting for more than 100 days (Moskophidis et al., 1993; Gallimore et al., 1998; Zajac et al., 1998). In this model of chronic virus infection, CD4+ T cells are crucial to sustain the virus-specific CD8+ T cell responses (Matloubian et al., 1994). Sustained upregulation of PD-1 and other co-inhibitory receptors such as 2B4, CTLA-4, and lymphocyte-activation gene 3 (Lag3) has become the defining characteristic of exhausted T (Tex) cells (Barber et al., 2006; Wherry et al., 2007; Blackburn et al., 2009; Crawford et al., 2014). These phenotypic changes are accompanied by a multistep loss of T cell effector functions (Speiser et al., 2014; Kahan et al., 2015; Wherry and Kurachi, 2015; McKinney and Smith, 2018). Dependent on the strength of PD-1 signaling CD8+ T lymphocytes gradually lose important effector functions (Wherry et al., 2003; Wei et al., 2013). Some are lost early (such as cytotoxicity, IL-2 production, and proliferation), whereas others (e.g., IFN-γ production) are maintained for a longer time (Wherry et al., 2003; Wei et al., 2013). Finally, Tex cells undergo apoptosis (Kahan et al., 2015). As an underlying mechanism of T cell exhaustion during chronic LCMV infection, PD-1 signaling impairs T cell motility facilitating engagement of inhibitory pathways in T cells (Zinselmeyer et al., 2013). In another experimental setting, PD-L1 blocking antibodies prolong the T cell migration arrest suggesting that PD-1 signaling in fact

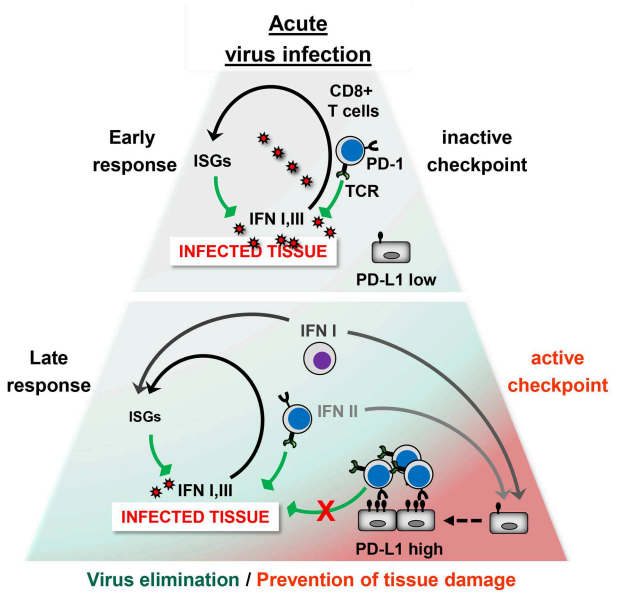

FIGURE 2 | The PD-1/PD-L1 checkpoint in acute virus infection. Early phase: The infected tissue produces type I IFNs and possibly type III IFNs, which strongly induce antiviral IFN-stimulated genes (ISGs) but only moderate PD-L1 levels. Antiviral CD8+ T cells eliminate virus-infected cells. At this stage, the PD-1/PD-L1 checkpoint activity is low and does not restrict the antiviral immune response. Late Phase: Type II IFN and TNF-α is secreted by CD8+ T cells and other immune cells. In addition, hematopoietic cells such as plasmacytoid DCs (pDCs) produce large amounts of type I IFN. This results not only in virus elimination but also increases PD-L1 expression. The high checkpoint activity downregulates terminal differentiation of antiviral CD8+ T cells. Ideally, the strength and quality of the CD8+ T cell response is balanced out in such a way that the viral intruder is eliminated without causing immunopathology.

enhances T cell motility (Honda et al., 2014). The reason for these contrasting results are unclear at the moment. Intriguingly, PD-1-regulated changes in several metabolic pathways occur at the very beginning of Tex cell development underlining the importance of these metabolic processes in the execution of the Tex program (Bengsch et al., 2016; Schurich et al., 2016; McKinney and Smith, 2018).

Several reports have found differences in the transcriptional program and epigenetic profile of Tex cells as compared to memory and effector T lymphocytes (Wherry et al., 2007; Doering et al., 2012; Pauken et al., 2016). In Tex cells derived from LCMV-infected mice, the *Pdcd1* regulatory region is completely demethylated and remains so even when virus titers decrease (Youngblood et al., 2011). They do not show antigen-independent persistence driven by IL-7 and IL-15, the hallmark of memory T cells, and instead require the continuous presence of their cognate antigen (Wherry and Ahmed, 2004; Shin et al., 2007). This can be explained by the observation that the TCR-induced transcription factors IRF4, BATF, and NFATc1 not only drive T cell exhaustion but also impair memory T cell development during chronic LCMV infection (Man et al., 2017). Recently, microRNA (miR)-155 has been identified as a key molecule that promotes long-term persistence of Tex cells (Stelekati et al., 2018).

T cells that have been rendered dysfunctional during persisting virus infections can be reinvigorated (Brooks et al., 2006a). Blockade of the PD-1/PD-L1 axis during chronic LCMV infection reinvigorates antiviral T cell functions and reduces viral load (Barber et al., 2006). Of note, CD8+ T cells also become exhausted in the absence of PD-1 (Odorizzi et al., 2015). These experiments show that other coinhibitory receptors contribute to T cell exhaustion. In line with this view, a combined blockade of PD-1 and LAG-3 or PD-1 and Tim-3 synergistically improves antiviral CD8+ T cell responses and viral control in mice with chronic LCMV infection (Blackburn et al., 2009; Jin et al., 2010). Reinvigorated CD8+ T cells in chronically LCMV-infected mice become exhausted again after termination of the PD-L1 blockade (Pauken et al., 2016; Sen et al., 2016; Turner and Russ, 2016). This finding indicates that inflexibility of the epigenetic regulation in Tex cells may limit the success of therapies using ICIs.

The studies of chronic LCMV infection in mice also relate to important human infectious diseases. In a recently established mouse model of HCV infection Tex cells are observed in the liver of mice infected with a newly identified Norway rat hepacivirus (NrHV), which belong to the same virus family as HCV (Billerbeck et al., 2017; Klenerman and Barnes, 2017). In NrHV-infected mice, CD4+ T cells were important to maintain the antiviral CD8+ T cell response similar to the LCMV model of chronic virus infection (Billerbeck et al., 2017). Blockade of the PD-1/PD-L1 axis in early chronic infection reduced the viral load whereas no beneficial effects were observed at later time points (Billerbeck et al., 2017). Moreover, ICIs blocking the PD-1 pathway can reinvigorate to some extent Tex cells in humans chronically infected with HBV or HCV (McKinney and Smith, 2016; Cox et al., 2017; Saeidi et al., 2018; Wykes and Lewin, 2018). Targeting the PD-1/PD-L1 pathway during retroviral infections has beneficial effects for virus control (Velu et al., 2015). PD-1 upregulation is linked to a loss of function in HIV-specific CD8+ T cells, which can be partially reversed *in vitro* by a blockade of the PD-1/PD-L1 axis (Day et al., 2006; Trautmann et al., 2006). Surprisingly, the context and timing of PD-1 blockade seems to be important for its functional outcome: PD-1 signaling inhibition during stimulation of naive $CD8^+$ T cells results in diminished activation, whereas PD-1 blockade during the T cell effector phase increases activation (Garcia-Bates et al., 2019). PD-1 blockade in rhesus macaques infected with simian immunodeficiency (SIV) rapidly increases the number and functional quality of virus-specific CD8+ T cells (Velu et al., 2009). Intriguingly, the combination of anti-PD-1 antibodies and antiretroviral therapy further improves antiviral CD8+ T cell function in SIV-infected rhesus macaques (Mylvaganam et al., 2018). This observation implies that directly acting antivirals (DAAs) reducing the viral load and ICIs releasing the brake in Tex cells synergistically increase antiviral T cell responses.

Recent data suggest that PD-1 expression does not necessarily reflect T cell failure but rather adaption of T cell function to chronic inflammation (Utzschneider et al., 2013, 2016; Speiser et al., 2014; Staron et al., 2014; Zehn et al., 2016; Barnes, 2018; Petrelli et al., 2018). In fact, at least two CD8+ Tex cell subsets

exist that act in concert to mount a partially effective CD8+ T cell response for control of chronic virus infection (Paley et al., 2012). Moreover, Tex cells have the capacity for self-renewal and are not entirely functionally inactive (Paley et al., 2012). The latter finding implies that Tex cells may represent a form of antiviral defense that is evolutionary adapted to the need to control a chronically replicating non-lytic virus with minimal collateral tissue damage and immunopathology. Moreover, experiments in mice with genetic ablation of PD-1 suggest that PD-1 is not required for induction of Tex cells (Odorizzi et al., 2015). In fact, PD-1 may play a pivotal role in maintaining Tex cells by preventing excessive stimulation that leads to proliferation and terminal differentiation (Odorizzi et al., 2015). After the elimination of HCV by DAAs, PD-1 expressing CD8+ T cell populations remain that display characteristics of memory cells including antigen-independent survival and proliferation after re-challenge with antigen (Wieland et al., 2017).

Latent Infection and Reactivation

Viruses that establish latent infection include the members of the family *Herpesviridae*. In contrast to chronic infection, latent infection is characterized by periodic suspension of virus replication. However, the blueprint of viral particles is preserved in the latently infected host cells enabling the virus to reactivate and resume virus production. It is a matter of debate whether reactivation from latent virus infection creates enough antigenic load to induce exhaustion of antiviral CD8+ T cells. Memory CD8+ T cells recognizing viral antigens in the context of chronic virus infections (e.g., HIV) more frequently express PD-1 than memory CD8+ T cells stimulated by virus periodically reactivating from latency, e.g., human cytomegalovirus (HCMV) (Petrovas et al., 2006). This finding is consistent with the idea that the amount of available antigen regulates PD-1 expression on reactive T cells (Petrovas et al., 2006). In accordance, increased virus replication in immunosuppressed patients with HCMV disease after allogeneic hematopoietic cell transplantation is associated with PD-1 upregulation on T cells (Gallez-Hawkins et al., 2009). In mice with a humanized immune system, HCMV reactivations induced by granulocyte-colony stimulating factor (G-CSF) resulted in a shift toward PD-1 expressing T cells (Theobald et al., 2018). Whether this phenotype corresponds to Tex cells is unclear, however. Upregulation of co-inhibitory receptors such as PD-1 on CD8+ T cells is tightly linked to activation and differentiation and not *per se* proof of T cell exhaustion (Legat et al., 2013). In addition, studies of HSV-1 infection in mice did not reveal evidence for functional impairment of virus-specific CD8+ T cells during latency and subsequent reactivations (Mackay et al., 2012).

PD-1 expression on brain Trm cells is maintained independently from antigen (Shwetank et al., 2017). Recently, it has been shown that Trm cells provide immunosurveillance in the human brain to eliminate neurotropic viruses (Smolders et al., 2018). In accordance, reactivation of HSV-1 from latently infected neurons of the mouse is controlled by CD8+ Trm lymphocytes (Liu et al., 2000; Khanna et al., 2003; Verjans et al., 2007). These immune cells provide IFN-γ which upregulates PD-L1 on HSV-1-infected neurons (Jeon et al., 2013). CD8+ T cells recognizing subdominant epitopes derived from HSV-1 proteins other than glycoprotein B (gB) but not CD8+ T cells specific for the dominant gB-derived epitope show a partial exhausted phenotype with increased PD-1 expression (Jeon et al., 2013). Blockade of PD-L1 resulted in increased survival of exhausted CD8+ T cells that were non-functional and not protective, however (Jeon et al., 2013). In contrast, it has been reported that HSV-1 LATs promotes functional exhaustion of CD8+ T cells specific for the dominant gB-derived epitope (Allen et al., 2011; Chentoufi et al., 2011).

During coevolution with their host, herpesviruses developed numerous mechanisms to evade the antiviral immune response such as modulation of programmed cell death (Raftery et al., 1999, 2001; Muller et al., 2004; Kather et al., 2010) and downregulation of MHC class I molecules (Schuren et al., 2016). Intriguingly, replication competent varicella-zoster virus (VZV) downregulates MHC class I and PD-L1 molecules in human brain vascular adventitial fibroblasts, perineurial cells, and human lung fibroblasts (Jones et al., 2016). In contrast, VZV upregulates PD-L1 in hematopoietic cells (Jones et al., 2019). The mechanism underlying VZV-associated downregulation of PD-L1 is posttranscriptional in nature but the VZV-encoded protein responsible has not yet been identified (Jones et al., 2016). VZV might target PD-L1 to increase the migration arrest of T cells (Honda et al., 2014). In this way, the virus could more efficiently spread from lung fibroblasts to T cells, which play crucial role in VZV dissemination to the skin (Arvin et al., 2010).

CONCLUDING REMARKS

It is a seductive proposition that a virus induces PD-1 ligands in order to inhibit and thus evade the host immune response. On the other hand, recent data on the regulation of PD-L1 expression during viral infection suggest that PD-L1 upregulation is rather a part of the normal innate response induced by IFNs and PRR signaling. The reason for this is still enigmatic. PD-L1 may have a yet not defined immunostimulatory role in the very early phase of viral infection. Later, it may adjust the quantity and quality of the antiviral CD8+ T cell response in such a way that virus is eliminated with minimal collateral tissue damage. The PD-1/PD-L1 axis may also be important to maintain antiviral Trm cells and Tex cells. Virus-induced PD-1 ligand expression as an immune evasion strategy should always be rigorously tested with this in mind.

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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Microbiota and Its Role on Viral Evasion: Is It With Us or Against Us?

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Viruses are obligate intracellular pathogens that require the protein synthesis machinery of the host cells to replicate. These microorganisms have evolved mechanisms to avoid detection from the host immune innate and adaptive response, which are known as viral evasion mechanisms. Viruses enter the host through skin and mucosal surfaces that happen to be colonized by communities of thousands of microorganisms collectively known as the commensal microbiota, where bacteria have a role in the modulation of the immune system and maintaining homeostasis. These bacteria are necessary for the development of the immune system and to prevent the adhesion and colonization of bacterial pathogens and parasites. However, the interactions between the commensal microbiota and viruses are not clear. The microbiota could confer protection against viral infection by priming the immune response to avoid infection, with some bacterial species being required to increase the antiviral response. On the other hand, it could also help to promote viral evasion of certain viruses by direct and indirect mechanisms, with the presence of the microbiota increasing infection and viruses using LPS and surface polysaccharides from bacteria to trigger immunosuppressive pathways. In this work, we reviewed the interaction between the microbiota and viruses to prevent their entry into host cells or to help them to evade the host antiviral immunity. This review is focused on the influence of the commensal microbiota in the viruses' success or failure of the host cells infection.

Keywords: microbiota, microbioma, viral evasion, microbiota-virome interaction, microbiota and antiviral immune defense

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INTRODUCTION

The mucosal surfaces of the human body contain complex communities of microorganisms collectively referred to as microbiota; these bacteria are a key factor in health and disease due to their participation in the development of the immune system and their host-protection against pathogens (Human Microbiome Project Consortium, 2012a,b; Lloyd-Price et al., 2016).

Viruses are a large and heterogeneous group of dependent biological agents that require the host-cell machinery to replicate. Most viruses are identified based on their capacity and mechanisms used to produce disease; however, healthy individuals harbor viral communities that do not cause directly known pathologies. These viral communities are known as the human virome (Rohwer et al., 2009). The coexistence of viruses and bacteria within the microbiome encourages the study of viral evasion mechanisms that provide immune system tolerance to these pathogens. These

mechanisms are undoubtedly also used during the pathophysiology of viral diseases (Abeles and Pride, 2014).

MICROBIOTA AGAINST VIRAL INFECTION

Since the discovery that gut bacteria instruct host immunity, i.e., they restrict pathogen proliferation, it would seem logical to think that the intestinal microbiota would also play a predominant role in viral etiology infection inhibition. Studies reveal that commensal bacteria are crucial in maintaining immune homeostasis and immune responses at mucosal surfaces (Ichinohe et al., 2011). Mucous membranes are the gateway to many pathogens, including viruses. For example, intestinal microorganisms promote maturation of the secondary lymphoid organs within the gastrointestinal tract, which is the first line of defense of the intestinal mucosa (Karst, 2016). Germ-free mice are unable to mount an efficient immune response against pathogens due to immature intestinal lymphoid structures (Hooper et al., 2012; Kamada and Núñez, 2014).

Given the complexity of the microenvironment in mucosal surfaces, it makes sense that the most studied bacteria and virus interactions are the ones involving the intestinal microbiome. The protective role of commensal bacteria, mainly probiotics, is well-established; however, in its interactions with viruses, more studies are needed. The *Lactobacillus* genus can inhibit murine norovirus (MNV) replication *in vitro,* which could be mediated by the increased expression of IFNβ and IFNγ. *In vivo* models show that these bacteria are decreased during MNV infection, though with the aid of retinoic acid treatment, it is possible to avoid this effect. It has been hypothesized that the antiviral effects of vitamin A (and consequently, retinoic acid) are mediated by the *Lactobacillus* genus due to interferon production (Lee and Ko, 2016).

Bacterial flagellin is efficient against rotavirus (RV) infection because it activates Pattern Recognition Receptors (PRR), TLR5 and NLRC-4, that stimulate the release of interleukin-22 (IL-22) and IL-18; the former induces normal epithelium proliferation, while the latter induces infected epithelial cell apoptosis (Zhang et al., 2014). *Bifidobacterium breve* and a mixture of this probiotic with galactooligosaccharides and fructooligosaccharides have a preventive effect against RV infection by increasing the production of IFNγ, IL-4, TNFα, and TLR2 expression, while also decreasing the tolerogenic response (Rigo-Adrover et al., 2018), thereby enhancing the mucosal defense against this pathogen.

Immunoregulation and reinforcement of the intestinal barrier through the relationship between commensal bacteria, probiotics, epithelial, and immune cells, are established physiological processes mediating the antiviral effects of the microbiota. The enteric microbiota regulates increased mucus production and synthesis of potential antiviral compounds, likereactive oxygen species and defensins, that inhibit local viral replication (Monedero et al., 2018).

Gut microbiota could also have distal protective effects on antiviral responses. There is evidence of the role of inflammasome activation in the immune defense against influenza virus infection (Allen et al., 2009; Ichinohe et al., 2009); it induces dendritic cell migration to the local lymph node to stimulate an influenza-specific T-cell response in the lung (Ichinohe et al., 2011; Wilks and Golovkina, 2012). The commensal gut microbiota regulates the respiratory mucosa immunity against respiratory influenza virus through the IgA secretion, and the proper activation of inflammasomes, Th1 cells, and CTLs, and through the upregulation of TLR7 signaling in the respiratory mucosa (Ichinohe et al., 2011; Wu et al., 2013). Steed et al. (2017) demonstrated that desaminotyrosine, a microbial metabolite, enhances type I interferon (IFN-I) signaling and protects against influenza pathogenesis.

Gut probiotics like *Lactobacillus paracasei* and *Lactobacillus plantarum* increase pro-inflammatory cytokines like IL-33, IL-1α, IL-β, IL-12, and IFNγ during influenza virus infection. There is also an increase in the presence of innate immune cells in the lungs such as NKs, macrophages, and dendritic cells. These probiotics were also able to diminish the inflammatory response in the lungs by an IL-10 increase, thereby controlling the antiviral response (Park et al., 2013; Belkacem et al., 2017). The crosstalk between the gut and airway bacteria through the gut-lung axis could explain how the intestinal bacteria are able to improve antiviral immunity since gut microbial metabolites could stimulate immune cells that can move to distal locations to mediate the antiviral response.

On the respiratory surface, airway bacteria protect against viral infections. *Staphylococcus aureus* stimulates the recruitment of peripheral $CCR2^{+}$ $CD11b^{+}$ monocytes and their subsequent maturation into M2 macrophages, through the activation of TLR2 signaling during influenza infection. This mechanism dampens influenza-mediated acute lung injury (Wang et al., 2013). The respiratory commensal bacteria, *Corynebacterium pseudodiphtheriticum* modulates the TLR3 antiviral response against Respiratory Syncytial Virus (RSV), enhancing the production of TNFα, IL-6, IFNγ, and IFNβ through the increase of T-cell subpopulations that produce these cytokines (Kanmani et al., 2017).

The vaginal mucosa is dominated by bacteria from the *Lactobacillus* genus. Vaginal microbial communities dominated by *Lactobacillus crispatus* were associated with a decreased HIV infection in South African women (Gosmann et al., 2017). *L. crispatus, Lactobacillus gasseri*, and *Lactobacillus vaginalis* inhibit HIV-1 replication in *ex vivo* cervico-vaginal tissue culture. These effects are mediated through acidification of the medium and lactic acid production, as well as their binding to the virus in order to reduce the free virions in the tissue (Ñahui Palomino et al., 2017). Lactic acid and acidic pH increase the production of anti-inflammatory cytokines, preventing the production of pro-inflammatory cytokines by epithelial cells and, with this, the inflammation that increases HIV acquisition (Hearps et al., 2017). Lack of the vaginal microbiome by antibiotic depletion leads to IL-33 increased production which suppresses IFNγ secretion, leading to Herpes Simplex Virus type 2 (HSV-2) susceptibility due to an impaired antiviral defense (Oh et al., 2016).

These findings demonstrate that commensal bacteria in different mucosal sites are part of the antiviral response against

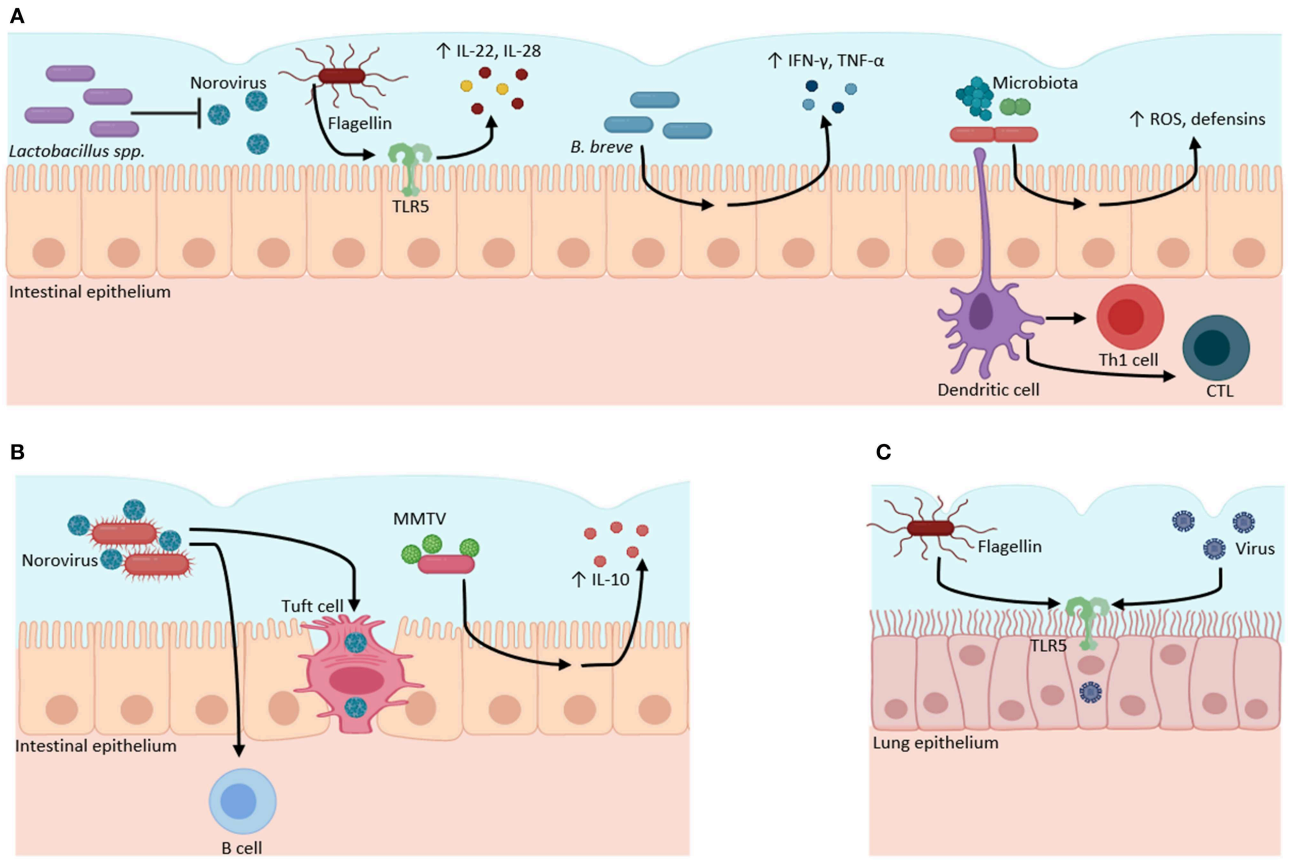

FIGURE 1 | Microbiota–virome interactions in mucosal surfaces. The microbiota has a dual role when it interacts with viruses. **(A)** Microbiota can have a protective role against viral infections. Bacteria from the Lactobacillus genus inhibit Norovirus infection. Bacterial flagellin activates TLR5 to produce inflammatory cytokines (IL-22, IL-18). *B. breve* stimulates the production of pro-inflammatory cytokines (IFN-γ, IL-4, TNFα) against viruses. The gut microbiota regulates the production of ROS and defensins, and the activation of Th1 and CTL against viral infections. **(B)** Microbiota can function as an evasion mechanism, where viruses can bind to bacterial structural molecules (such as LPS) or bacterial pili or membranes to induce immunotolerance through the increase of anti-inflammatory cytokines (IL-10) and to infect host cells. **(C)** Bacterial flagellin increases the infectivity of influenza, Measles, Ebola, Lassa, and Vesicular stomatitis viruses through TLR5 activation in lung epithelial cell culture. Created with BioRender.com.

pathogenic viruses; nevertheless, there is much yet to define in the mechanisms through which they can achieve this (**Figure 1A**).

MICROBIOTA AS PROMOTERS OF VIRAL INFECTIONS

Despite the significant evidence available about the role of the microbiota in the regulation of the mucosal immune system and the host protection from viral infections, it is also known that, through microbiota rich mucosal surfaces, different viruses enter host cells most efficiently. Furthermore, viruses escape the immune response to establish chronic infections. Then, contrary to the known benefits of gut microbiota, intestinal viruses take advantage of gut bacteria to trigger replication at favorable transmission sites (Kuss et al., 2011).

Human and murine norovirus (MNV) require the presence of bacteria to infect B cells since the lack of both bacteria by antibiotic treatment and B cells in $Rag^{-/-}$ mice inhibit the infection by norovirus (Jones et al., 2014; Baldridge et al., 2015). MNV also targets intestinal tuft cells by the CD3001f receptor and antibiotics reduce the specific genes for these cells in the colon. The MNV needs the colonic commensal microbiota to regulate these epithelial cells to utilize them as a reservoir for its chronic infection (Wilen et al., 2018). Commensal bacteria from the human gut, such as *Enterobacter faecium, Klebsiella* spp., *Bacillus* spp., *Bacteroides thetaiotaomicron, L. plantarum*, and *L. gasseri*, among others, bind human norovirus through bacterial pili and membranes, possibly through HBGA-like (histo-blood group antigens) molecules, sialylated gangliosides, and lipopolysaccharides (LPS), which can facilitate the entry of these viruses and the development of the infection (Almand et al., 2017). It is yet to be elucidated the exact mechanisms and molecules this virus utilizes to bind on bacterial surfaces; however, these interactions are a good example of viruses exploiting commensal bacterial to promote their infectivity (**Figure 1B**).

The intestinal microbiota enhances mouse mammary tumor virus (MMTV), poliovirus, and mammalian orthoreovirus (reovirus) infections (Kane et al., 2011; Kuss et al., 2011). MMTV

vertical transmission to offspring's via milk is thought to rely upon TLR4 activation, a PRR for bacteria LPS (Rassa et al., 2002; Jude et al., 2003). The retrovirus MMTV relies on the microbiota interaction to evade the immune response. It binds LPS and induces immune tolerance through a TLR4/MyD88 pathway to induce IL-10 (Kane et al., 2011).

Poliovirus interacts with surface polysaccharides on specific microbes, enhancing host cell binding via the poliovirus receptor (PVR) (Kuss et al., 2011; Robinson et al., 2014). Poliovirus particles are also able to bind to LPS and peptidoglycan (Robinson and Pfeiffer, 2014). Moreover, microbiota-harboring mice support more efficient poliovirus replication (Kuss et al., 2011). Also, the reovirus utilizes bacterial envelope components to enhance virion stability (Berger et al., 2017). Both peptidoglycan and LPS improve viral, and ISVPs (infectious subvirion particles) thermostability; ISVPs are produced when the virus encounters intestinal proteases and play a part in the initial infection steps (Bodkin et al., 1989; Berger and Mainou, 2018).

A recent study showed how human milk oligosaccharides (HMOs) correlate with neonatal RV G10P[11] infection and an increase in abundance of *Enterobacter/Klebsiella*. This neonatal RV evolved to bind HMOs to possibly enter into epithelial cells or to be stabilized by them (Ramani et al., 2018). Considering that HMOs are considered to have a beneficial effect due to their prebiotics effect for bacteria like *Bifidobacterium,* it is remarkable that pathogenic viruses and bacteria can take advantage of this prebiotic to increase their infectivity. Additionally, the commensal microbiota promotes RV infection and affects the immune response to the infection. Antibiotic treatment reduces its infectivity and increases IgA-producing cells, suppressing RV entry (Uchiyama et al., 2014). Vancomycin treatment in healthy adults improves RV vaccine immunogenicity and RV shedding through the increase of Proteobacteria and a reduction in Bacteroidetes (Harris et al., 2018). These studies show that the complete commensal microbiota downregulates the antiviral response to RV infection and only particular taxa can enhance the immunity against viruses.

Recently, it was reported that bacterial flagellin promotes viral infection in an *in vitro* model using lentiviral pseudoviruses encoding the glycoproteins of influenza, Measles, Ebola, Lassa, and Vesicular stomatitis virus in pulmonary epithelial cell culture through TLR5 and NF-κB activation (Benedikz et al., 2019). This finding is particularly exciting since previously, it was reported that flagellin had a protective effect against RV infection in mice (Zhang et al., 2014). The dual effect of flagellin could be due to the differences in the microenvironment and models used to study the interaction between the viruses and bacteria (**Figure 1C**). These studies exemplify how much is unknown in the interplay of bacteria and viruses.

VIRUSES AS PART OF THE HUMAN MICROBIOME

The intestine contains other types of organisms, besides bacteria, that can influence mucosal and systemic immune responses such as viruses (Minot et al., 2012; Kernbauer et al., 2014; Norman et al., 2015). To interpret the role of the microbiota within viral infections, we must also consider the impact that the virome may play in this interaction. A recent study approximated that in healthy humans, there are 45% of mammalian viruses that are part of the virome without a clinical outcome (Rascovan et al., 2016; Olival et al., 2017). However, similar to bacteria, resident viruses modulate the immune responses (Freer et al., 2018).

Enteric human virome has also been linked to diseases. For example, enteric eukaryotic viruses can be associated with gastroenteritis, enteritis, or colitis (Norman et al., 2015). Bacteriophages perturb the bacterial community, interplay with the host immune system, and an antagonistic relationship between bacteria and bacteriophages during inflammatory bowel disease has been reported (Duerkop and Hooper, 2013; Virgin, 2015). Also, bacteriophages contribute to the spread of antibiotic resistance genes among bacteria; they form a reservoir of these genes within the microbiome (Muniesa et al., 2013; Quirós et al., 2014). In Crohn's disease, a reduction in viral diversity is part of its characteristic dysbiosis (Abeles and Pride, 2014).

Changes in the intestinal virome are significant in AIDS and HIV enteric disease pathogenesis. Reciprocal transactivation between HIV-1 and other human viruses have been reported (White et al., 2006). Monaco et al. found a relation between the enteric adenovirus sequence expansion and the advanced HIV/AIDS stage (Monaco et al., 2016). Also, AIDS alters the commensal plasma virome since an increase in the proportion of anelloviruses has been reported (Li et al., 2013). In this study, the presence of viral sequences from HIV, HCV, hepatitis B virus (HBV), human endogenous retroviruses (HERV), and GB virus C (GBV-C) in the plasma virome of HIV subjects was also found.

HSV-2 may alter vaginal epithelial integrity, which favors HIV infection and transmission (Shannon et al., 2017). Furthermore, it induces genital inflammation and, in the genital tract mucosa, it increases HIV susceptive target cells (Rebbapragada et al., 2007). Epidemiological studies report a coincidence in different populations of women who have a high incidence of HSV-2 infection and an increased HIV risk (Shannon et al., 2017). Viral-bacterial interactions involving HSV, human cytomegalovirus (HCMV), and Epstein–Barr virus type 1 (EBV-1) might contribute to the development of periodontitis, since HSV infects T-lymphocytes and monocytes/macrophages, EBV-1 infects B-lymphocytes, and HCMV infects monocytes/macrophages and T-lymphocytes, which may cause an impaired immune response against bacteria (Contreras and Slots, 2003; Elamin et al., 2017). HSV may promote subgingival attachment and colonization by periodontopathic bacteria using the capsid proteins as receptors for bacteria (Bakaletz, 1995; Contreras and Slots, 2003). This is similar to one of the mechanisms by which commensal bacteria collaborate with viral infections. Chronic periodontitis has also been related to the natural history of HPV in patients with base of tongue cancers (Tezal et al., 2009), since it facilitates the life cycle of HPV infection in the periodontal pocket (Shipilova et al., 2017). This represents a clear example of a virus-bacteria-virus interaction that ends in

increased susceptibility to the disease, in this case, head and neck cancer.

LIMITATIONS OF STUDIES EVALUATING BACTERIA-VIRUSES' INTERACTIONS

It was supposed that bacteria removal, by antibiotics or the lack of these microorganisms in germ-free models, would increase the predisposition to viral infections; on the other hand, it was found that microbiota ablation decreases the infectivity of pathogenic viruses. Experimental systems to evaluate the role of the gut microbiota during enteric viral diseases included two strategies: the infection of germ-free mice and the administration of treatments to eliminate the commensal microbiota in mice prior to a viral infection (Karst, 2016). However, there are several problems with germ-free animals, such as defects in mucosal immune development and changes in intestinal morphology, while the antibiotic treatment has some disadvantages—antibiotics do not remove the entirety of the commensal microorganisms (Wilks and Golovkina, 2012), some gut species are unculturable so its complete absence can't be proved (Schmeisser et al., 2007), there is currently evidence of antimicrobial resistance of some bacterial groups (Pogue et al., 2015).

The study of the effects of the intestinal microbiota on the host immune system requires precisely defined experimental approaches that are complex, and the requirement of samples limits *in vivo* analysis. Also, the study of the microbiome suggests that there is significant variability among individuals, this indicates that microbiomes are dynamic "fingerprints", though they can change depending on environmental challenges (Bogdanos et al., 2015).

Improvement of *in vitro* and *ex vivo* cultures to simulate more accurately the *in vivo* conditions of microbiome-virome interactions is needed to be able to understand the complexity of this relationship. Otherwise, these models are too simplistic in their approaches, and they should only be used as a first encounter in order to further elucidate the mechanisms of these relations. "Omics" approaches are essential methods to unravel these interactions since pathogenic viruses not only interact with one type of bacteria, but with hundreds of them. Further studies of the relationship between these microorganisms need to take into consideration these approaches to improve our understanding of the complexity of mucosal surface microenvironments.

CONCLUSIONS AND PERSPECTIVES

Although the role of the host-microbiome in human health has been a topic of interest in recent years, its role in the immune response in the context of the susceptibility to different strains of viruses is an important new consideration. Most viruses access the human body through mucosal surfaces that are traditionally described as rich in a diversity of commensal pathogens. In those sites, viruses interact with hundreds of different commensal bacteria, which are part of the host immune defense. Since the discovery of the protective role of the microbiota, it is easy to imagine that bacteria interact with viruses to eliminate or reduce their infectivity, ensuring the homeostasis of the mucosal sites. However, viruses have developed mechanisms to take advantage of the microbiota, and thereby, evade the immune system. So previous considerations of viruses as the sole grounds of different pathologies is not entirely accurate. It is important to remember the complex interaction within the microenvironment and how they determine the outcomes of disease. Therefore, the commensal microbiota could have a fundamental role against viral infections, but also viruses have evolved to interact with the microbiota, use it, and facilitate viral infection, so based on these observations the microbiota can be in itself a mechanism of viral evasion.

There is also a great need for the development of techniques that allow the characterization of these interactions. There are few translational studies, and the experimental models used have several deficiencies.

In this mini-review, we show how current investigations are just starting to untangle the complex world of the microbiome-virome interactions. While it is undeniable that bacteria aid in the antiviral response to certain viruses, they are also, without a doubt, used as a way of entry by them. This makes it complicated to define the role of the microbiota as a friend or foe in this context.

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Evolutionary and Structural Overview of Human Picornavirus Capsid Antibody Evasion

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Picornaviruses constitute one of the most relevant viral groups according to their impact on human and animal health. Etiologic agents of a broad spectrum of illnesses with a clinical presentation that ranges from asymptomatic to fatal disease, they have been the cause of uncountable epidemics throughout history. Picornaviruses are small naked RNA-positive single-stranded viruses that include some of the most important pillars in the development of virology, comprising poliovirus, rhinovirus, and hepatitis A virus. Picornavirus infectious particles use the fecal–oral or respiratory routes as primary modes of transmission. In this regard, successful viral spread relies on the capability of viral capsids to (i) shelter the viral genome, (ii) display molecular determinants for cell receptor recognition, (iii) facilitate efficient genome delivery, and (iv) escape from the immune system. Importantly, picornaviruses display a substantial amount of genetic variability driven by both mutation and recombination. Therefore, the outcome of their replication results in the emergence of a genetically diverse cloud of individuals presenting phenotypic variance. The host humoral response against the capsid protein represents the most active immune pressure and primary weapon to control the infection. Since the preservation of the capsid function is deeply rooted in the virus evolutionary dynamics, here we review the current structural evidence focused on capsid antibody evasion mechanisms from that perspective.

Keywords: picornavirus, capsid, antibody, genetic variability, structure, vaccine

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PICORNAVIRUS HISTORICAL RELEVANCE

Picornaviruses have been pivotal in the foundations of virology. Original research on "ultra-filterable infectious agents" such as foot-and-mouth disease virus (FMDV) and poliovirus (PV) began the era of animal virology (Loeffler and Frosch, 1898; Eggers, 1999). The development of cell cultures for PV replication led to Salk's inactivated and Sabin's attenuated vaccines (Enders et al., 1949). The first animal virus engineered into an infectious clone (Racaniello and Baltimore, 1981) and the first virus synthesized outside the cell was PV (Molla et al., 1991).

Although vast knowledge has been gained, picornaviruses still challenge our understanding. The still open fundamental questions and public health challenges picornaviruses pose reflect that we are far from a conclusive comprehension (Holm-Hansen et al., 2016; Li et al., 2017; Zarocostas, 2018). In the following review, we examine how these agents evade host antibodies (Abs) based on their biological and evolutionary properties, with the spotlight on human picornaviruses.

CLASSIFICATION AND CLINICAL IMPACT ON HUMAN HEALTH

Picornaviridae is a large family of vertebrate viruses that produce both clinically asymptomatic infections but often mild and fatal disease. Their current classification includes more than 30 genera and 75 species (Zell et al., 2017), including several human picornaviruses. The genus *Enterovirus* comprises seven species infecting humans (enterovirus A-to-D and rhinovirus A-to-C). This genus contains poliovirus (PV), coxsackieviruses A/B (CVA/B), enteroviruses (EV), echoviruses (E), and rhinoviruses (RV). Further serological classification results in hundreds of serotypes. Hepatitis A virus (HAV) is the sole human-virus species in the genus *Hepatovirus*. Other human picornaviruses include members of the genera *Cardiovirus* (Saffold virus—SAFV), *Cosavirus* (CoSV), *Parechovirus* (Ljungan virus—LV), *Kubovirus* (Aichi virus—AiV), and *Salivirus* (Salivirus A—SaVA) (Nielsen et al., 2013).

RVs are airborne pathogens, while other enteroviruses and HAV use the fecal–oral route (Yin-Murphy and Almond, 1996). RVs cause the common cold, the most prevalent infectious disease worldwide, resulting in uncountable lost days from school and work. Epidemics occur yearly with outbreaks in the winter and spring (Drysdale et al., 2017). PV infection targets the central nervous system, destroying nerves and motor neurons, resulting in paralytic poliomyelitis. Until the PV worldwide eradication program based on global vaccination, polio epidemics have been the cause of high morbimortality (Minor, 2014). The so-called "non-polio enteroviruses" (CVs, EVs, and Es) cause several diseases with high morbimortality including meningitis, myocarditis, poliomyelitis-like syndrome, pancreatitis, and possibly the onset of diabetes. Outbreaks are common and have been considered to have pandemic potential (Zhang et al., 2015; Pons-Salort et al., 2018). Hepatitis produced by HAV is a mild disease producing liver damage usually leading to total recovery, but rare, severe cases are fatal in older age individuals. HAV produces large outbreaks probably due to its long 2 to 3 week incubation time (Jacobsen and Wiersma, 2010).

GENOME ORGANIZATION

Picornaviruses have single-stranded RNA positive-sense genomes (∼7–9 kb) that serve as mRNA for viral protein synthesis (Baltimore, 1971) (**Figure 1A**). Their RNA holds a single ORF encoding a polyprotein precursor for all viral proteins. Importantly, the 5′-end of the genome is covalently bound to the Viral-Protein-genome (VPg) (Crawford and Baltimore, 1983), the primer for viral RNA synthesis (Nomoto et al., 1977). Two untranslated regions (UTR), 5′UTR and 3′UTR, flank the ORF and contain virus-specific RNA secondary structural elements implicated in replication and providing host specificity (Kloc et al., 2018). The 5′UTR bears an internal ribosomal entry site (IRES) and polypyrimidine tract (PPT) that elicit host ribosomes and PPT-binding protein for translation (Martinez-Salas et al., 2018). The 3′UTR finishes in a poly(A) tail that mimics host mRNA tail conferring genome stability.

From 5′-to-3′, the ORF comprises three regions: (i) P1, encoding the structural capsid viral proteins (VP4–VP2–VP3–VP1), while, in some picornaviruses, also codifying a short leader L-protein; (ii) P2, encoding the viral non-structural proteins 2A−2B−2C; and (iii) P3 encoding the viral non-structural proteins 3A−3B−3C−3D (Palmenberg, 1990) (**Figure 1B**). The non-structural proteins' central role is replication, translation, and hijacking host-cell machinery (**Figure 2**). In particular, 3D is the RNA-dependent RNA polymerase (RdRp or 3Dpol) that synthesizes the virus genome and 3B (which is VPg) acts as its primer, being the only non-structural protein in the virion (Palmenberg, 1990).

PICORNAVIRUS CAPSID ANATOMY

Picornaviruses were the first human viruses to be structurally defined at the atomic level (Rossmann et al., 1985). To date, several structures of human picornaviruses have been unveiled including HRV, PV, HAV, CVB, E, CVA, EV, SAFV, and AiV (Hogle et al., 1985; Rossmann et al., 1985; Filman et al., 1989, 1998; Muckelbauer et al., 1995; Zhao et al., 1996; Lentz et al., 1997; Hendry et al., 1999; Verdaguer et al., 2000, 2003; Stuart et al., 2002; Zhang et al., 2004; Venkataraman et al., 2008; Plevka et al., 2010, 2012; Zocher et al., 2014; Liu et al., 2015, 2016; Ren et al., 2015; Zhu et al., 2015; Mullapudi et al., 2016). Moreover, the structure of FMDV has been disclosed (Logan et al., 1993; Lea et al., 1994). All picornaviruses have a naked 30 nm icosahedral capsid composed of 60 identical tightly packed protomers (**Figure 1E**). Early in particle morphogenesis, immature protomers contain VP1 and VP3 together with VP0, the precursor of VP4 and VP2 (Jiang et al., 2014) (**Figure 1B**). Virus assembly likely goes through a dodecahedral pathway (Li et al., 2012), by the association of pentamers formed by five immature protomers leading to the icosahedral particle, defining 5-, 3-, and 2-fold symmetry axes. Upon genome encapsidation, VP0 is generally auto-catalytically cleaved into VP2 and VP4 generating the mature capsid (except for *Parechovirus* and *Kubovirus*) (**Figure 2**).

The larger proteins (VP1–3) form the external and internal capsid surface. These proteins have a common fold, the "jelly-roll," formed by two 4-strand anti-parallel β-sheets and two helices (**Figure 1D**). Conversely, the small VP4 is located inside the capsid only and usually appears myristoylated at its N-terminus (Paul and Schultz, 1987; Belsham et al., 1991) (**Figure 1C**). The capsid external surface displays a rugged topography. Main surface features include (i) a principal protrusion built by the interaction of copies of VP1 forming a star-shaped 5-fold vertex, (ii) a 5-fold surrounding valley called the "canyon," (iii) a VP2 loop protuberance or the "puff," (iv) a VP3 loop rise or the "knob," and (v) a large 2-fold depression (Muckelbauer et al., 1995). Loop differences result in distinctive surface traits between picornaviruses. Finally, some picornaviruses exhibit a lipid molecule, the "pocket factor," bound

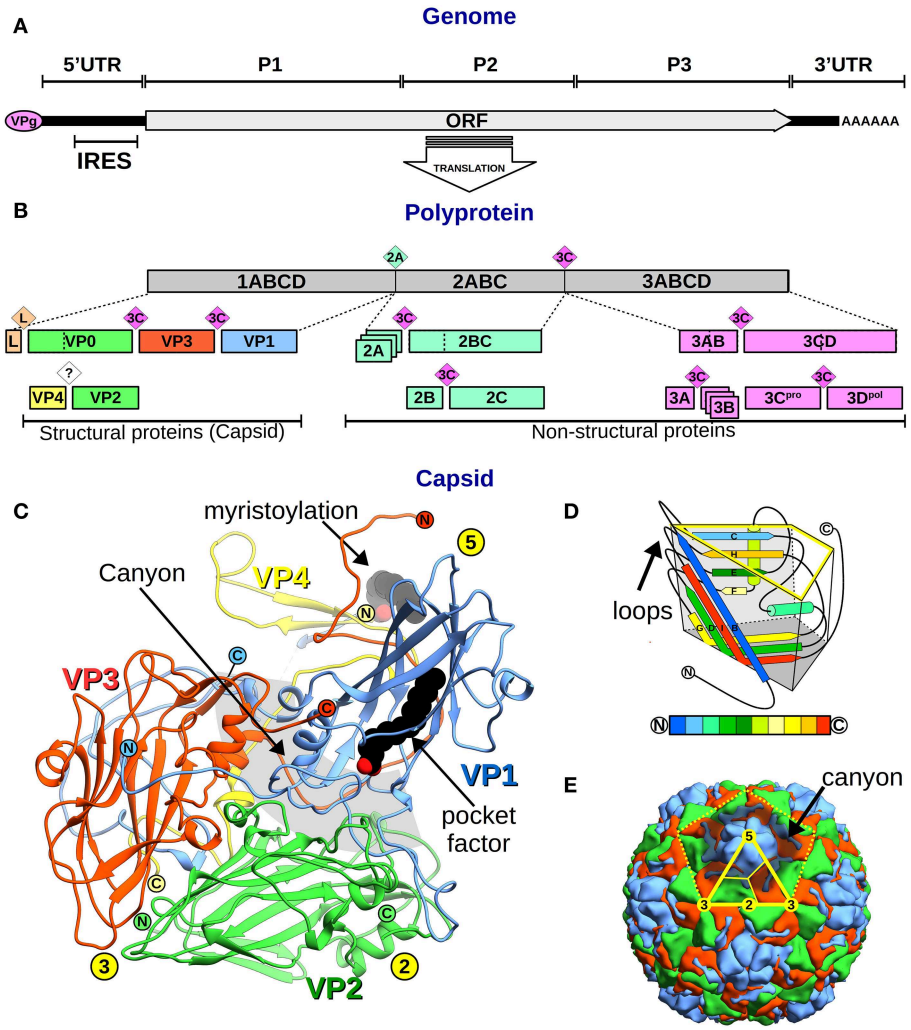

FIGURE 1 | Picornavirus genome, proteins, and capsid organization. **(A)** Representation of the picornavirus genome, the VPg, and the polyA tail, showing the single ORF location. The position of the P1–3 regions, the flanking 5′ and 3′UTR, and the IRES are indicated. **(B)** A bar diagram showing the polyprotein (gray box) and the proteolytic cascade that leads to all picornaviral proteins (colored boxes). Boxes include the protein names following the genome-ORF regions' nomenclature (number–letters) or the VP1–4 nomenclature for the structural proteins. Colored rhombi indicate cleavage points and are labeled with the corresponding protease name. **(C)** Overall view of the canonical picornavirus protomer with the proteins VP1 (blue), VP2 (green), VP3 (red), and VP4 (yellow). The protein N- and C-termini are indicated as encircled N and C letters, and yellow circles show the 5-,–3, 2-fold symmetry axes positions. Lipid components as the VP4 myristoylation and the "pocket factor" are depicted as black spheres. The "canyon" region is shown as a gray circular segment shadow. **(D)** Schematics of the "jelly roll" fold of VP1–3 proteins inscribed in a trapezoidal prism where the yellow highlighted face corresponds to the external capsid surface, and the dark gray base faces the inner capsid. The secondary structure elements are colored from N- to C-terminus according to the color code bar below. External loops and N- and C-terminus are indicated. **(E)** Overall view of the picornavirus capsid showing the outer surface of VP1 (blue), VP2 (green), and VP3 (red). The yellow dotted line indicates the boundaries of one pentamer. The solid yellow line marks the icosahedral asymmetric subunit and thinner lines separate proteins following the trapezoidal schematics shown in **(D)**. Symmetry 5-, 3-, 2-fold symmetry axes are indicated in yellow circles.

in a cavity located inside the VP1 jelly-roll, which has been observed to play a role in particle stability (**Figure 1C**).

RECEPTORS AND TROPISM

Picornavirus cell infection starts with its attachment to cell receptors (**Figure 2**). Therefore, virus-receptor usage is critical for tropism and its evolution can change virus targets at the level of cells to host ranges. The capsid binding sites of picornavirus receptors can be used to classify them into canyon binders and non-canyon binders.

Canyon binders are members of the immunoglobulin superfamily including: (i) ICAM-1 used by HRV and CVA (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989; Kolatkar et al., 1999; Xiao et al., 2001; Baggen et al., 2018), (ii) the PV receptor (PVR) (Mendelsohn et al., 1989; He et al., 2000; Strauss et al., 2015), (iii) the coxsackie and

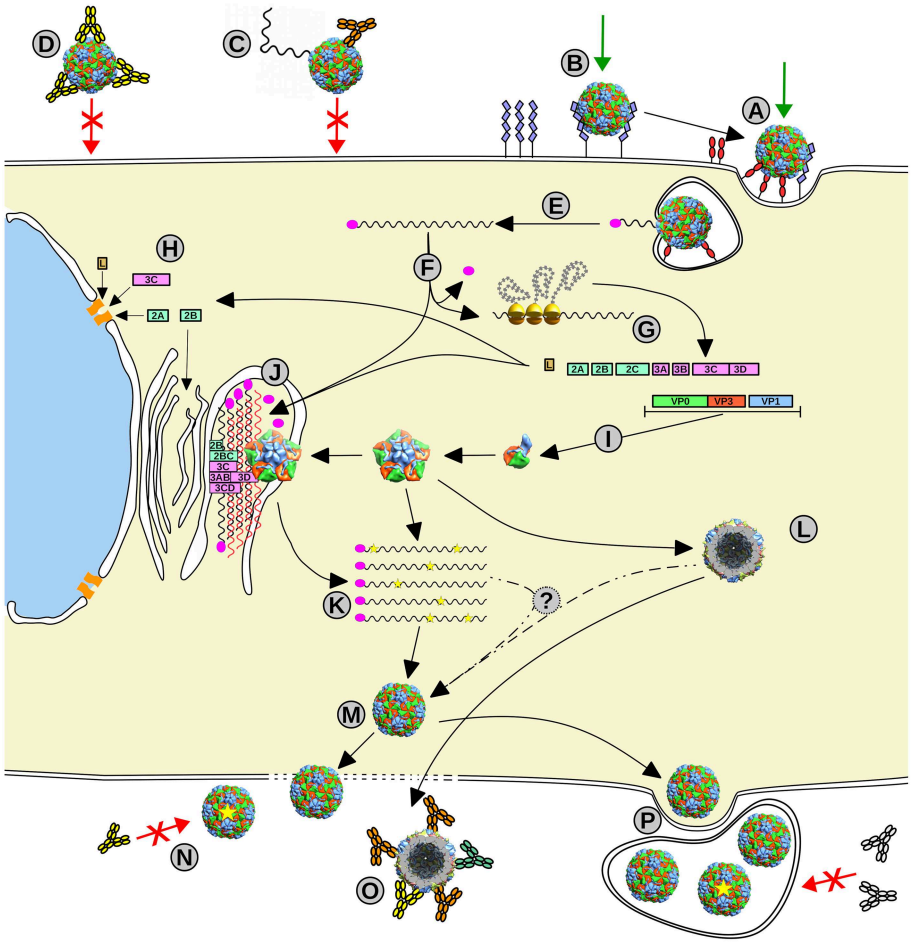

FIGURE 2 | Picornavirus life cycle. (A, B) Picornavirus uses different receptors to enter the cell, some implicated in the signaling internalization (A), meanwhile others can act as carriers that transport the viral particle to meet the primary receptor (B). (C, D) This infection event can be impeded by the action of specific neutralizing antibodies that can destabilize the viral particle (C) or opsonize or stabilize the particle to impair receptor binding or conformational changes required for infection (D). (E) Once the virus enters the cell, the viral RNA delivery mechanism is triggered, and the viral genome (black wavy line) is released into the cytoplasm. (F) Upon removal of VPg (magenta oval), the genome starts the IRES-driven translation leading to the production of the viral polyprotein. (G) The proteolytic cascade produces all viral proteins, structural and non-structural. (H) Some proteins act by hijacking the host cellular systems such as the nuclear pore, the cell translation machinery, and the apoptotic systems and initiate the remodeling of the internal cell membranes. (I) The structural proteins assemble into the capsid intermediates, the protomer and the pentamer, and also procapsids (L). (J) The formed replication complex assembled from non-structural proteins and modified internal membranes firing the picornaviral genome replication by the 3D polymerase *via* RNA complementary (red wavy lines) and using VPg as a primer. (K) The new progeny genomes including eventual mutations (yellow stars). (M) Mature virions assemble from pentamers that surround and package the new viral genomes. Viral particles escape from the cell by cell lysis or budding within membranes that can protect the viral progeny (P). (N) Some progeny virus with mutations in their capsids (yellow star) may escape from to the action of specific NAbs. (O) Empty capsids can act as molecular decoys for Abs to protect the infecting particles from neutralization.

adenovirus receptor (CAR) used by CVBs (Bergelson, 1997; He et al., 2001; Organtini et al., 2014; Lee et al., 2016), (iv) $\alpha_v\beta_3$ and $\alpha_v\beta_6$ integrin used by some CVAs (Roivainen et al., 1994; Williams et al., 2004; Shakeel et al., 2013), and (v) the α2 subunit of VLA-2 used by E1 and E8 (Bergelson et al., 1992; Xing et al., 2004). Canyon binders' apical domain engages in the binding into the canyon, triggering conformational changes essential for infection, leading to the altered-particle (A-particle) conformational state (Greve et al., 1989; Xing et al., 2004; Xiao et al., 2005; Shakeel et al., 2013; Organtini et al., 2014; Strauss et al., 2015). These changes have been observed to depend on the number of binding events that stimulate the viral particle (Lee et al., 2016). Engagement of several receptors is known to bring the viral 5-fold vertex close to the cell membrane (Bubeck et al., 2005).

Non-canyon binders attach to the virus surface elsewhere outside the canyon, tethering the virus to the cell surface eventually signaling for virus internalization. Importantly, they are diverse in molecular characteristics. These receptors include (i) the LDL-receptor used by HRV-C (Verdaguer et al., 2004); (ii) the decay-accelerating factor (DAF), receptor of many echoviruses and CVBs (Bergelson et al., 1994, 1995; Pettigrew et al., 2006; Plevka et al., 2010; Pan et al., 2011; Yoder et al.,

2012); (iii) P-selectin glycoprotein ligand-1 (PSGL-1) used by EV71 (Nishimura et al., 2009); and (iv) scavenger receptor B2 (ScaRB2), a receptor of EV71 (Yamayoshi et al., 2009; Zhou et al., 2018). This group possibly includes heparan sulfate used by several enteroviruses (Escribano-Romero, 2004; Zautner et al., 2006; McLeish et al., 2012; Tan et al., 2013; Nishimura et al., 2015) and cadherin-related family member 3 (CDHR3) used by HRV-C (Watters and Palmenberg, 2018). Non-canyon binders rarely induce substantial conformational changes due to their interaction, and some may not be essential for infection since few mutations allow or limit their usage (Pan et al., 2011; McLeish et al., 2012; Lee et al., 2013). This alternative receptor usage can modify the infection mechanism, as seen in the case of CVB3 binding to DAF which signals the trafficking of the attached virus to tight junctions where the virus can meet CAR (Coyne and Bergelson, 2006).

POPULATION DYNAMICS AND GENETIC VARIABILITY

Picornaviruses display a great potential for adaptation and evolution, which is primarily dictated by their high mutation rate. Viral progenies are huge in population size and they have short generation times. Thus, the RNA virus population is a dynamic cloud of mutants where the average-consensus sequence of all variants represents the "genotype." The mutation rate is the number of genetic changes, such as point mutations, insertions, or deletions introduced during viral replication. The first mutation rate measurement on RNA viruses was reported for CVA, disclosing a value of 1 mutation every 10,000 nucleotides copied (Eggers and Tamm, 1965).

Natural selection may have shaped picornaviral mutation rates in response to extremely dynamic ecosystems (Elena and Sanjuán, 2005). Therefore, this natural low replicative fidelity results in populations that quickly adapt to unexpected changes in the environment, such as immune pressure (Andino and Domingo, 2015). These observations have been conceptualized in the light of quasispecies evolution (Domingo et al., 2012). This adaptive capacity can be impaired by altering viral mutation rates (Vignuzzi et al., 2006). Indeed, the first support of the role of replicative fidelity in viral pathogenesis was observed in picornaviruses. Two groups isolated the first antimutator variant of an RNA virus, by serially passaging PV in the presence of ribavirin (Pfeiffer and Kirkegaard, 2003; Vignuzzi et al., 2006). The resistant variant contained a 3Dpol single point mutant (G64S) relatively resistant to lethal mutagenesis leading to (i) populations with lower mutation rates, (ii) reduced genetic diversity, and (iii) attenuated phenotype in mice (Vignuzzi et al., 2006). Recently, it has been suggested that this attenuation could be partly an outcome of a decrease in the replication speed (Fitzsimmons et al., 2018). Moreover, genetic engineering of viral polymerases has also identified several low-fidelity variants, called mutator variants (Thompson et al., 2007; Gong and Peersen, 2010; Gnädig et al., 2012; Rozen-Gagnon et al., 2014). For instance, using FMDV as a model, low-fidelity variants were found to increase mutation frequencies and render these viruses more susceptible to mutagenesis (Xie et al., 2014). Moreover, the same residue in the 3Dpol of FMDV is responsible to increase or decrease fidelity (Rai et al., 2017). Thus, picornaviral mutator variants were proven to increase mutation frequencies, decrease viral fitness, and also display an attenuated phenotype. Ostensibly, picornavirus mutation rates have been tuned to be near an upper limit (Crotty et al., 2001) yet evading population extinction by the accumulation of deleterious mutations by harmonizing (i) genetic integrity, (ii) genetic diversity, and (iii) replicative speed.

In addition to the classical view of single virus infectious unit of picornaviruses, structures containing many viral genomes support the existence of collective infectious units (Sanjuán, 2017). Lipid vesicles have been observed in HAV and EV infections (Feng et al., 2013; Chen et al., 2015; Kirkegaard, 2017). This current evidence incorporates the vesicle release and transmission to the standard lytic release and transmission of free virions, opening the debate on the "social evolution" of picornaviruses. Social evolution has been proposed to reduce detrimental mutations and negative interactions among the individuals within the population with direct implications for viral evolution, genetic diversity, and viral fitness (Bordería et al., 2015).

RECOMBINATION

During infection, RNA virus genomes can interchange nucleotide sequences resulting in genetic variation by recombination resulting in unpredictable advantages (Simon-Loriere and Holmes, 2011). This phenomenon was discovered in cells co-infected with PV escape mutants, resistant to antisera and guanidine, resulting in recombinant infectious PV (Ledinko, 1963). Recombination is widespread at intra-typic and inter-typic levels (Lukashev, 2005, 2010), often preceding the emergence of novel evolutionary lineages of picornaviruses (McWilliam Leitch et al., 2009, 2010; Meijer et al., 2012). For instance, there is evidence about recombinants of Sabin-related polioviruses harboring homologous sequences of other species of enteroviruses (Arita et al., 2005; Combelas et al., 2011; Bessaud et al., 2016).

Interestingly, recombination-deficient variants of PV have been identified. These viruses carry amino acid changes in the in 3Dpol that reduce recombination without conferring other detectable replication deficiencies. These non-recombining viruses accumulate a higher number of detrimental mutations, presumably by an inability to purge deleterious mutations, and fewer beneficial mutations (Xiao et al., 2016, 2017). Lately, the combined approach of mathematical modeling and experimental evolution experiments have predicted the frequency of recombination of picornaviruses such as PV and EV71 (Stern et al., 2017; Woodman et al., 2018).

ANTIBODY RESPONSE AGAINST PICORNAVIRUS INFECTION

Innate immune response detects foreign RNA using sensing proteins such as RIG-I, MDA-5, and Toll-like Receptor-3. These mediators act in the early control of picornaviral infection (Slater

et al., 2010). Nevertheless, the adaptive response mediated by Abs plays the definitive role in the resolution of the infection. Several pieces of evidence support this view as (i) patients with agammaglobulinemia develop chronic infections (Wilfert et al., 1977; McKinney et al., 1987; Kainulainen et al., 2010; Bucciol et al., 2018), (ii) humoral response mediates the protective effect of picornavirus vaccines (Grant et al., 2017; Sun et al., 2017), (iii) mice lacking B-cells cannot clear enteroviral infections (Mena et al., 1999), and (iv) passive immunization with Abs is an efficient treatment of HAV infection (Stapleton, 1992), although their effectiveness for enteroviral neonatal infections is disputed (Yen et al., 2015). Therefore, it appears to be clear that an effective humoral immune response represents the final host weapon to shortcut the viral infection.

ANTIBODY ESCAPE MUTANTS

Picornaviruses' high mutation rates permit the rapid escape from the immune system. The change of residues in the exterior capsid surface overcomes the intense pressure exerted by host Abs. These properties were used to locate capsid antigenic sites by testing neutralizing Abs (nAbs) escape mutants *in vitro* (Minor et al., 1986; Sherry et al., 1986; Stapleton and Lemon, 1987). Successful progeny virus rely on capsid functionality. Therefore, the preservation of the architecture and receptor binding restrict viable mutations. The exposed jelly-roll loops can accommodate mutations easier than secondary structure elements and protein–protein interacting surfaces (Murray et al., 1988; Usherwood and Nash, 1995). Several structural studies by cryoEM revealed the way nAbs bind to solvent-exposed loops of VP1–3 by interacting with critical residues. Complexes of viruses with Fab-Abs fragments can display 1-Fab:1-protomer ratio following the icosahedral symmetry. Nevertheless, when epitopes are close to the symmetry axis, lower binding ratios are observed due to Fab–Fab steric hindrance (Lee et al., 2013). Three mechanisms of neutralization are interpreted from these structures: (i) destabilization of the virion by triggering conformational changes upon Fab binding, which is accompanied by the "pocket factor" release when present (Smith et al., 1996; Plevka et al., 2014; Dong et al., 2017; Zheng et al., 2019), (ii) stabilization of virions by cross-linking of protomers to prevent conformational changes for infection (Ye et al., 2016), (iii) virus aggregation by antibody cross-linking particles (Mosser et al., 1989), and (iv) opsonization that can interfere with virus-cell attachment and receptor binding (Lee et al., 2013; Wang et al., 2017).

THE CANYON HYPOTHESIS

Hiding the receptor-interacting surface from Abs surveillance is the hypothetical function of the canyon (Rossmann, 1989). Therefore, the canyon is the result of an evolutionary process to preserve and protect the critical residues required for host-cell receptor recognition. Conversely, accessible areas can mutate to disguise the virus from the humoral immune response. Nevertheless, some observations have challenged this view, proposing the receptor-binding site topology as an uncoating mechanism that dictates receptor binding to trigger the uncoating event (Smith et al., 1996). These views are not strictly incompatible; hence, both pictures contribute to the paradigm of picornavirus capsid evolution. Here, capsid topology arose out of and continues to be shaped by the interplay of host environmental pressure, random genome mutations, and fixation of mutations when beneficial.

PICORNAVIRUS ANTIBODY DECOY PARTICLES

Picornaviruses are known to produce a significant amount of procapsids during the infectious cell cycle, which appears as an inefficient way to replicate (Shingler et al., 2015). This wastefulness looks aggravated considering each polyprotein translation event would lead to a single protomer. Finally, the so-called procapsid may be an off-pathway particle (Cifuente et al., 2013). Although counterintuitive in appearance, the function of the procapsid could be to act as an immune decoy to enhance the infectivity of mature virions providing an evolutive advantage (Shingler et al., 2015; Liu et al., 2016). In this regard, empty Dane particles also have been proposed to be decoy particles for the hepatitis B virus (Rydell et al., 2017).

Several picornavirus procapsids are larger particles compared to the mature virion and similar in shape to the A-particle. Procapsids have some viral epitopes more accessible and consequently can bind nAbs more efficiently. This phenomenon has been observed for the procapsid and mature virion of EV71, revealing that they are antigenically distinct (Shingler et al., 2015). Moreover, a non-nAb has been structurally solved in complex with procapsids but not disclosed for the mature virion (Hewat and Blaas, 2006), which suggests that procapsid may also lead to effects in the modulation of non-nAbs immune response.

VACCINES

Inactivated vaccines for PV, HAV, and FMDV are shown to prevent associated illnesses by inducing specific antibody defenses (Salk, 1957). Nevertheless, it was the live-attenuated oral PV vaccine (OPV) responsible for most of the success in controlling polio epidemics. PV attenuation was obtained by serial passage in cell lines from non-human hosts (Sabin, 1965). Rare cases of vaccination-derived paralytic disease can occur as well as vaccines shedding of virulent poliovirus revertant (Platt et al., 2014).

New ideas for picornavirus vaccines, currently under development, exploit evolutionary concepts including (i) codon usage, (ii) mutational robustness, (iii) modification of translational efficiency, and (iv) RdRp fidelity manipulation (Burns et al., 2006; Mueller et al., 2006; Coleman et al., 2008; Le Nouën et al., 2014; Tulloch et al., 2014; McDonald et al., 2016). In this regard, synonymous codon deoptimization was first applied for PV (Burns et al., 2006; Mueller et al., 2006) and other picornaviruses such as FMDV (Diaz-San Segundo et al., 2016). Interestingly, increasing the CpG and UpA dinucleotide frequencies using synonymous codon substitutions leads to

increased activation of the immune system (Tulloch et al., 2014). The maintenance of phenotype despite changing the genotype (mutations), or mutational robustness, can be altered by codon substitutions, correlating less robust viruses with attenuation in mice (Lauring et al., 2012). This approach was further extended using synonymous codons that upon mutation became stop codons (Moratorio et al., 2017). The increase in stop codon mutations in codon-engineered CVB3 during replication led to a loss of infectivity *in vitro* and attenuation *in vivo*. These new methods can improve the safety of already existing live-attenuated vaccines and can be broadly applied by re-coding any viral genome (Jorge et al., 2015).

Further elucidation of the mechanisms underlying these phenotypes could be used for rational codon rewiring in combination with increasing CpG and UpA frequencies to activate innate host immunity (Kumagai et al., 2008; Tulloch et al., 2014).

FINAL REMARKS

The control and eradication of pathogenic picornaviruses is an ongoing problem. Picornavirus evolution, ruled by high mutation rates and recombination, has made this viral group genetically and antigenically highly variable. Moreover, changes in virulence represent an unpredictable threat. Important lessons drawn from the polio eradication battle indicate the necessity of a new generation of vaccines (Agol et al., 2016). A holistic approach based on big data and mathematical modeling combining views from structural and evolutionary biology, cellular, and molecular immunology will allow a better understanding of picornavirus–host interactions. This knowledge could have the potential to foresee possible outbreaks and changes on viral virulence. Furthermore, these models may redefine the way new vaccines and antiviral therapies will be designed.

AUTHOR CONTRIBUTIONS

JC and GM conceived the review project, designed figures, wrote the manuscirpt, and approved it for publication. They both contributed equally to the work.

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REVIEW
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Strategies for Success. Viral Infections and Membraneless Organelles

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Regulation of RNA homeostasis or "RNAstasis" is a central step in eukaryotic gene expression. From transcription to decay, cellular messenger RNAs (mRNAs) associate with specific proteins in order to regulate their entire cycle, including mRNA localization, translation and degradation, among others. The best characterized of such RNA-protein complexes, today named membraneless organelles, are Stress Granules (SGs) and Processing Bodies (PBs) which are involved in RNA storage and RNA decay/storage, respectively. Given that SGs and PBs are generally associated with repression of gene expression, viruses have evolved different mechanisms to counteract their assembly or to use them in their favor to successfully replicate within the host environment. In this review we summarize the current knowledge about the viral regulation of SGs and PBs, which could be a potential novel target for the development of broad-spectrum antiviral therapies.

Keywords: RNAstasis, RNA granules, membraneless organelles, stress granules, P-Bodies, anti-viral host immune response

INTRODUCTION

RNA plays key roles in all biological systems where RNAstasis is a *central processing unit* in the regulation of gene expression in eukaryotic cells (Sharp, 2009). RNAstasis include synthesis, modification, protection, storage, release, transportation and degradation of different types of RNA (mRNA, tRNA, rRNA, siRNA, miRNA, lncRNA, piRNA, snRNA, snoRNA, smRNA) and metabolic processes mediated by RNA–protein complexes called RNA granules. Depending on its localization, RNA granules are found in the nucleus, in the nucleolus, paraspeckles, nuclear speckles and Cajal bodies; or in the cytoplasm, as stress granules (SGs) and processing bodies (PBs). All are membraneless organelles (i.e., lack an enclosing membrane, MLOs) to allow for rapid exchange of components with the surrounding cellular environment (Fay and Anderson, 2018). MLOs contain a heterogeneous mixture of nucleic acids and proteins that present low-complexity regions (LCRs) and intrinsically disordered regions (IDRs) regulated by post-translational modifications (Ramaswami et al., 2013; Panas et al., 2016; Wheeler et al., 2016). MLO biogenesis has been shown to be via liquid–liquid phase separation (LLPS) process, supporting the high flexibility and quick adaptive responses to environmental stresses required for function (reviewed in Fay and Anderson, 2018).

After several rounds of translation, an mRNA undergoes degradation as a way of turnover. Indeed, it is suggested that mRNA degradation is tightly dependent on translation (Bicknell and Ricci, 2017).

However, under conditions of cellular stress, the cell responds by mounting a robust response causing the shutoff of protein synthesis in order to protect the mRNA so that translation can resume once the stress disappears. Repression of gene expression induces the assembly of RNA granules such as SGs and PBs, which are involved in mRNA triage and untranslated mRNA storage, respectively. By using single mRNA imaging in living human cells, it has been recently reported that a single mRNA can interact with both SGs and PBs (Wilbertz et al., 2018; Moon et al., 2019). However, while Wilbertz et al. showed that an mRNA preferably moves from a SG to a PB, Moon et al. showed a dynamic and bidirectional exchange of a single mRNA to multiple SGs and PBs (Wilbertz et al., 2018; Moon et al., 2019). Despite their distinctive organization and unique molecular markers, SGs and PBs share molecular components which could allow the dynamic shuttling of an mRNA between them (Kedersha et al., 2005).

Viral infections are a major trigger of cellular stress and, thus, viruses have evolved diverse mechanisms aimed to modulate host RNAstasis with a direct impact in the assembly of different RNA granules while counteracting mRNA decay machineries in order to ensure viral replication (Poblete-Durán et al., 2016; Toro-Ascuy et al., 2016). In this review, we provide an update on the current knowledge of the different strategies used by several virus families to modulate the RNA granules assembly/disassembly, specifically SGs and PBs, in order to promote a successful viral infection (see **Figure 1**).

VIRAL FAMILIES AND STRESS GRANULES

SGs are translationally silent membraneless organelles with a diameter between 0.1 and 4 μm. Canonical or *bona fide* SGs contain mRNA, RNA-binding proteins, and components of the 40S ribosomal subunit. Many proteins involved in SG assembly are RNA binding proteins that favor mRNA stability (TIA-1, TIAR, HuR), mRNA metabolism (G3BP-1, G3BP-2, DDX6, SMN, Staufen1, DHX36, Caprin1, ZBP1, HDAC6, ADAR), signaling proteins (mTOR, RACK1) and interferon-stimulated gene (ISG) products (PKR, ADAR1, RIG-I, RNase L, and OAS (reviewed in Poblete-Durán et al., 2016). Recently, Nunes et al. generated an open access electronic resource containing all SGs-recruited protein reported to date (available at https://msgp.pt/) (Nunes et al., 2019). Its assembly is typically a consequence of translation repression upon phosphorylation of the translation initiation factor eIF2α by environmental stress such as heat shock, UV irradiation, oxidative stress, viral infection, and even upon treatment with several drugs (see **Table 1**). Most of these stresses are sensed by the eIF2α kinases PKR, which is activated by double-stranded RNA during viral infection (Williams, 2001); PERK, which is activated upon accumulation of misfolded protein in the ER and during hypoxia (Harding et al., 2000); HRI, which is activated by oxidative stress and heme deprivation (Han et al., 2001); and GCN2, which is activated by aminoacid deprivation and UV irradiation (Jiang and Wek, 2005). However, SGs can also be formed by inhibitors of translation that target other components of the translation machinery (**Table 1**) or by overexpression of SG-associated proteins such as TIA-1/TIAR or G3BP-1 (Kedersha et al., 1999; Tourrière et al., 2003). In addition to its role in mRNA triage, SGs have been described as signaling centers. Recruitment of signaling proteins to SGs allow the crosstalk between multiple stress cascades including translational control pathways, prevention of apoptosis and innate immune responses against viral infections (reviewed in Kedersha et al., 2011; Onomoto et al., 2014; Mahboubi and Stochaj, 2017).

Here, we summarize how viruses modulate SG accumulation in order to maintain viral protein synthesis and particles production.

Double-Stranded DNA (dsDNA) Viruses

Herpesviridae

All members of the *Herpesviridae* family that have been studied prevent the accumulation of SGs. Herpes simplex virus type 1 (HSV-1) infection upregulates and relocalizes to the cytoplasm the SG components TTP, TIAR, and TIA-1 but does not induce SG assembly (Esclatine et al., 2004). The virion host shutoff (vhs) protein, an mRNA endonuclease, has been shown to be essential in SGs blockade as vhs-deficient HSV-1 (Δvhs) infected cells do trigger SG assembly (Esclatine et al., 2004; Dauber et al., 2011, 2016). HSV vhs is thought to facilitate viral mRNA translation throughout the viral cycle by reducing host mRNAs and preventing viral mRNA overload (Dauber et al., 2016). Δvhs-induced SGs accumulation correlates with increased PKR activation (Sciortino et al., 2013; Dauber et al., 2016; Burgess and Mohr, 2018), but while a group observed higher eIF2α phosphorylation (Pasieka et al., 2008; Burgess and Mohr, 2018), others did not (Dauber et al., 2011, 2016). This phenotype could be in part due the reduced levels of the late-expressed dsRNA binding protein Us11, that has been shown to block PKR activation (Mulvey et al., 2003; Dauber et al., 2011). Burgess and Mohr showed that dsRNA accumulates and partially localizes to Δvhs-induced SGs (Burgess and Mohr, 2018). Furthermore, they show that SGs are not assembled neither PKR is phosphorylated in Δvhs-infected cells upon treatment with ISRIB (see **Table 1**) or in absence of G3BP-1 or TIA-1. Based on these observations, the authors suggest that Δvhs-enhanced PKR activation is a consequence of SG assembly due to dsRNA accumulation (Burgess and Mohr, 2018). Interestingly, other HSV proteins have also been involved in SGs regulation, although it is not clear whether they all act cooperatively, or during different stages of the viral cycle. HSV-1 ICP27 has been shown to prevent formation of arsenite-induced SGs by inhibiting PKR and eIF2α phosphorylation (Sharma et al., 2017). On the other hand, overexpression of HSV-1 ICP8 protein, a G3BP binding partner, blocks arsenite-induced SG assembly (Panas et al., 2015). Similar to HSV-1, SGs do not assemble during herpes simplex virus 2 (HSV-2) infection and its blockade is mediated by the vhs protein (Finnen et al., 2012; Dauber et al., 2014). HSV-2 vhs protein

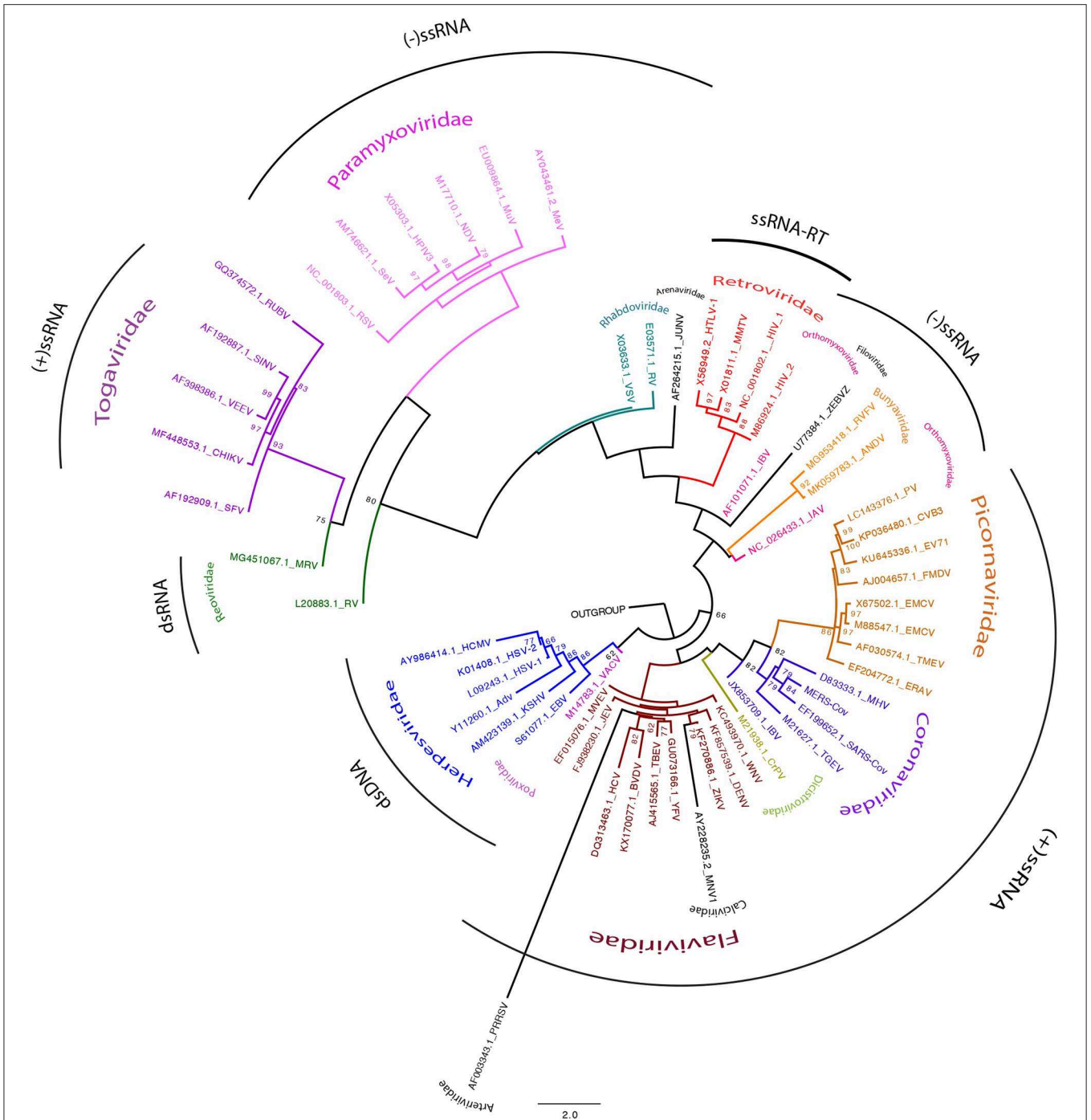

FIGURE 1 | Viral families tree. Phylogenetic tree showing 56 sequences representing all viral families described to modulate RNA granules assembly. The chosen sequences were "gene encoding to superficies structural protein." The sequences were selected from NCBI databases (https://www.ncbi.nlm.nih.gov/nuccore/). Alignment were performed by MUSCLE (http://www.drive5.com/muscle/) (Edgar, 2004). Phylogenetic tree was constructed with MEGA6 (http://www.megasoftware.net) and IQ-TREE on the IQ-TREE web server (http://www.cibiv.at/software/iqtree/) (Trifinopoulos et al., 2016) by using the maximum-likelihood (ML) method. Robustness of tree topologies was assessed with 1,000 bootstrap replicates. Phylogenetic tree was constructed using ML inference with the general time reversible (GTR)_G nucleotide substitution model. Viral families are showed in different colors. Genomes by clade are grouped by black arch.

has also been shown to shutoff protein synthesis by depleting mRNAs (Smith et al., 2000). Wild Type (WT) HSV-2 impairs arsenite-induced SGs despite increased eIF2α phosphorylation, but not pateamine (eIF2α-independent)-induced SGs, indicating that vhs can disrupt or modify SGs independently of eIF2α phosphorylation (Finnen et al., 2012). Further investigation

TABLE 1 | List of drugs/stressors used to induce or disassemble SGs and PBs.

Class	Drug/stressor	Effect	Mechanism	References
	Pateamine-A	Induces SG assembly	Interacts with eIF4A disrupting the eIF4F complex	Bordeleau et al., 2005; Kedersha et al., 2006
	Hippuristanol	Induces SG assembly	Inhibits eIF4A RNA binding activity	Bordeleau et al., 2006; Mazroui et al., 2006
Translation inhibitors	Cycloheximide	Disassembles both SGs and PBs	Inhibits eEF2-mediated translation elongation	Obrig et al., 1971; Mollet et al., 2008
	Selenite	Induces non-canonical SG assembly	Enhances 4EBP-1 binding to eIF4E, thus disrupting the eIF4F complex	Fujimura et al., 2012
	Sorbitol	Induces SG assembly	Causes osmotic stress, which enhances 4EBP-1 binding to eIF4E, thus disrupting the eIF4F complex	Patel et al., 2002
	Arsenite	Induces SG and PB assembly	Induces HRI-mediated eIF2α phosphorylation*	McEwen et al., 2005
	Dithiothreitol (DTT)	Induces SG assembly	Induces PERK-mediated eIF2α phosphorylation	Oslowski and Urano, 2011; Dimasi et al., 2017
	Heat-Shock	Induces SG assembly and inhibits PBs	Induces HRI-mediated eIF2α phosphorylation	McEwen et al., 2005; Aulas et al., 2017
eIF2α kinases Stressors	Poly I:C	Induces SG assembly	Induces PKR-mediated eIF2α phosphorylation	Weissbach and Scadden, 2012
	Bortezomib and MG132 (proteosome inhibitors)	Induce SG assembly	Induce HRI(Bortezomib)- and GCN2(MG132)-mediated eIF2α phosphorylation	Mazroui et al., 2007; Fournier et al., 2010
	Thapsigargin	Induces SG assembly	Induces PERK-mediated eIF2α phosphorylation	Kimball et al., 2002
eIF2α modulators	ISRIB	Inhibit SG assembly	Prevents eIF2B inhibition, maintaining translation initiation despite eIF2α phosphorylation	Sidrauski et al., 2015
	Salubrinal	Induces SG assembly	Blocks eIF2α dephosphorylation	Boyce et al., 2005
Others	1,6-Hexanediol	Disassembles and induces PB and SG assembly	Disrupt weak hydrophobic interactions causing quick disassembly of granules that reappear after a few minutes**	Wheeler et al., 2016; Kroschwald et al., 2017
	Zn^{+2}	Stress- inducible second messenger	Induces reversible multimerization, phase separation and SG recruitment of TIA-1	Rayman et al., 2018

Arsenite can also induce SG assembly independent of HRI. In Drosophila, which lacks HRI, induces the PEK eIF2a kinase (Farny et al., 2009). In addition, Sharma et al. demonstrated that arsenite can also induce phosphorylation of PKR and SG assembly in HeLa and BCBL-1 cells (Sharma et al., 2017).

**It has been shown to also alter many other cellular structures (Wheeler et al., 2016).*

revealed that (i) vhs localizes to SGs, (ii) vhs not only inhibits SG assembly but also disrupts pre-assembled SGs, and (iii) vhs endoribonuclease activity is required in SGs modulation (Finnen et al., 2016). Interestingly, TIA-1 was shown to egress before G3BP in the course of vhs-mediated SGs disassembly, which could be explained by the G3BP-enriched core SG structure (Jain et al., 2016; Niewidok et al., 2018). Based on these results, the authors proposed that HSV-2 vhs modify SGs by directly or indirectly degrading mRNA. Human cytomegalovirus (HCMV) inhibits the assembly of SGs but induces the unfolded protein response (UPR) (Isler et al., 2005a). Typically, this ER stress response leads to eIF2α phosphorylation via PERK activation, but HCMV limits eIF2α phosphorylation without diminishing PERK activation (Isler et al., 2005b). Marshall et al. showed that infection with HCMV lacking pTRS1 and pIRS1, dsRNA-binding proteins linked to PKR pathway inhibition, results in increased levels of eIF2α phosphorylation and the reduction of viral protein synthesis (Marshall et al., 2009). Both proteins have identical amino-terminal regions and share 35% of similarity in their carboxy-terminal regions, suggesting that HCMV pTRS1 and pIRS1 have redundant roles in evading dsRNA-mediated antiviral response. Ziehr et al. demonstrated that lack of both proteins also results in PKR activation and SG assembly, and that expression of either pTRS1 or pIRS1 is necessary and sufficient to prevent PKR activation, eIF2α phosphorylation and SG assembly (Ziehr et al., 2016). Furthermore, pTRS1 PKR binding domain (PDB) was shown to be critical to accomplish those three phenotypes suggesting that the main mechanism of HCMV to inhibit SG assembly is through PKR antagonism. Strikingly, pTRS1 transfection interferes with arsenite-induced SG assembly in WT and PKR-depleted cells, but pTRS1-ΔPBD does not, suggesting that pTRS1 could also obstruct SG assembly promoted by other eIF2α kinases and that its PDB is crucial for it. Kaposi's sarcoma-associated herpesvirus (KSHV) does not lead to SG accumulation via the viral protein ORF57 and SOX which are able to restrict arsenite-induced SG assembly independently (Sharma et al., 2017). ORF57, the HSV-1 ICP27 homologous, inhibits PKR/eIF2α phosphorylation by directly interacting with PKR via its N-terminal dsRNA-binding domain, and with PACT via its two N-terminal RNA-binding motifs, thus obstructing PKR binding to both dsRNA and PACT. The mechanism and the spatiotemporal regulation of SG assembly

by the viral shutoff exonuclease SOX is unclear, but it might be related to its intrinsic RNA endonuclease activity similarly to HSV-2 vhs (Glaunsinger and Ganem, 2004; Sharma et al., 2017). Expression of Epstein–Barr virus (EBV) protein EB2, the counterpart of KSHV ORF57 and HSV-1 ICP27, does not abolish SG assembly, neither PKR/eIF2α phosphorylation, indicating that this specific ability to regulate SGs is not conserved along herpesviruses (Sharma et al., 2017). Further research is necessary to define the effect of EBV on SG assembly.

Poxviridae

Unlike herpes viruses, Vaccinia virus (VACV), a member of *Poxviridae* family, exploits SG components to favor viral protein production (reviewed in Liem and Liu, 2016). VACV redistributes proteins from the host translation machinery and SGs, such as eIF4E, eIF4G, G3BP, and Caprin1 into viral replication factories (RFs) assembled in the cytoplasm of the host cell (Katsafanas and Moss, 2004, 2007). Notably, TIA-1 is not recruited into these replication foci (Walsh et al., 2008). How VACV redistributes each of these components remain unclear, but evidences have shown that VACV ssDNA-binding protein I3 associates and recruits eIF4G to ssDNA formed within the RFs (Zaborowska et al., 2012). Furthermore, it was shown that G3BP-1 and Caprin1 associate with nascent VACV DNA by mass spectrometry (Senkevich et al., 2017). Despite the disruption of canonical SGs for its own benefit, infection with the replication-defective VACV lacking E3L leads to the accumulation of granule-like structures around the RFs, named antiviral granules (AVGs), that arrest viral translation (Simpson-Holley et al., 2010). AVGs contain proteins that are typically found in SGs such as TIA-1, eIF3b, G3BP-1, and USP10, but they are not affected by cycloheximide, a drug that induce the disassembly of *bona fide* SGs (Simpson-Holley et al., 2010). AVG assembly requires eIF2α phosphorylation via PKR activation (Simpson-Holley et al., 2010; Pham et al., 2016), process that is inhibited in presence of E3L (Chang et al., 1992). Furthermore, TIA-1 is an essential component of AVGs, as in its absence these antiviral granules are not formed even if PKR and eIF2α are phosphorylated (Simpson-Holley et al., 2010). Interestingly, WT VACV infection also induces AVGs assembly that repress viral protein synthesis but to negligible levels (Rozelle et al., 2014). Recently, another mutant VACV lacking C7L/K1L was shown to induce AVG assembly (Liu and McFadden, 2014). AVGs accumulation was abolished and abortive infection was rescued in ΔC7L/K1L in SAMD9-depleted cells, suggesting that C7L/K1L antagonize SAMD9 host protein antiviral function. Even though SAMD9 localize to both ΔE3L and ΔC7L/K1L VACV induced-AVGs, infectivity neither AVG assembly is blocked with ΔE3L VACV in SAMD9-depleted cells, suggesting a different mode of organization of the granules induced by both mutants (Liu and McFadden, 2014). ΔC7L/K1L-dependent AVGs assembly is independent of eIF2α phosphorylation, in contrast to ΔE3L AVG accumulation (Liu and McFadden, 2014). Viral mRNA was shown to colocalize with AVGs during ΔC7L/K1L VACV infection, thus limiting translation of viral proteins (Sivan et al., 2018), as well as dsRNA, TIA-1 and the viral protein E3L (Meng and Xiang, 2019). Despite of that, TIA-1 is not required for ΔC7L/K1L-mediated AVG assembly as it is on ΔE3L (Meng and Xiang, 2019). The role of each viral system, E3L and C7L/K1L, to prevent formation of AVGs in the context of viral infection remain to be studied.

Double-Stranded RNA (dsRNA) Viruses

Reoviridae

Rotavirus replication, the prototypical member of the *Reoviridae* family, also occurs in viral replication factories and upon infection, synthesis of cellular proteins is reduced while viral protein production is maintained. Accumulation of viral dsRNA in the cytoplasm causes a persistent PKR-dependent eIF2α phosphorylation, even when eIF2α phosphorylation is not required for viral replication (Montero et al., 2008; Rojas et al., 2010). Despite that, rotavirus infection does not induce SG assembly; instead it changes the cellular localization of SG components (Montero et al., 2008). TIA-1 is relocalized to the cytoplasm, eIF4E distributes more homogeneously in the cytoplasm, and PABP is translocated to the nucleus through the viral protein NSP3 (Montero et al., 2008). Recently, Dhillon et al. determined that rotavirus remodels SGs by excluding some of their proteins, such as G3BP-1, hnRNP A1, and ZBP1, and then recruits these atypical granules to viral replication factories (Dhillon and Rao, 2018; Dhillon et al., 2018). It will be of interest to understand how rotavirus selectively excludes these specific SG components. In contrast, uncoating of the mammalian orthoreovirus (MRV) during the early stage of infection leads to eIF2α phosphorylation by the action of at least two eIF2 kinases, suggesting that MRV infection is a complex process that induces different types of stresses to the cell (Qin et al., 2009). Phosphorylation of eIF2α triggers SG accumulation (Qin et al., 2009). MRV cores are then recruited into the assembled SGs, a step that depends on synthesis of viral mRNA. As the infection proceeds, assembled SGs are disrupted in order to allow efficient synthesis of viral proteins, despite the sustained levels of eIF2α phosphorylation (Qin et al., 2011). Like rotavirus, MRV replication occurs in viral replication factories that grow in the perinuclear region (Rhim et al., 1962). The non-structural μNS viral protein associates with σNS, σ2, μ2, and λ1 are recruited into viral replication factories. μNS viral protein has been shown to localize with SGs, although is unable to independently prevent SGs accumulation (Carroll et al., 2014). Interestingly, the SG components G3BP-1, Caprin1, USP10, TIAR, TIA-1, and eIF3b were found to localize to the outer peripheries of viral replication factories (Choudhury et al., 2017). σNS and μNS were shown to be responsible for their redistribution as well as for disruption of the SG assembly. In addition, in the absence of G3BP-1 the other recruited SGs-associated protein, except for eIF3b, do not localize to RFs. The recruitment mode is thought to be as follows: Caprin1, USP10, TIAR, and TIA-1 interact with G3BP-1 which binds to σNS via its RNA recognition (RRM) and an arginine/glycine-rich (RGG) motifs. Then, σNS partner with μNS for RF localization, carrying all the other proteins with it (Choudhury et al., 2017).

Positive-Sense Single Stranded RNA ((+) ssRNA) Viruses

Picornaviridae

Members of the *Picornaviridae* family also modulate SGs accumulation during replication. Poliovirus (PV) regulates SGs in a time-dependent manner; at early times the 2A proteinase induces assembly of SGs (Mazroui et al., 2006; Chen et al., 2008) that are later disassembled by the 3C proteinase through G3BP-1 cleavage (White et al., 2007). Despite of *bona fide* SG disruption, atypical SGs (aSGs) that contain TIA-1 and viral RNA, but no eIF4G nor PABP, still accumulate later in the course of PV infection (Piotrowska et al., 2010; White and Lloyd, 2011). A similar temporal control of SG assembly is exhibited by Coxsakievirus B3 (CVB3) and Enterovirus 71 (EV71). CVB3 2A proteinase induces SG assembly as early as 3 h post infection (hpi) in an eIF2α phosphorylation-independent manner (Wu et al., 2014; Zhai et al., 2018). It has been described that they have an antiviral role, inhibiting the biosynthesis of CVB3 (Zhai et al., 2018). However, at 6 hpi CVB3 induces the assembly of granules that do not contain G3BP-1 or eIF4G, likely because of G3BP-1 cleavage (Fung et al., 2013; Zhai et al., 2018). In the case of EV71, canonical SGs are assembled early during infection dependent on the PKR-eIF2α pathway (Zhu et al., 2016), but are dispersed at late stages of infection (Yang et al., 2018b; Zhang et al., 2018). Yet, atypical SGs in which TIA-1, TIAR, Sam68, and viral RNA are persistently aggregated in an eIF2α independent and cycloheximide-resistant manner remain during infection. Yang et al. showed that EV71 2A protease expression is enough for atypical SGs induction through the cleavage of eIF4GI and *bona fide* SGs blockage by abolishing eF4GI-G3BP-1 interaction (Yang et al., 2018a,b). In contrast, Zhang et al. reported that EV71 3C protease alone is sufficient to inhibit canonical SGs accumulation during late stages of infection through G3BP-1 cleavage at amino acid Q326 (Zhang et al., 2018). Interestingly, cells infected with EV71-2AC110S (a cleavage-deficient 2A protease) do form canonical SGs in which viral RNA is aggregated, suggesting that EV71 blocks *bona fide* SGs but induce atypical SGs to facilitate viral translation by stalling only cellular mRNAs (Yang et al., 2018b; Zhang et al., 2018). Unlike already mentioned picornaviruses, encephalomyocarditis virus (EMCV) infection does not induce SG assembly at all, and cleavage of G3BP-1 is the mechanism for their disruption (Ng et al., 2013). On the other hand, the leader (L) protein of Theiler murine encephalomyelitis virus (TMEV) and mengovirus (a strain of EMCV) inhibit SG assembly without cleaving G3BP-1 (Borghese and Michiels, 2011; Langereis et al., 2013). A mutant mengovirus, in which the Zn-finger domain of L is disrupted, induces antiviral G3BP-1 aggregations in which Caprin-1 and PKR are recruited, resulting in PKR activation and viral replication inhibition (Langereis et al., 2013; Reineke et al., 2015). Foot and Mouth Disease Virus (FMDV) does not induce SG assembly despite strongly shutoff cap-dependent translation and G3BP-1 dephosphorylation at Ser-149 (Ye et al., 2018; Visser et al., 2019), suggesting that FMDV infection regulates the cellular stress response. In fact, G3BP-1, eIF4G, eIF3, and eIF2α protein levels are downregulated and eIF4E-BP and PKR are dephosphorylated during FMDV infection (Ye et al., 2018). Ye et al. showed that G3BP-1 cleavage by 3C protease impairs SG assembly (Ye et al., 2018) while Visser et al. argued that L protease catalytic activity is responsible for the impairment of SG assembly in infected cells, without affecting PKR signaling (White et al., 2007). In addition, Ye et al. reported that the 3C-induced cleavage of G3BP-1 inhibits the NF-κB-dependent induction of antiviral immune responses (Ye et al., 2018). By using a reporter system, it has been shown that G3BP-1 negatively regulates viral translation by interacting with a structure located at domain 4 of the viral IRES (Galan et al., 2017). Furthermore, the G3BP-1 S149A substitution impairs the negative effect of G3BP-1 on IRES translation, suggesting that G3BP-1 is an antiviral protein whose activity depends on its phosphorylation (Ye et al., 2018). Instead, FMDV induces the nuclear-to-cytoplasm translocation of Sam68 via a proteolytic cleavage of its C-terminal domain mediated by 3C protease (Lawrence et al., 2012). Interestingly, Sam68 and TIA-1 colocalize in transient cytoplasmic granule-like structures in infected cells. Moreover, Sam68 interacts with FMDV IRES and Sam68 knockdown leads to a reduction in virus production, suggesting that Sam68 is a proviral factor (Lawrence et al., 2012; Rai et al., 2015). Similar to FMDV, Equine Rhinitis A virus (ERAV) infection also disrupts SG assembly via L-protease mediated cleavage of G3BP-1 and G3BP-2, suggesting that this is a conserved mechanism among aphtoviruses. However, despite G3BP-1 cleavage at multiple positions during FMDV and ERAV infections, the products differ in molecular weight, suggesting that they do not induce identical cleavages of G3BP-1 (Visser et al., 2019).

Togaviridae

Among viruses of *Togaviridae* family, Chikungunya virus (CHIKV) is the only member know to block SG assembly. G3BP-1 is sequestered by nsP3 in cytoplasmic foci (Fros et al., 2012) while G3BP-2 colocalizes with nsP2/3 in complexes different from viral replication factories (Scholte et al., 2015). Recently, it has been shown that dsRNA foci, nsP3-like granules and nsP1-coated structures are in close proximity, suggesting that CHIKV not only sequesters G3BP-1/-2 proteins in order to impair SGs assembly, but also to support viral replication (Remenyi et al., 2018). CHIKV dsRNA was shown to be undetectable in G3BP-1/-2 double knock-out (dKO) cells, indicating that G3BPs play key roles in RNA replication and formation of viral replication complexes (Kim et al., 2016). In contrast, Venezuelan Equine Encephalitis virus (VEEV) replication is not affected by G3BP-1/-2 dKO (Kim et al., 2016), but the SG-associated proteins FXR1, FXR2, and FMR1 have been shown to be essential factors for VEEV replication and protein production. Interestingly, VEEV infected cells contain both large and small plasma membrane-bound FXR-nsP3 complexes containing viral genomic RNA, suggesting a role of FXRs in viral replication and protection of viral genomic RNA from degradation during transport to the plasma membrane (Kim et al., 2016). Semliki Forest Virus (SFV) induces SG assembly early during infection in an eIF2α phosphorylation-dependent manner (McInerney et al., 2005). Nevertheless, at late stages of infection nsP3

promotes SG disassembly by sequestering G3BP-1 to sites of viral replication, which correlates with an increase in viral RNA levels (McInerney et al., 2005; Panas et al., 2012). Consistently, infection with a non-G3BP-1 binding SFV promotes a persistent accumulation of SGs containing G3BP-1 and TIA-1, which correlates with an attenuation in viral infection (Panas et al., 2015). On the other hand, Sindbis virus (SINV)-derived vectors induce PKR activation and the subsequent assembly of SGs containing TIA-1, eIF4E, and eIF4G (Venticinque and Meruelo, 2010). Furthermore, viral nsP2, nsP3, and nsP4 colocalize with aggregates containing G3BP-1 (Frolova et al., 2006; Gorchakov et al., 2008; Cristea et al., 2010) while nsP3 also interacts with G3BP-2. In 2011, Mohankumar et al. revealed that SINV infection induces the phosphorylation of eIF2α which correlates with a strong shutoff of *de novo* protein synthesis and 4E-BP1 dephosphorylation. Moreover, the authors demonstrated that SINV replication does not require the PI3K/Akt/mTOR pathway, and that later during infection, SINV suppresses Akt/mTOR activation in HEK cells (Mohankumar et al., 2011). Similar to CHIKV, G3BP-1/-2 dKO significantly reduce SINV replication rates and plaque size. However, FXR1/2 and FMR1 triple knockout only induces a delay in viral particles production (Kim et al., 2016). Finally, it has been suggested that G3BP-1 plays a potential role in the encapsidation of Rubella virus (RUBV) due to the colocalization of RUBV genomic RNA, the non-structural viral protein P150 and G3BP-1 aggregates (Matthews and Frey, 2012).

Flaviviridae

West Nile virus (WNV), a member of the *Flaviviridae* family, was the first virus described to block SG assembly. The 3′ stem loop in the (–) RNA, which is the site of initiation for nascent genome RNA synthesis, captures the SG components TIA-1 and TIAR, suggesting that they have a role in viral replication (Li et al., 2002). In addition, TIA-1 and TIAR colocalize with viral replication complexes containing dsRNA and NS3 viral protein in the perinuclear region (Emara and Brinton, 2007). Although WT WNV impedes SGs assembly, the chimeric WNV W956IC induces PKR-dependent SG assembly due to the high levels of viral RNA that are produced (Courtney et al., 2012). Remarkably, WNV inhibits arsenite, but not heat shock, or DTT-induced SG assembly. High levels of GSH (antioxidant) has been shown to counteract arsenite-induced SGs, as during WNV infection even low levels of PERK-mediated eIF2α phosphorylation upregulate ATF4 and Nrf2, transcription factors that induce antioxidant gene expression (Basu et al., 2017). Similar to WNV, TIA-1, and TIAR colocalize with viral replication complexes containing dsRNA and NS3 in Dengue Virus type 2 (DENV-2) infected cells (Emara and Brinton, 2007). In addition, a quantitative mass spectrometry study revealed that DENV-2 RNA interacts with the SG components G3BP-1/2, Caprin1, and USP10 (Ward et al., 2011). It has been shown that DENV infection generates a non-coding subgenomic flaviviral RNA (sfRNA) that binds to G3BP-1/2 and Caprin1, impairing its ability to induce the translation of interferon Stimulated Genes (ISGs) mRNAs in response to DENV infection (Bidet et al., 2014). Recently, it has been described that Zika virus (ZIKV) infection blocks SG assembly (Amorim et al., 2017; Basu et al., 2017; Hou et al., 2017; Bonenfant et al., 2019) despite a strongly induced translational shutoff and activation of both PKR- and UPR-induced phosphorylation of eIF2α, suggesting that ZIKV impairs SG assembly downstream of eIF2α phosphorylation (Hou et al., 2017). In addition, ZIKV infection impairs arsenite-, poly I:C and hippuristanol, but not DTT-, Pateamine A- and Selenite-induced SG assembly (Amorim et al., 2017; Hou et al., 2017; Bonenfant et al., 2019) without affecting levels of SG-nucleating proteins (Amorim et al., 2017; Bonenfant et al., 2019). Hou et al. showed that expression of ZIKV NS3, NS4, NS2B-3 or capsid protein are sufficient to inhibit SG assembly (Hou et al., 2017). Interestingly, during ZIKV infection the host proteins YB-1 and Ataxin-2 are redistributed to the nucleus, while HuR and TIA-1 are redistributed to the cytoplasm of infected cells (Bonenfant et al., 2019). Moreover, TIAR is partially redistributed to sites of viral replication in the perinuclear zone, as seen on its colocalization with NS1 and viral RNA (Amorim et al., 2017). Furthermore, G3BP-1 and HuR are isolated with replication complexes, but only G3BP-1 interacts with viral dsRNA (Hou et al., 2017; Bonenfant et al., 2019). G3BP-1, Caprin-1, TIAR, Ataxin-2 and YB-1 knockdown negatively affects virus production, while HuR and TIA-1 knockdown resulted in an increase of viral titers (Hou et al., 2017; Bonenfant et al., 2019). Specifically, G3BP-1 knockdown also decreases genomic RNA and viral protein levels, while HuR knockdown increases genomic RNA and protein level (Bonenfant et al., 2019). Together, these data suggest a possible proviral role of the SG components G3BP-1, Caprin-1, TIAR, Ataxin-2, and YB-1 in ZIKV replication (Hou et al., 2017; Bonenfant et al., 2019). Similar to ZIKV, Japanese encephalitis virus (JEV), Murray Valley Encephalitis Virus (MVEV) and Yellow Fever Virus (YFV) capsid-expressing cells showed a significantly impairment in hippuristanol-induced SG assembly (Hou et al., 2017). Specifically, it has been shown that JEV core protein blocks SG assembly through an interaction with Caprin-1, resulting in the recruitment of other SG components such as G3BP-1 and USP10 (Katoh et al., 2013). A JEV virus carrying a non Caprin-1-binding core protein is less pathogenic in mice and exhibits lower propagation *in vitro* than WT virus, suggesting that SGs blockade is crucial to facilitate viral replication (Katoh et al., 2013). Analogous to WNV and DENV, Tick-Borne Encephalitis virus (TBEV) sequesters TIA-1 and TIAR to viral replication factories (Albornoz et al., 2014). In particular, TIA-1 binds viral RNA and acts as a negative regulator of TBEV translation, suggesting that TIA-1 function is independent of SG assembly (Albornoz et al., 2014). In addition, TBEV infection induces eIF2α-dependent SG assembly containing the canonical SGs markers G3BP-1, eIF3, and eIF4B (Albornoz et al., 2014). On the other hand, Bovine Viral Diarrhea virus (BVDV) impairs the assembly of arsenite-induced SGs and despite viral N-terminal protease (Npro) interaction with several SG components (such as YB-1, IGFBP2, DDX3, ILF2, and DXH9), this is not the mechanism by which BVDV blocks SG assembly (Jefferson et al., 2014). Hepatitis C virus (HCV) relocalizes G3BP-1, PABP1, ATX2, DDX3, TIA-1, and TIAR to viral replication factories in lipid droplets (LDs) (Ariumi et al., 2011; Garaigorta et al., 2012). In particular, DDX3 activates IKKα during HCV infection to induce LDs biogenesis

(Li et al., 2013). Importantly, Garaigorta et al. reported that G3BP-1, TIA-1, and TIAR are required for viral RNA and protein synthesis early during infection, while G3BP-1, DDX3, and TIA-1 play a role in viral particle assembly (Garaigorta et al., 2012; Pène et al., 2015; Valiente-Echeverría et al., 2015). In addition, they showed that HCV induces SG assembly in a PKR-dependent manner in order to impair the translation of antiviral ISGs (Garaigorta et al., 2012). Ruggieri et al. showed that HCV induces an oscillation between SG assembly and disassembly as a result of balance between dsRNA-dependent PKR activation with the subsequent phosphorylation of eIF2α and the antagonist effect of GADD34-mediated dephosphorylation of eIF2α (Ruggieri et al., 2012). This tight balance allows HCV to chronically infect cells without affecting cell survival (Ruggieri et al., 2012). In addition, two other SG components have been related to HCV replication: Staufen1 and YB-1. Staufen1 is involved in cellular mRNA transport, translation and decay, and negatively regulates the assembly of SGs (Thomas et al., 2009). Despite YB-1 being a general translational repressor, it regulates SG assembly by inducing G3BP-1 mRNA translation through its interaction with the 5′UTR of the mRNA (Somasekharan et al., 2015). In 2016, Dixit et al. showed that Staufen1 interacts directly with PKR and NS5B, and that this interaction is required to inhibit PKR activation during HCV infection to allow viral RNA translation. In addition, the interaction of Staufen1 with NS5B suggests a role of Staufen1 in HCV replication, which is in accordance with a strong reduction in viral RNA and NS5A and NS5B protein levels in cells transfected with and Staufen1-siRNA (Dixit et al., 2016). Moreover, Wang et al. demonstrated that YB-1 knockdown reduces the phosphorylation status of NS5A, which is crucial for the NS5A-mediated regulation of RNA replication and virus assembly (Wang et al., 2015). Also, YB-1 interacts with NS5A in an YB-1 phosphorylation-dependent manner and this interaction is crucial for NS5A protein stability during HCV infection. Interestingly, YB-1 is phosphorylated by Akt at serine 102 and is known that HCV infection and NS5A expression activate the PI3K/Akt signaling (Wang et al., 2015). Together, these observations could explain the oscillation of SG assembly/disassembly detected in HCV-infected cells (Ruggieri et al., 2012) and how SG assembly and SG components are necessary for HCV RNA replication, assembly and egress (Ariumi et al., 2011; Garaigorta et al., 2012; Pager et al., 2013).

Dicistroviridae

Cricket Paralysis Virus (CrPV) is the only described member of *Dicistroviridae* family that regulates SG assembly. CrPV 1A protein impairs the assembly of arsenite-, Pateamine A-, and heat shock-induced SGs containing Rox8 and Rin, *Drosophila* homologs of TIA-1 and G3BP-1 respectively, demonstrating that there is a conserved mechanism in insect and human cells (Khong and Jan, 2011; Khong et al., 2016). In addition, CrPV-induced inhibition of SG assembly is not due to a cleavage of Rox8 or Rin despite 3C proteinase sequestration in SGs (Khong and Jan, 2011).

Coronaviridae and *Arteriviridae*

Transmissible gastroenteritis virus (TGEV), a member of the *Coronaviridae* family, induces SG assembly later during infection (Sola et al., 2011). The SGs component PTB binds to TGEV genomic and subgenomic RNA and colocalize with TGEV-induced aggregates containing TIA-1 and TIAR (Sola et al., 2011). In addition, Xue et al. described that PERK-mediated eIF2α phosphorylation during TGEV infection is detrimental for viral replication due to the global translational repression induced by activation of the IFN pathway (Xue et al., 2018). On the other hand, Mouse Hepatitis Coronavirus (MHV) induces the aggregation of TIAR early during infection in an eIF2α phosphorylation-dependent manner and, in contrast to TGEV, translational shutoff induced by MHV enhanced viral replication (Raaben et al., 2007). Moreover, MHV infection does not induce the expression of factors necessary to dephosphorylate eIF2α such as CHOP and GADD34 (Bechill et al., 2008). MHV N protein strongly impairs the IFN-induced PKR signaling activation, suggesting a viral regulation of the cellular antiviral response (Ye et al., 2007). Recently, Middle East Respiratory Syndrome Coronavirus (MERS-CoV) was shown to impair SG assembly even when viral dsRNA alone activates PKR-mediated SG assembly, suggesting that the virus protects its viral dsRNA from PKR (Rabouw et al., 2016; Nakagawa et al., 2018). Rabouw et al. showed that viral protein p4a antagonizes PKR activity through its dsRNA-binding motif and inhibits partially arsenite-dependent SG assembly, suggesting that p4a suppresses PKR but no other pathways of the cellular stress response (Rabouw et al., 2016). In addition, MERS-CoV replication is significantly impaired in cells depleted of TIA-1 or G3BP-1/-2, suggesting a potential proviral role of these SG components (Nakagawa et al., 2018). Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) induces a strong inhibition of host protein synthesis mediated by the nsp1 viral protein, which interacts with the 40S ribosomal subunit, impairing 80S formation (Narayanan et al., 2008; Kamitani et al., 2009). In addition, SARS-CoV infection induces PKR-mediated eIF2α phosphorylation, while GCN2 protein levels decreased in infected cells (Krahling et al., 2008). Finally, Infectious Bronchitis Coronavirus (IBV) induces PERK and eIF2α phosphorylation at early times post infection, while induces GADD34 expression and the subsequent eIF2α dephosphorylation at late stages of the course of infection in order to maintain viral protein synthesis (Wang X. et al., 2009; Liao et al., 2013). Interestingly, *IfnB* mRNA, but not IFN protein was detected in the supernatant of IBV infected cells, probably due to a 5b-mediated inhibition of general protein synthesis (Kint et al., 2016). However, although it has been described that SARS-CoV and IBV regulate eIF2α phosphorylation in infected cells, it has not been evaluated whether it result in SG assembly or blockade. In contrast, it is known that Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), a member of the *Arteriviridae* family, induces canonical SG assembly mediated by PERK and eIF2α phosphorylation in infected cells (Zhou et al., 2017).

Caliciviridae

Members of the *Caliciviridae* family block SG assembly by targeting G3BP-1. Although Feline Calicivirus (FCV) infection results in eIF2α phosphorylation, viral 3C-like NS6 proteinase cleaves G3BP-1, thus impeding SG assembly in infected cells (Humoud et al., 2016). Similarly, Murine Norovirus 1 (MNV1)

induces a shutoff of global translation by triggering the phosphorylation of eIF4E and eIF2α in a PKR-dependent manner, without inducing SG assembly (Royall et al., 2015; Brocard et al., 2018; Fritzlar et al., 2019). Interestingly, MNV1 infected cells showed a redistribution of G3BP-1 to sites of viral replication closely to the nucleus, colocalizing with NS5 (Fritzlar et al., 2019) or NS3 viral protein (Brocard et al., 2018). Together, these observations showed that MNV impairs SG assembly by sequestering G3BP-1, thus, uncoupling the cellular stress response (Brocard et al., 2018; Fritzlar et al., 2019). Although Humoud et al. showed that MNV does not impair arsenite-induced SGs, recently Fritzlar et al. demonstrated the opposite (Humoud et al., 2016; Fritzlar et al., 2019).

Negative-Sense Single Stranded ((–) ssRNA) Viruses

Orthomyxoviridae

Influenza A virus (IAV) and Influenza B virus (IBV), members of the *Orthomyxoviridae* family, block SG assembly during infection. IAV disrupts SGs accumulation by expressing three different proteins: the host-shutoff protein polymerase-acidic protein-X (PA-X), the nucleoprotein (NP), and the non-structural protein 1 (NS1) (Khaperskyy et al., 2014). PA-X inhibits SG assembly in an eIF2α-independent manner, and requires its endoribonuclease activity for this function (Khaperskyy et al., 2014). It causes nuclear relocalization of PABP1, a phenotype that has been observed with other viral host-shutoff proteins (Khaperskyy et al., 2014). In addition, it depletes poly(A) RNAs from the cytoplasm but promotes its accumulation in the nuclei (Khaperskyy et al., 2014). A recent publication demonstrates that PA-X selectivity degrades host RNAs by selecting transcripts that have undergone splicing, and that can interact with cellular proteins involved in RNA splicing (Gaucherand et al., 2019). NP can block arsenite-induced SGs accumulation in an eIF2α-independent manner, but its effect depends on its expression levels (Khaperskyy et al., 2014). In contrast, NS-1-mediated inhibition of SG assembly depends on the PKR pathway; NS-1 binding to dsRNA inhibits PKR autophosphorylation and subsequent eIF2α phosphorylation (Khaperskyy et al., 2011). Interestingly, the SGs-associated proteins RAP55, DDX3, and NF90 have been shown to interact with both NP and NS-1, which could represent the cell's attempt to inhibit IAV infection or most the virus hijacks these host proteins to block SG assembly (Wang P. et al., 2009; Mok et al., 2012; Li et al., 2016; Raman et al., 2016). NP and DDX3 are recruited to SGs during ΔNS1 IAV infection (Onomoto et al., 2012; Raman et al., 2016), but in presence of NS1 NP localizes to PBs instead, suggesting that NS1 is essential for NP escape from SGs (Mok et al., 2012). Normally NF90 leads to SG accumulation by directly binding and activating PKR, but in presence of IAV NS1, NF90 binds preferentially to it rather than PKR, suggesting that NS1 also suppress PKR activation by blocking NF90-PKR interaction (Wen et al., 2014; Li et al., 2016). Similarly, IBV requires NS1 in order to restrict SG assembly (Núñez et al., 2018). The vRNA sensor retinoic acid inducible gene I (RIG-I) is recruited to SGs and induces IFN response during ΔNS1 IAV and IBV infections (Onomoto et al., 2012; Núñez et al., 2018). Furthermore, RIG-I was shown to associate with DDX6, which upon binding to vRNA stimulated RIG-I IFN induction (Núñez et al., 2018).

Arenaviridae

Infection with Junin virus (JUNV), a member of the *Arenaviridae* family, inhibits SG assembly in mock and arsenite-treated cells by impairing eIF2α phosphorylation. To do so, the presence of either NP or glycoprotein precursor (GPC) is required, as they both block SGs accumulation when expressed individually in cells (Linero et al., 2011). Recently, JUNV NP was found to interact with PKR, G3BP-1, eIF2α, hnRNP A1, and hnRNP K (King et al., 2017), as well as with DDX3 (Loureiro et al., 2018). Upon infection, PKR expression increases but is targeted to viral replication factories (RFs) together with NP, G3BP-1, dsRNA, PKR, phosphorylated PKR, RIG-I, and MDA-5 (King et al., 2017; Mateer et al., 2018). Despite the high levels of PKR activation, JUNV fails to induce eIF2α phosphorylation, maybe due its sequestration to the RFs via NP (King et al., 2017). Lassa virus and lymphocytic choriomeningitis virus (LCMV) NPs also interact with G3BP-1, eIF2α, and DDX3 (King et al., 2017; Loureiro et al., 2018). PKR interaction with LCMV NP occurs but weakly than with JUNV NP, which is reflected in the lack of PKR upregulation and colocalization with NP, and the increased eIF2α phosphorylation level compared to JUNV infection (King et al., 2017).

Rhabdoviridae

The vesicular stomatitis virus (VSV), member of the *Rhabdoviridae* family, promotes eIF2α phosphorylation and downregulates the synthesis of cellular proteins while maintaining viral production (Dinh et al., 2012). Under these conditions, it forms aSGs that contain PCBP2, TIA-1 and TIAR, but no eIF3 or eIF4A. VSV RNA, phosphoprotein (P) and NP are also part of these atypical SG-like structures, whose induction requires ongoing viral protein synthesis and viral replication (Dinh et al., 2012). Interestingly, assembly of aSGs and *bona fide* arsenite-induced SGs can occur simultaneously, revealing that VSV infection suppresses the accumulation of *bona fide* antiviral SGs and utilize SGs-associated components for its own benefit (Dinh et al., 2012). In contrast, Rabies virus (RABV) effectively replicate in cells that assemble SGs upon infection (Nikolic et al., 2016). The observed SGs contain G3BP-1, TIA-1 and PABP, and their accumulation is dependent on PKR-induced eIF2α phosphorylation, suggesting that they are canonical SGs. Notably, PKR and TIA-1 depletion enhances viral replication, revealing that they have an antiviral effect but that is not strong enough to completely stop RABV infection. RABV-induced SGs locate adjacent to viral RFs. Interestingly, viral mRNA but no viral genomic RNA is transported from RFs to SGs, suggesting that RABV may be using SGs to modulate viral transcription and replication (Nikolic et al., 2016).

Paramyxoviridae

Respiratory Syncytial Virus (RSV), member of the *Paramyxoviridae* family, replicate in viral replication factories

(RFs) which have been observed to interact with SGs. However, seemingly contradictory findings have been reported for RSV. Lindquist et al. showed that RSV replication induces SG assembly in ∼10–25% of the infected cells, and that they enhance RFs formation and viral replication (Lindquist et al., 2010, 2011). SGs accumulation requires PKR activation, which induces eIF2α phosphorylation, however PKR depletion did not affect RSV replication (Lindquist et al., 2011). Contrastingly, Groskreutz et al. reported that RSV infection activates PKR but does not trigger eIF2α phosphorylation due to PKR sequestration by the RSV NP (Groskreutz et al., 2010). Two other groups reported that RSV induce SG aggregation in ∼1% (Hanley et al., 2010) and ∼5% (Fricke et al., 2012) of the infected cells, revealing that, in general, RSV inhibits their assembly. Sequestration to RFs of the O-linked *N*-acetylglucosamine transferase (OGT), a factor involved in SGs regulation, and the presence of the 5′ extragenic trailer sequence of the RSV genome have been associated with SGs suppression (Hanley et al., 2010; Fricke et al., 2012). Measles virus (MeV) infection does not induce SG assembly due to the PKR inhibitory effect of the viral accessory protein C (Okonski and Samuel, 2012). To block PKR autophosphorylation, MeV C protein requires the presence of the dsRNA binding protein and the SGs-component ADAR1, as WT MeV infection induce PKR activation and SGs accumulation in ADAR1 depleted cells (Toth et al., 2009; Li et al., 2012; Okonski and Samuel, 2012). Furthermore, infection with ΔC-MeV produces large amounts of dsRNA that activate PKR and induce SGs assembly, suggesting that the C protein may utilize ADAR1 to downregulate the viral dsRNA produced during replication (Pfaller et al., 2013). Both ADAR1 and C protein colocalize with SGs (Okonski and Samuel, 2012). Similar to RSV, Sendai virus (SeV) induces SGs accumulation in just a fraction of the cells (5–15%) and the 5′ trailer region of its sequence has been implicated in SG assembly prevention via interaction with TIAR (Iseni et al., 2002). Like MeV, the SeV C protein is required to impair SG assembly in control and arsenite-treated cells, although C protein expression alone is not able to do so (Yoshida et al., 2015). ΔC-SeV assembled SGs contain RIG-I and unusual viral RNA species. Recently, the effect on SG assembly of three more paramyxoviruses have been studied. Newcastle disease virus (NDV) infection trigger the canonical SGs assembly to arrest host mRNAs and boost viral replication (Oh et al., 2016; Sun et al., 2017). SG assembly is dependent on PKR/eIF2α phosphorylation and its suppression (by depleting TIA-1 or TIAR proteins) reduces viral protein synthesis but increases cellular protein synthesis (Sun et al., 2017). Accordingly, cellular mRNAs have been shown to be predominately recruited to SGs compared to viral mRNAs (Sun et al., 2017). Strikingly, RIG-I is also recruited to the assembled SGs, which induces IFN production as an antiviral response (Oh et al., 2016). Likewise, Mumps virus (MuV) infection promotes SG assembly dependent on PKR activation despite weak eIF2α phosphorylation (Hashimoto et al., 2016). PKR, G3BP-1 and TIA-1 depletion reduces MeV-SGs and increased IFN response, but did not alter viral titers, suggesting that MuV replication occurs independently of the presence or absence of SGs. Conversely, Human Parainfluenza Virus Type 3 (HPIV3) infection leads to assembly of SGs that seem to have a poor antiviral role, as it is able to replicate in presence of SGs although viral protein expression and particle production is improved when SG assembly is constrained by knockdown of PKR, G3BP-1 or expression of a non-phosphorylatable eIF2α (Hu et al., 2018). SG assembly is due eIF2α PKR-dependent phosphorylation triggered by viral mRNAs, which can be shielded, and therefore block SGs assembly, by HPIV3 RFs (Hu et al., 2018).

Bunyaviridae

Since our last review (Poblete-Durán et al., 2016), no new reports have been published on how members of the *Bunyaviridae* family modulate SG assembly. Briefly, Rift Valley fever virus (RVFV) infection inhibit SG assembly despite attenuation of the Akt/mTOR signaling pathway which leads to the arrest of cap-dependent translation (Hopkins et al., 2015). It has been shown that non-structural protein from the S segment of Orthobunyaviruses, Hantaviruses and Phleboviruses (Kohl et al., 2003; Jaaskelainen et al., 2009), as well as glycoprotein Gn and the capsid N protein from Hantaviruses (Alff et al., 2006; Cimica et al., 2014; Matthys et al., 2014), inhibit IFN response.

Interestingly, the Andes Hantavirus (ANDV) N protein inhibits PKR dimerization, but this lack of activation does not stop protein translation (Wang and Mir, 2014). In contrast, RVFV infection promotes a protein translation shutoff due to PKR degradation by NSs protein (Habjan et al., 2009; Ikegami et al., 2009).

Filoviridae

Ebola virus (EBOV), member of the *Filoviridae* family, inhibits the assembly of SGs and instead sequesters the SGs-associated proteins eIF4G, eIF3, PABP, and G3BP-1, but no TIA-1 into granules within the viral replication factories (Nelson et al., 2016). These inclusion-bodies (IB) granules do not require eIF2α phosphorylation, do not disassemble with cycloheximide, and do not block translation. Furthermore, arsenite, heat and hippuristanol can still induce *bona fide* SGs accumulation, suggesting that sequestration of SGs proteins in IB granules may be released upon stress. EBOV VP35 was found to be the protein that prevents the *bona fide* SG assembly late in infection, and its C-terminal domain is critical for this function (Le Sage et al., 2016). VP35-CTD contains an inhibition of interferon regulatory factor 3 (IRF3) domain that is responsible for blocking PKR activation during EBOV infection (Schumann et al., 2009). However, when expressed in sufficient high levels, VP35 can block arsenite-induced SGs without reducing the levels of eIF2α phosphorylation, suggesting that VP35 suppress SG assembly by using an alternative way to PKR (Le Sage et al., 2016). VP35 can interact with several SG-associated proteins such as G3BP-1, eIF3, and eEF2 and it is targeted to viral replication factories, suggesting that it may be blocking SG assembly by relocating SG constituents (Le Sage et al., 2016; Nelson et al., 2016).

Single Strand RNA Retroviruses (ssRNA-RTs)

Retroviridae

Viruses belonging the *Retroviridae* family integrate its retrotranscribed (+)ssRNA into the host chromosomal DNA (Fields et al., 2013). The Human T-Lymphotropic Virus 1 (HTLV1) Tax protein blocks SG assembly by interacting with the SG components histone deacetylase 6 (HDAC6) (Legros et al., 2011) and USP10 (Takahashi et al., 2013). Similarly, the Human Immunodeficiency Virus Type I (HIV-1) also blocks SG assembly despite eIF2α phosphorylation, through an interaction between Gag and the eukaryotic elongation factor 2 (eEF2) (Valiente-Echeverría et al., 2014; Poblete-Durán et al., 2016). HIV-1 Gag also disassembles pre-formed arsenite-induced SGs by interacting with G3BP-1 (Valiente-Echeverría et al., 2014) and selenite-induced atypical SGs by interacting with eIF4E (Cinti et al., 2016; Poblete-Durán et al., 2016). Notably, G3BP-1 was shown to act as a restriction factor that inhibits viral replication by interacting with HIV-1 genomic RNA (gRNA) in macrophages (Cobos Jiménez et al., 2015). Recently, Rao et al. described a novel HIV-1 NC-induced SGs containing G3BP-1, TIAR, eIF3, PABP, and poly(A) mRNAs that are no longer disassembled by Gag or CA (Rao et al., 2017). HIV-1 NC expression significantly increased eIF2α phosphorylation, inhibiting protein synthesis and reducing viral particle production. In addition, NC was shown to interact with G3BP-1, TIAR, and Staufen1 even in absence of RNA. The inability of NC to assembly SGs in G3BP-1 depleted cells suggest that their interaction is required to promote NC-induced SG accumulation (Rao et al., 2017). The authors also showed that Staufen1 counteracts NC-induced PKR-dependent eIF2α phosphorylation and translational shutoff (Rao et al., 2017). Interestingly, while HIV-1 impairs SGs assembly, the virus favors the assembly of Staufen1-containing HIV-1 dependent ribonucleoproteins (Abrahamyan et al., 2010). Indeed, it has been reported that HIV-1 is unable to dissociate or block arsenite-induced SG assembly in Staufen1 knock-out cells, suggesting that the recruitment of Staufen1 is crucial for HIV-1 SGs blockade. In addition, in absence of Staufen1 the HIV-1 genomic RNA colocalizes with TIAR in arsenite-induced SGs, which correlates with a significant reduction of Gag protein levels possibly due to the robust eIF2α phosphorylation induced by HIV-1 infection (Rao et al., 2019b). Besides these data, Soto-Rifo et al. showed that DDX3, eIF4GI, and PABPC1 form a pre-translation initiation complex with the HIV-1 genomic RNA to promote viral translation (Soto-Rifo et al., 2014; Poblete-Durán et al., 2016). In contrast to HIV-1, HIV-2 infection does not block SG assembly and the viral genomic RNA recruits TIAR in a different type of RNA granules where it is suggested that the transition from translation to packaging occurs (Soto-Rifo et al., 2014). On another hand, Bann et al. showed that Mouse Mammary Tumor Virus (MMTV) Gag interacts and colocalizes with the SGs component YB-1 in small cytoplasmic foci in an RNA-dependent manner. Interestingly, these foci also contain viral RNA and are insensitive to cycloheximide treatment. It is suggested that YB-1 plays key roles in MMTV as YB-1 knockdown results in a significant reduction in viral particle production (Bann et al., 2014).

In summary, information about virus-host interaction mediated by membraneless organelles in order to ensure viral replication can be found summarized in **Table 2**.

VIRAL INFECTIONS AND PROCESSING BODIES

PBs are membraneless organelles and their diameter range between 150 and 340 nm (Cougot et al., 2012). PBs contain proteins involved in mRNA decapping machinery (Dcp1/2, LSm1-7, Edc3 proteins), scaffolding proteins (GW182, Ge-1/Hedls), deadenylation factors (Ccr1, Caf1, Not1), nonsense-mediated decay (NMD) proteins (SMG5-6-7, UPF1) and translation control factors (CPEB, eIF4E-T) (reviewed in Poblete-Durán et al., 2016). PBs are constitutively assembled in the cytoplasm of the cell, but their size and number increases upon cellular stress (Kedersha et al., 2005). Initially it was suggested that PBs were sites of mRNA decay, but recent evidences suggest that instead, P-bodies are sites of long-term mRNA storage and decapping enzymes (reviewed in Luo et al., 2018). Recent studies have shown that PBs participate in the storage of a selection of mRNAs; transcripts involved in regulatory processes are enriched while mRNAs that encode proteins that support basic cell functions are excluded (Hubstenberger et al., 2017; Standart and Weil, 2018).

Interestingly, viruses have been shown to modulate PB assembly by degrading and/or relocating PB-associated components, thus avoiding their accumulation. Here we summarize how viruses modulate PBs.

Double-Stranded DNA (dsDNA)Viruses

Adenoviridae

In order to accumulate late mRNAs, adenovirus decreases the number of PBs in the cell by relocalizing several PB components, such as DDX6, LSm1, Ge-1, Ago2, and Xrn1 to aggresomes, where proteins are inactivated or degraded (Greer et al., 2011). Aggresome formation is induced by the viral protein E4 11K, which was found to specifically bind DDX6, suggesting that this interaction may redistribute DDX6 (Greer et al., 2011). Recently, the PB-associated protein PatL1 was also shown to localize within aggresomes following E4 11K protein expression (Friedman and Karen, 2017).

Herpesviridae

In contrast to the aforementioned adenovirus, expression of PB-associated proteins and PBs accumulation increase upon HCMV infection, but viral mRNA is not targeted to them, suggesting that HCMV mRNAs escape translation repression (Seto et al., 2014). HCMV-induced PB assembly requires cellular but no viral RNA synthesis and was independent of the translational status of the cell (Seto et al., 2014). KSHV prevents PB assembly during latent and lytic infection thanks to the activation of the cytoskeletal regulator RhoA GTPase (RhoA) (Corcoran et al., 2012, 2015). How RhoA disperse PBs is unknown, but in both

TABLE 2 | Virus families that modulate SGs.

Genome	Virus family	Virus	SGs induction	SGs blockade	Mechanism	References
dsDNA	*Herpesviridae*	Herpes simplex Virus type 1 (HSV-1)	No	Yes	vhs is required for inhibition of SG assembly dependent on PKR	Esclatine et al., 2004; Dauber et al., 2011, 2016; Sciortino et al., 2013
					vhs-dependent SGs inhibition is independent on eIF2α phosphorylation	Dauber et al., 2011, 2016
					vhs-dependent SG inhibition is dependent on eIF2α phosphorylation, dsRNA partially localizes to SGs, and SG assembly activates PKR	Burgess and Mohr, 2018
					ICP27 inhibits phosphorylation of PKR/eIF2α and blocks SG assembly	Sharma et al., 2017
					ICP8 interacts with G3BP and blocks SG assembly	Panas et al., 2015
		Herpes simplex virus type 2 (HSV-2)	No	Yes	HSV-2 inhibits SG assembly independent on eIF2α phosphorylation	Finnen et al., 2012
					vhs is required for disruption of canonical and arsenite-induced SGs	Finnen et al., 2012
					vhs inhibit SG assembly and disrupt pre-assembled SGs, and its endoribonuclease activity is required	Finnen et al., 2016
		Cytomegalovirus (HCMV)	No	Yes	HCMV inhibits eIF2α phosphorylation	Isler et al., 2005b
					HCMV inhibits SG assembly	Isler et al., 2005a
					Lack of viral proteins pIRS1 and pTRS1 increase levels of eIF2α phosphorylation	Marshall et al., 2009
					pTRS1 or pIRS1 inhibits SG assembly dependent on PKR. Transfected pTRS1 also prevent SG assembly independent on PKR	Ziehr et al., 2016
		Kaposi's sarcoma-associated herpesvirus (KSHV)	No	Yes	ORF57 interacts with PKR inhibiting its binding to dsRNA and its activation, which impairs eIF2α phosphorylation and SG assembly	Sharma et al., 2017
					SOX inhibits arsenite-induced SG assembly	Sharma et al., 2017
		Epstein–Barr virus (EBV)	ND	ND	EB2 overexpression does not abolish SG assembly neither PKR/eIF2α phosphorylation	Sharma et al., 2017
	Poxviridae	Vaccinia virus (VACV)	Yes (AVGs)	Yes	G3BP-1, Caprin 1, eIF4G, eIF4E, PABP are sequestered into RFs	Katsafanas and Moss, 2004
					VACV lacking E3L induce AVGs which require TIA-1 expression	Simpson-Holley et al., 2010
					ΔE3L induced AVGs requires eIF2α phosphorylation	Pham et al., 2016
					WT VACV spontaneously form AVGs but to negligible levels	Rozelle et al., 2014
					ΔC7L/K1L VACV induce AVGs assembly dependent on SAMD9 host protein and independent of eIF2α phosphorylation	Liu and McFadden, 2014
					Viral mRNA is recruited to ΔC7L/K1L VACV induced AVGs	Sivan et al., 2018
					TIA-1 is not required for ΔC7L/K1L-mediated AVG assembly	Meng and Xiang, 2019
dsRNA	*Reoviridae*	Rotavirus	No	Yes	Rotavirus inhibits SG assembly independent on eIF2α phosphorylation, but changes the localization of TIA-1, eIF4E, and PABP	Montero et al., 2008
					Relocalizes ADAR1, Caprin1, CPEB, eIF2α, 4EBP1, PKR, and Staufen1 to RFs, and selectively excludes G3BP-1 and ZBP1	Dhillon and Rao, 2018; Dhillon et al., 2018
		Mammalian orthoreovirus (MRV)	Yes (canonical)	Yes	SGs are formed during the early stage of infection but disassembled in later stages independent on eIF2α phosphorylation	Qin et al., 2009, 2011

(Continued)

TABLE 2 | Continued

Genome	Virus family	Virus	SGs induction	SGs blockade	Mechanism	References
					μNS is recruited to SGs	Carroll et al., 2014
					MRV relocalizes G3BP-1, Caprin1, USP10, TIAR, TIA-1, eIF3b to RFs via G3BP1-oNS-uNS interaction	Núñez et al., 2018
(+)ssRNA	*Picornaviridae*	Poliovirus (PV)	Yes (canonical and atypical)	Yes	2A-protease mediated SGs assembly	Mazroui et al., 2006
					3C protease-mediated G3BP-1 cleavage	White et al., 2007
					Induces aggregates containing TIA-1 and viral RNA	Piotrowska et al., 2010
		Encephalomyocarditis virus (EMCV)	No	Yes	Cleavage of G3BP-1	Ng et al., 2013
		Coxsakievirus B3 CVB3	Yes (canonical and atypical)	Yes	Cleavage of G3BP-1	Fung et al., 2013
					2A protease-mediated SGs assembly	Zhai et al., 2018
					2A protease-mediated eIF4G cleavage	Wu et al., 2014
		Theiler's murine encephalomyelitis virus (TMEV)	No	Yes	Inhibition of SG assembly mediated by Leader protein (L)	Borghese and Michiels, 2011
		Enterovirus 71 (EV71)	Yes (canonical and atypical)	Yes	2A protease-mediated inhibition of SGs	Zhu et al., 2016; Yang et al., 2018b
					PKR-eIF2α phosphorylation- dependent SG assembly mediated by 2A protease	Zhang et al., 2016, 2018; Zhu et al., 2016
					Cleavage of eIF4GI mediated by 2A protease, abolishing eIF4GI and G3BP-1 interaction	(Yang et al., 2018a,b)
					Cleavage of G3BP-1 mediated by 3C protease	Zhang et al., 2018
		Foot-and-Mouth Disease Virus (FMDV)	No	Yes	Shuts-off host cap-dependent translation mediated by eIF2α downregulation and PKR dephosphorylation	Ye et al., 2018
					Cleavage of G3BP-1 and Sam68 mediated by 3C protease	Lawrence et al., 2012; Ye et al., 2018
					Cleavage of G3BP-1 mediated by Leader protease	Visser et al., 2019
		Equine Rhinitis A virus (ERAV)	ND	Yes	Cleavage of G3BP-1 and G3BP-2 by Leader protease	Visser et al., 2019
		Mengovirus, a strain of EMCV	No	Yes	Leader protein (L) inhibits SG assembly	Langereis et al., 2013; Reineke et al., 2015
	Togaviridae	Semliki Forest Virus (SFV)	Yes (canonical)	Yes	Induces eIF2α phosphorylation	McInerney et al., 2005
					nsP3 sequesters G3BP-1 and G3BP-2 into RFs	Panas et al., 2012
					G3BP-1 binding by nsP3 is necessary for SGs blockage	Panas et al., 2015
		Chikungunya virus (CHIKV)	No	Yes	Nsp3 sequesters G3BP-1 to RFs	Fros et al., 2012; Remenyi et al., 2018
					G3BP-2 colocalizes with nsP2/nsP3 complexes	Scholte et al., 2015
		Rubella virus (RUBV)	Yes	No	Accumulation of G3BP-1	Matthews and Frey, 2012
		Venezuelan equine encephalitis virus (VEEV)	ND	ND	nsP3 interacts with FXRs to facilitate viral RFs formation	Kim et al., 2016
		Sindbis virus (SINV)	Yes (canonical)	Yes	Nsp4 interacts with G3BP-1	Cristea et al., 2010
					Induces PKR-dependent SGs assembly	Venticinque and Meruelo, 2010
					Nsp3 protein interacts with G3BP-1 and G3BP-2	Kim et al., 2016

(Continued)

TABLE 2 | Continued

Genome	Virus family	Virus	SGs induction	SGs blockade	Mechanism	References
	Flaviviridae	West Nile Virus (WNV)	No	Yes	Viral RNA captures TIA-1 and TIAR	Li et al., 2002
					Increased GSH levels inhibit arsenite-induced SGs	Basu et al., 2017
		Dengue Virus (DENV)	No	Yes	Viral RNA colocalizes with TIA-1 and TIAR	Emara and Brinton, 2007
					3′UTR interacts with G3BP-1, G3BP-2, Caprin 1 and USP1	Ward et al., 2011
		Zika Virus (ZIKV)	No	Yes	ZIKV impairs SG assembly downstream of eIF2α phosphorylation	Hou et al., 2017
					Expression of ZIKV capsid, NS3, NS2B-3, or NS4A protein inhibits SG assembly. Capsid protein interacts with G3BP-1 and Caprin-1	Hou et al., 2017
					Relocalizes Ataxin-2, HuR and YB-1. G3BP-1 and TIAR localize at viral RFs	Bonenfant et al., 2019
		Yellow Fever Virus (YFV)	ND	Yes	Ectopically expressed capsid protein blocks hippuristanol-induced SGs	Hou et al., 2017
		Murray Valley Encephalitis Virus (MVEV)	ND	Yes	Ectopically expressed capsid protein blocks hippuristanol-induced SGs	Hou et al., 2017
		Tick-borne encephalitis virus (TBEV)	Yes (canonical)	No	Induces eIF2α phosphorylation	Albornoz et al., 2014
					Sequesters TIA-1 and TIAR to RFs	Albornoz et al., 2014
		Japanese encephalitis virus (JEV)	No	Yes	Core protein interacts with Caprin 1	Katoh et al., 2013
					Ectopically expressed capsid protein blocks hippuristanol-induced SGs	Hou et al., 2017
		Bovine viral diarrhea virus (BVDV)	No	Yes	Impairs arsenite-induced SGs assembly	Jefferson et al., 2014
		Hepatitis C virus (HCV)	Yes	Yes	G3BP-1, Ataxin-2, PABP1, DDX3, TIA-1, and TIAR are recruited to lipid droplets	Ariumi et al., 2011; Garaigorta et al., 2012
					Induces SGs dependent on PKR and IFN	Garaigorta et al., 2012
					GADD34-mediated SGs disassembly	Ruggieri et al., 2012
					DDX3 binds viral 3'UTR	Li et al., 2013
					DDX3 and G3BP-1 localize with HCV core protein	Pène et al., 2015
					Staufen 1 inhibits PKR activation	Dixit et al., 2016
	Dicistroviridae	Cricket paralysis virus (CrPV)	No	Yes	3C protease is sequestered to SGs	Khong and Jan, 2011
					CrPV-1A protein disrupts Pateamine A, arsenite and heat shock-induced SGs assembly	Khong et al., 2016
	Coronaviridae	Mouse hepatitis coronavirus (MHV)	Yes	No	Induces eIF2α phosphorylation	Raaben et al., 2007; Bechill et al., 2008
					N protein impairs PKR and eIF2α phosphorylation during IFN treatment	Ye et al., 2007
		Transmissible gastroenteritis virus (TGEV)	Yes	No	PTB localization in SGs correlates with an increase in viral replication	Sola et al., 2011
					Induces PERK-dependent eIF2α phosphorylation	Xue et al., 2018
		Middle East Respiratory Syndrome Coronavirus (MERS-CoV)	No	Yes	4a protein inhibits PKR-dependent SG assembly by binding and sequestering dsRNAs from PKR	Rabouw et al., 2016; Nakagawa et al., 2018
		Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)	ND	ND	Nsp1 induces translational shutoff by impairing 80S formation	Narayanan et al., 2008; Kamitani et al., 2009
					Induces PKR and eIF2α phosphorylation	Krahling et al., 2008
		Infectious Bronchitis Coronavirus (IBV)	ND	ND	Induces PKR and eIF2α phosphorylation at early stages of infection and inhibits eIF2α phosphorylation at later stages	Wang X. et al., 2009; Liao et al., 2013
					Viral 5b protein induces host translational shutoff	Kint et al., 2016

(Continued)

TABLE 2 | Continued

Genome	Virus family	Virus	SGs induction	SGs blockade	Mechanism	References
	Arteriviridae	Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)	Yes (canonical)	ND	Induces PERK-dependent eIF2α phosphorylation and subsequent SGs assembly	Zhou et al., 2017
					Induces Mnk1-mediated eIF4E phosphorylation	Royall et al., 2015
					Induces PKR and eIF2α phosphorylation, but translation repression is PKR-independent	Fritzlar et al., 2019
					G3BP-1 is sequestered to RFs even in presence of arsenite treatment	Fritzlar et al., 2019
					G3BP-1 colocalizes with Nsp3 in a perinuclear zone but not in presence of arsenite treatment	Brocard et al., 2018
		Feline Calicivirus (FCV)	No	Yes	NS6-mediated G3BP-1 cleavage	Humoud et al., 2016
(-)ssRNA	*Orthomyxoviridae*	Influenza A virus (IAV)	No	Yes	NS1 restrict SG assembly dependent on eIF2α while NP and PA-X block SG assembly in an eIF2α-independent manner	Khaperskyy et al., 2014
					PA-X requires its endoribonuclease activity to inhibit SGs, and relocalizes PABP1 to the nucleus	Khaperskyy et al., 2014
					PA-X selectively degrades host spliced RNAs	Gaucherand et al., 2019
					NS-1 inhibits PKR activation by binding to dsRNA	Khaperskyy et al., 2011
					NS1 interacts with RAP55 and its RNA and PKR binding sites are required for the interaction and to inhibit SGs	Mok et al., 2012
					NS1 and NP interact with DDX3	Raman et al., 2016
					NP and RIG-I are recruited to SGs on ΔNS1 IAV infection, and IAV genomic RNA is sufficient to form SGs	Onomoto et al., 2012
					NS1 interacts with NF90 and restricts its binding to PKR. The NS1 RNA-binding domain and the NF90 double-stranded RNA binding domain are required	Wen et al., 2014; Li et al., 2016
					NF90 binds NP independently of RNA binding	Wang P. et al., 2009
		Influenza B virus (IBV)	No	Yes	NS1 is required to inhibit SG assembly. RIG-I and DDX6 interact and colocalize to ΔNS1-induced SGs	Núñez et al., 2018
	Arenaviridae	Junin Virus (JUNV)	No	Yes	NP and GPC individually impair arsenite-induced SGs by inhibiting eIF2α phosphorylation	Linero et al., 2011
					NP interacts with G3BP-1, PKR, hnRNP A1, and hnRNP K, G3BP-1 and eIF2α. NP sequesters PKR into RFs	King et al., 2017
					NP interacts with DDX3	Loureiro et al., 2018
					dsRNA activates PKR and colocalizes with RFs	Mateer et al., 2018
	Rhabdoviridae	Vesicular stomatitis virus (VSV)	Yes (atypical)	Yes	Inhibit canonical SGs but induces SGs-like structures containing PCBP2, TIA1, TIAR, and viral RNA, P and NP proteins	Dinh et al., 2012
		Rabies virus	Yes (canonical)	No	SG assembly is dependent on PKR and they locate close to RFs. Viral mRNA is transported to RFs	Nikolic et al., 2016
	Paramyxoviridae	Respiratory Syncytial Virus (RSV)	Yes (canonical)	Yes	10–25% of infected cells form SGs dependent on PKR	Lindquist et al., 2010, 2011
					Just 1% of infected cells form SGs. The 5'trailer region of the RSV genome is required to inhibit SGs	Hanley et al., 2010
					Just 5% of infected cells form SGs. Sequestration of OGT into RFs suppresses SGs accumulation	Fricke et al., 2012
		Measles virus (MeV)	No	Yes	Viral C protein inhibits SG assembly by blocking PKR activation but requires the presence of ADAR1	Okonski and Samuel, 2012

(Continued)

TABLE 2 | Continued

Genome	Virus family	Virus	SGs induction	SGs blockade	Mechanism	References
					MeV C protein reduces the dsRNA in the cytoplasm to inhibit PKR activation	Pfaller et al., 2013
		Sendai virus (SeV)	Yes (canonical)	Yes	5–15% of infected cells form SGs. The trailer RNA region captures TIAR and inhibit SGs accumulation	Iseni et al., 2002
					Viral C protein is required to inhibit SG assembly	Yoshida et al., 2015
		Newcastle disease virus (NDV)	Yes (canonical)	No	NDV replication induces canonical SGs which contain vRNA(+) and RIG-I	Oh et al., 2016
					SG assembly is dependent on PKR/eIF2α pathway. SGs contain cellular mRNA but no viral mRNA	Sun et al., 2017
		Mumps virus (MuV)	Yes (canonical)	No	SG assembly is dependent on PKR. MuV replicates independently of the presence or absence of SGs	Hashimoto et al., 2016
		Human parainfluenza virus type 3 (HPIV3)	Yes	No	PKR-dependent SGs are induced by viral mRNA. SGs have an inhibitory role in HPIV3 replication.	Hu et al., 2018
	Bunyaviridae	Rift Valley fever virus (RVFV)	Yes	Yes	Attenuate Atk/mTOR signaling	Hopkins et al., 2015
		Andes hantavirus (ANDV)	ND	Yes	N protein inhibits PKR activation	Wang and Mir, 2014
	Filoviridae	Ebola virus	Yes (IB granules)	Yes	Ebola inhibits canonical SGs but form IB granules within RFs that contain eIF4G, eIF3, PABP, and G3BP-1, but no TIA-1	Nelson et al., 2016
					VP35 inhibit canonical and stress-induced SGs, and its C-terminal domain is required. VP35 interacts with G3BP-1, eIF3, and eEF2	Le Sage et al., 2016
ssRNA-RT	*Retroviridae*	Human T-cell Leukemia virus (HTLV-1)	No	Yes	Tax interacts with HDAC6 and USP10	Legros et al., 2011; Takahashi et al., 2013
		Human immunodeficiency virus type 1 (HIV-1)	No	Yes	Staufen 1 and Gag-mediated blockade of SGs assembly	Abrahamyan et al., 2010
					Gag interacts with eEF2 to block SGs assembly	Valiente-Echeverría et al., 2014
					G3BP-1 interact with Gag to disassembly preformed SGs	Valiente-Echeverría et al., 2014
					gRNA promote pre-translation initiation complex assembly	Soto-Rifo et al., 2014
					Gag interacts with eIF4E to promote disassembly of SGs	Cinti et al., 2016
					Ectopically expressed NC protein induces eIF2α phosphorylation and interacts with SGs proteins.	Rao et al., 2017
		Human immunodeficiency virus type 2(HIV-2)	Yes	No	gRNA and TIAR colocalizes in SGs	Soto-Rifo et al., 2014
		Mouse Mammary Tumor Virus (MMTV)	ND	ND	YB-1 interacts with Gag and gRNA in cytoplasmic foci	Bann et al., 2014

cases its activated by the p38/MK2 pathway, which is triggered by the viral gene G-protein-coupled receptor (vGPCR) during lytic infection, and by the viral kaposin B (KapB) protein during latency (Corcoran et al., 2012, 2015). Furthermore, on both cycles PBs inhibition causes stabilization of AU-rich containing RNAs, a *cis-acting* RNA element usually present in mRNAs coding cytokines, growth factors and proto-oncogenes, increasing their protein synthesis during infection (Corcoran et al., 2012, 2015).

Double-Stranded RNA (dsRNA) Viruses

Reoviridae

Similarly, rotavirus has also been shown to suppress PB assembly (Montero et al., 2008; Bhowmick et al., 2015). Upon infection, it reduces the cytosolic levels of Xrn1, Pan3, and Dcp1a, but no GW182 in a time-dependent manner (Bhowmick et al., 2015). Furthermore, it has been shown that rotavirus infection induces the relocalization of Xrn1, Dcp1a, and PABP to the nucleus (Montero et al., 2008; Bhowmick et al., 2015), and to sequester most of the PB components into RFs, with the exception of DDX6, Edc4, and Pan3 (Dhillon and Rao, 2018; Dhillon et al., 2018), and to accelerate Pan3 decay by the NSP1 protein (Bhowmick et al., 2015).

Positive-Sense Single Stranded RNA ((+) ssRNA) Viruses

Flaviviridae

The relationship between (+)ssRNA and PB components has been extensively studied. Members of the Flavivirus genus of

the *Flaviviridae* family generates a subgenomic flavivirus RNA (sfRNA) as a product of genomic RNA incomplete degradation by the exonuclease Xrn1 (Pijlman et al., 2008; Silva et al., 2010). In that process, Xrn1 stalls on highly structured sequences in the 3′ UTR, thus inhibiting Xrn1 activity and resulting in the accumulation of uncapped cellular mRNAs (Pijlman et al., 2008; Moon et al., 2012). Interestingly, sfRNA colocalizes with Xrn1 in PBs and is essential for viral-mediated cytopathogenesis (Pijlman et al., 2008). The flavivirus WNV reduces PB assembly during the course of infection through the sequestration of several PB components such as LSm1, GW182, Xrn1, DDX3, and DDX6 to viral replication factories (RFs) (Chahar et al., 2013). Similarly, DENV infection reduces PBs accumulation through an interaction of DENV 3′UTR with DDX6 (Ward et al., 2011). In addition, it has been shown that Dcp1b colocalizes with viral dsRNA, suggesting that DENV RNA replication occurs within PBs (Dong et al., 2015). LSm1, another PBs component, interacts with DENV RNA both *in vitro* and *in vivo* during DENV-2 infection, colocalizing with sites of viral replication (Dong et al., 2015). Moreover, downregulation of LSm1 negatively affects viral RNA accumulation and particle production, suggesting that LSm1 plays key roles in the early stages of the viral replicative cycle, such as translation and replication (Dong et al., 2015). In 2016 Balinsky et al. showed that NS4A and NS3 interact with IRAV, which is a constituent of PBs and is induced by DENV infection in an interferon-dependent manner. Interestingly, IRAV is relocalized to viral RFs in HEK-293T cells and in monocyte-derived macrophages (Balinsky et al., 2017). MOV10 is also relocalized to viral RFs, as shown by its colocalization with NS3. Both IRAV and MOV10 are restriction factors for DENV, as viral replication is significantly enhanced in KO and KD cells, respectively (Balinsky et al., 2017). ZIKV infection does not affect the morphology, localization or number of PBs per cell (Hou et al., 2017), however both DDX6 and DGCR8 are upregulated in ZIKV-infected neurospheres (Garcez et al., 2017). Interestingly, nonsense-mediated mRNA decay (NMD) transcripts are stabilized in ZIKV-infected cells, suggesting that NMD pathway is impaired in infected cells. In addition, ZIKV capsid interacts with several NMD components, including nuclear Upf1, which is targeted for proteasomal degradation (Fontaine et al., 2018). Downregulation of Upf1 prior to infection significantly increases RNA viral levels and consequently viral production, suggesting that Upf1 regulates early stages of ZIKV infection (Fontaine et al., 2018). Similar to the other flavivirus mentioned above, HCV induces the relocalization of several PB components such as DDX6, LSm1, Xrn1, PatL1, Ago2, Dicer, and DDX3 to sites of viral replication in lipid droplets (Ariumi et al., 2011; Berezhna et al., 2011; Pager et al., 2013) in order to promote viral replication (Ariumi et al., 2007, 2011; Berezhna et al., 2011; Pager et al., 2013). In particular, the decapping activators DDX6, LSm1, and PatL1 are crucial for the transition from translation to replication of HCV RNA (Scheller et al., 2009; Jangra et al., 2010). Although PB constituents play key roles during HCV infection, the presence of PBs is not necessary for efficient viral replication (Berezhna et al., 2011; Pérez-Vilaró et al., 2012). Analysis of liver biopsies from HCV-infected patients confirmed that HCV decreases the number of PB *in vivo* independent of the viral genotype, the inflammation status of the sample donor or whether the infection is chronical or recent. Interestingly and contrary to previous reports, DDX6 was shown to not be recruited to sites of viral replication at lipid droplets (Pérez-Vilar et al., 2015), although its role in HCV replication in patient samples remains to be elucidated. This article by Pérez-Vilaró et al. is the first evidence and confirmation of a direct relationship between a viral-induced pathogenesis and PB modulation. Furthermore, they reported differences in PB composition since DDX6 do not colocalize with Dcp1 in human hepatocytes *in vivo*, highlighting the potential variations between cell line-based experiments and analysis of human or animal models (Pérez-Vilar et al., 2015). Although HCV and BVDV do not generate a sfRNA from their 3′UTR as other flavivirus such as DENV, Moon et al. showed that Xrn1 stalls while attempting to degrade the 5′UTR of both HCV and BVDV, thus Xrn1 activity is repressed. This dysregulation causes a stabilization of mRNAs encoding proteins involved in innate immune responses and transcription factors, which have short half-life in uninfected cells (Moon et al., 2015). In addition, they suggest that HCV-mediated Xrn1 repression induces a feedback mechanism that prevents the initial steps of 5′-3′ decay, such as deadenylation and decapping (Moon et al., 2015).

Picornaviridae

The picornaviruses PV and CVB3 3C proteases cleave Dcp1a and target Xrn1 and Pan3 for proteasomal degradation, thus resulting in the total disruption of PBs (Dougherty et al., 2011; Poblete-Durán et al., 2016). In addition, CVB3 2A protease relocalizes AUF1 (also known as hnRNP D) from the nucleus to the cytoplasm of infected cells and 3C protease cleaves AUF1 (Wong et al., 2013). Moreover, AUF1 knockdown significantly enhances viral RNA abundance, suggesting that AUF1 is a restriction factor for CVB3 replication. Interestingly, AUF1 binds to the 3′UTR of viral genome containing AU-rich sequences and likely targets it for degradation, thus CVB3 counteracts AUF1-induced degradation of the viral RNA genome by targeting AUF1 for degradation (Wong et al., 2013). In addition, it has been reported that MOV10 is a restriction factor for EMCV and CVB3 replication. Thus, both EMCV and CVB3 viral 3C protease induces the cleavage of MOV10 to counteract its antiviral activity (Cuevas et al., 2016). Enterovirus 71 (EV71) increases the number of PBs at early stages but disrupt PBs at late stages of infection (Wang et al., 2016; Zhu et al., 2016; Yang et al., 2018b). In addition, EV71 2C protease reduces the expression of APOBEC3G (A3G) by targeting to degradation via the autophagy-lysosome pathway. Surprisingly, A3G antiviral activity does not depend on its cytidine deaminase activity but restricts viral replication by binding to the viral 5′UTR thus displacing PCBP1, which is essential for the replication of picornaviruses such EV71 (Li et al., 2018). In contrast, MOV10 is a positive regulator of EV71 replication through its binding to a cloverleaf-like structure and the IRES of viral RNA, facilitating replication and viral protein synthesis (Wang et al., 2016). Upon EV71 infection MOV10 co-localized with PBs and it is suggested that

MOV10 relocalization is a host response to impair viral MOV10 recruitment (Wang et al., 2016).

Dicistroviridae and *Togaviridae*

CrPV, a member of the *Dicistroviridae* family, selectively disrupts GW182/Dcp1 but not Ago1/Ago2 aggregates, suggesting that they play differential roles during infection (Khong and Jan, 2011), while that RNAs of SINV, a virus that belong to the *Togaviridae* family, interact with HuR in order to stabilize and avoid the cellular mRNA decay machinery (Sokoloski et al., 2010). It was reported that Upf1 knockdown increases SINV and Semliki Forest Virus (SFV) RNA replication. Thus, Upf1 has an antiviral activity, probably by promoting viral RNA degradation. In addition, Upf1-mediated inhibition of viral replication also involved other NMD components, such as Smg5 and Smg7 (Balistreri et al., 2014). On other hand, the coronavirus MHV induces the degradation of several cellular mRNAs encoding translation-related factors with a concomitant translational shut off and increase in the number of PBs per cell (Raaben et al., 2007). The mechanism behind MHV-induced regulation of mRNA decay is still unknown. Similarly, SARS-CoV Nsp1 promotes mRNA downregulation as a consequence of global mRNA degradation in order to maximize viral RNA translation (Huang et al., 2011). In contrast, TGEV infection decreases significantly the number of PBs by an unknown mechanism (Sola et al., 2011).

Negative-Sense Single Stranded ((–) ssRNA) Viruses

Orthomyxoviridae and *Bunyaviridae*

The negative-strand RNA virus IAV, member of *Orthomyxoviridae* family, suppresses PB assembly via interaction of NS1 and RAP55; and prevents the sequestering of NP in the PBs (Mok et al., 2012). In contrast, the *Bunyaviridae* hantavirus nucleocapsid protein (N) avoids the 5′ cap degradation of cellular mRNAs, protecting them from Dcp1a/Dcp2-mediated decapping which allows the viral transcripts to escape from PBs (Mir et al., 2008) (reviewed in Poblete-Durán et al., 2016).

Paramyxoviridae and *Rhabdoviridae*

Recently, it was shown that the infection of RSV, member of *Paramyxoviridae* family, decreases the number of Dcp1-containing puncta late in infection, suggesting that RSV disassemble PBs over time or that Dcp1 is excluded from PBs (Dickey et al., 2015); while that during early stages of VSV infection, virus belong to *Rhabdoviridae* family, Dcp1/PBs accumulation is unaltered, but further work is necessary to determine its effect on later times (Dinh et al., 2012).

Single Strand RNA Retroviruses (ssRNA-RTs)

Retroviridae

Abrahamyan et al. reported that HIV-1 expression dramatically decreases the number of PBs (Abrahamyan et al., 2010), but the relationship between HIV-1 and the PB components is still controversial. It has been shown that HIV-1 gRNA localize to PBs (Chable-Bessia et al., 2009; Nathans et al., 2009; Martin et al., 2011), however, that observation has not been reproduced by others (Abrahamyan et al., 2010; Phalora et al., 2012). Similarly, some groups have reported that DDX6, LSm1, GW182, Xrn1, DGCR8, Dicer, and Drosha are antiviral factors, while other researchers argue that Ago2 and DDX6 are proviral factors (Chable-Bessia et al., 2009; Nathans et al., 2009; Martin et al., 2011; Bouttier et al., 2012; Reed et al., 2012). APOBEC3 has been shown to have an anti-HIV-1 activity, but it is targeted for degradation by the viral protein Vif (Poblete-Durán et al., 2016). Interestingly, Chen et al. showed that MOV10 protects A3G from Vif-mediated degradation by interfering with the interaction between Vif and the ubiquitin CBF-β-Cullin 5-ElonginB-ElonginC complex (Chen et al., 2017). In addition, MOV10 increases the incorporation of A3G in HIV-1 viral particles, enhancing the antiviral effect of A3G (Chen et al., 2017). Recently, it has been shown HIV-1 infected monocyte-derived macrophages (MDMs) show diminished levels of Upf1, Upf2, and SMG6 proteins, accordingly with their restrictive role in HIV-1 replication due to their ability to inhibit genomic RNA expression (Rao et al., 2019a). Interestingly, Reed et al. demonstrated that the first assembly intermediate (AI) in which Gag interacts with genomic RNA—containing HIV-1 Gag, GagPol, and Vif (Lingappa et al., 1997)—are formed by the recruitment of DDX6 and ATP-binding cassette protein E1 (ABCE1), suggesting that HIV-1 hijack PB-components to promote viral assembly (Reed et al., 2012; Barajas et al., 2018). The Feline Immunodeficiency Virus (FIV) also co-opts a cellular RNA granule containing DDX6, ABCE1, and Dcp2 to assembly immature capsids, suggesting that this is a mechanism conserved between primate and non-primate lentiviruses (Reed et al., 2018). It has been reported that HTLV-1 Tax protein interferes with host NMD by its interaction with Upf1 and INT6 (Mocquet et al., 2012). In addition, Tax increases the localization of Upf1 in PBs, causing an increase in their size and number. Interestingly, viral RNA is sensitive to degradation via NMD and it has been shown that a fraction of viral genomic RNA co-localized with PBs (Mocquet et al., 2012; Nakano et al., 2013). Fiorini et al. reported that Tax stabilizes the SMG5 and Upf1 interaction, which inhibits Upf1 recycling for another round of NMD (Mocquet et al., 2012). Moreover, Tax inhibits Upf1 binding to its substrate and also destabilizes Upf1 during both unwinding and translocation, promoting its dissociation from the substrate. Together, these observations suggest that Upf1 is targeted by Tax both before and during Upf1-mediated decay (Fiorini et al., 2018). In contrast, Nakano et al. argued that Rex protein is the main protein responsible for NMD inhibition in HTLV-1 infected cells and that Tax effect on NMD is not as significant as the effect of Rex protein (Nakano et al., 2013).

A summary of how viruses modulate PBs can be found in **Table 3**.

CONCLUDING REMARKS

The knowledge about MLOs has increased exponentially during the last few years (reviewed in Guzikowski et al., 2019).

TABLE 3 | Virus families that modulate PBs.

Genome	Virus family	Virus	PB induction	PB blockade	Mechanism	References
dsDNA	*Adenoviridae*	Adenovirus	No	Yes	Relocalization of DDX6, LSm1, Ge-1, Ago2, and Xrn1 to aggresomes dependent on E4 11K viral protein	Greer et al., 2011
					Relocalization of Pat1b to aggresomes dependent on E4 11K viral protein	Friedman and Karen, 2017
	Herpesviridae	Cytomegalovirus (HCMV)	Yes	No	Increased Dcp1a, Edc4, DDX6, and Rap55 protein levels and PB accumulation, but viral mRNA is not sequestered	Seto et al., 2014
		Kaposi's sarcoma-associated herpesvirus (KSHV)	No	Yes	PB disruption during lytic replication requires RhoA activation, mediated by vGPCR activation pathway (vGPCR-MK2)	Corcoran et al., 2012
					PBs disruption during latency requires RhoA activation, mediated by Kaposin B activation pathway (KapB-MK2-hsp27-p11RhoGEF)	Corcoran et al., 2015
dsRNA	*Reoviridae*	Rotavirus	No	Yes	Xrn1, Dcp1, and Pan3, but not GW182 protein levels are reduced. NSP1 triggers Pan3 decay. Xnr1 and Dcp1 are translocated to the nucleus	Bhowmick et al., 2015
					PABP is relocalized to the nucleus dependent on the viral protein NSP3	Montero et al., 2008
					Most of PBs-associated proteins, except DDX6, Edc4, and Pan3, are recruited into RFs	Dhillon and Rao, 2018; Dhillon et al., 2018
(+)ssRNA	*Flaviviridae*	West Nile virus (WNV)	No	Yes	LSm1, GW182, DDX6, DDX3, and Xrn1 are sequestered to RFs	Emara and Brinton, 2007; Chahar et al., 2013
		Dengue Virus (DENV)	No	Yes	LSm1, GW182, DDX6, DDX3, MOV10, and Xrn1 are sequestered to RFs	Emara and Brinton, 2007; Chahar et al., 2013
					IRAV and MOV10 localizes to RFs and associates with DENV NS4A and NS3	Balinsky et al., 2017
					LSm1 binds to the Dengue virus RNA 3' UTR and localizes to viral RFs	Dong et al., 2015
		Zika Virus	No	No	DDX6 and DGCR8 are upregulated in ZIKV-infected neurospheres	Garcez et al., 2017
					ZIKV does not affects PBs abundance, morphology or localization	Hou et al., 2017
					ZIKV capsid protein specifically targets nuclear Upf1 for degradation via the proteasome	Fontaine et al., 2018
		Yellow fever virus (YFV)	Yes*	No	sfRNA co-localizes at PBs and inhibits Xrn1 activity	Silva et al., 2010
		Kunjin virus (KUNV), Australian strain of DENV	Yes*	No	sfRNA co-localizes at PBs and inhibits Xrn1 activity	Pijlman et al., 2008; Moon et al., 2012
		Hepatitis C virus (HCV)	Yes*	Yes	DDX6, LSm1, Xrn1, PATL1, Ago2, Dicer, and DDX3 localize to lipid droplets	Ariumi et al., 2011; Berezhna et al., 2011; Pager et al., 2013
					Dcp1 and GW182 not localize to viral factories	Pérez-Vilaró et al., 2012
					DDX6 did not colocalize at lipid droplets in hepatocytes from HCV-infected patients	Pérez-Vilar et al., 2015
					XRN1 stalls during exonucleolytic decay of the 5' UTRs of HCV	Moon et al., 2015
		Bovine Viral Diarrhea Virus (BVDV)	ND	ND	XRN1 stalls during exonucleolytic decay of the 5′ UTRs of BVDV	Moon et al., 2015
	Picornaviridae	Poliovirus (PV)	No	Yes	3C protease-mediated cleavage of Xrn1, Dcp1a, and Pan3	Dougherty et al., 2011
					Protease 2A inhibits PB assembly	Dougherty et al., 2015
		Coxsackievirus B3 (CVB3)	No	Yes	3C protease-mediated cleavage of Xrn1, Dcp1a, and Pan3	Dougherty et al., 2011
					Cytoplasmic redistribution and cleavage of AUF1, mediated by 2A and 3C protease, respectively	Wong et al., 2013

(Continued)

TABLE 3 | Continued

Genome	Virus family	Virus	PB induction	PB blockade	Mechanism	References
					Cleavage of MOV10 by 3C protease	Rao et al., 2019b
		Encephalomyocarditis virus (EMCV)	ND	ND	Cleavage of MOV10 by 3C protease	Cuevas et al., 2016
		Enterovirus 71 (EV-71)	Yes	Yes	Disrupts DDX6 and Dcp1a foci	Zhu et al., 2016; Yang et al., 2018b
					EV71 2C protein reduces the expression of A3G through autophagy–lysosome pathway	Li et al., 2018
					MOV10 promotes viral RNA replication and IRES-dependent translation	Wang et al., 2016
					MOV10 co-localizes with PBs upon EV71 infection	Wang et al., 2016
	Dicistroviridae	Cricket paralysis virus (CrPV)	Yes	Yes	Disrupts aggregates containing GW182 and Dcp1	Khong and Jan, 2011
	Togaviridae	Sindbis virus (SINV)	No	Yes	Viral RNA interacts with HuR	Sokoloski et al., 2010
					Upf1 is a restriction factor for SINV	Balistreri et al., 2014
		Semliki Forest Virus (SFV)	ND	ND	Upf1 is a restriction factor for SFV	Balistreri et al., 2014
	Coronaviridae	Mouse Hepatitis Coronavirus (MHC)	Yes	No	Induces host translational shutoff	Raaben et al., 2007
		Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)	ND	ND	SCoV nsp1-mediated promotion of host mRNA degradation	Kamitani et al., 2009; Huang et al., 2011
		Transmissible Gastroenteritis Virus (TGEV)	No	Yes	Decreases the number of PBs	Sola et al., 2011
(-)ssRNA	*Orthomyxoviridae*	Influenza virus A (IAV)	No	Yes	NS1 interacts with RAP55, Ago1, Ago2, and Dcp1a	Mok et al., 2012
	Bunyaviridae	Hanta virus	Yes*	No	Cap snatching occurs in PBs	Mir et al., 2008
	Paramyxoviridae	Respiratory Syncytial Virus (RSV)	ND	ND	Reduction of Dcp1 puncta over time	Dickey et al., 2015
	Rabdhoviridae	Vesicular stomatitis virus (VSV)	ND	ND	Dcp1 puncta are not affected	Dinh et al., 2012
ssRNA-RT	*Retroviridae*	Human Immunodeficiency virus type 1 HIV-1	ND	Yes	HIV-1 genomic RNA interacts with DDX6, Ago2, and APOBE3G	Nathans et al., 2009
					Relocalization of PBs out of zones where genomic RNA accumulates	Abrahamyan et al., 2010
					The first assembly intermediate where Gag interacts with viral RNA contains DDX6 and ABCE1	Reed et al., 2012; Barajas et al., 2018
					Overexpression of MOV10 inhibits HIV-1 replication	Burdick et al., 2010
					MOV10 inhibits the degradation of APOBEC3G through interference with the Vif-mediated ubiquitin–proteasome pathway	Dong et al., 2015
					Downregulation of Upf1, Upf2, and SMG6 in infected monocyte-derived macrophages	Rao et al., 2019a
		Feline Immunodeficiency Virus (FIV)	ND	ND	Assembly intermediates are formed by DDX6, Dcp2, and ABCE1	Reed et al., 2018
		Human T-cell lymphotropic virus type I (HTLV-1)	Yes	No	Tax inhibits NMD by targeting Upf1 and INT6	Mocquet et al., 2012; Fiorini et al., 2018
					Tax increases the localization of Upf1 in PBs	Mocquet et al., 2012
					A fraction of viral RNA colocalizes in PBs	Mocquet et al., 2012; Nakano et al., 2013
					Rex inhibits Upf1 activity	Nakano et al., 2013

**Maintain PB endogenous.*

Consequently, the historically proposed roles of SGs and PBs in mRNA storage and degradation have been challenged. SGs and PBs assembly have been shown to contribute to cell survival, inducing translational arrest and delay apoptosis by sequestration of RACK1 from JNK, while that SGs may inhibit growth signaling by diverting TORC1 from its active location at lysosomes (Arimoto et al., 2008; Kedersha et al., 2011; Thedieck et al., 2013; Arimoto-Matsuzaki et al., 2016). Single-molecule imaging studies have revealed that mRNAs can transiently interact or move between SGs and PBs (Wilbertz et al., 2018; Moon et al., 2019), and more interestingly, that mRNA translation and degradation dynamics are equivalent for MLO-engaged and "free" mRNAs, suggesting that their sequestration into granules does not regulate translation and decay (Wilbertz et al., 2018).

Recently, Niewidok et al. revealed the existence of a relative immobile nanocore within SGs and a mobile outer liquid phase that allows a dynamic exchange of protein and mRNA between SGs (Niewidok et al., 2018). Importantly, the tight interaction between SG-nanocores and prion-like domains present in many SG and PB components has been proposed as a potential origin of neurotoxic protein aggregates that are linked to the progression of different neurodegenerative diseases (reviewed in Ramaswami et al., 2013; Dobra et al., 2018), growing MLOs as an attractive therapeutic target for the treatment of such syndromes. A similar interest is rising regarding MLOs and viral infections. As reviewed here, cellular and viral-induced MLOs have been associated with a spatiotemporal regulation of viral replication, viral assembly and host immune evasion (Schuster et al., 2018). MLOs allow the concentration of viral proteins and viral genome in the cytosol of infected cells, enabling a dynamic exchange and adaptation to changing environmental conditions. Despite all the information presented in this review about the modulation of the MLOs assembly, many molecular details of how some viral families proceed to subvert the MLOs remain unknown. Why some viruses promote or inhibit the MLO assembly is well-characterized. In many cases, these MLOs serve as viral factories recruiting cellular proteins to ensure efficient viral replication, while that the co-option of proteins involved in antiviral response (e.g., interferon-stimulated genes) could have a detrimental effect. On the other hand, the dynamism in the MLOs assembly/disassembly over the course of a viral infection would allow the chronicity of the infection (Ruggieri et al., 2012). Today, we know that not only proteins, but also RNA modifications have gained importance in the fate of mRNAs inside the cell. These RNA modifications (e.g., m6A, m5C) have been involved in triaging mRNAs to RNA granules (Anders et al., 2018) which have also been widely identified in the RNA genomes of several viruses such as HIV-1, ZIKV, HCV, IAV, KSHV, and SV40 (Reviewed in Pereira-Montecinos et al., 2017; Manners et al., 2018). Thus, the possibility to develop drugs that upregulate these pathways, the generation of molecules that disaggregate or target crucial viral -MLO interactions arises to impair viral replication (Jackrel et al., 2014). Furthermore, due to the shared mechanism that different viruses use to modulate MLOs, drugs targeting MLOs could eventually serve as broad-spectrum antivirals for infectious diseases.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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