THE NATURAL KILLER CELL INTERACTOME IN THE TUMOR MICROENVIRONMENT: BASIC CONCEPTS AND CLINICAL APPLICATION

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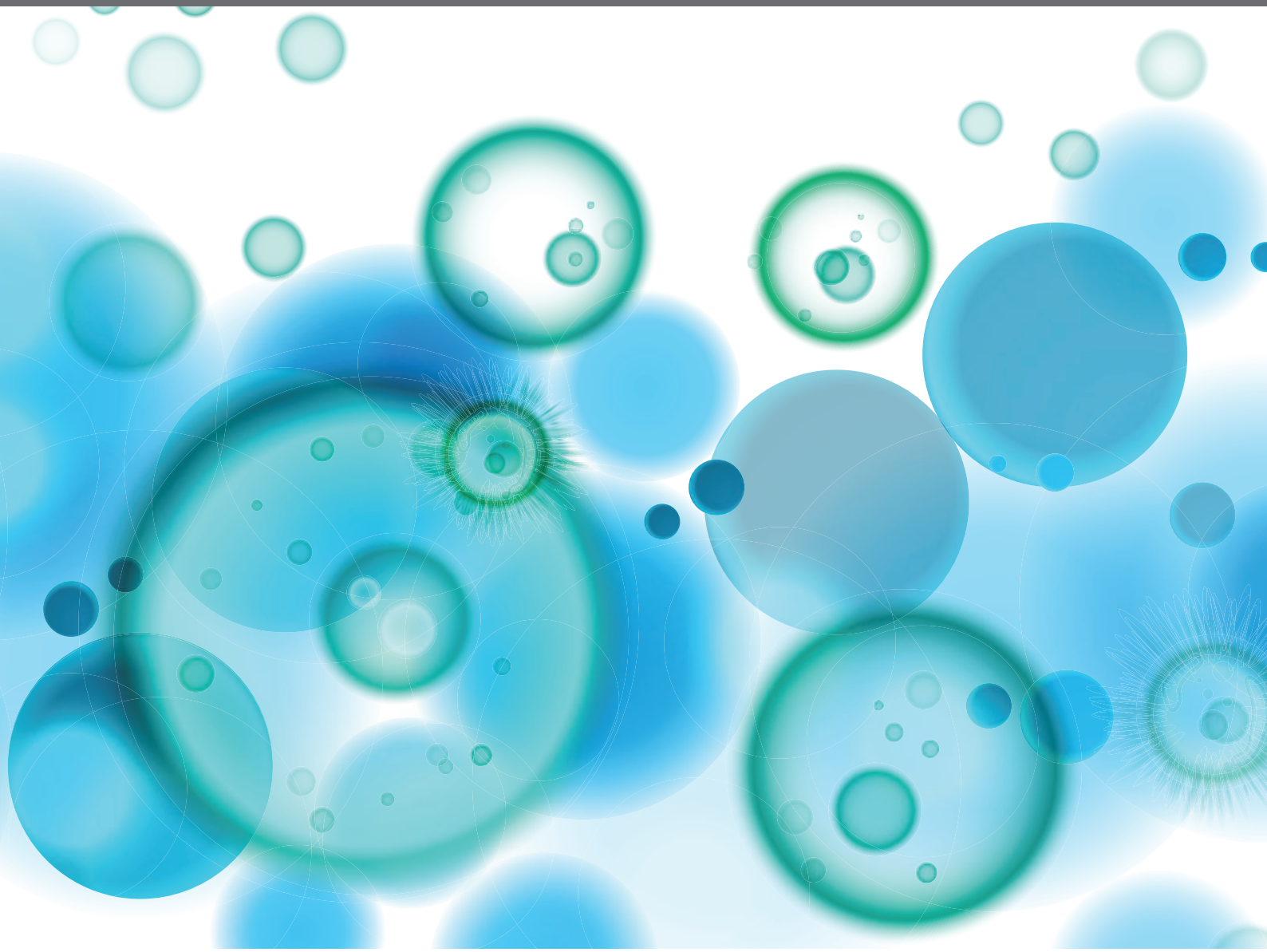

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THE NATURAL KILLER CELL INTERACTOME IN THE TUMOR MICROENVIRONMENT: BASIC CONCEPTS AND CLINICAL APPLICATION

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EDITORIAL
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Editorial: The Natural Killer Cell Interactome in the Tumor Microenvironment: Basic Concepts and Clinical Application

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Editorial on the Research Topic

The Natural Killer Cell Interactome in the Tumor Microenvironment: Basic Concepts and Clinical Application

NK cell activity is impaired in cancer patients, supporting the use of adoptive NK cell therapy, which is becoming a credible immunotherapy for hematological malignancies. This is even more so the case after the presentation of the first clinical study using anti-CD19 NK CAR cells, which showed a good clinical activity in the absence of toxicity. The possibility of targeting solid tumors is being studied by numerous laboratories, but the tumor microenvironment supports immune suppression. Unveiling the molecular and cellular mechanisms explaining this immunosuppression is a major goal.

For this special issue, we pointed to several specific subjects, such as the metabolic interactions of NK cells with tumor targets that would regulate their function or novel molecular strategies for generating off-the-shelf NK cell cancer immunotherapies. A total of 10 manuscripts have been accepted for publication, of which five are original research and five are reviews or minireviews.

Regarding the original research articles, Alvarez et al. have described the indirect contribution of the PD-1/PD-L1 system to the regulation of NK cell exhaustion using an *in vivo* murine model. They showed that a PD-1 blockade increased $CD8^+$ T cell activation rates, which competed for IL-2 and resources with NK cells, retarding their activation but also their subsequent exhaustion. Federici et al. developed an exhaustive work characterizing NK-cell derived extracellular vesicles (NKEVs), separating true exosomes from microvesicles. They demonstrated that NKEVs supported immune activation, modulating the expression of key stimulatory molecules in monocytes and in T cells. They also demonstrated that the amount of NKEVs is reduced in the plasma of melanoma patients compared with healthy donors. The Martin Villalba's group, in collaboration with clinicians and the group of Alexia et al., investigated the antitumor effect of the new immunoadjuvant Polyoxidonium (PO) in breast cancer patients. They demonstrate that PO increases activation markers in dendritic cells, favoring T-cell activation. In addition, PO increased the degranulation capacity of NK cells, showing positive clinical effects in a percentage of patients. Diaz et al. performed an interesting clinical study on a cohort of 60 young and pediatric patients with hematological malignancies. Patients were engrafted with haploidentical stem cells after T- and

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B-cell depletion. Improved outcomes correlated with a rapid expansion of mature $CD56^{dim}$ NK cells early after transplantation, suggesting a positive graft vs. leukemia effect. Directly entering in the off-the-shelf NK cells for cancer immunotherapy, Fernández et al. described the manufacturing in GMP conditions of allogeneic NKG2D CAR T cells. To avoid undesirable graft vs. host reactions, $CD45^{-}$ memory T cells were used, and good expansions of active CAR T cells were obtained.

Regarding reviews, Nersesian et al. published a comprehensive work describing the typical immunosuppressive tumor microenvironment (TME) of ovarian tumors and how adoptive NK cell treatment can help to revert this situation, alone or in combination with other immunotherapies. In fact, at least nine clinical trials using this approach in ovarian cancer patients are currently ongoing. Terrén et al. put the point on the molecular mechanisms that explain the inactivation of NK cell function by the TME through the modulation of NK cell metabolism. This review has attracted interest, being the most read paper of the collection at the moment, with more than 6,000 views. Villalba et al. describe strategies to improve NK cell anti-tumoral function, including combination with mAbs to induce ADCC, "arming" NK cells with antibodies, or the use of metabolic drugs that could increase tumor sensitivity to activated NK cells. Burger et al. described a promising field for NK cell-based therapy; CAR NK cells would be used for the treatment of glioblastoma, with the tumor antigen HER2 as the main target at the moment. Finally, and in relation with the first original article mentioned at the beginning of this editorial, Lanuza et al. reviewed the available experimental evidences regarding the role of immune checkpoints in NK cell function during physiological and pathological (cancer) conditions, arriving at the conclusion that the main checkpoint molecules targeted in T cells (CTLA-4, PD-1, LAG-3) have low impact in NK cell function during physiological conditions. This might be an additional advantage when using adoptive allogeneic NK cell transfer in the treatment of cancer.

As stated in the presentation of the special issue, we can therefore consider that this collection of reviews and original articles highlights significant advances made in the field of NK cell-based therapy and indicate potential new useful directions, always keeping in mind the benefit of patients and the improvement of their quality of life.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. AA wrote the first draft; MV revised the reviews section and JP revised the original papers section, proposing a final integrated text.

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REVIEW
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Naturally Killing the Silent Killer: NK Cell-Based Immunotherapy for Ovarian Cancer

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Ovarian cancer (OC) is diagnosed in ∼22,000 women in the US each year and kills 14,000 of them. Often, patients are not diagnosed until the later stages of disease, when treatment options are limited, highlighting the urgent need for new and improved therapies for precise cancer control. An individual's immune function and interaction with tumor cells can be prognostic of the response to cancer treatment. Current emerging therapies for OC include immunotherapies, which use antibodies or drive T cell-mediated cancer recognition and elimination. In OC, these have been limited by adverse side effects and tumor characteristics including inter- and intra-tumoral heterogeneity, lack of targetable antigens, loss of tumor human leukocyte antigen expression, high levels of immunosuppressive factors, and insufficient immune cell trafficking. Natural killer (NK) cells may be ideal as primary or collateral effectors to these nascent immunotherapies. NK cells exhibit multiple functions that combat immune escape and tumor relapse: they kill targets and elicit inflammation through antigen-independent pathways and detect loss of HLA as a signal for activation. NK cells are efficient mediators of tumor immune surveillance and control, suppressed by the tumor microenvironment and rescued by immune checkpoint blockade. NK cells are regulated by a variety of activating and inhibitory receptors and already known to be central effectors across an array of existing therapies. In this article, we highlight interactions between NK cells and OC and their potential to change the immunosuppressive tumor microenvironment and participate in durable immune control of OC.

Keywords: natural killer cell, immunotherapy, ovarian cancer immunology, oncoimmunology, tumor microenvironment, high grade serous ovarian cancer

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OVARIAN CANCER

Ovarian cancer (OC) is the leading cause of death from gynecologic malignancies with a 5-year survival of <50% (1). The majority of cases are diagnosed at advanced stages (III and IV), when treatment becomes especially challenging, earning OC its "silent killer" moniker. Beyond stage III, OC disseminates into the peritoneum, and patients present with bloating from a buildup of ascitic fluid in the abdomen (2). Current standard of care includes de-bulking surgery followed by a combination of platinum- and taxane-based chemotherapy. Despite these aggressive treatments, recurrence occurs in 60–70% of patients within 2–5 years; most will eventually succumb to OC (3).

Epithelial OC represents the majority of malignant ovarian tumors and arises from the epithelium of the ovary and fallopian tube. OC tumors are broadly divided into two subtypes: type I and II; these classifications carry both prognostic and predictive value (4). These subtypes are differentially responsive to the available treatments, and whether they may inform the development and application of more precise treatment, including immunotherapy, is the subject of active investigation. Type I OC includes low-grade serous, endometrioid, clear cell, and mucinous carcinomas that together account for ∼30% of ovarian tumors (4, 5). This subset is typically genetically stable and slow-growing. By contrast, type II OC is genetically unstable, more aggressive and accounts for the majority of OC mortality (4, 5). This subtype exclusively includes high-grade serous carcinomas, represents 70% of all ovarian tumors, and will be the focus of this review (6–8).

NK CELLS

NK cells are lymphocytes that reside in the peripheral blood and are highly efficient anti-tumor effectors (9–11). They comprise 5–10% of circulating lymphocytes and kill target cells without previous sensitization. NK cells differentiate from the common lymphoid progenitor in the bone marrow, are most closely related to T cells, and have similar abilities to expel perforin and granzymes for direct target cell killing (12, 13). In addition, they signal for target cell apoptosis through Fas and TRAIL pathways and secrete pro-inflammatory cytokines including IFN-γ and TNF (12). NK cells can be characterized into an array of categories based on the presence and density of surface markers expressed with some studies reporting up to 30,000 in one individual (14). At a superficial level they can be categorized into two main populations based on CD16 and CD56–expression; with CD56bright/CD16– functioning to primarily produce cytokines in the circulating blood, and CD56dim/CD16+ performing cytotoxicity in the tissues (12).

Rather than direct detection of antigens through a germline-rearranged receptor (the domain of T and B cells), NK cells recognize putative targets based on their expression of stress-induced ligands, upregulated on the cell surface consequent to DNA damage and heat shock, and in response to stimulation by environmental factors, including cytokines and chemokines (15–17). To avoid unwanted auto-aggression, NK cells are also sensitive to inhibition by "self" human leukocyte antigen (HLA) class I molecules; it is the net outcome of incoming activating and inhibitory signals that determines the NK cell response to a putative target (12). A complete summary of the receptors and cytokines produced by NK cells is beyond the scope of this review and can be found elsewhere (18–20). Here, we limit our discussion to those identified as relevant in OC, summarized in **Table 1**.

NK cell responsiveness varies within and between individuals, via a process called "education," "licensing," or "arming." The killer immunoglobulin-like receptors (KIR) interact with conserved epitopes on HLA (41). KIR and HLA genes are both highly polymorphic and polygenic and their loci segregate independently (42). Thus, the availability of binding pairs differs between people, with consequences on NK cell reactive potential (42). NK cells expressing KIR that can bind to "self" HLA (licensed) are highly responsive to targets lacking "self" HLA—a process termed "missing self" recognition. NK cells that do not express self-HLA specific receptors are "unlicensed" and require potent stimulation for reactivity. Unlicensed NK cells are especially protective against HLA-expressing tumors because they are refractory to the inhibitory signals sent by HLA (43, 44). Thus, education potentiates a spectrum of NK cell reactivity, establishing, at the repertoire level, an array of functions to target HLA-sufficient and HLA-deficient target cells (19, 20).

NK cells are capable of memory or adaptive functions, whereby previous sensitization leads to expansion and retention of a population of NK cells that respond more rapidly and robustly to secondary challenges with the same virus or hapten (45, 46). These "adaptive" NK cells have undergone epigenetic alterations leading to a distinct phenotype and enhanced function (47–49). While additional research is required to fully characterize this cell population, unbound inhibitory receptors, NKp44, NKp46, NKG2C, and CD57 receptors are consistently overexpressed (47–50). Noteworthy, these same adaptive features can be achieved by cytokine cocktail stimulation in the absence of a specific antigen; cytokine-induced adaptive cells exhibit increased expression of activating receptors including NKp30, NKG2D, NKp44, NKp46, and TRAIL (51). Thus, while a memory response may be generated toward a specific pathogen, it may be the environment or steric changes in the HLA-peptide complex that drive NK cell adaptive functions (45). For cancer immunotherapy, this is noteworthy as it opens the possibility to train highly effective NK cells without strict requirements for antigen restriction.

THE TUMOR MICROENVIRONMENT AND IMMUNOSUPPRESSION IN OVARIAN CANCER

The tumor microenvironment (TME) includes the tumor, stroma, and local immune cells. The TME is dynamic and can promote or suppress tumor invasion and metastasis. The immune composition in the TME is now known to be an important predictor of response to therapy in a variety of solid tumor types, including OC. Several factors, processes, and cellular subsets contribute to the development of the TME including innate and adaptive immune cells, intercellular signaling, and tumor intrinsic factors such as gene mutational burden (**Figure 1**) (52).

Good prognosis for OC and its treatment are correlated with immune cell infiltration in the TME, and the majority of studies focused on infiltrating T cells (53) (**Figure 2**). These so-called "hot" tumors represent two scenarios: (1) immune inflamed tumors, where T cells can directly contact malignant cells; and (2) immune excluded tumors, where T cells are restricted to the stroma surrounding malignant cells, with limited access for ligation and killing. In contrast, "cold" or "non-inflamed" tumors lack T cell infiltration. Reproducibly, patients whose OC tumors

TABLE 1 | NK cell receptors and their relevance to ovarian cancer.

Receptor	Ligand	OC relevance	References
CYTOKINE RECEPTORS			
IL-2R	IL-2	NK cells isolated from OC patient ascitic fluid demonstrated reduced proliferation in response to interleukin-2 (IL-2)	(21)
IL-10R	IL-10	In OC patient ascitic fluid increased IL-10 expression relates to advanced stages (III/IV)	(22)
IL-12R	IL-12	Human PBMCs, isolated from OC patients, stimulated by IL-12 demonstrate enhanced activation and proliferation of functional NK cells	(23)
IL-15R	IL-15	Increased levels in OC patient ascitic fluid were associated with increased NK cell cytotoxicity	(24)
IL-21R	IL-21	Mice treated with IL-21 demonstrated delayed tumor appearance and reduced OC tumor size	(25, 26)
TGF-βR	TGF-β	Increased TGF-β expression in OC tumors has been associated with progression and metastasis	(27–30)
ACTIVATING RECEPTORS			
2B4 (CD244)	CD48	Downregulation of 2B4 and hyporesponsiveness of 2B4+ NK cells to MHC class I -negative targets in OC patient ascitic fluid	(31–34)
CD16 (FcRγIII)	Fc portion of antibodies	Decreased expression has been identified in NK cells isolated from OC patient ascitic fluid	(21)
CD69	Undefined	Increased expression has been identified in NK cells isolated from OC patient ascitic fluid	(24)
DNAM1 (CD226)	CD155, CD112	Decreased DNAM1 expression and hypo-responsiveness of DNAM1+ NK cells to MHC class I – negative targets in NK cells isolated from OC patient ascitic fluid	(24, 31–33, 35)
NKG2D	NKG2D ligands – various, including MIC-A/B, ULBP1-6	NKG2D was downregulated on NK cells isolated from OC patient ascitic fluid	(24, 35, 36)
NKp30 (CD337)	Various, including B7-H6, CMV pp65 tegument protein, BAG6, heparan sulfate	Decreased NKp30 expression on NK cells isolated from OC patient ascitic fluid	(35, 37)
NKp44 (CD336)	Various, including proliferating cell nuclear antigen (PCNA), platelet-derived growth factor (PDGF), mixed-lineage leukemia-5 (MLL-5), viral hemagglutinins	Decreased NKp44 expression on NK cells isolated from OC patient ascitic fluid	(24, 33, 38)
NKp46 (CD335)	Various, including complement factor P, heparin sulfate, viral hemagglutinins	Decreased NKp46 expression on NK cells isolated from OC patient ascitic fluid	(24, 35)
TRAIL	TRAIL-R	TRAIL-R downregulated on OC cells isolated from OC patient tumors	(39)
INHIBITORY RECEPTORS			
KIR2DL1 (CD158a)	MHC-C2 group ligands	Decreased expression on NK cells isolated from OC patient ascitic fluid	(33)
KIR2DL2 (CD158b)	MHC-C1 group ligands (major); some binding to MHC-C2 group ligands	Decreased expression on NK cells isolated from OC patient ascitic fluid	(33)
KIR2DL3 (CD158b)	MHC-C2 group ligands	Decreased expression on NK cells isolated from OC patient ascitic fluid	(33)
KIR3DL1 (CD158e)	MHC-B alleles with the Bw4 motif	Decreased expression on NK cells isolated from OC patient ascitic fluid	(33)
PD-1	PD-L1	PD-1 overexpression on NK cells isolated from OC patient ascitic fluid	(40)

are infiltrated by T cells respond better to therapy and have better prognosis (54, 55): 55% of patients with T cell infiltration reached a 5-year survival of 38% compared to just 4.5% in patients without T cell infiltration (54).

Tumor transcriptome analysis has revealed correlations of OC sub-classifications with T cell infiltration, neoantigen burden and patient prognosis, termed C1, C2, C4, and C5 (8). Among them, the C2 "immunoreactive" subtype is well-infiltrated and associated with the best prognosis (8). Intermediate T cell infiltration and prognoses are associated with the C1 "mesenchymal" and C4 "differentiated" subtypes, and the C5 "proliferative" has the lowest T cell infiltration and conveys the poorest prognosis (8). Despite the mutational burden found in many OC—a feature that can predict priming and infiltration of lymphocytes—they remain immunologically "cold," and infiltrating T cells are not universally reactive against tumor antigens (56, 57). This highlights an urgent need to better understand how other cellular and acellular players contribute to tumor growth and treatment success.

An additional barrier to immunotherapy and effective anti-cancer reactivity is the immunosuppressive nature of the TME, which advances with OC progression (58). Dendritic cells

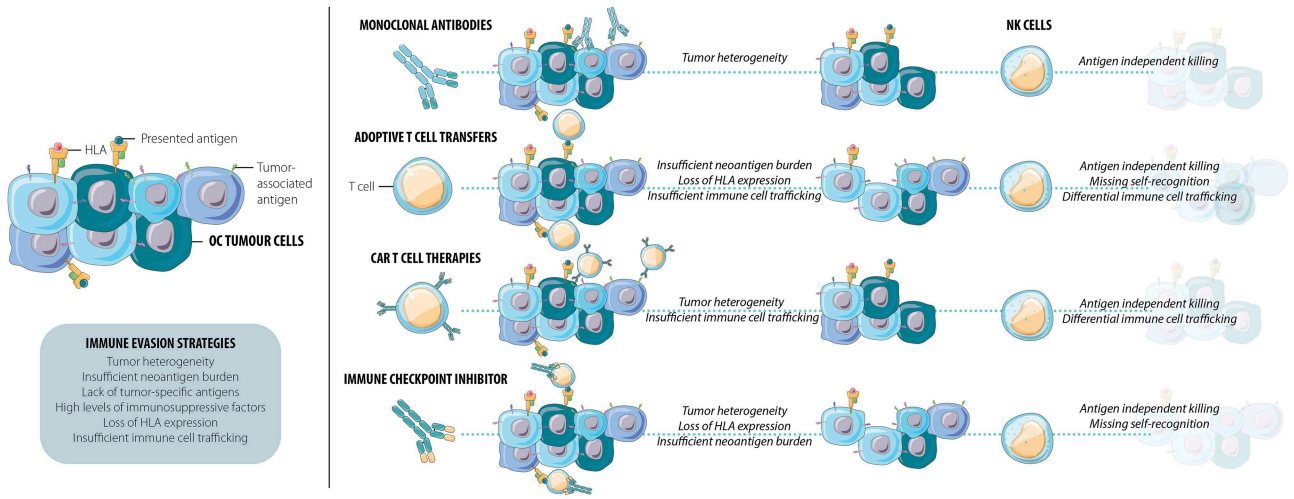

FIGURE 1 | Current immunotherapies frequently result in the persistence of a resistant cell population. This immune evasion of OC tumor cells can be facilitated by tumor heterogeneity, insufficient neoantigen burden, lack of tumor-specific antigens, high levels of immunosuppressive factors, loss of HLA, and/or insufficient immune cell trafficking. NK cells exhibit multiple functions that combat immune escape and tumor relapse: they kill targets and elicit inflammation through both antigen-specific and antigen-independent pathways and detect loss of HLA as a signal for activation. As efficient mediators of tumor immune surveillance and control, NK cells may be able to kill the cells many current immunotherapies leave behind.

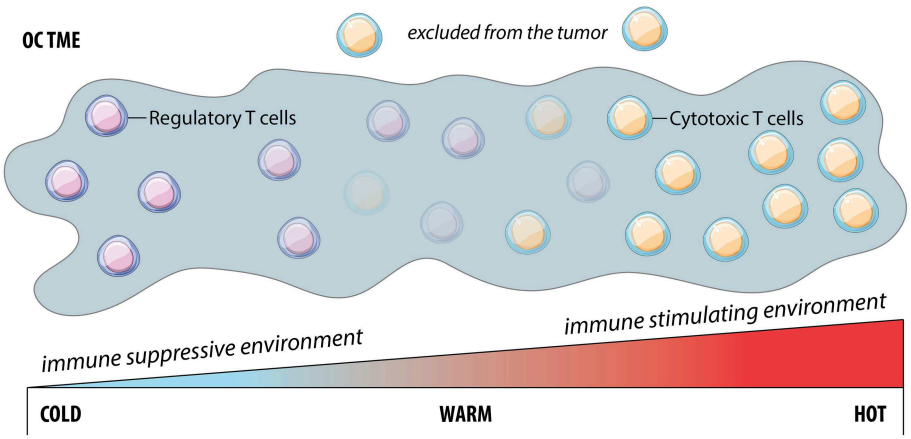

FIGURE 2 | The cold, warm or hot tumor microenvironment is a continuum. Cold tumors are characterized by the lack of cytotoxic T cells and are typically associated with an immune suppressive environment. Conversely, hot tumors are well infiltrated by cytotoxic T cells and are associated with an immune stimulating environment and better prognosis than the prior. However, the characterization of tumors is not dichotomous, but rather exists as a sliding scale of cytotoxic T cell infiltration with "warm" tumors representing a situation where although T cells exist, they are excluded and therefore ineffective at producing an efficient anti-tumor response.

(DCs) are required for the activation of anti-tumor T cells but also have the ability to release immunosuppressive cytokines. In OC, DCs are dysfunctional and immature due to high levels of VEGF and IL-10 in the TME (59–61). These DCs are not only unable to activate cytotoxic T cells, but also function to induce regulatory T cell (Treg) differentiation, further promoting immune suppression (62). Moreover, patient ascitic fluid contains high levels of the Treg recruiting chemokine, CCL22 (63). Unsurprisingly, Treg accumulate in the ascites isolated from late stage OC patients (64, 65). Treg release IL-6 and IL-10 which induces expression of B7-H4 on macrophages and subsequently leads to cytotoxic T cell cycle arrest (65). Discussed in the following section, these impacts extend beyond suppression of T cell immune responses and likely also interfere with productive, anti-tumor NK cell function (**Figure 3**).

NK Cells in the OC Tumor Microenvironment

The important contributions of NK cells to cancer control were identified through mouse models deficient in NK cells or key NK cell activating receptors (66, 67). The protective effect of NK cells was further supported by studies in humans that correlated poor NK cell function to cancer susceptibility, progression and metastases in a variety of both solid and

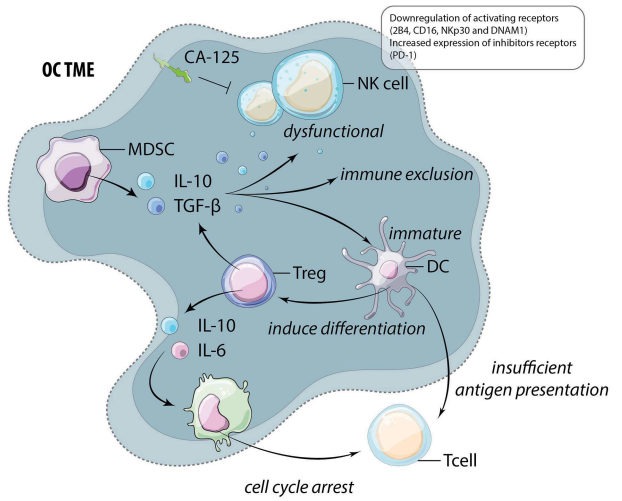

FIGURE 3 | The immunosuppressive TME of OC. Myeloid derived suppressor cells (MDSC) and regulatory T cells (Treg) release high levels of immunosuppressive cytokines including IL-10 and TGF-β. These cytokines function to alter and suppress the function of multiple cells within the TME including natural killer (NK) cells, dendritic cells (DC), and macrophages (M). These cells frequently feedback onto lymphocytes by inducing further Treg differentiation and preventing anti-tumor cytotoxic T cell function through insufficient antigen presentation and the induction of cell cycle arrest.

hematologic cancers (68, 69). In OC, the prognostic value of infiltrating NK cells has been debated—NK cells and the NK cell-like population, innate-like lymphocytes (ILCs), have been associated with both tumor progression and control (70–72). NK cells co-infiltrate with cytotoxic T cells and are strongly associated with patient survival (71, 73). One study stratified OC patients into three subgroups based on infiltration of T cells and other lymphocytes, primarily NK cells, and reported differential 5-year survival rates: T + NK cells (90%), NK cells only (63%) and neither T nor NK cell infiltration (0%) (70). Even when they are present in OC, NK cells are dysfunctional: they exhibit reduced proliferation, decreased cytolytic function and decreased inflammatory cytokine production compared to the same patient's peripheral blood NK cells (74, 75) (Summarized in **Table 1**). Hence, a better understanding of how to enumerate, assess and control NK cells will be required to maximize their infiltration, function and reactive potential in the TME.

Alterations in NK cell phenotype and function occur as a result of the products of a growing ovarian tumor, related ascites, and a variety of immunosuppressive cytokines produced by myeloid derived suppressor cells (MDSC) and Treg (76). For example, macrophage migration inhibitory factor (MIF) overexpression has been reported in OC and correlates with tumor progression (77). MIF downregulates transcription of the NK activating receptor NKG2D (37, 77, 78). MIF is also associated with increased expression of the inhibitory checkpoint, B7-H6, which is associated with overall poorer prognosis in OC (34, 79). Similarly, TGF-β overexpression can suppress CD16-triggered NK cell IFN-γ production (80) and, together with IL-10, has been shown to decrease the inflammatory cytokine production and cytotoxicity of various effector cells including NK cells (81–84). These alterations include a downregulation of activating receptors 2B4, CD16, NKp30, DNAM1, and an upregulation of the inhibitory checkpoint receptor PD-1 (21, 31, 32, 37, 40). Finally, despite upregulation of the early activation marker, CD69, NK cells expressing it remain poorly cytolytic (31). In addition to cytokine mediated suppression, CA-125, an antigen overexpressed in 80% of OC, can directly protect tumor cells from NK cell-mediated cytotoxicity by preventing the formation of an immune synapse, regardless of the repertoire of receptors expressed on the NK cells (79, 85).

Taken together, the available research indicates that NK cells interact dynamically with the OC TME and are highly sensitive to the immunoregulation it drives. Infiltration of NK cells into a tumor may only partially predict outcomes of OC. Better understanding the mechanisms through which the TME contributes to immune exclusion and dysregulation will be required for precise tumor control and to maximize NK cell reactivity.

OC IMMUNOTHERAPY: A FOCUS ON THE ROLES AND POTENTIAL OF NK CELLS

Successful immunotherapy requires restoration of immunity in the immunosuppressive TME and complete targeting of a heterogeneous tumor. Anti-tumor immunity can be driven by *ex vivo* re-stimulation of lymphocytes, engineering cells for direct targeting of specific tumor-associated antigens or turning off immune suppression (86–90). Antibody-based therapies can redirect immune cells by blocking their function, or for antibody-dependent cell-mediated cytotoxicity (ADCC), a process for which NK cells are major effectors. While the majority of immunotherapeutic approaches have been developed with a goal of supporting or reinvigorating antigen-specific anticancer activity, they can also support the function of NK cells, whose functional features can complement and extend the breadth of OC immunotherapy (**Figure 1**). In the following sections, we highlight current approaches to cancer immunotherapy, their potential interactions with NK cells and the opportunities to maximize anti-tumor immunity by recruiting NK cells.

Cytokine-Based Immunomodulation

Recognizing that immunosuppression is a major hindrance for lymphocytes to proceed in anti-cancer activity, approaches with cytokines to induce local and/or systemic inflammation have been tested. A strategy to elicit and improve immune cell activation in humans was first attempted using a variety of activating cytokines including IL-2, IL-12, IL-15, IFN-α, and IFN-γ (91).

IL-2 was one of the earliest cytokines tested for improving anti-tumor immunity. Although early clinical trials were limited by toxicity and activation of Treg, they provided an important proof of concept that stimulating T and NK cells can impact tumor progression. Since then, research has focused on strategies to improve IL-2 safety including low-dose IL-2. In patients with

platinum-sensitive advanced OC, low-dose IL-2 in combination with 13-cis-retinoic acid improved clinical outcomes and increased lymphocyte and NK cell counts (92). As low-dose IL-2 can activate Treg, current efforts are testing constructs that selectively bind to NK cells to support anti-tumor immunity without driving Treg proliferation (93, 94).

Similar disappointing and toxicity-related issues were reported in many trials of activating cytokines. Research resulting in the development of analogs and oncolytic strategies for local delivery may provide the required specificity to bring cytokines safely into clinical use. IL-15 is similar to IL-2 but more specific in that it binds cytotoxic T cells and non-terminally differentiated NK cells to enhance cell cytotoxicity and proliferation. Further, the toxicity of IL-15 is less than that of IL-2, but the concentrations of IL-15 required to drive efficient anti-tumor function remain toxic. Ongoing efforts involve IL-15 "superagonists," which deliver the IL-15 signal in complex with the IL-15 receptor alpha subunit or its biologically-relevant fragments, and/or fused in dimers with an IgG1Fc molecule to stabilize the complex. In each instance, these superagonists more closely replicate the biologically-potent delivery of IL-15, exhibit longer *in vivo* half-lives, and drive lymphocytes (including NK cells) for anti-cancer activity without marked toxicity (95).

ALT-803 is an IL-15 superagonist that potently enhances NK functionality *in vitro* and *in vivo* against OC cell lines (96). After ALT-803 treatment, NK cells isolated from OC patient ascitic fluid exhibited greater degranulation (CD107a) and IFN-γ production (24). Several clinical trials are ongoing evaluating the efficacy of ALT-803 and other IL-15-based therapies, alone and in combination with other immunotherapies including three for patients with OC: NCT03054909, NCT03197584, and NCT03127098 (97). It is expected that the addition of IL-15 and its related superagonists will support NK cell proliferation and development. Metrics to understand NK cell recruitment to the OC TME, persistence and NK cell reactivity (i.e., with *ex vivo* restimulation) will enlighten subsequent clinical trials by indicating how NK cell reactivity can be improved further. Furthermore, studies to understand whether the cytokine milieu varies with defined OC subtypes might help to predict how NK cells will be recruited and effective in patients with OC.

Checkpoint Blockage and Antigen Insufficiency

A high mutational burden creates a challenge for antigen-targeted immunotherapies, but it creates an opportunity for immune-mediated OC recognition. Tumors with high mutational burdens may have increased neoantigen levels, against which antigen-specific T cells may be activated. Many studies have predicted that resistant tumors may lack the neoantigen burden required to mount an effective T cell response (98). One recent investigation profiling tumor and T-cells isolated from the ascites of three OC patients identified that while a high mutational burden was indeed present, only 1.3% of these mutations were recognized by tumor-associated T cells (99). As expected, the presence of neoantigen-reactive T cells (rather than T cells with non-specific reactivity) is predictive of improved prognosis in OC (99, 100). This highlights the importance of the immunogenicity of antigens and an adequate repertoire of tumor-reactive T cells, rather than the overall tumor mutational burden in predicting OC outcomes (101). However, the immune-suppressive TME can still interfere with T cell-mediated tumor recognition.

High mutational burdens have been associated with improved responses to immune checkpoint therapies in both melanoma and non-small cell lung cancers (102, 103). Unexpectedly, despite a significant mutational load being present in pre-treatment surgical samples of OC patients, overall response rates to anti-PD1 treatment in clinical trials have been largely disappointing, ranging from 11–24% (104, 105). These clinical trials were mainly conducted in OC patients that progressed despite conventional treatments—these tumors likely had established immune evasion strategies; earlier intervention with immunotherapy may have achieved better outcomes. Regardless, these clinical trials highlight that a subset of OC patients can respond to anti-PD1 treatment, but the majority have innate or acquired resistance or lack T cells with appropriate anti-tumor reactivity (106).

Various changes in the TME have been identified that may be associated with anti-PD1 resistance including genomic mutations and downregulation of HLA and its associated processing and presentation pathway components (106, 107). These alterations have been reported in both melanoma and lung tumors treated with anti-PD1 (106, 107). Specifically, the acquired genomic mutations provided protection against T-cell mediated killing via loss of IFN-γ or HLA class I components (108, 109). It is not yet known whether anti-PD1 therapy drives similar genetic alterations in OC, but in recurrent OC the expression of HLA genes was negatively correlated with expression of PD-L1, suggesting two mutually exclusive pathways to immune evasion (110). This loss of HLA class I expression, together with insufficient T cell-mediated recognition of tumor antigens, may contribute to the insufficiency of anti-PD1 for complete OC clearance. Strategies to augment NK cell function, particularly those NK cell populations that recognize cells with DNA damage and loss of components of the HLA processing and presentation pathway (i.e., the "licensed" NK cell population), may improve OC treatment and the outcome of anti-PD1 therapy, and complement existing strategies aimed at maximizing T cell-mediated OC control. Likewise, phenotyping of tumor-infiltrating NK cells or the tumors themselves for expression of checkpoints and HLA expression may assist in predicting how NK cells may be functional or inhibited against tumor killing.

Checkpoint inhibitors interfere with inhibitory signaling that prevents anti-tumor reactivity; their application enables lymphocytes to proceed in anticancer cytotoxicity. Although anti-PD1 therapies were designed to rescue T cells from immunosuppression, PD-1 has been found to be expressed on NK cells isolated from OC and other tumor types (40, 111, 112). PD-1 expression is not universal on NK cells however, with studies reporting highly variable PD-1 expression peripheral NK cells in healthy donors, from 0 to 50% (40, 113). In patients with OC however, PD-1 expression on peripheral blood NK cells is

increased suggesting that the presence of a tumor could induce its expression (40).

Despite inconsistent expression on NK cells, anti-PD1 therapies have demonstrated the potential to simultaneously support T and NK cell responses in the TME, suggesting that PD-1 blockade could indirectly influence NK cell function, or that PD-1 expression could be dynamic on NK cells in response to the TME. A recent study investigated the therapeutic effect of anti-PD1 therapy on NK cells using several mouse cancer models and concluded that NK cells were crucial to anti-tumor responses (114). Additionally, OC xenograft studies have demonstrated that both NK cell persistence and cytotoxicity can be improved with PD-L1 blockade (115). Hence, NK cells may be contributing to the outcomes of checkpoint inhibition therapy, even if they have not been expressly studied for this purpose. Recognizing this potentially important feature, clinical trials have also begun to investigate the impact of anti-PD-1 treatment on preventing or reversing NK cell exhaustion in the TME (NCT03241927), but this is not yet standard in clinical trials. Given that NK cells may contribute to the anti-tumor immunity driven by checkpoint inhibition therapies, metrics to assess their prevalence and function may help to illuminate the larger picture of anti-OC immunity.

Some studies have identified an immunoregulatory population of NK cells that exhibit the PD-1 ligand PD-L1 in patients with cancer, but its function has not been determined (116). In antigen presenting cells *cis* expression of PD-L1 and PD-1 permits regulation of PD-1 signaling (117); whether this also occurs in NK cells is unknown. An alternative mechanism, demonstrated in a mouse model, involves PD-L1-mediated editing of dendritic cells, limiting the extent of their interactions with T cells and development of productive anti-tumor T cell response (118). With this in mind, expression of PD-L1 (and its control by anti-PDL1 antibodies) may have significant impacts on direct NK cell function and its interactions with neighboring cells; this warrants further investigation.

In addition to PD-1, other immune checkpoints, including KIR, NKG2A, and TIGIT are being explored as targets for immunotherapy (119, 120). Like PD-1, TIGIT is expressed on both T and NK cells and suppresses anti-tumor effector function in both (120). While its mechanism of action in T cells was recognized, it was only recently identified that they also prevent or reverse NK cell exhaustion (120). TIGIT blockade improved prognosis in murine T and B-deficient, NK-sufficient xenograft models against several human tumor cell lines including colon, breast, melanoma, and fibrosarcoma (120). Anti-TIGIT antibodies are now undergoing clinical trials and just one includes OC (NCT036286770). This trial does not plan measurements of NK cell phenotype or function; future studies should include this as an important outcome measure.

Like TIGIT and PD-1, NKG2A, and the inhibitory subset of KIRs prevent excessive inflammation but may interfere with productive anti-cancer activity. For NK cells, NKG2A and KIR convey an important signal of "self" upon binding with HLA class I. The importance of preventing NK cell inhibition is clear in patients undergoing hematopoietic cell transplantation for acute myelogenous leukemia, where "uninhibitable" populations of NK cells predict for less relapse and greater overall survival (19, 121–123). These observations inspired the creation of antibodies against KIR and NKG2A, which are now being tested against other hematologic and solid malignancies, but not yet in OC.

HLA-E, the ligand for NKG2A, is a non-classical and ubiquitous HLA molecule that has been found at high expression levels in OC tumors (124). As HLA-E overexpression is negatively correlated with survival and exhibited in the majority of tumor types, blocking this NKG2A/HLA-E inhibition could enhance immunotherapy across an array of tumors, including OC (125, 126). In tumors exhibiting high-density HLA-E expression, infiltrating CD8+T and NK cells exhibit high NKG2A and PD-1, suggesting adoption of a phenotype highly sensitive to inhibition in the TME (126). Although OC-infiltrating NK cells' NKG2A expression has not been expressly measured, the abundance of HLA-E on OC tumors would suggest that preventing inhibition of NK cells via NKG2A may enable strong anti-tumor reactivity.

Experimentally, NKG2A surface expression has been eliminated on NK cells by blocking its export from the endoplasmic reticulum using a protein expression blocker construct (126). Primary human NK cells engineered in this way lacked NKG2A surface expression and more efficiently controlled growth of human tumor xenografts in mice (126). Toward a similar goal, a monoclonal antibody, anti-NKG2A (monalizumab) has been delivered for direct delivery to patients. In addition to boosting NK cell cytotoxicity against targets expressing HLA-E, anti-NKG2A has also been demonstrated to augment the function of T cells expressing NKG2A, providing an opportunity to activate both NK and T cells; both were shown to contribute to control of tumor xenografts in mice (127). Inclusion of NKG2A and HLA-E measurements in OC tumors from patients could help to ascertain whether a patient might benefit from anti-NKG2A therapy.

The anti-KIR antibody IPH2101 (lirilumab) aims to interfere with inhibition via the KIR2DL1/2/3 receptors. *In vitro*, lirilumab functions to enhance killing of tumor cells by NK cells (128). Unfortunately, efficacy for this monoclonal antibody has been poor in clinical trials for patients with hematologic malignancies (129), a finding that corresponds with decreased surface density of KIR molecules and "detuning" or diminishment of missing self-responsiveness (130). IPH2101 has not yet been tested against OC, but the available information suggests that further improvements, such as interrupting inhibitory signaling without altering receptor expression, preventing the loss of cell surface KIR through the trogocytosis prompted by IPH2101, or stratifying patients based on particular KIR haplotypes or KIR allotypes likely to be sensitive to lirilumab, will be necessary to gain efficacy against OC.

By combining checkpoint inhibitors, it may be possible to augment the NK cell anti-tumor response—by relieving two or more inhibitory signals, or by rebalancing immunity toward activation by blocking inhibition while triggering activation. These approaches could lower the threshold for NK cell activation and provide a failsafe to target a tumor that evolves away from dependence on PD-L1 and/or HLA expression. Unlike T cells, where TIGIT and PD-1 are often co-expressed, TIGIT and PD-1 expression was nearly mutually exclusive on NK

cells, suggesting that checkpoint blockade for both molecules simultaneously may permit rescue of a larger population of NK cells from inhibition and exhaustion (120). Simultaneous blockade of NKG2A and triggering of ADCC using the anti-EGFR antibody cetuximab enhanced *in vivo* control of tumor xenografts more efficiently than either antibody alone (127). In a phase II trial (NCT02643550), monalizumab was given to patients with squamous cell carcinoma of the head and neck after chemotherapy and alongside standard-of-care treatment with cetuximab (127). This combination was deemed safe, with interim results indicating an improvement from the addition of monalizumab compared with historical control subjects treated with cetuxumab only, but further studies will be required to formally draw this conclusion.

Clinical trials are now testing combinations of anti-PD1 with novel checkpoint inhibitor therapies including anti-KIR2D antibody (lirilumab) (NCT01714739), anti-NKG2A (monalizumab) (NCT02671435, NCT03822351, NCT02557516, NCT03794544, NCT03833440), and anti-TIGIT (MTIG7192A) (NCT02794571, NCT03119428, NCT03563716, NCT03628677) (127). Theoretically, further strategies to appraise the tumor's expression of PD-L1 (which is the current standard of care for checkpoint therapies in other cancers), HLA and TIGIT ligands may inform the rational combination of checkpoint inhibitors to maximize NK cell function.

Adoptive and Adaptive NK Cell Therapies

Recognizing the potential for NK cells to participate in immune-mediated cancer control, a thrust in NK cell-based cancer therapies is adoptive transfer of NK cells (131, 132). Since NK cells do not require HLA matching to a specific patient, it is feasible and safe to transfer cells across allogeneic barriers. This opens the possibility of transferring NK cell lines (i.e., NK-92) or *ex vivo*-expanded NK cells from third-party donors (25). Efforts are underway to create cell lines—including those based on NK-92 cells—which may enable direct targeting of OC based on defined criteria (133, 134). In addition, clinical protocols are in place for virtually unlimited expansion of primary NK cells for adoptive transfer. Together, these efforts open the possibility of off-the-shelf NK immunotherapy. A complete summary of all clinical trials employing NK and NK-related cells for treatment of OC is shown in **Table 2**.

Early attempts at inducing NK cells for anti-cancer function included priming of lymphokine activated killer (LAK) and cytokine induced killer (CIK) cells. LAK and CIK cells originate from naïve lymphocytes which are "activated" or "induced" by IL-2 alone (LAK) or following IFN-γ stimulation *ex vivo* (CIK) (136, 137). In clinical trials, LAK cells exhibited limited clinical response and high rates of peritoneal fibrosis (138–140). The addition of IFN-γ stimulation to LAK cells to create CIK cells substantially improved both proliferation and cytotoxicity. CIK cells are characterized by the expression of a CD3+CD56+ cell phenotype and functional properties of NK cells (141). CIK cells were used in a recent phase III study of adoptive transfer following primary debulking and carboplatin/paclitaxel chemotherapy of OC (141, 142). Results from this clinical trial were positive with progression free survival improving from 22.2 months in the control group to 37.7 months among CIK-treated patients (142).

A completed early phase II study used allogeneic, haploidentical donor NK cells in combination with high-intensity chemotherapy to treat patients with recurrent OC (143). NK cell effects were difficult to differentiate from those of the chemotherapy and limited efficacy was attributed by the authors to a significantly increased number of Treg. Overcoming immunosuppression, including that driven by Treg, will indeed be important to ensuring the efficacy of NK cell-based immunotherapy. One strategy is to combine haploidentical donor NK cells with cytokines, with the goal of reversing the suppressive immune TME, enabling NK cell anticancer reactivity to proceed. Supporting this, the authors of the aforementioned phase II study have recently reported the ability to stimulate NK cells to overcome the soluble immunosuppressive environment from OC patient ascites *in vitro* with a combination of stimulatory IL-12, IL-15, and IL-18 (144). Indeed, this cytokine cocktail has been known to induce an "adaptive" population of highly-functional NK cells (145).

"Adaptive" NK cell population, first identified following cytomegalovirus infection but now known to be long-lived effector cells with potent cytotoxic ability (47). A recent study converted NK cells from a patient with OC to a cytotoxic CD56superbrightCD16+ subset that upon autologous transfer efficiently controlled growth of an autologous OC xenograft in a mouse (146). These adaptive NK cells are refractory to inhibition by Treg, implying a mechanism for their enhanced function (147). In a current Phase I clinical trial, FATE-NK100 cells, which are primary NK cells isolated from haploidentical cytomegalovirus-seropositive donors, are being transferred to patients with OC (NCT03213964).

Identifying the ideal source of NK cells for adoptive immunotherapy is a field of active study. Compared with mature and *in vitro* differentiated NK cells, an alternate approach is to transfer NK cells derived from umbilical cord blood (UCB) stem cells (NCT03539406). UCB contains a high proportion of immunologically-naïve NK cells that can be easily recovered and exhibit functions similar to peripheral blood NK cells. They produce similar amounts of IFN-γ and TNF (148–150). Research has also highlighted some potential weaknesses of UCB-derived NK cells: the relative immaturity of this NK cell population is associated with lower cytotoxicity, lower expression of perforin, granzyme B and KIR, and higher expression of NKG2A (150, 151). The processes for NK cell differentiation, the relative immaturity of NK cells from UCB and how they can be further differentiated or potentiated using cytokine cocktails or stimulation remain to be studied.

While selection of where to source NK cells from continues to elicit debate, once transferred into a patient, persistence, expansion, and homing/trafficking of the NK cells has proven an additional challenge. Unsurprisingly these factors have been identified as key to anti-tumor efficacy (143, 152, 153). Several factors including modifying cryopreservation and cytokine stimulation techniques can have profound impacts on homing, persistence and expansion of NK cells in *in vivo* (154). Indeed, a variety of strategies are being tested to improve NK cell potency

TABLE 2 | Current NK cell-based adoptive cell immunotherapies under clinical trial for the treatment of ovarian cancer.

NK cell intervention	Phase, date, (Status)	Study population (*n*)	Primary outcomes	Results	Reference/Clinical trial identifier
Allogeneic NK cells (with IL-2)	Phase II, 2008–2010 (Terminated due to toxicity)	Ovarian cancer, fallopian tube cancer, peritoneal cavity cancer (12)	To evaluate the *in vivo* expansion of an infused allogeneic natural killer (NK) cell product	PR (3), SD (8), PD (1)	NCT00652899
Allogeneic NK cells (with IL-2)	Phase II, 2010–2014 (Completed)	Ovarian cancer, fallopian tube cancer, primary peritoneal cancer, breast cancer (13)	Response Rate by RECIST [Time Frame: Month 3]	N/A	NCT01105650
Cord Blood Cytokine Induced Killer Cells	Phase I, 2012–2014 (Completed)	Ovarian (4), colon (4), rectal (5), hepatocellular (2), gastric (1), pancreatic (1), lung (1), esophagus (1)	Response Rate by RECIST	CR (1, HCC, 1 esophageal), PR (2 ovarian), PD (1 HCC), SD (10, averaging 11.4 months)	(135)
Radiofrequency ablation and Cytokine-induced Killer Cells	Phase II, 2015–2016 (Active, not recruiting)	Ovarian carcinoma (50)	Recurrence-free survival [Time Frame: 1 year]	N/A	NCT02487693
NK cells with cryosurgery	Phase I/II, 2016 (Recruiting)	Recurrent ovarian cancer	Response Rate by RECIST	N/A	NCT02849353
FATE-NK 100 (CMV+ donor NK cells with IL-2)	Phase I, 2017–2019 (Recruiting)	Epithelial ovarian cancer, Fallopian tube cancer, Primary peritoneal cancer (*estimated* 16)	Maximum Tolerated Dose of FATE-NK100 [Time Frame: 1 Year]	N/A	NCT03213964
Primary NK cells	Phase I/II, 2018 (Recruiting)	Lung cancer, breast cancer, colon cancer, pancreatic cancer, ovarian cancer (*200*)	Incidence of toxicity induced by NK infusion [Time Frame: 6 months]	N/A	NCT03634501
NKG2D-Ligand Targeted CAR-NK	Phase I, 2018 (Recruiting)	Solid tumors (*estimated* 30)	Number of Adverse Events [Time Frame: from day 0 to month 4]	N/A	NCT03415100
6B11-OCIK	Phase I, 2018 (Not yet recruiting)	Recurrent platinum-resistant ovarian cancer (*estimated* 10)	Progress-free survival [Time Frame: 3 years]	N/A	NCT03542669
Allogeneic NK cells	Phase I, 2018 (Not yet recruiting)	Recurrent ovarian cancer, recurrent fallopian tube cancer, recurrent primary peritoneal cancer (*estimated* 12)	Incidence of treatment emergent adverse events [Time Frame: 6 months]	N/A	NCT03539406
Anti-Mesothelin CAR-NK	Phase I, 2018 (Not yet recruiting)	Epithelial ovarian cancer (*estimated* 30)	Occurrence of treatment related adverse events as assessed by CTCAE v4.0 [Time Frame: Day 3-Year 2 after injection]	N/A	NCT03692637

SD, stable disease; PR, partial response; CR, complete response; PD, progressive disease.

in vivo. Interestingly, a recent study describes the use of a NK-cell-recruiting protein conjugated antibody that is cleaved into CXCL16 upon interaction with the tumor surface. This created a chemokine gradient resulting in increased NK cell tumor infiltration in a mouse model of pancreatic cancer (155). Similar strategies could be employed for other solid tumors including OC. Likewise, inclusion of extensive immune phenotyping, including that which would identify the type(s) and relative differentiation of NK cells invading tumors in patients, with stratifications based on outcomes and tumor subtypes, might better inform precise application of adoptive NK cell therapies.

Antigen Targeting With Antibodies and CAR-NK Cells

T cells engineered to express a chimeric antigen receptor (CAR)-T cells targeting CD19, a tumor antigen present on B-cell malignancies, provided the groundwork, and rationale for development of T cells engineered to target a specific antigen. Likewise, antibodies against CD20 and the HER2 receptor on lymphoma and breast cancer, respectively, provided the first proof-of-principle that tumors can be tagged for recognition and elimination by the immune system. Noteworthy, many of these antibody therapies rely on NK cells to mediate ADCC, and their efficacy has been inversely correlated to the extent of inhibitory KIR expression on NK cells (43, 156, 157). The list of targetable antigens in OC has been growing, and currently includes CA-125, FOLR1, EPCAM, MUC-1, and NY-ESO-1 (158, 159). Unfortunately, immunotherapies targeting these specific antigens have been largely ineffective. Currently, two CAR-T cell therapies have been approved by the FDA (160); neither is applicable to OC. For patients with OC, CAR-T cells against CA-125 are being developed and have shown promise against human

xenograft models and plans to evaluate their safety in in-human phase I clinical trials have been reported (161, 162).

There are limitations to the universal use of CAR-T cells, which may be directly addressed by NK cells: CAR-T cells take weeks to produce, are difficult to generate as autologous, and expensive, making them impractical for patients requiring quick treatment for aggressive tumors or standard of care therapy (163). Moreover, allogeneic CAR-T cells pose the risk of graft-vs.-host disease (GvHD), even when HLA-matched, due to minor mismatches (164).

Since NK cells can be delivered from allogeneic sources, they are readily available and relatively more cost-effective. Importantly, NK cells are not associated with GvHD, and therefore CAR-NK cells may be a safer alternative to CAR-T cells for engineered cell therapy. Preliminary data supports the safety and efficacy of CAR-NK cell therapy, which may be attributed to the relatively short lifespan of NK cells, which lowers the risk of long-term autoimmunity and toxicity (165). Moreover, the pre-existing tolerance conveyed by germline-encoded NK cell inhibitory receptors (i.e., KIR, NKG2A) may restrict their reactivity to the tumor and damaged cells, making them less likely to convey off-target and toxic effects than CAR-T cells. Two CAR-NK cells currently under development include CARs against CD24 and mesothelin.

CD24, a cancer stem cell marker, is rarely expressed in non-hematologic healthy tissue, and associated with poor clinical outcome in OC patients (166, 167). Recently published work tested a genetically engineered CAR-NK92 against CD24, and demonstrated its to ability kill OC cells *in vitro*, in addition to producing high levels of IFN-γ upon co-culture with CD24 expressing OC cell lines (168). Researchers indicate that future *in vivo* experiments will be conducted to further evaluate efficacy.

One of the most developed CAR-NK cells is engineered against mesothelin, the receptor for CA-125. CA-125 is overexpressed in ∼80% of patients and elicits an effective T cell response *in vitro* (169). A recent study evaluating various CAR constructs against mesothelin demonstrated their cytotoxic potential *in vitro* and further proved to be less toxic than their CAR-T cell counterparts *in vivo* while retaining similar anti-tumor effects (170). Based on early success *in vivo*, there are plans to evaluate mesothelin targeting CAR-NK cells in a phase I clinical trial (NCT03692637).

Despite its high expression on OC tumors, antibodies against CA-125 have not proven efficacious in OC patients (171), suggesting that exclusively targeting CA-125 is insufficient to target the heterogeneity associated with primary OC. "Antigen escape" in which the target antigen is lost through downregulation, acquired gene mutation or outgrowth of tumor subpopulations, occurs as a result of the selective pressure applied by antigen-targeting therapies like CAR-T cells and antibodies. This likely underlies the incomplete tumor control by existing antigen-specific immunotherapies and is facilitated by the tumor heterogeneity (172, 173). Complete control of OC will likely require simultaneous targeting of more than one feature.

In addition to CA-125, several therapeutics targeting NK cells to specific OC-associated antigens to activate ADCC are emerging. These include monoclonal and bi-specific antibodies, antibody-drug conjugates alone, and in combination with CAR-engineered NK cells (165, 174). One notable study found that the bispecific monoclonal antibody anti-EpCAM x anti-CD3 simultaneously activated T and NK cells with a strong enough interaction to overcome the immunosuppressive TME found in malignant ascites (175). Antibody-drug conjugates have also been developed for OC patients (176). Of these, Mirvetuximab Soravtasine (IMGN83), an anti-folate receptor alpha antibody conjugated to a cytotoxic maytansinoid, is the most developed and has demonstrated efficacy as a single agent *in vivo* (177). Unfortunately, this efficacy did not translate into clinical efficacy: a phase III study in platinum resistant OC patients concluded that progression free survival was not superior to standard chemotherapy (178, 179). Limited efficacy in antibody-based therapies, including IMGN83, may be due to one or several strategies including acquired resistance and/or an insufficient immune response.

NK cells from cancer patients often exhibit downregulation of the Fc receptor CD16 required for ADCC, resulting in reduced efficacy of antibody-based approaches (180, 181). To overcome this challenge and augment NK cell-mediated ADCC, researchers are inhibiting the metalloproteinases that cleave CD16 (182), or specifically engineering NK cells or cell lines for permanent expression of CD16 to facilitate ADCC (183).

The combination of antigen-agnostic reactivity (i.e., through germline-encoded activating receptors) together with the abilty to recruit NK cells for responsiveness against specific tumor antigens (i.e., through CAR or antibodies) may provide the required heterogeneous immune response required to combat the highly heterogeneous cancer that is OC. Cancer phenotyping to understand this heterogeneity—with extensions in predicting the ideal configuration of NK immunity—would assist in developing more precise approaches to OC immunotherapy.

FUTURE RECOMMENDATIONS

Immunotherapies that focus and predict the specific ligands and ligand combinations for NK cells are likely to enhance OC clearance and control. NK cells can provide a multifaceted approach to meet the challenge of heterogeneity in OC tumors. That NK cells can serve both antigen-specific (i.e., ADCC, CAR) and antigen-agnostic roles (i.e., DNA damage, cell stress) (15, 184), in detecting and eliminating tumors is a strength of NK-based immunotherapies, especially against the highly-heterogeneous OC.

Enhancing or replacing NK cell function in OC is both a feasible and logical strategy that complements several existing immunotherapies. To maximize NK function and activation within the TME, further research is warranted to first identify the relevance of NK cells for the outcome of OC patients. Toward the inclusion of NK cells as key players in immunotherapy we have synthesized the following recommendations for those researching how the OC TME interacts with cancer therapies. These recommendations should be considered when developing

fundamental, translational and clinical studies to contribute to the growing body of knowledge surrounding NK cell relevance in OC.

1. NK cells as outcome measures
   It is crucial that NK cells be included as outcome measures in clinical trials evaluating immunotherapies. For example, anti-PD-1 therapies characterize T cell populations and neglect the evaluation of other lymphocytes. Likewise, NK cells are important mediators of ADCC; therefore, NK cell function should be considered in antibody-based approaches.
2. Further characterization of NK cells in OC
   Identifying phenotypic classification and prognostic value of NK in OC may also aid in the stratification of patients for therapy. For example, identifying KIR immunogenomic status may allow identification of immunotherapeutic responders from non-responders based on immunogenetics and NK cell licensing. Inspiration for this can be drawn from investigations in other cancers that have shown important contributions of NK cell education and inhibition in contributing to therapeutic outcomes (19).
3. Measure leukocyte reactivity and compositions in the TME
   Efficient tumor control by immune mechanisms involves the collaboration of innate and adaptive lymphocytes. Thus, strategies to better understand immune function in the tumor will identify shortcomings in the current approaches and keys to next-generation immunotherapies.

CONCLUSIONS

Limited treatment options, no effective screening strategies, high recurrence, and poor overall survival emphasize the need for improving therapeutic strategies to combat OC. It is clear that current treatments are unable to control OC progression. As with several other hard-to-treat tumors, researchers and oncologists are turning to immunotherapy to treat OC. Immune cell infiltration carries both predictive and prognostic value in OC; however, the complex relationship between the immune system and tumor remains a topic of active study. This investigation has revealed an extremely immunosuppressive TME in OC that results in the dysregulation of immune effector cells leading to immune evasion and tumor progression.

While immunotherapies have encountered several challenges, strategies are being developed to improve their efficacy. Cytokine treatments now focus on enhancing specificity and safety. The development of cytokine superagonists and oncolytic virus delivery strategies can theoretically provide the required specificity to bring these into clinical use. Similarly, adoptive cell transfer therapies are being enhanced to establish feasibility and efficacy. Most promising are combination immunotherapies designed to target and activate multiple immune pathways.

These advances demonstrate promise to improve immunotherapies and minimize associated toxicities; however, without strategies to efficiently identify OC responders, immunotherapies will continue to yield disappointing results. The ability to properly stratify patients relies on understanding of a patient's underlying immunity and the pre-existing immune TME. It also requires an in depth understanding into how these factors interact with and respond to chemo-, radio- and immuno-therapies. By focusing and targeting immunotherapies to individual pathways, including those which enhance functionality of just one cell type or one immune cell pathway, the therapeutic impact may be limited. The broad functions of NK cells make them amenable for immunotherapy because they can mediate tumor killing using a variety of mechanisms, including ADCC and germline-encoded receptors, with minimal toxicity. Alone, or in combination with existing strategies, NK cells hold great promise for treatment of OC.

AUTHOR CONTRIBUTIONS

JB and SN conceptualized the review. SN, HG, JT, SG, and JB wrote and edited the review. SN, HG, and SG generated the tables.

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NK Cell Metabolism and Tumor Microenvironment

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Natural Killer (NK) cells are characterized by their potential to kill tumor cells by different means without previous sensitization and have, therefore, become a valuable tool in cancer immunotherapy. However, their efficacy against solid tumors is still poor and further studies are required to improve it. One of the major restrictions for NK cell activity is the immunosuppressive tumor microenvironment (TME). There, tumor and other immune cells create the appropriate conditions for tumor proliferation while, among others, preventing NK cell activation. Furthermore, NK cell metabolism is impaired in the TME, presumably due to nutrient and oxygen deprivation, and the higher concentration of tumor-derived metabolic end products, such as lactate. This metabolic restriction of NK cells limits their effector functions, and it could represent a potential target to focus on to improve the efficacy of NK cell-based therapies against solid tumors. In this review, we discuss the potential effect of TME into NK cell metabolism and its influence in NK cell effector functions.

Keywords: NK cell, metabolism, glucose, glycolysis, amino acid, hypoxia, tumor microenvironment, TME

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INTRODUCTION

Natural Killer (NK) cells are a promising tool in cancer immunotherapy. Their activation is driven by a balance between activating and inhibitory signals, so they are able to exert antitumor responses without prior sensitization. NK cells have demonstrated their potential in the treatment of several malignancies. However, the efficacy of these cells to treat solid tumors is still unsatisfactory (1–3). One of the main reasons for this limitation is the immunosuppressive effect of the tumor microenvironment (TME). In the TME, several tumor and tumor-associated cells produce and secrete factors that directly or indirectly prevent NK cell activation, including interleukin (IL)-6, IL-10, transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), and idoleamine 2,3-dioxygenase (IDO) (4, 5). Through these cytokines and factors, tumors are able to downmodulate NK cell activating receptors, such as NKp30, NKp44, or NKG2D (3, 4, 6–8), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (9). Furthermore, in the TME, NK cells receive signals from inhibitory receptors such as CD94/NKG2A, which bind to HLA-E exposed on the surfaces of several solid tumors including lung, pancreas, stomach, colon, head and neck, and liver tumor tissues (10). These immunosuppressive mechanisms mainly alter the balance between activating and inhibitory signals of NK cells, a step that is decisive for NK cell activation. Nonetheless, it should be also considered the effect of the TME in NK cell metabolism, which is essential to display full effector functions (11).

It is now accepted that the metabolic profile of NK cells is different under certain pathologies, such as obesity (12, 13) or viral infection (14). Also, it can be modified by several processes,

including education (15, 16), maturation (17), or cytokine stimulation (18–28). The latter is especially relevant because of the potential use of cytokine-stimulated and/or expanded NK cells for adoptive cell therapy in cancer treatment (29–32). In the tumor context, multiple factors converge to modulate NK cell metabolism. For instance, it is known that TGF-β downregulates the expression of activating receptors and effector functions (6, 33–37), but it also limits metabolic changes that accompany cell activation (18, 34). Recently, it has been demonstrated that TGF-β decreases IL-2-induced mitochondrial metabolism, including oxidative phosphorylation (OXPHOS) and maximal respiration in human NK cells (18). Another example of the impact of TME in NK cell metabolism comes from a recent report from patients with colorectal liver metastasis. The authors found that tumor-infiltrating liver-resident NK cells showed signs of mitochondrial stress, including decreased mitochondrial mass and increased reactive oxygen species (ROS) production (38). This reduced mitochondrial metabolism may be an important limitation for NK cell functionality in the TME. In this review, we will discuss how TME could shape NK cell metabolism and thus impair their antitumor activity (**Figure 1**).

TUMOR-DERIVED METABOLITES

Besides immunosuppressive cytokines, in the TME there is also an accumulation of tumor-derived metabolites, such as adenosine and lactate that limit antitumor responses. Extracellular adenosine concentration is increased in the hypoxic conditions of tumors, and it plays an important role in immune modulation (39). Hypoxia promotes the release of ATP and AMP, and the ectonucleotidases CD39 and CD73 catalyze the conversion of extracellular ATP to AMP, and AMP to adenosine (40). The stimulation of NK cells through the adenosine A_{2A} receptor ($A_{2A}R$), the predominant subtype of adenosine receptor expressed in these cells, has been found to suppress their effector functions (40–43). Following this, it has been reported that adenosine inhibits cytotoxic activity of mouse lymphokine-activated killer (LAK) cells (44). A more recent article has shown that adenosine impairs metabolic activity of IL-12/15-stimulated human NK cells by inhibiting their OXPHOS and glycolytic capacity (45). Interestingly, it was reported that there is an elevated interferon γ (IFNγ) production in the presence of adenosine, along with a decrease in NK cell cytotoxicity, suggesting that the metabolic requirements for these two NK cell effector functions are not the same. Indeed, IL-15-stimulated NK cells showed reduced killing of A549 cancer cells in the presence of adenosine (45). A possible explanation for the elevated IFNγ production may be the downregulation of the *GAPDH* gene observed in IL-12/15-stimulated NK cells exposed to adenosine (45). It has been shown that GAPDH can bind to IFNγ mRNA and prevent its translation (46). However, this transcript-arresting mechanism has not been defined in NK cells yet, and it has to be considered that other mechanisms involved in the regulation of IFNγ production may explain these results.

On the other hand, lactate and low pH have been found to decrease cytotoxic activity of NK cells (47). Exposure of NK cells to lactic acid blocked their IFNγ production following PMA/Ionomycin stimulation (48). A more comprehensive analysis revealed that lactic acid inhibits the upregulation of nuclear factor of activated T cells (NFAT), which is involved in IFNγ transcription (48). Additionally, Brand et al. have also shown that lactic acid uptake by murine NK cells leads to intracellular acidification and to an impaired energy metabolism (measured as intracellular ATP levels) (48). Similar results were obtained in liver-resident NK cells treated with lactic acid, in which intracellular pH and ATP decreased, promoting apoptosis (38). The accumulation of lactate in the TME is mainly due to the metabolic reprogramming of tumors, characterized by primarily using glucose for glycolytic metabolism rather than metabolizing it via OXPHOS. This accelerated glycolysis of cancer cells, induced by multiple factors such as hypoxia and oncogenes (49), may represent a considerable obstacle for NK cell activity, since it is not only causing lactate accumulation but also reducing glucose availability in the TME. Considering that NK cells strongly rely on glucose metabolism to exert their effector functions, as we will discuss in the next section, limiting their key fuel may seriously dampen their antitumor activity. However, not only tumor cells but also many immune cells undergo metabolic reprogramming upon activation, a process that may be especially relevant in the context of the TME and have a significant impact in the tumor progression (50).

GLUCOSE RESTRICTION

Lymphocytes require glucose to survive and its consumption is increased following activation, to support energetic and biosynthetic demands (51). Glucose can be utilized by NK cells for ATP and NADPH generation through different metabolic pathways, or as a carbon source for other biomolecules such as amino acids and fatty acids (19). It has been reported that NK cells express GLUT1, GLUT3, and GLUT4 (15, 21, 22, 52, 53), three glucose transporters from the GLUT family. Additionally, RNA expression of GLUT8 and H^+/myo-inositol co-transporter (HMIT or GLUT13) has been also measured in human NK cells (16). However, most studies have been focused on GLUT1, so the expression and regulation of the rest of glucose transporters of the GLUT family are unknown. Upon cytokine-stimulation, NK cells increase GLUT1 expression (21, 22), which is consistent with the augmented glucose uptake and glycolysis that accompanies cell activation (17, 21, 23). Several groups have studied the correlation between the glycolytic pathway and the functionality of activated NK cells, and have shown its relevance in the production of IFNγ and granzyme B, cytotoxicity and proliferative capacity (21, 23–25, 54). These findings are in accordance with those obtained in other lymphocytes. It has been demonstrated that glucose deprivation dampens T cell antitumor activity (46, 55, 56), and that metabolic competition in the TME can regulate cancer progression by impairing antigen-specific responses of tumor-infiltrating T cells (57). Therefore, it is reasonable to hypothesize that in the TME, tumor-driven glucose restriction may reduce glycolysis of NK cells and thus impair their antitumor functions.

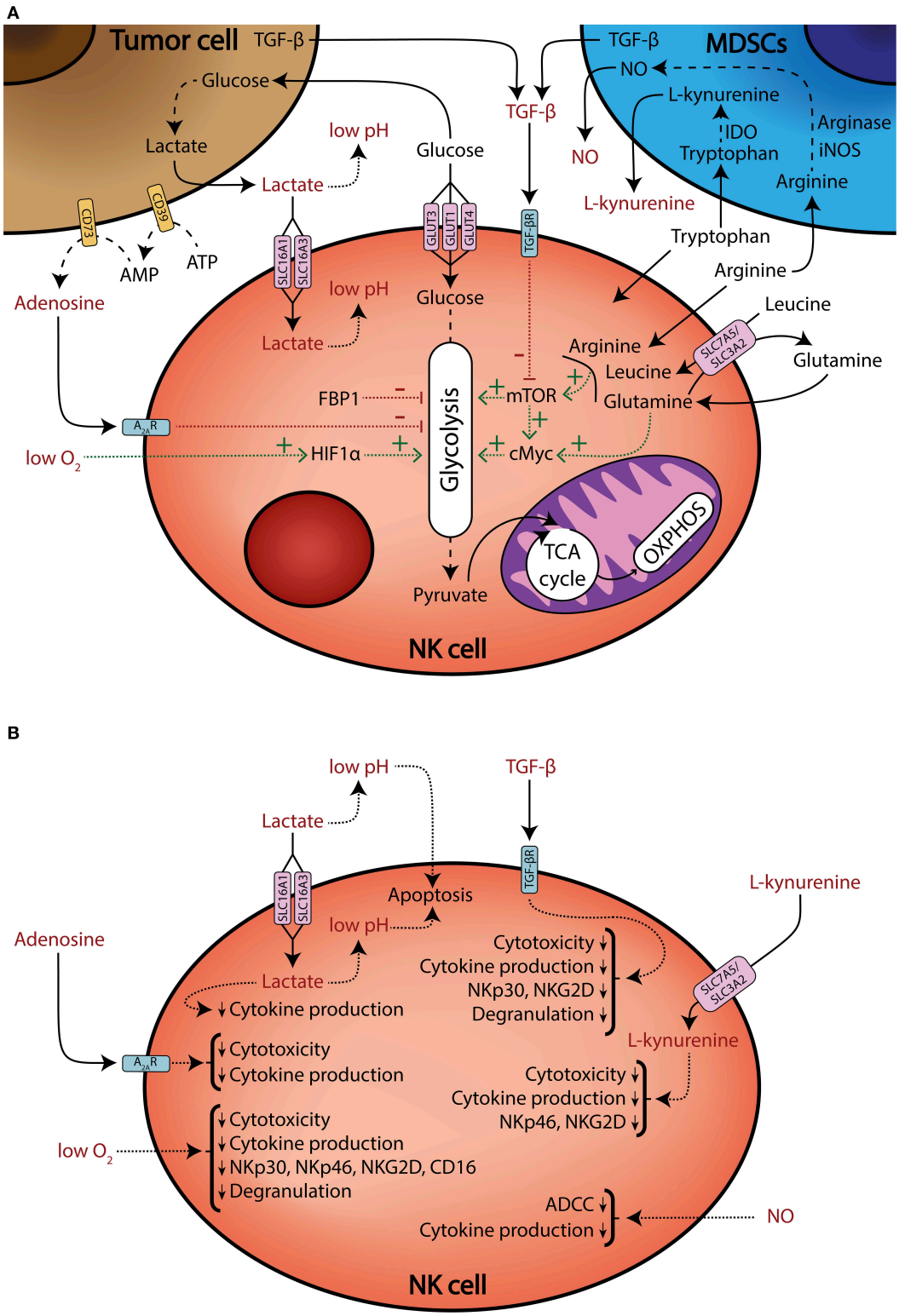

FIGURE 1 | Tumor microenvironment shapes NK cell metabolism and effector functions. **(A)** Schematic representation of multiple factors that modulate NK cell metabolism (black), and factors that modulate metabolism and/or negatively affect effector functions (red). NK cells compete for nutrients against tumor and myeloid-derived suppressor cells (MDSCs). Tumor cells consume large amounts of glucose and produce lactate, which is transported into NK cells through the SLC16A1 and SLC16A3 transporters, impairing ATP production. Tumor cells also generate extracellular adenosine through the ectonucleotidases CD39 and CD73.

(Continued)

FIGURE 1 | Extracellular adenosine inhibits NK cell oxidative phosphorylation (OXPHOS) and glycolytic capacity. Additionally, tumor cells increase amino acid consumption and MDSCs upregulate arginase, IDO, and iNOS enzymes, thus generating an amino acid depleted environment and releasing the immunosuppressive metabolites NO and L-kynurenine. Some amino acids and their transport are necessary to sustain mTOR and cMyc signaling, which promote glycolysis. Moreover, mTOR signaling can be also impaired by TGF-β secreted by tumor cells and MDSCs. The glycolytic pathway can be also modulated by the FBP1 enzyme, which is found to be upregulated in the tumor-infiltrating NK cells of some cancers. Finally, high oxygen consumption by tumor cells and disorganized vascularization can generate hypoxic regions. Hypoxia impairs NK cell effector functions, but also sustains HIF1α, which promotes glycolytic metabolism. Solid lines: release or uptake of different nutrients, metabolites, and other factors. Dashed lines: metabolic processing of substrates. Dotted lines: factors that promote or sustain (green lines), or inhibit (red lines), specific pathways. **(B)** A schematic representation of several metabolites and other factors present in the TME that limit NK cell effector functions.

Cong et al. have addressed this issue by investigating NK cells in a murine model of lung cancer. They have found lower glycolytic rates in NK cells from the lung cancer microenvironment, which also presented attenuated cytotoxicity and cytokine production. Furthermore, Cong et al. have described the increased expression of fructose-1,6-bisphosphatase (FBP1), an enzyme that inhibits glycolysis, in NK cells of the lung cancer microenvironment. More importantly, they have demonstrated that NK cell effector functions can be restored during tumor promotion by inhibiting FBP1 (58). These findings represent a good example of how metabolism can be modulated to improve NK cell antitumor responses.

On another front, Assmann et al. thoroughly analyzed the metabolic reprogramming of cytokine-stimulated NK cells, and described the relevance of citrate-malate shuttle and its regulation by sterol regulatory element-binding proteins (SREBP). They found that SREBP activity is crucial to maintain elevated glycolytic rates and effector functions, including cytotoxicity and IFNγ and granzyme B production (19). Remarkably, some SREBP inhibitors may be increased in the TME, such as 27-hydroxycholesterol, which is found to be elevated in patients with breast, gastric and colorectal cancers (11, 59–62). Considering this fact and that citrate-malate shuttle relies on glucose metabolism, it would be interesting to study the modulation of SREBP in tumor-infiltrating NK cells and to test whether this metabolic pathway configuration is conserved in the TME, where glucose concentration is diminished. Also, it would be of utmost interest to understand the effect of TME in the modulation of other mediators linked to both NK cell metabolism and function, such as AMP-activated protein kinase (AMPK), glycogen synthase kinase 3 β (GSK-3β), diacylglycerol kinases (DGK), regulatory factor X 7 (Rfx7), or inositol-requiring enzyme 1 α (IRE1α) and its substrate X-box-binding protein 1 (XBP1) (63–69). Following this line, it would be also worthwhile to further investigate the role of the mechanistic (or mammalian) target of rapamycin (mTOR), a central metabolic regulator that promotes, among others, the glycolytic pathway. Several authors have pointed the relevance of mTOR for NK cell activation and metabolic reprogramming, and the negative effect of mTOR inhibition in terms of NK cell functionality (12, 17, 21, 23, 26, 70–72). mTOR is sensitive to nutrient availability (73) and can be repressed by TGF-β, which inhibits NK cell metabolism and functionality (18, 33, 34). It is therefore presumable that in the nutrient-deprived TME, where there is also a higher production of TGF-β, mTOR may be inhibited, thereby limiting NK cell effector functions.

AMINO ACID DEPLETION

In addition to glucose, amino acids are also an important fuel for many cellular processes. Tumors show increased amino acid consumption (74) and synergize with tumor-associated cells to create a nutrient-depleted microenvironment. It has been reported that low arginine concentration impairs the proliferation and IFNγ production of the NK-92 cell line and primary human NK cells (75, 76). In addition, mTOR signaling has been found to be inhibited in leucine-depleted media (27, 28). As previously mentioned, mTOR plays a key role in the modulation of the glycolytic pathway, so the impairment of its signaling cascade may lead to diminished effector functions. Similarly, arginine and glutamine levels also affect mTOR signaling (77). Additionally, mTOR has been found to sustain the initial expression of cMyc, a transcription factor that supports the metabolic reprogramming (including the elevated glycolytic rates) required for the functional responses of IL-2/12-stimulated mouse NK cells (27). Moreover, glutamine and amino acid transport through the SLC7A5 transporter are also required for a sustained expression of cMyc (27).

Our current knowledge indicates that amino acid availability may be necessary for NK cell functionality, although the main role of some amino acids could be to maintain the signaling of other metabolic regulators, such as mTOR or cMyc, rather than to be used as a fuel. Indeed, Loftus et al. have reported that glutaminolysis can be inhibited without reducing NK cell functional responses (27). In this line, a previous report indicated that receptor-induced IFNγ production of murine NK cells was not impaired by limiting concentrations of glutamine (24). These findings suggest that targeting amino acid metabolism may enhance NK cell-based therapies, by impairing tumor fuel supply without reducing NK cells' functionality. However, it is necessary to thoroughly explore metabolic requirements of NK cells to validate this hypothesis and to design better therapeutic strategies.

Nonetheless, it should be also considered that amino acid consumption by tumor and tumor-associated cells leads to the accumulation of immunosuppressive catabolites in the TME. Myeloid-derived suppressor cells (MDSCs) upregulate arginase and inducible nitric oxide synthase (iNOS). Both enzymes use arginine as substrate, and the latter catabolizes its conversion to nitric oxide (NO) (6, 78). It has been found that NO impairs NK cell antibody-dependent cellular cytotoxicity, and that the inhibition of iNOS in a mouse model of breast cancer can rescue this function (79). Furthermore, tumor and tumor-associated dendritic cells and fibroblasts show increased expression of

IDO, which catabolizes the conversion of tryptophan to L-kynurenine (80, 81). L-kynurenine can be transported through the SLC7A5 transporter (82), and has been found to inhibit NK cell proliferation (81, 83). Also, L-kynurenine inhibits IL-2-induced upregulation of NKp46 and NKG2D receptors and cytokine production of human NK cells *in vitro* (84).

HYPOXIA

Most solid tumors show high oxygen consumption and disorganized vascularization, which leads to the generation of regions permanently or transiently subjected to hypoxia (85). Cells adapt to this hypoxic conditions through the hypoxia-inducible family of transcription factors (HIFs) that modulate a wide range of genes (86). In particular, most dysregulated genes of NK cells under hypoxia are related to metabolic and biosynthetic processes (87), which is consistent with the results obtained in other cells in which HIF-1α promotes or sustains glycolytic metabolism (88–91). In human NK cells, IL-15-priming synergistically acts with short term hypoxia to induce the upregulation of genes involved in the glycolytic pathway (20). Given the relevance of glycolysis for NK cell effector functions, these findings could suggest that these cells may partially conserve their functionality in hypoxic environments. Indeed, available data argue that hypoxia limits, but does not completely block, NK cell responses (92, 93). However, it is still unclear whether HIF-1α and its effect on glycolytic activity play a significant role on NK cells effector functions. It has been reported that *Hif1a*$^{-/-}$ NK cells have normal metabolic and effector functions in response to IL-2/12 stimulation (27). It would be interesting to study the relationship between HIF-1α, metabolism and effector responses of NK cells under hypoxia. Noteworthy, under hypoxic conditions, there is also a modulation of genes related to immunomodulatory functions and a downregulation of activating receptors, such as NKp30, NKp46, or NKG2D, that contributes to the diminished effector functions (87, 94). Also, hypoxia promotes tumor immune evasion through other mechanisms, such as degrading NK cell-derived granzyme B by autophagy (95). Interestingly, it has been reported that IL-2-priming can increase the expression of the above mentioned activating receptors and improve NK cell cytotoxicity, thereby overcoming the inhibitory effects of hypoxia (93, 96). Considering that there are several antitumor therapeutic strategies based on NK cell stimulation with different cytokines, including IL-2, IL-12, IL-15, IL-18, and IL-21 (29, 31, 32, 97), it would be of great interest to further analyze the effector functions of these cytokine-primed and expanded NK cells under hypoxic conditions.

OTHER FACTORS INFLUENCING NK CELL METABOLISM

As before mentioned, NK cell metabolism can be modulated by multiple factors, and some of them may have a relevant impact on the anti-cancer therapeutic efficacy. A recent report revealed that obesity impaired mTORC1 activation and limits NK cell antitumor responses (12). The same article has shown that NK cells from obese individuals have a reduced metabolic response following cytokine stimulation (12). Thus, therapies based in the administration of interleukins may show reduced efficacy in these patients. In contrast, a previous report indicated that NK cells from obese children displayed higher levels of glycolysis and mTORC1 activation both at basal levels and upon cytokine stimulation (13). These data suggest that NK cell metabolism may be differentially regulated in adults and children. It would be also interesting to explore NK cell metabolism in other pathologies, such as viral infection, and test whether the metabolic reprogramming is maintained after a prolonged period. Chronic infections have been related to the exhaustion of effector lymphocytes, and exhausted T cells show a different metabolic pattern (98, 99). It has been described that NK cells can also become exhausted during tumor progression or chronic infections (100, 101). Therefore, it would be of great interest to study metabolic requirements of exhausted NK cells, and furthermore, test whether metabolic reprogramming induced by viral infections and tumors could drive NK cell exhaustion.

CONCLUDING REMARKS

NK cell effector functions are supported by their metabolism. For instance, IFNγ production could be metabolically regulated at different levels, during transcription, translation, or post-translational processing (54). In the restrictive TME, NK cell metabolism and antitumor responses are impaired (38, 58). Similarly, it has been found that tumor cells compete with T cells for glucose, and this metabolic competition in the TME has been described as a driver of cancer progression (57). In accordance, others have demonstrated that highly glycolytic tumors impair T cell antitumor responses through multiple mechanisms (102). Hence, considering that glucose is a key fuel for NK cells, it is presumable that the metabolic competition of the TME will dampen their effector functions. In addition to glucose, reduced amino acid availability and the accumulation of tumor-derived metabolites may also have a substantial impact on NK cell functionality. Thus, targeting tumor metabolism could be a good option to improve the efficacy of NK cell-based therapies. Alternatively, or additionally, NK cell metabolism may be modified to compete more efficiently for nutrients in the TME, or to be less susceptible to the hypoxia-driven inhibition. For instance, stimulation with different cytokine combinations can upregulate the expression of several nutrient transporters, glycolysis and OXPHOS (11, 33, 103), which can be a good option to enhance NK cell metabolic competitiveness, fitness and plasticity, and thus improve their effector functions in the TME. NK cell stimulation with IL-2, IL-15, or IL-18 has been found to increase the expression of amino acid transporters (28, 72). IL-15-priming sustains murine NK cells production of IFNγ in glutamine-free media, and during the inhibition of fatty acid oxidation with etomoxir (24). Similarly, pretreatment with ALT-803, an IL-15 superagonist, confers some metabolic resistance to the inhibition of glycolysis or mTORC1 with

2-deoxy-D-glucose or rapamycin, respectively (25). However, a continuous exposure to IL-15 can lead to NK cell exhaustion, which is accompanied by a reduction in mitochondrial activity (104). It is therefore necessary to continue exploring NK cell metabolism to understand how it could be modified to resist the metabolically restrictive TME and preserve the effector functions. Undoubtedly, immunometabolism is a fascinating field of research that has been demonstrated to be relevant for NK cell effector functions. Further studies will reveal more precisely how TME shapes NK cell metabolism, which represents an attractive target to focus on to improve NK cell-based immunotherapies.

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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GMP-Compliant Manufacturing of NKG2D CAR Memory T Cells Using CliniMACS Prodigy

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Natural killer group 2D (NKG2D) is a natural killer (NK) cell-activating receptor that recognizes different stress-induced ligands that are overexpressed in a variety of childhood and adult tumors. NKG2D chimeric antigen receptor (CAR) T cells have shown potent anticancer effects against different cancer types. A second-generation NKG2D CAR was generated by fusing full-length human NKG2D to 4-1BB costimulatory molecule and CD3ζ signaling domain. Patient-derived CAR T cells show limitations including inability to manufacture CAR T cells from the patients' own T cells, disease progression, and death prior to return of engineered cells. The use of allogeneic T cells for CAR therapy could be an attractive alternative, although undesirable graft vs. host reactions may occur. To avoid such adverse effects, we used $CD45RA^-$ memory T cells, a T-cell subset with less alloreactivity, as effector cells to express NKG2D CAR. In this study, we developed a protocol to obtain large-scale NKG2D CAR memory T cells for clinical use by using CliniMACS Prodigy, an automated closed system compliant with Good Manufacturing Practice (GMP) guidelines. $CD45RA^+$ fraction was depleted from healthy donors' non-mobilized apheresis using CliniMACS CD45RA Reagent and CliniMACS Plus device. A total of 10^8 $CD45RA^-$ cells were cultured in TexMACS media supplemented with 100 IU/mL IL-2 and activated at day 0 with T Cell TransAct. Then, we used NKG2D-CD8TM-4-1BB-CD3ζ lentiviral vector for cell transduction (MOI = 2). NKG2D CAR T cells expanded between 10 and 13 days. Final cell products were analyzed to comply with the specifications derived from the quality and complementary controls carried out in accordance with the instructions of the Spanish Regulatory Agency of Medicines and Medical Devices (AEMPS) for the manufacture of investigational advanced therapy medicinal products (ATMPs). We performed four validations. The manufacturing protocol here described achieved large numbers of viable NKG2D CAR memory T cells with elevated levels of NKG2D CAR expression and highly cytotoxic against Jurkat and 531MII tumor target cells. CAR T cell final products met release

criteria, except for one showing *myc* overexpression and another with viral copy number higher than five. Manufacturing of clinical-grade NKG2D CAR memory T cells using CliniMACS Prodigy is feasible and reproducible, widening clinical application of CAR T cell therapies.

Keywords: NKG2D CAR, memory T cells, automated production, large-scale, clinical-grade, CliniMACS prodigy

INTRODUCTION

Redirected chimeric antigen receptor (CAR) T cells (CART) have shown effective potency against hematologic tumors (1, 2). Second-generation CARs are hybrid receptors comprising a recognition domain, normally derived from a single-chain antibody fragment (scFv), fused to costimulatory, and cytotoxic signaling domains that enhance T cell function (3, 4). This restricts CAR T cells to recognize a single tumor antigen in a defined set of tumors, such as CD19 in B-cell malignancies. CD19-specific CAR T cell therapy for the treatment of CD19-positive B cell malignancies such as B-cell acute lymphoblastic leukemia (B-ALL), B-cell non-Hodgkin lymphoma (NHL), or chronic lymphocytic leukemia (CLL) has had remarkable success (5–8), resulting in their recent US Food and Drug Administration (FDA) approval. However, relapse of leukemia through CD19 loss variants in leukemia/lymphoma patients and immunosuppressive microenvironment or lack of tumor-associated antigens (TAAs) in solid tumors (9–11) represents major challenges for CAR T cell therapies. These inconveniences along with antigen-loss escape make it necessary to focus in other possible TAAs (9).

Natural killer group 2D (NKG2D) is an activating receptor expressed on different immune effector cells [natural killer (NK), CD8, and γδ T cells], although is in the NK cells where it has a main role in tumor surveillance. Ligands for NKG2D receptor, namely, MIC-A, MIC-B, and the UL-16 binding proteins, are expressed in 70% of human cancers including leukemia, osteosarcoma, or Ewing sarcoma (11–13), whereas their expression in healthy tissues is rare. We have produced a second-generation NKG2D CAR by fusing the full-length extracellular domain of human NKG2D to 4-1BB, which provides a costimulatory signal, and CD3ζ signaling domain. Thus, through the expression of this CAR, T cells acquire NK cell anti-tumor specificity while maintaining T-cell ability to expand and persist *in vivo*. The main advantages of this CAR are (1) the recognition of different ligands, widening clinical application, and potentially avoiding tumor immune escape by single antigen loss, and (2) it is a fully human CAR, causing less immunogenicity.

Most clinical trials use autologous T cells to express CARs; however, owing to low T-cell numbers, poor quality, or rapid disease progression, manufacturing of patient-derived CAR T cells is not always possible. To overcome these limitations, we propose here the use of allogeneic CAR T cells. Allogeneic cells expressing CARs have been infused into patients after a hematopoietic stem cell transplantation (HSCT) from the same healthy donor (14, 15). Nevertheless, the universal availability of large numbers of healthy donor T cells to express CARs and their infusion into patients without the requirement of a prior HSCT would be major challenges of CAR T cell immunotherapy. One potential risk of the use of allogeneic T cell-based therapies is the T-cell response against normal tissue: graft-vs.-host disease (GvHD). To avoid undesirable GvH reactions, T-cell products lacking an alloreactive T-cell receptor (TCR) are needed. Several methods have attempted to intensify graft-vs.-tumor (GvT) effects while minimizing GvH responses to lower toxicity and improve the outcome of treatment (14, 16, 17). One approach to enrich non-allogeneic T cells is by using antigen-experienced memory T cells for CAR transduction (16, 18). Predictably, the vast majority of T cells with a memory phenotype are likely to have encountered antigens other than the allogeneic type. Thus, selection for memory phenotype cells should enrich for a non-alloreactive repertoire. Indeed, memory T cells showed less potential to generate GvHD in murine models (19, 20), in part owing to non-alloreactive TCR enrichment along with the evidence that memory T cells are less likely to traffic to GvHD organs such as the gastrointestinal tract. Different extracellular markers can be used to differentiate naïve from memory T cells. Commonly, naïve T cells are $CD45RA^{+}CD45RO^{-}CCR7^{+}CD62L^{+}$, central memory T cells (T_{CM}) are $CD45RA^{-}CD45RO^{+}CCR7^{+}CD62L^{+}$, effector memory T cells (T_{EM}) are $CD45RA^{-}CD45RO^{+}CCR7^{-}CD62L^{-}$, and effector cells are $CD45RA^{+}CD45RO^{-}CCR7^{-}CD62L^{-}$ (21). Thus, one marker to roughly distinguish naïve from memory T cells is CD45RA (22). CD45RA is expressed on naïve T cells and a minor population of T memory stem cells (T_{SCM}) (21), whereas CD45RO is expressed on memory T cells (22). $CD45RA^{+}$ naïve T cells have high potential for alloreactivity against recipient-specific antigens upon adoptive transfer, causing clinical GvHD (23, 24). In contrast, $CD45RA^{-}CD45RO^{+}$ T cells exert a memory response to prior pathogens or vaccines and can mediate GvT effects without inducing GvHD (19, 25).

In the present study, we describe the manufacturing process to produce large-scale NKG2D CAR memory T cells from healthy donors for clinical use. In CAR T cell therapies, besides the designing of genetic constructs, the choice of effector cells to transduce, and the clinical trial design, the methods used to produce CAR T cells are key for clinical success. Thus, detailed description of each step along the manufacturing process and the full analysis of CAR T cell products composition at every step are essential. In fact, and according to the Good Manufacturing Practice (GMP) manufacturing standard, during the manufacturing procedure, in-process controls are carried out at different times. Optimization of manufacturing protocols to improve reproducibility, cost-effectiveness, and scalability will enable a broad application of CAR T cell therapies.

The NKG2D CAR memory T cells showed in this study were manufactured after 10–13 days of *ex vivo* processing, described in detail below, including activation with TransAct and IL-2, transduction with an NKG2D-CD8TM-4-1BB-CD3ζ lentiviral vector at multiplicity of infection (MOI) = 2, and expansion in CliniMACS Prodigy device. The NKG2D CAR memory T cells collected after this process fulfilled the release criteria with respect to safety, purity, and potency established in the protocols adhered to the guidelines of the current GMP (26–28). The manufacturing process developed in this study allows the automated GMP-compliant production of large doses of clinical-grade NKG2D CAR T cells in a short time and provides a robust and flexible base for further optimization of NKG2D CAR T cells manufacturing for their clinical application in different tumor types.

MATERIALS AND METHODS

Starting Material

Non-mobilized apheresis was obtained from healthy donors at the Bone Marrow Transplant and Cell Therapy Unit (BMTCT) of Hospital Universitario La Paz (HULP) by using CliniMACS Plus device (Miltenyi Biotec). All donors gave their written informed consent in accordance with the Declaration of Helsinki protocol, and the study was performed according to the guidelines of the local ethics committee. All donors comply with the requirements regarding quality and safety for donation, obtaining, storage, distribution, and preservation of human cells and tissues under the Spanish specific regulation. $CD45RA^+$ cells were depleted by immunomagnetic separation using CliniMACS CD45RA Reagent (701-46) and CliniMACS Plus system, both from Miltenyi Biotec, following manufacturer instructions. $CD45RA^-$ cells were either processed immediately or stored at 2–8°C for subsequent processing no later than 24 h after depletion. The viability and purity of $CD45RA^-$ fraction were analyzed by flow cytometry (FCM) before activation, transduction, and expansion.

Construction and Production of Lentiviral Vector

The HL20i4r-MNDantiCD19bbz lentiviral vectors were derived from the clinical vector CL20i4r-EF1a-hgcOPT27 but expressed an NKG2D CAR. The anti-CD19-4-1BB-CD3ζ CAR designed by Imai et al. (29) was used as backbone to build the NKG2D CAR construct. It contained the extracellular domain of NKG2D (designed by Wai-Hang Leung and Wing Leung), the hinge region of CD8a, and the signaling domains of 4-1BB and CD3ζ. The cassette was driven by a prMND. The viral supernatant was produced according to GMP guidelines by transient transfection of HEK293T cells with the vector genome plasmid and lentiviral packaging helper plasmids pCAGG-HIVgpco, pCAGG-VSVG, and pCAG4-RTR2. Lentiviral plasmids were kindly provided by Dr. Byoung (St. Jude Children's Research Hospital). The virus supernatants were harvested and filtered through 0.22 μm filters. Virus supernatant was concentrated by ultracentrifugation and titrated on HeLa cells by serial dilution followed by a quantitative polymerase chain reaction (qPCR) to determine vector genome copy number.

Manufacturing of Clinical-Grade NKG2D CAR T Cells

Activation, transduction, and expansion of $CD45RA^-$ cells were performed on the CliniMACS Prodigy using Tubing set TS520 (170-076-600) and T-cell transduction (TCT) process. In detail, at day 0, cultivation was initiated with 10^8 $CD45RA^-$ cells in a total volume of 70 mL of TexMACs GMP medium (170-076-306) + 100 IU/mL of MACS GMP human recombinant IL-2 (170-076-147). MACS GMP TransAct CD3/CD28 Kit (170-076-156) was used for a 24-h activation at a final dilution of 1:17.5, as recommended by the manufacturer. At the following day, cells were transduced with NKG2D-4-1BB-CD3ζ lentiviral particles at MOI = 2. The vector was diluted in 10 mL of medium in a 150-mL transfer bag, which was attached to the CliniMACS Prodigy by sterile welding. The vector was automatically transferred in the culture chamber, and the vector bag was further rinsed with 20 mL of medium to bring the total culture volume to 100 mL. Residual TransAct was removed by an automated culture wash on day 4. Cells were then expanded for 10–13 days before being harvested. Sampling was performed at days +6 and +8 for in-process controls including cell counts, cytotoxicity, and FCM. At the end of the expansion, cells were automatically collected in 0.9% sodium chloride solution supplemented with 0.5% human serum albumin (Albutein 20%, Grifols) and transferred into a sterile bag. Release quality controls were performed at the end of the process.

Analysis of Viability and Surface Immunophenotype by FCM

At day +6 and between days +8 and +10, as in-process controls, and at harvest, as release controls, NKG2D CAR memory T cell products were counted in a CELL-DYN Emerald hematology analyzer (Abbott) and analyzed for their viability, immunophenotype, NKG2D CAR expression, and activation status by FCM. The following anti-human fluorochrome-labeled monoclonal antibodies (mAb) were purchased from BioLegend: CD45RA-APC (Clone H100, 304111), CD3-PE/Cy7 (Clone HIT3a, 300316), CD4-APC/Cy7 (Clone OKT4, 317417), CD8 FITC (Clone KK1, 344703), NKG2D-PE (Clone 1D11, 320806), CD4 PerCP (Clone OKT4, 31743125), PD-1 APC (Clone EH12.2H7, 329907), Tim-3 APC Cy7 (Clone F38-2E2, 345025), CD25 APC (Clone BC96, 302610), and CD127 PE/Cy7 (Clone A019D5, 351320). Anti-human CD45RO-APC-Vio770 (Clone REA611, 130-114-083) was purchased from Miltenyi Biotec. Anti-human CCR7 PE (Clone 3D12, 552176) was purchased from BD Biosciences. The viability was tested by using DAPI or 7AAD as dead cell exclusion markers. Cells were analyzed using FACS CANTO II (BD Biosciences) and FlowJo v10.5.3 software (TreeStar). To ensure the expression of NKG2D CAR in the manufactured NKG2D CAR T cell products, we performed western blot with an antibody detecting CD3ζ. Total peripheral blood mononuclear cells (PBMC), activated and expanded NK cells (NKAE), untransduced $CD45RA^-$, and NKG2D CAR T cells were pelleted and frozen at −80°C. Cell lysates were obtained by incubating cell pellets with RIPA (Millipore, 20188) supplemented with phosphatase inhibitor (PhosSTOP,

04906845001) and a cocktail of protease inhibitors (cOmplete Mini, 11836153001), both from Roche. Proteins were quantified using Bradford reagent (Bio-Rad Laboratories, 500-0205) and measuring absorbance at 595 nm in a Victor Plate Reader. Cell lysates were then mixed with the Laemmli sample buffer (Bio-Rad Laboratories, 161-0747), and equal amounts of protein (20 μg) were loaded on 4–15% Mini-PROTEAN TGX Gels (Bio-Rad Laboratories, 456-1086). Gels were transferred to polyvinylidene difluoride (PVDF) membranes. Blots were incubated with the mouse anti-human CD3ζ (BD Biosciences, 551033) or rabbit anti-human β-actin (Cell Signaling Technology, 4967S) primary antibodies at 4°C overnight. Horseradish peroxidase (HRP)-conjugated anti-mouse (Agilent, P0447) and anti-rabbit (Agilent, P0448) were used as secondary antibodies. The membranes were developed by enhanced chemiluminescence and exposed on Clarity Western ECL substrate (Bio-Rad Laboratories, 170-5060). The immunoblotting images were analyzed using the Image Lab software.

Effector Function

The mechanism of action of CAR cells is complex, and there are no standardized methods to determine the degree of action. Although methods are available to determine potency, comparison of results is not easy owing to the absence of standardization (26). In our study, to test the cytotoxicity of manufactured NKG2D CAR T cells, conventional 4-h europium-TDA assays (PerkinElmer, AD0116) were performed as previously described (30) using a 20:1 effector to target ratio. The NKG2DL-expressing cell lines Jurkat and 531MII were used as targets. Cytotoxicity assays were performed on days +6 and +8 and at the end of the process. The 531MII primary osteosarcoma cell line was kindly provided by Dr. Patiño-García (Centro de Investigación Médica Aplicada (CIMA), Universidad de Navarra, Spain) and was cultured in minimum essential medium (MEM; GIBCO, 22571-020) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, 10270-098) and penicillin–streptomycin (P/S; GIBCO, 15140-122). The T-ALL Jurkat cell line was acquired from American Type Culture Collection (ATCC) and kept in culture in Roswell Park Memorial Institute (GIBCO, 61870-010) and 10% FBS and P/S. Both cell lines were routinely tested for mycoplasma.

Analyses of Non-cellular Impurities

The detection of non-cellular impurities was carried out in accordance with the methodology recommendations of Chapter 2.6.21 and 2.6.7 of the European Pharmacopeia (Eu Ph) for mycoplasma and Chapter 2.6.14 for endotoxins. A DNA-binding dye-based qPCR system was employed for the detection of mycoplasma DNA in cell cultures. The assay was developed by the Genomics Unit in collaboration with the Monoclonal Antibodies Unit, both from CNIO, to detect 16s rRNA gene sequences from up to 70 *Mollicutes* species. Both specificity and sensitivity were extensively tested through benchmarking with established commercial systems (MTC-NI System, Millipore/GEN-PROBE Cat. No. 4573 and MycoAlert™ PLUS Mycoplasma Detection Kit, Lonza Cat. No. LT07-705). The Clinical Microbiology and Parasitology Service of HULP carried out the endotoxin test Endosafe-PTS (Charles River) to quantify endotoxin levels at day +8 and in final products.

Microbiological Tests

At days +6 and between days +8 and +10 as in-process controls and at the end of manufacturing protocol, NKG2D CAR memory T cell products were tested for sterility according to Eu Ph 2.6.1. The microbiological tests were developed by the Clinical Microbiology and Parasitology Service of HULP by conventional microbiology techniques. In summary, sample tests were inoculated into separate culture media, and the growth of viable microorganisms was tested after several days. When a rapid result was required, Gram staining was used as a non-culture method, although it is a less sensitive technique than techniques based on culture (26).

Genetic Tests, Genome Integrated Vector Copy Number, and Determination of Replication Competent Lentivirus in the Supernatant

Genetic tests and determination of vector copy number (VCN) and replication competent lentivirus (RCL) in the supernatant were carried out at days +6 and between days +8 and +10 as quality controls during process validation and at the end of the manufacturing process between days 10 and 13. To rule out chromosomal aberrations caused by lentiviral transduction, comparative genome hybridization (CGH) analysis was performed as previously described (30). Genome integrated lentiviral copy number and viral particles in supernatant were measured by qPCR according to Christodoulou et al. (31) using TaqMan Universal PCR Master Mix (Thermo Fisher; 4304437) and LightCycler 480 (Roche) after viral RNA extraction with RNeasy (Qiagen, 74104) and cDNA retrotranscription with Superscript II (Thermo Fisher, 18064014). The lack of oncogenic effects of the NKG2D CAR T cell products was verified using reverse transcriptase (RT)-PCR to detect c-*MYC* and telomerase (*TERT*) expression. Total RNA was isolated from the PBMCs using the RNeasy kit from Qiagen (PN 74104), followed by reverse transcription using SuperScript™ IV First-Strand Synthesis System from Thermo Fisher (PN 18091050). The resulting cDNA was amplified with the following specific TaqMan probes: Hs00972650_m1 (TERT), Hs00153408_m1 (MYC), and Hs02800695_m1 (HPRT1, housekeeping) from Life Technologies and the LightCycler 480 System from Roche. Finally, the data were analyzed by the comparative Ct methods as previously described (32). The genetic tests were performed at the Institute of Medical and Molecular Genetics of HULP (INGEMM).

Effects of Cryopreservation on NKG2D CAR T Cells

As infusion of freshly manufactured CAR T cells is not always possible, we wanted to determine if cryopreservation could have a negative impact on viability, NKG2D CAR expression, and cytotoxicity of NKG2D CAR T cells. To this aim, spare CAR T cells were frozen at a concentration of 2.5–3 $\times$ 10^5 cells/μL

either by using HypoThermosol, M199 media supplemented with 10% human serum albumin and 5% dimethyl sulfoxide (DMSO), or in autologous plasma supplemented with 5% DMSO. One year after cryopreservation, NKG2D CAR T cells were thawed and evaluated for viability, NKG2D expression, and CD45RA$^-$ purity by FCM and for cytotoxicity by europium-TDA as described above.

Statistics Analyses

All statistical analyses in this study were performed using GraphPad Prism. Except indicated in another way, results are shown as median and interquartile range.

RESULTS

Manufacturing Process: Activation, Transduction, and Expansion

CD45RA$^-$ cells from four different donors were activated, transduced, and expanded in CliniMACS Prodigy in four different experiments. In-process tests were carried out at days +6 and +8. At the end of culture (between days +10 and +13), cells were harvested, and quality/release assays performed. A schema of the different steps for NKG2D CAR T cells manufacturing and the quality tests conducted along the process is shown in **Figure 1**.

Purity of CD45RA$^-$ Starting Cells

Non-mobilized apheresis from four different healthy donors were obtained and depleted for CD45RA$^+$ cells at CliniMACS Plus. After depletion of CD45RA$^+$ cells, median of purity of CD45RA$^-$ population was 99.8 (range 99.7–99.9), and median of viability was 97.9 (range 97.7–99.9). Data of CD45RA$^-$ purity and viability from each experiment are shown in **Table 1**.

Transduction Efficiency

We transduced CD45RA$^-$ cells 1 day after cell activation. A lentiviral construct encoding for NKG2D CAR was used at MOI = 2. As CD45RA$^-$ cells only have basal levels of NKG2D receptor expression, we considered that the expression of NKG2D observed by FCM in NKG2D CAR T cell products corresponds to NKG2D CAR. Our target goal was to achieve ≥50% transduction of total cells. This goal was achieved for all four final products. Data from NKG2D CAR expression along the process are shown in **Table 2**. Representative dot plots of NKG2D staining at the different times are shown in **Figure 2A**. The anti-NKG2D antibody that we use for FCM does not discriminate between the NKG2D endogenous receptor and the NKG2D CAR. In order to analyze the expression of NKG2D CAR in the transduced cells, we performed a western blot using an anti-CD3ζ antibody to detect the CAR protein. NKG2D CAR protein is 40 kDa, whereas endogenous CD3ζ is 16 kDa. As shown in **Figure 2B**, bands corresponding to the NKG2D CAR were only observed in those cell lysates from transduced CD45RA$^-$ cells, whereas they were absent in the different negative controls (activated and expanded NK cells, PBMC, and CD45RA$^-$ untransduced cells). Additionally, PCR analysis using specific primers for endogenous NKG2D and NKG2D CAR genes further confirmed these results (**Supplementary Figure 1**).

TABLE 1 | Purity and viability of CD45RA$^-$ starting cells.

Validation	% Viability	% of CD45RA$^-$
#1	98.1	99.8
#2	99.9	99.9
#3	97.7	99.9
#4	97.7	99.7

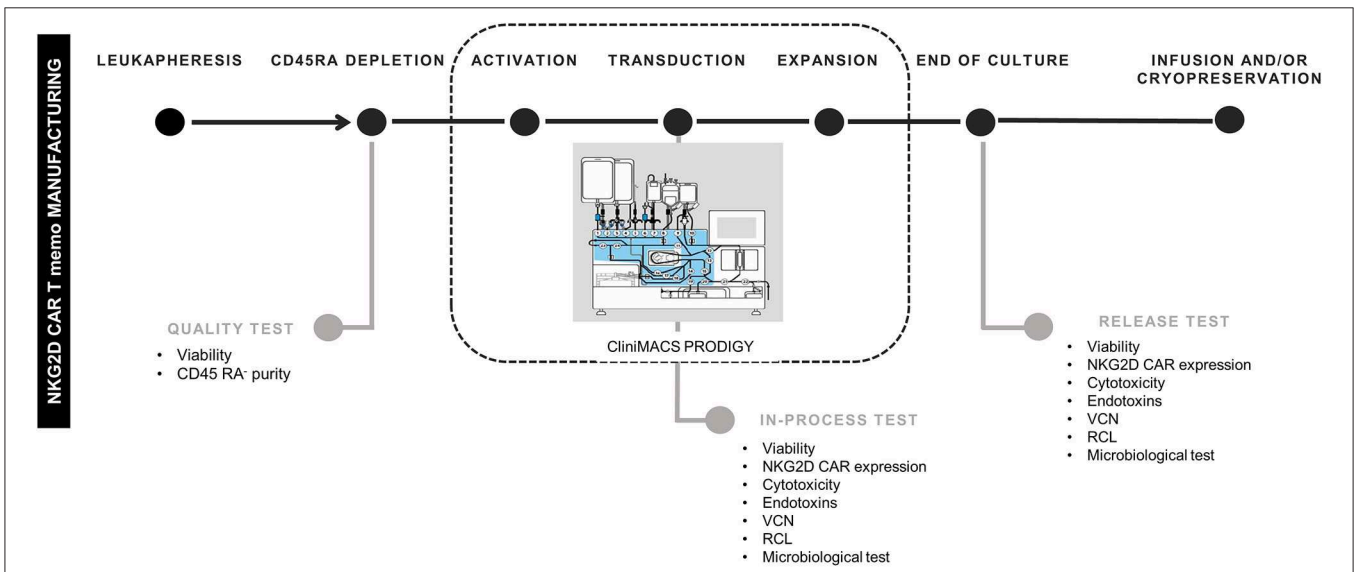

FIGURE 1 | Schema of NKG2D CAR memory T-cell manufacturing process. NKG2D, Natural Killer Group 2D; CAR, chimeric antigen receptor. The schema of CliniMACS Prodigy is Copyrighted © 2015 by Miltenyi Biotec GmbH, used with permission.

Expansion

After CD45RA$^+$ depletion, 10^8 of CD45RA$^-$ cells were transferred into a sterile bag and connected to CliniMACS Prodigy for further processing. The number of cells recovered after CD45RA depletion exceeded this limit for all experiments. For the final products, the fold expansion ranged from 13.4 to 38.6; thus, in all cases, the total number of cells obtained was enough to perform a clinical treatment in a multiple-dose regimen. Data of cell expansion from each experiment are shown in **Figure 3**.

TABLE 2 | Data from transduction efficiency and viability.

	% NKG2D CAR expression			Viability		
Validation	**Day +6**	**Day +8**	**Final**	**Day +6**	**Day +8**	**Final**
#1	73	60.5	60.6	85	82.5	86.3
#2	41	43	55	73	77	65
#3	24	82	87.4	70	83	81.4
#4	62	75	91	80	84	82

NKG2D, natural killer group 2D; CAR, chimeric antigen receptor.

Immunophenotype

Starting and final CAR T cell products were analyzed for viability and CD3, CD4, and CD8 contents. Naïve and memory populations were also identified by using CD45RA and CCR7 markers. The activation/exhaustion status of starting and final cells was analyzed by CD25, PD-1, and TIM-3 markers. The presence of CD4$^+$CD25$^+$CD127$^{low/neg}$ (Tregs) was also analyzed in the starting and final products. Both starting

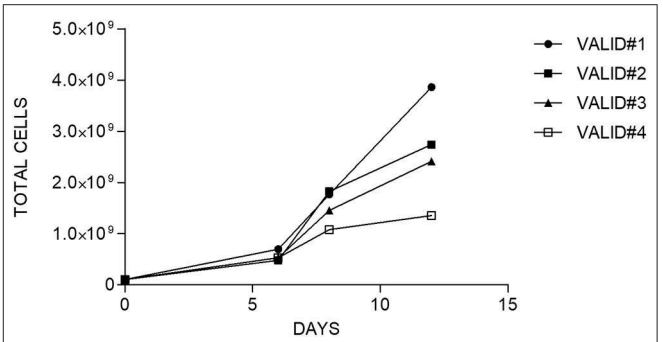

FIGURE 3 | Fold expansion of cells along the time in each manufacturing validation.

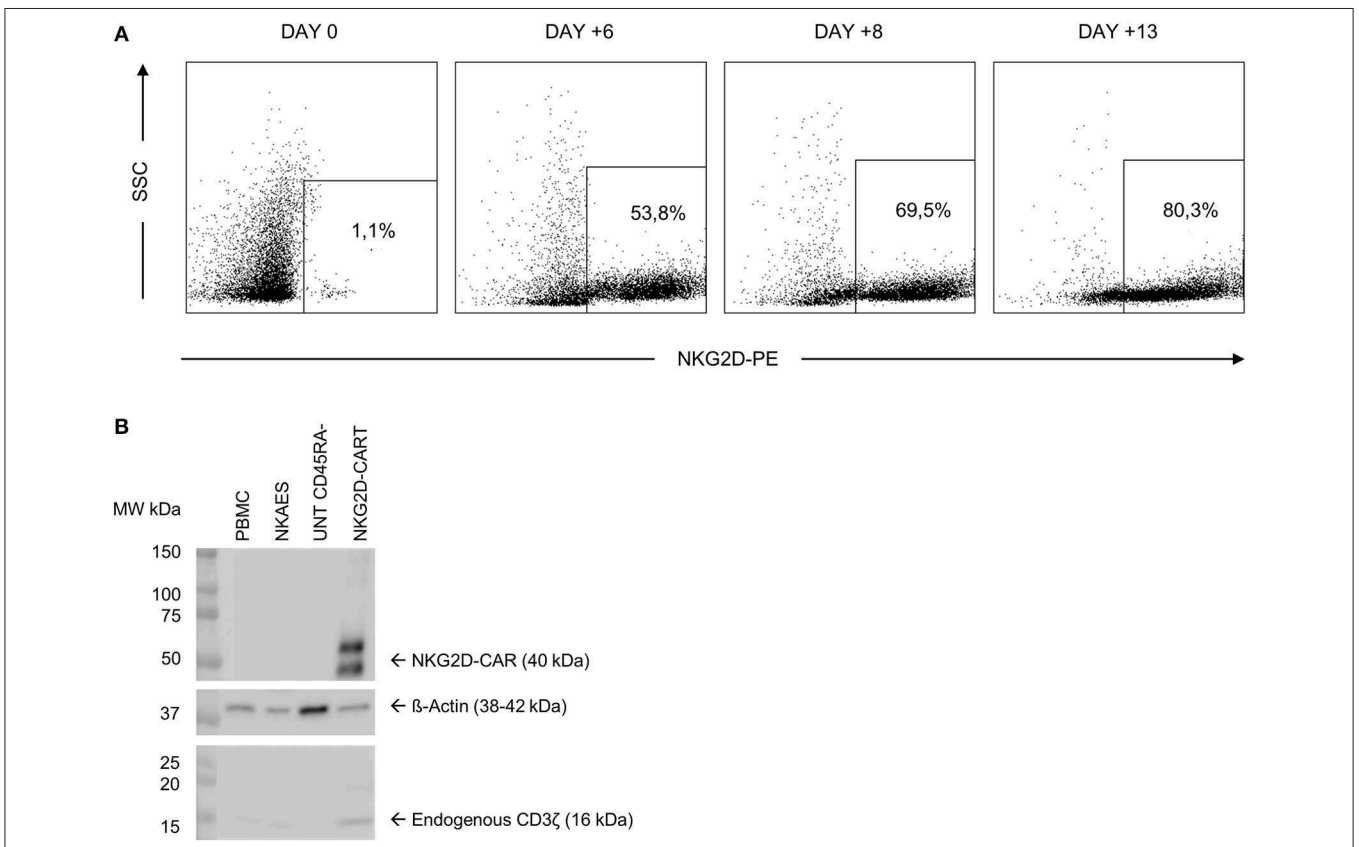

FIGURE 2 | NKG2D CAR expression in CD45RA$^-$ cells after transduction. **(A)** Representative FCM data showing an increase in NKG2D CAR expression along the manufacturing process. **(B)** Analysis of NKG2D CAR expression in transduced cells by western blot detecting CD3ζ. NKG2D, natural killer group 2D; CAR, chimeric antigen receptor; FCM, flow cytometry.

CD45RA$^-$ cells and final NKG2D CAR memory T cell products were CD3$^+$ and showed an enrichment in CD4$^+$ vs. CD8$^+$ T cells. Before and after manufacturing process, T cells were negative for CD45RA and CCR7, indicating an effector memory (T_{EM}) phenotype. Tim-3 and CD25 activation/exhaustion markers were upregulated in final NKG2D CAR memory T cell products compared to starting CD45RA$^-$ cells; however, PD-1 expression was downregulated at the end of the process (**Figure 4**). Additionally, only a low proportion of T_{regs} (CD4$^+$CD25$^+$CD127$^{low/neg}$) was found on starting CD45RA$^-$ cells and final NKG2D CAR T cells compared with total PBMC (**Supplementary Figure 2**).

Effector Function

Lysis ability of NKG2D CAR T cells was tested against the NKG2DL-expressing cell lines Jurkat (T-ALL) and 531MII (metastatic osteosarcoma) by performing conventional 4-h europium-TDA assays. Although donor variability was observed, all final NKG2D CAR T cell products analyzed could target Jurkat and 531MII cells with a percentage of cytotoxicity ≥20%, thus meeting the established requirements. For validation #1, owing to technical issues, cytotoxicity against Jurkat cells was only tested using cryopreserved NKG2D CAR T cells. Cytotoxicity of final NKG2D CAR T cells against Jurkat was higher (median 80%, range 28.2–100%) than against 531MII cells (median 42.3%, range 20–74.6%) for all analyzed products, although this difference was not statistically significant. Data of cytotoxicity levels from each experiment are shown in **Table 3**. Additionally, the cytotoxicity of manufactured NKG2D CAR T cells against Jurkat cells is shown in **Supplementary Video 1**.

Safety and Purity Tests

To meet regulatory specifications (acceptable thresholds in parentheses), samples were taken at days +6 and between days

TABLE 3 | Cytotoxicity of NKG2D CAR memory T cells against Jurkat and 531MII target cells.

Validation	% Cytotoxicity vs. Jurkat	% Cytotoxicity vs. 531MII
#1	NR	74.6
#2	100	19.5
#3	79.8	42.3
#4	28.2	NR

NKG2D, natural killer group 2D; CAR, chimeric antigen receptor; NR, non-reproducible experiment.

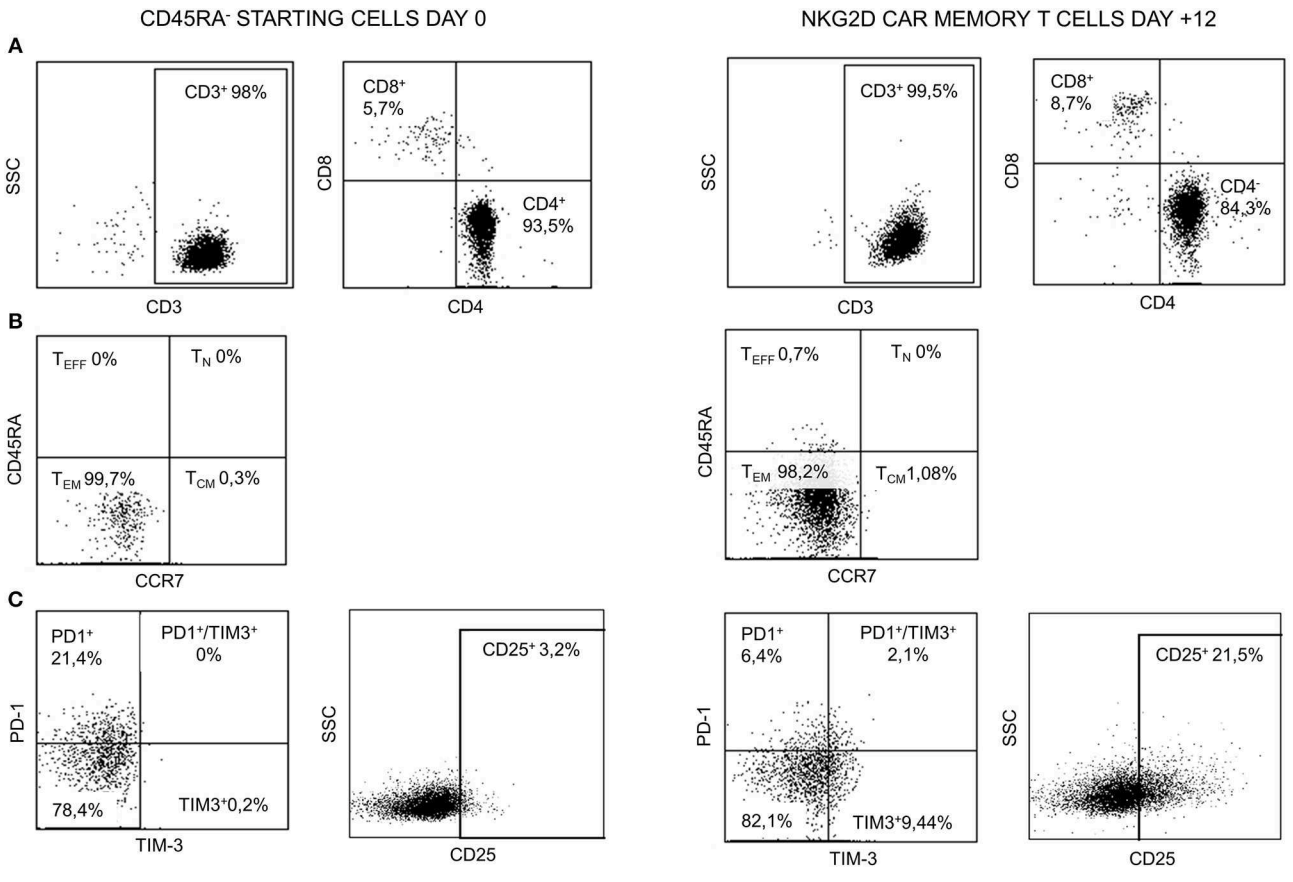

FIGURE 4 | Representative FCM data of starting CD45RA$^-$ cells (day 0) and NKG2D CAR memory T cell products at the end of manufacture process (day +12). **(A)** CD3, CD4, and CD8 contents. **(B)** Naïve/memory phenotype. **(C)** Expression of activation/exhaustion markers. FCM, flow cytometry; NKG2D, natural killer group 2D; CAR, chimeric antigen receptor.

+8 and +10 as in-process controls and at the end of process as release controls and were evaluated for VCN (≤5 copies/cell), free lentiviral particles in the supernatant (LVPS) (≤0.05%), oncogenic gene expression (no overexpression), and genetic stability (normal CGH). A Gram stain (no organisms seen) as a quick method and microbiological tests [0 colony-forming unit (CFU)] ensured no bacterial contamination. Other release controls performed included those relating to the purity of the final product: measurement of endotoxin levels, whose limits for administration depend on the product and the parenteral administration route, where the pyrogenic threshold dose of endotoxin per kilogram of body mass in a single hour in the case of transduced cells is 5 IU/kg/h. All final products analyzed fulfilled the specifications except for validation #3, which showed VCN of 12 instead of ≤5 copies/cell, and for validation 4#, which showed overexpression of myc. Complete data regarding genetic tests are shown in **Table 4**. All samples showed no microbiological contamination. Endotoxin levels were below 5 IU/kg/h, and the presence of mycoplasma was undetectable (**Table 5**).

Stability of Cryopreserved NKG2D CAR T Cells

CliniMACS Prodigy allows the production of sufficient number of CAR T cells to be administered in multiple doses. Although the first dose could be administered right after harvesting, spare cells need to be cryopreserved for future infusions. To explore if cryopreservation could have a negative impact on manufactured CAR T cells, we tested three different freezing media and evaluated cell counts, viability CD45RA$^-$ purity, NKG2D expression, and cytotoxicity of cryopreserved NKG2D CAR T cells 1 year after freezing. We observed that those NKG2D CAR T cells cryopreserved in autologous plasma +5% DMSO showed the highest viability and cytotoxicity indicating that, whenever possible, this should be the freezing media of preference, followed by M199 + 10% albumin and 5% DMSO (**Table 6**).

TABLE 4 | Results from genetic tests.

Validation	VCN	LVPS	CGH	*tert* expression	*myc* expression
#1	NA	Undetectable	Normal	No overexpression	No overexpression
#2	3.6	Undetectable	Normal	No overexpression	No overexpression
#3	12.3	Undetectable	Normal	No overexpression	No overexpression
#4	2.4	Undetectable	Normal	No overexpression	*myc* overexpression

VCN, vector copy number; LVPS, free lentiviral particles in the supernatant; CGH, comparative genome hybridization.

TABLE 5 | Results from sterility tests.

Validation	Gram staining	Mycoplasma	Endotoxins EU/mL
#1	Negative	Negative	NA
#2	Negative	Negative	0.019
#3	Negative	Negative	0.0035
#4	Negative	Negative	0.01

DISCUSSION

In the last decades, CAR T cell-based immunotherapies have demonstrated to be an effective and safe approach for cancer treatment. The clinical success of two CART19 cell products (Kymriah^{TM} and Yescarta^{TM}) for the treatment of B cell malignancies has led to their recent FDA and European Medicines Agency (EMA) approval, emphasizing the great potential of this technology. Targeting myeloid and non-B lymphoid cell hematological malignancies such as T-ALL, biphenotypic, and infant leukemia or solid tumors has been much harder, owing to the lack of specific antigens and the immunosuppressive tumor microenvironment (33–36). We present data on NKG2D CAR, which has the ability to recognize multiple ligands that are overexpressed in different tumor types including AML, T-ALL, and sarcomas (12, 13, 37–39). In fact, D. Sallman et al. have recently reported a case of remission in a patient suffering from relapsed/refractory AML after multiple infusions of autologous T cells redirected with a first-generation CAR recognizing NKG2DL (40). Aside from CAR specificity, choice of signaling domains and selection of effector cell subset to transduce, manufacturing process, and full characterization of final CAR T cell products are essential for clinical success. In the present study, we validate and provide detailed description of our manufacturing protocol and the characteristics of NKG2D CAR T cells. We show the feasibility of producing large numbers of allogeneic NKG2D CAR memory T cells using a 10–13 days' protocol, which includes activation with TransAct reagent, transduction with an NKG2D-4-1BB-CD3ζ lentiviral vector and expansion with rhIL-2 in CliniMACS Prodigy, an automated closed system compliant with GMP guidelines.

The main objectives of this study were (1) to show the feasibility and reproducibility of automated manufacturing of GMP-grade NKG2D CAR T cells in an academic institution and (2) to demonstrate that manufactured NKG2D CAR T cells meet the requirements established by the Spanish Regulatory Agency for clinical use. A total of four manufacturing processes were completed, and the CAR T cell products obtained were analyzed

TABLE 6 | Stability of manufactured NKG2D CAR memory T cells after cryopreservation.

Freezing medium	% Viability	% NKG2D	% CD45RA$^-$	% Cytotox vs. Jurkat	% Cytotox vs. 531MII
M199 + ALB + DMSO V4	47.9	61.5	99.2	55.2	17.3
Hypothermosol v4	14.1	61.5	99.2	NA	NA
Auto plasma + DMSO V5	74.6	69.9	97.1	78.6	60.3

NKG2D, natural killer group 2D; ALB, albumin; DMSO, dimethyl sulfoxide.

at three different time points: day +6 and between days +8 and +10, as in-process controls, and at the end of culture, between days 10 and 13, when quality tests were performed to analyze if NKG2D CAR memory T cell products met release criteria.

We used CD45RA$^-$ cells from healthy donors to produce our NKG2D CAR T cells in order to develop a safe allogeneic therapy. The lack of alloreactivity of CD45RA$^-$ cells has been explored before in preclinical studies from our group and others (18, 30). Furthermore, Maschan et al. have described the safety of low-dose infusions of CD45RA$^-$ lymphocytes in mismatched-related HSCT (41). Recently, our group has reported the safety of high-dose infusions of donor-derived CD45RA$^-$CD45RO$^+$ T cells after haploidentical transplantation (42).

The manufacturing process starts with a non-mobilized apheresis from a healthy donor followed by depletion of CD45RA$^+$ cells in CliniMACS Plus device. Although CD45RA$^+$ cells can be currently depleted at CliniMACS Prodigy, at the moment when these experiments were carried out, the software to do so was unavailable. After depletion, CD45RA$^-$ cells were tested for viability and purity. All CD45RA$^-$ cell products showed viability >95%. Purity of CD45RA$^-$ cells after depletion of CD45RA$^+$ subset was at least 99.7%, indicating that carryover of naïve T cells was minimal and meets the established criteria for further processing to obtain NKG2D CAR T cells. Activation, transduction, and expansion were conducted in CliniMACS Prodigy. Median of fold expansion was 24.4 (range 13.5–38.6), and the mean of total cells obtained was 2.44×10^9 (range 1.35×10^9-3.86×10^9). These expansion data are in line with other manufacturing protocols using CliniMACS Prodigy (43, 44). Additionally, according to the number of CAR T cells that have been infused in other clinical trials (45, 46), the number of NKG2D CAR T cells we achieved would have been enough to treat patients in a multiple-dose base. Over the manufacturing procedure, viability of the harvested cells has shown to be robust and above 80% except for validation 2, which showed a viability of 65%. During the process, a decrease in viability was observed on day +6 compared with that observed in starting cells, and this temporary drop on viability after transduction has been already reported by other groups (44, 47, 48). Some authors have reported that NKG2D CAR T cells may induce fratricide, hindering the expansion and the viability of cultured cells (49, 50). Additionally, a CD4/CD8 ratio bias and enhanced effector memory differentiation have been described when using PBMCs as starting cells to express NKG2D CAR. The fold NKG2D CAR T cell expansion observed in this study, along with the viability of the final cell products, suggests that no NKG2D CAR T cell-mediated fratricide is occurring during the manufacturing protocol. This observation could be related to the T-cell subset used as starting cells, as CD45RA$^-$ compartment is already enriched in CD4$^+$ T cells with an effector memory and central memory phenotypes. However, more experiments need to be performed to explore the susceptibility of different T-cell subsets to NKG2D CAR T cell-mediated fratricide to confirm this hypothesis. To further explore if fratricide could be taking place in our experiments, the expression of NKG2DL on NKG2D CAR T cells expanded at small scale was analyzed by FCM. No upregulation of NKG2DL was observed in these cells (data not shown). Nevertheless, we only analyzed the expression of NKG2DL at day +8 post-activation, and it has been described that activated T cells upregulate NKG2DL in a temporary manner, specially between days 2 and 5 upon activation (49, 50). Thus, with our data, we cannot totally rule out an upregulation of NKG2DL and, consequently, a fratricide phenomenon in other moments of the culture. A more detailed study of NKG2DL expression kinetics on NKG2D CAR T cells along the manufacturing procedure would shed light on this question.

Activated CD45RA$^-$ cells were lentivirally transduced with MOI = 2 because small-scale preclinical data using the same vector achieved transduction efficiencies higher than 95% (30). This MOI of 2 is low compared with that of other works where MOIs of 5–10 are reported (43, 48). We used a fluorochrome-labeled anti-NKG2D mAb for CAR detection by FCM, as untransduced CD45RA$^-$ cells only have basal expression of the NKG2D receptor, and then we can consider that the NKG2D expression observed in manufactured cells comes from the CAR (30). The expression of NKG2D CAR in transduced CD45RA$^-$ cells was further confirmed by western blot and PCR analysis. As previously observed in small-scale experiments, the expression of NKG2D increased during the expansion of cells. In two out of four batches, NKG2D CAR expression was over 80%, whereas the other two achieved an expression of 55 and 60.6%. These expression values (55–60.6%) are comparable with those reported in other publications (43, 51) and were enough to efficiently eliminate Jurkat and primary osteosarcoma cells (531MII) at a 20:1 effector to target ratio. Owing to technical issues, some cytotoxicity assays were non-reproducible, and thus, potency of NKG2D CAR T cells could not be evaluated at some time points either during manufacturing procedure or at the end of culture. Nevertheless, those cytotoxicity assays that were reproducible also fulfilled the specification for potency, indicating manufactured NKG2D CAR T cells are cytotoxic against the target cells.

At the end of the activation–transduction–expansion protocol, different quality tests need to be performed to ensure safety and purity of manufactured CAR T cells before they are administered in patients. Sterility tests were negative, and no mycoplasma was detected. The concentration of bacterial endotoxins was within the limits set by Eu Ph for intravenous injectable products in all validations. Genetic stability of NKG2D CAR T was confirmed by normal CGH, indicating no chromosomal aberrations are caused by lentiviral transduction. Three out of four validations showed <5 genome integrated vector copies, fulfilling the specifications required. However, in validation #3, up to 12.3 genome integrated vector copies were detected. These data are striking, as a MOI of 2 was used in all experiments and does not match the hypothesis that one viral particle is able to infect one cell. Despite that higher-than-expected VCN was found in these cells, the percentage of NKG2D CAR positive cells in this validation was 87%,

indicating transduction efficiency was not above the usual levels. Additionally, CGH and expression of *myc* and *tert* oncogenes were normal in this batch, suggesting that even though more than five copies were integrated, they caused no genetic alterations. To rule out a potential oncogenic effect of NKG2D CAR T cells, the expression of *myc* and *tert* oncogenes was analyzed. All validations showed no overexpression of these genes except for validation #4, which presented overexpression of *myc,* and consequently did not fulfill the specifications required. Although *myc* overexpression in NK cell products has been previously demonstrated to be safe and to induce no complications nor secondary neoplasia in patients (52), it would be important to be aware and increase monitoring of these cell products to ensure safety before being administered to patients. The specification of the percentage of RCL in the supernatants is established at a maximum of 0.05%. All NKG2D CAR T cell products remained under that limit, indicating that there is no potential risk of virus infection after infusion.

In summary, the data here reported demonstrate the feasibility and reproducibility of a manufacturing protocol to obtain clinical-grade large-scale NKG2D CAR CD45RA$^-$ T cells in CliniMACS Prodigy system. NKG2D CAR T cells met the release criteria for expansion, NKG2D CAR expression, cytotoxicity, and sterility, although grade of expansion and product characteristics showed variability. Most importantly, the manufacturing process described here shows flexibility and admits further improvements for future NKG2D CAR T cell trials.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee from Hospital La Paz. Non-mobilized apheresis was obtained from healthy donors at the Bone Marrow Transplant and Cell Therapy Unit (BMTCT) of Hospital Universitario La Paz (HULP) by using CliniMACS Plus device (Miltenyi Biotec). All donors gave their written informed consent in accordance with the Helsinki protocol, and the study was performed according to the guidelines of the Ethics Committee from Hospital La Paz. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LF, IM, and AP-M: conception and design. LF, AF, IM, DL, AM, and AR: development of methodology. LF, AE, MV, LC, AF, AL, MG, IM, RP, and AP-M: acquisition of data (acquired and managed patients, provided facilities, etc.). LF, AF, IM, AE, JM-L, and AP-M: analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis). LF, AF, IM, and AP-M: writing, review, and/or revision of the manuscript. LF, AF, IM, and AE: administrative, technical, or material support (i.e., reporting or organizing data, constructing databases). LF and AP-M: study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02361/full#supplementary-material

Supplementary Figure 1 | PCR showing expression of endogenous NKG2D receptor and NKG2D CAR in transduced cells. NKG2D CAR expression is absent in CD45RA$^-$ cells and PBMC negative controls.

Supplementary Figure 2 | Representative FCM data of $CD4^+CD25^+CD127^{low/-}$ (Treg) content in total PBMC from a healthy donor (first row), starting CD45RA$^-$ cells from validation 2 (second row), and NKG2D CAR T cells from validation 4 at the end of manufacturing process (third row).

Supplementary Video 1 | 3.35 h Time-lapse showing how NKG2D CAR memory T cells (labeled in orange with CMTMR) recognize, bind, and eliminate Jurkat cells (labeled in green with CFSE).

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Haploidentical Stem Cell Transplantation in Children With Hematological Malignancies Using $\alpha\beta^+$ T-Cell Receptor and $CD19^+$ Cell Depleted Grafts: High $CD56^{dim}/CD56^{bright}$ NK Cell Ratio Early Following Transplantation Is Associated With Lower Relapse Incidence and Better Outcome

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We prospectively analyzed outcomes of haploidentical hematopoietic stem cell transplantation using $\alpha\beta^+$ T-cell receptor/$CD19^+$ depleted grafts. Sixty-three transplantations were performed in 60 patients. Twenty-eight patients were diagnosed with acute lymphoblastic leukemia (ALL), 27 patients were diagnosed with acute myelogenous leukemia, and in eight other hematological malignancies were diagnosed. Twenty-three were in first complete remission (CR), 20 in second CR, 20 beyond second CR. Four patients developed graft failure. Median time to neutrophil and platelet recovery was 14 (range 9–25) and 10 days (range 7–30), respectively. The probability of non-relapse mortality (NRM) by day +100 after transplantation was 10 ± 4%. With a median follow-up of 28 months, the probability of relapse was 32 ± 6% and disease-free survival was 52 ± 6%. Immune reconstitution was leaded by NK cells. As such, a high $CD56^{dim}/CD56^{bright}$ NK cell ratio early after transplantation was associated with better disease-free survival (DFS) (≥3.5; 77 ± 8% vs. <3.5; 28 ± 5%; $p = 0.001$) due to lower relapse incidence (≥3.5; 15 ± 7% vs. <3.5; 37 ± 9%; $p = 0.04$). T-cell reconstitution was delayed and associated with severe infections after transplant. Viral reactivation/disease and presence of venooclusive disease of liver in the non-caucasian population had a significant impact on NRM. $\alpha\beta^+$ T-cell receptor/$CD19^+$ cell-depleted

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haploidentical transplant is associated with good outcomes especially in patients in early phase of disease. A rapid expansion of "mature" natural killer cells early after transplantation resulted on lower probability of relapse, suggesting a graft vs. leukemia effect independent from graft-vs.-host reactions.

Keywords: T-cell depletion, NK cells, haploidentical transplantation, immune reconstitution, acute leukemia, children

INTRODUCTION

Nowadays, allogeneic hematopoietic stem cell transplantation is considered a curative option for pediatric patients with high-risk leukemia at diagnosis or after relapse. However, an important proportion of those patients lack a suitable human leucocyte antigen (HLA) match donor or delay for donor availability may be unacceptable (1). For patients needing an allogeneic transplant in a timely manner, the use of haploidentical relative donors has been considered a suitable approach (2, 3). Donor T-cells in the graft facilitate T-cell reconstitution but they are also responsible for graft-vs.-host disease (GvHD). Currently, several haploidentical transplant platforms using "*ex-vivo*" T-cell depletion (TCD) without using post-transplant GvHD pharmacological prophylaxis have gained clinical relevance (4, 5).

Natural killer (NK) cells are have been detected in the first weeks following TCD haploidentical transplantation and the pattern of expressed killer cell immunoglobulin-like receptors (KIRs) was similar to that originally found in the donor (6). However, NK cell phenotype was altered and cytotoxicity was lower compared with their donors in pediatric patients receiving $CD3^+/CD19^+$ depleted haploidentical transplantation (7). The mentioned TCD haploidentical transplantation platform was associated with encouraging results especially in patients in the early phase of disease (8). KIR-B haplotype donors conferred a rapid NK cell expansion early after transplant, which resulted in lower probability of relapse. Using grafts from younger donors resulted in an improved immune reconstitution in all lymphocyte subsets (9) however, viral reactivation affected a significant proportion of patients.

A new approach to improve delayed immune reconstitution has been recently developed, based on selective depletion of $\alpha\beta^+$ T lymphocytes, and of B cells (10). Previous T-cell depleted strategies resulted in loss of lymphocyte cell subsets that may play a positive role in the recipient. T cells displaying the αβ T-cell receptor (TCR) are responsible for GVHD. However, T cells with the γδ receptor chains have no alloreactive capacity, and may contribute to important anti-infectious activity and to a possible anti-leukemia effect. A recent publication using the mentioned haploidentical transplant platform are mainly focused on early immune reconstitution especially on $\gamma\delta^+$ T cells subsets (10).

However, few studies have reported a detailed analysis of prognostic factors and outcomes in this type of transplantation considering early immune reconstitution and its impact on transplant outcomes (10, 11). The aim of this observational prospective study was to analyze the outcomes and risk factors for survival of pediatric patients who received an haploidentical relative donor transplant using $\alpha\beta^+$ TCR/$CD19^+$ depleted grafts.

PATIENTS, MATERIALS AND METHODS

Patient and Transplant Characteristics

Patients were enrolled in the study from November 2012 to November 2018.

All consecutive patients, younger than 20 years old, diagnosed with high-risk hematological malignancies, in need of an allogeneic transplantation, and in good clinical condition who lacked either a matched related donor or a matched unrelated donor, were included. Indications for allogeneic hematopoietic transplantation for children with acute lymphoblastic leukemia (ALL) included: poor cytogenetics, induction failure (defined as no remission at 1 month following induction treatment), persistent minimal residual disease tested by PCR before transplant with a cutoff point of 10^{-3} and 2nd complete remission (CR) or beyond. For acute myeloid leukemia (AML) patients transplant criteria included intermediate or poor risk characteristics at diagnosis and also 2nd CR or beyond. Primary refractory AML was defined as the failure to achieve a response after one or two cycles of induction. Refractory ALL was defined as the failure to achieve a cytological remission response after induction chemotherapy and second-line rescue chemotherapy. The only exclusion criterion was a poor clinical condition, defined as a Lansky score lower than 60%.

Patients, parents, and/or their legal guardians gave written informed consent in accordance with the Helsinki Declaration.

Sixty-three transplants were performed in 60 patients. Twenty-eight patients had ALL, 27 AML, five advanced myelodysplastic syndrome (MDS), two Hodgkin disease, and one non-Hodgkin lymphoma. Twenty-three were in first CR, 20 in second CR and 20 in more than second CR or active disease at time of transplantation. Twenty-two patients had MRD positive at time of transplant. Haploidentical was the first transplant in 49 cases. In 14 cases, one or two previous stem cell transplantations and subsequent chemotherapy courses before haploidentical transplantation were performed.

Patient and transplantation characteristics are shown in **Table 1**.

KIR Genotyping and KIR Ligand

Fifteen human KIR genes and two pseudogenes were analyzed by PCR with a KIR typing kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The KIR A haplotype was defined by the absence of 2DS1, 2DS2, 2DS3, and 3DS1 and the presence of 2DS4 as the only KIR-activating receptor. The KIR B haplotype was determined by the presence of any activating genes except 2DS4.

TABLE 1 | Patient, donors, and transplant characteristics.

Number	63
Patient age, years	
Median (range)	9 (1–19)
Patient weight, kg	
Median (range)	30 (8–136)
Patient gender	
Male	41 (65%)
Female	22 (35%)
Patient lansky score	
90–100	55 (87%)
<90	8 (13%)
Patient CMV serostatus	
Positive	40 (64%)
Negative	23 (36%)
Patient ethnicity	
White caucasian	47 (75%)
White non-caucasian (Hispanic/North Africans)	16 (25%)
Disease	
Acute myeloid leukemia	27 (43%)
Acute lymphoblastic leukemia	28 (44%)
Myelodisplastic syndrome/refractory cytopenia childhood	5 (8%)
Hodgkin disease	2 (3%)
Non-Hodgkin Lymphoma	1 (2%)
Disease status at transplant	
1st CR	23 (36%)
2nd CR	20 (32%)
3rd CR or active disease	20 (32%)
Minimal residual disease status	
Positive	22 (35%)
Negative	41 (65%)
Donor age, years	
Median (range)	40 (10–54)
Donor gender	
Male	27 (43%)
Female	36 (57%)
Donor CMV serostatus	
Positive	42 (67%)
Negative	21 (33%)
Donor KIR genotype	
KIR B	58 (92%)
KIR A	5 (8%)
Donor KIR B score	
Neutral	30 (52%)
Better	21 (36%)
Best	7 (12%)
KIR ligand-ligand matching	
Match	33 (52%)
Mismatch	30 (48%)
Transplant number	
1st transplant	49 (78%)
2nd transplant	10 (16%)
3rd transplant	4 (6%)
Graft composition	
CD34$^+$ cells × 10^6/Kg median (range)	7.59 (2.06–16.56)
CD3$^+$ cells × 10^6/Kg median (range)	5.89 (1.30–46.26)
CD3$^+$ TCRαβ cells × 10^5/Kg median (range)	0.01 (0.01–0.78)
CD3$^+$ TCRγδ cells × 10^6/Kg median (range)	5.64 (0.13–46.17)
CD3$^-$CD56$^+$ cells × 10^6/Kg median (range)	32.20 (0.18–139.54)
CD3$^-$CD19$^+$ cells × 10^5/Kg median (range)	0.04 (0.01–1.34)
Median follow-up of survivors, months (range)	28 (4–72)

The KIR ligand HLA-C allotypes (C1 and C2) and the HLA-B allotypes (Bw4) were determined using high-resolution PCR-sequence-based typing.

We also determined KIR B-content scores for all donors according to the system proposed by Cooley et al. (12) (www.ebi.ac.uk/ipd/kir/donor_b_content.html). Criteria for donor selection have been previously reported (8, 13). Briefly, donors were chosen based on KIR B haplotype, higher B-content score, younger age, and NK alloreactivity (KIR-Ligand model). Donors were parents (mother in 34 and father in 27) or siblings in 2. Donor characteristics are also shown in **Table 1**.

Donor Hematopoietic Stem Cell Mobilization, Collection, Graft Manipulation Procedure and Infusion

Donor mobilization has been previously described (8, 9, 14). Briefly, mobilization started on day 5 of the conditioning regimen at a G-CSF dose of 10 μg/kg/day subcutaneously. Based on the volume, the dose may be split into two injection sites. Progenitor cells collections were performed by leukapheresis. In all, 66 products were obtained by large-volume leukapheresis procedure according to established protocols of the center using a continuous flow blood cell separator (Spectra Optia MNC v.3.0. Terumo BCT, Lakewood, CO; USA or COBE Spectra TM, v.6.1, by Caridian BCT Europe, Garching, Germany) on the fifth day of mobilization and the day before infusion. Apheresis was carried out via bilateral peripheral veins whenever possible, or otherwise by a central venous catheter. During leukapheresis, between 3 and 5 blood volumes were processed. Acid citrate dextrose (ACD-A) was used as an anticoagulant with a ratio of 14:1. Leukapheresis products were also analyzed for expression of the CD34$^+$ antigen as previously reported (8). Concurrent plasma (200–300 mL), was collected for products to be stored overnight after receipt into the processing facility. A unique identification and labeling system has been used to track leukapheresis product from collection to infusion according to FACT/JACIE guidelines. A target dose $\geq 5.0 \times 10^6$ CD34$^+$ cells/kg after selection containing $\leq 25.0 \times 10^3$ CD3$^+$ αβ$^+$ TCR cells/kg was desired. If after two collections, the minimum required dose CD34$^+$ cell dose ($>2.0 \times 10^6$ per kg) were reached, no more collections were performed.

T-cell depletion was performed using CliniMACS Plus device or the fully automated Prodigy device after manipulations in a laminar-flow cabinet located in a clean room certified

for sterile manipulations. Clinical grade reagents, disposable kits, and instrumentation were from Miltenyi Biotec (Bergisch Gladbach, Germany).

Before depletion procedure, cell composition of the apheresis product were analyzed by flow cytometry (cell count and cell subpopulation CD34$^+$, CD3$^+$ αβ$^+$, CD3$^+$ γδ$^+$, CD19$^+$, and CD3-CD56$^+$ cells, and theirs viability), to adjust the doses of total nucleated cells (TNC) and αβ$^+$ T cells to the maximal capacity of $<60 \times 10^9$ TNC and $<24 \times 10^9$ αβ$^+$ T-cells in case of CliniMACS system (CliniMACS system, Miltenyi Biotec, Germany), or $<45 \times 10^9$ TNC and $<20 \times 10^9$ αβ$^+$ T-cells in case of Prodigy device (Prodigy system, Miltenyi Biotec, Germany), according to the manufacturer's instructions.

We use the CliniMACS Prodigy device together with the CliniMACS Prodigy TS310, CliniMACS TCR αβ -biotin reagent, CliniMACS Anti-Biotin reagent, CliniMACS CD19 reagent, and CliniMACS PBS/EDTA, all of them obtained from Miltenyi Biotec GmbH (Bergisch-Gladbach, Germany).We selected the process "LP TCT αβ−19 Depletion" normal scale; this process uses the following reagents: CliniMACS TCR αβ -biotin reagent, CliniMACS Anti-Biotin reagent, CliniMACS CD19 reagent (1 vial of each), and CliniMACS PBS/EDTA; a CliniMACS TS310 tubing set was installed and the liquids were connected as prompted by the device. The cells passed through a magnetic field through which they will be separated. Thus, αβ$^+$ T and CD19$^+$ lymphocytes are removed/eliminated/retained, while all unlabeled cells are recovered for subsequent transplantation to the patient.

At the end of the procedure, quality control by flow cytometry was carried out in the final product, such as cell counts, viability studies, and aerobic and anaerobic cultures before and after immunomagnetic depletion.

In all, 66 depletion procedures were performed (48 out of by the CliniMACS Plus system (72.72%) and 18 depletions were performed with CliniMACS Prodigy device (27.27%) according to the availability of both devices. None of the apheresis products exceeded the maximal capacity/limits of TNC or αβ$^+$ T cells established by the manufacturer at the beginning of the manipulation procedure. Median recovery of CD34$^+$ were 81.92% with CliniMACS Plus and 73.70% with CliniMACS Prodigy, without reaching statistical significance. There were no significant differences in terms of the T cells depletion efficacy assessed by logarithmic descent (4.41 vs. 6.03, $p = 0.42$). The median infused doses of αβ$^+$ T cells/kg cells were 3.89×10^3 and 2.18×10^3 in each group. However, there was a higher number of infused γδ$^+$ T-cells/Kg recipient body weight using CliniMACS Prodigy (71.90×10^3/kg) vs. CliniMACS Plus 15.13×10^6/kg ($p = 0.015$). Data on cell composition of final apheresis products after depletion procedures using both systems are provided in **Supplemental Table 1**. All cellular products were freshly infused on day 0.

Transplantation Protocol

The conditioning regimen consisted of fludarabine (25 mg/m^2 day from days −6 to −2), busulfan (3.2–4.8 mg/kg/day, according to patient body weight, from days −6 to −4) and thiotepa (5 mg/kg/day from days −3 to −2). Methylprednisolone was administered on days −6 to −2 (5 mg/kg). No serotherapy was given as part of transplant conditioning.

For those patients who required a second haploidentical transplant due to primary graft failure, the conditioning regimen used consisted of fludarabine 40 mg/m^2/day from days −5 to −3, thymoglobulin 2 mg/kg/day from days −5 to −3, and melphalan 120 mg/m^2 on day −1. Cyclosporine was used as pharmacological GvHD prophylaxis from day −1 until engraftment and it was tailored as soon as possible after transplant whenever acute GvHD was not present.

Assessment of Engraftment, Chimerism Status, Antibodies and Flow Cytometry Analysis for Immune Reconstitution Following Transplantation

Myeloid recovery was defined as the first of 3 consecutive days on which the absolute neutrophil count was $\geq 0.5 \times 10^9$/L. Platelet recovery time was considered the first of 3 consecutive days on which a platelet count above 20×10^9/L was achieved, with no transfusion requirements.

Donor chimerism was evaluated by the short tandem repeat PCR method at the time of engraftment and monthly following transplantation or when clinical condition required. Donor chimerism was determined for whole blood and cell subsets. For chimerism analysis in sub-populations, CD34$^+$ progenitor cells from bone marrow, and T lymphocytes from peripheral blood were purified by immunomagnetic methods (CD34 or CD3 beads; Miltenyi Biotec). Phenotyping of NK cells, T lymphocytes, T lymphocyte subsets, B lymphocytes, NKT lymphocytes, and dendritic cells (DCs) was performed on fresh samples of whole blood by multiparametric flow cytometry as previously described (7, 8).

The following fluorochrome-labeled monoclonal Abs against human Ags were obtained from Becton Dickinson (San Jose, CA, USA): CD3 PE-Cy7, CD20-PE, CD45-FITC, lineage-FITC, HLADR-APC-Cy7, CD11c-PECy5, CD45RAPE-Cy5, and CCR7-PE. Fluorochrome-labeled monoclonal Abs against CD19-PE, CD56-APC, and CD25-PE were obtained from Beckman Coulter (Fullerton, CA, USA). Fluorochrome-labeled monoclonal Abs against BDCA4-APC were obtained from Miltenyi Biotec.

Study Design, Definitions and Statistical Analysis

The major study endpoints were disease-free survival (DFS), cumulative incidence of relapse and non-relapse mortality (NRM). DFS was defined as time from transplantation to relapse/progression or death for any cause, whichever occurred first.

Acute GvHD (aGvHD) was graded according to standard criteria, whereas chronic GvHD (cGvHD) was defined as mild, moderate and severe according to NIH criteria (15) for chronic GvHD. Relapse was defined as morphologic or clinical evidence of recurrence in the peripheral blood, bone marrow, or extramedullary sites. NRM was defined as any cause of death other than disease. The probability of DFS was calculated from

TABLE 2 | Engraftment and supportive care.

Outcomes	Value
Engraftment (on days)	**Median (range)**
Time to neutrophils	14 (9–25)
Time to platelet ≥ 20 × 10^9/L	10 (7–30)
Time to platelet ≥ 50 × 10^9/L	13 (7–35)
Time to platelet ≥ 100 × 10^9/L	16 (9–50)
Supportive care (on days)	
Time on fever	1 (0–12)
Time on antibiotics treatment	18 (3–85)
Time on RBC transfusion	2 (0–20)
Median time on platelets transfusion	2 (0–54)
Length of hospital stay	15 (10–86)

TABLE 3 | Transplantation-related toxicity.

Toxicity	
Mucositis	***N* = 41**
Grade 1–2	28 (44)
Grade 3–4	13 (21)
Nausea and vomiting	***N* = 23**
Grade 1–2	10 (16)
Grade 3–4	13 (21)
Diarrhea	***N* = 25**
Grade 1–2	15 (24)
Grade 3–4	10 (16)
Organ toxicity (grade 3–4)	***N* = 12**
Hepatic (including VOD/SOS)	7 (11)
Cardiac	0 (0)
Neurologic	4 (6)
Renal	1 (2)
Pulmonary	0 (0)
Engraftment syndrome	17 (27)
Thrombotic microangiopathy	3 (5)

the time of transplantation by means of the Kaplan–Meier product limit method (16).

Cumulative incidence was used to estimate the relapse and NRM probabilities (17). Death in remission was treated as a competing event to calculate the cumulative relapse incidence. Relapse was considered to be the competing event for calculating cumulative incidence of NRM. Gray's test was used to assess differences between relapse incidence and NRM. The multivariate analysis of survival was evaluated using proportional hazard regression model (18). Hazard ratios (HRs) were calculated with a 95% confidence interval (95% CI). A $P < 0.05$ was considered statistically significant. The following variables were included in the analysis as covariates: patient and donor age, diagnosis, diseases status at time of transplantation, cell graft composition, KIR ligand mismatch status, donor KIR haplotype, KIR B score, and kinetics of immune reconstitution. End points were calculated at the time of last contact, loss of follow-up or death. Statistical analyses of data were performed using statistical package SPSS for Macintosh (software version 20.0; IBM Corporation, Armonk, NY, USA) and the R software package for Macintosh (version R 3.3.3).

RESULTS

Engraftment Kinetics, Supportive Care and Toxicity Profile

Four patients developed primary graft failure. All of them were rescued using a second allogeneic transplant. The median time to neutrophil recovery for engrafted patients was 14 days (range; 9–25). The median time to platelet engraftment was 10 days (range; 7–30). Data on engraftment kinetics are provided in **Table 2**. All patients achieved full donor chimerism at time of engraftment. Patients required platelet transfusions for a median time of 2 days (range; 0–54) and the median duration of the red blood cell transfusions was 2 days (range; 0–20). Data on supportive care are also provided in **Table 2**.

The toxicity profile is shown in **Table 3**. Seventeen patients (27%) developed engraftment syndrome (ES). Seven patients (11%) developed veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) of liver.

This complication was significantly associated to ethnicity. Incidence of VOD/SOS was 4% in Caucasians whereas in non-Caucasians rose to 29% (OR 9.17, range 1.58–52.6; $p = 0.005$). The median hospitalization time after hematopoietic cell infusion was 15 days (range; 10–85).

Immune Reconstitution

The immune reconstitution was characterized in all patients with engraftment by a rapid increase in NK cells. The NK cell counts peaked on day +30 [median of 250/μL (range; 1–2,046)]. The median number of $CD56^{dim}$ NK cells on day +30 was 210/μL (3–2,040) whereas the median number of $CD56^{bright}$ NK cells on day +30 was 40/μL (10–350). The median $CD56^{dim}/CD56^{bright}$ NK cell ratio was 3.5 on day +30 (range; 1–45). After day 30, the NK cell population slowly decreased, being a median of 185 (8–1,381) cells/μL on day +180. Ultimately, the NK cell count remained steady at ∼160–180 cells/μL.

T-cell recovery was delayed with a median number of 155 $CD3^+$ cells/μL (range, 11–1,694) on day +30, 185 cells/μL (range, 3–4,740) on day +60, 293 cells/μL (range, 5–6,250) on day +90, and 490 cells/μL (range, 18–3,250) on day +180.

There was a relationship between the infused $CD34^+$ cell dose and T-cell recovery ($r = 0.540$; $p = 0.03$). The reconstitution of B lymphocytes was also delayed, starting with a median of 2 cells/μL (range, 0–414) on day +30 and progressively increasing until reaching 90 (range, 2–4,927) on day +90, and 240 (range, 2–9,99) on day +180. We observed a gradual increase in the DC population over time, with a median of 20 cells/μL (range, 2–760) on day +30 and 34 (range, 2–270) on day +180. Detailed data of lymphoid subpopulations recovery and kinetics are shown in **Supplemental Table S2** and **Supplemental Figure S1**.

Acute and Chronic GvHD

Thirty-five patients developed aGvHD (grade 1, $n = 10$; grade 2, $n = 7$; grade 3, $n = 10$; and grade 4, $n = 8$). The probability

TABLE 4 | Causes of death.

Cause	*N* = 22 (%)
Relapse	11 (50%)
Infections	5 (23%)
Severe VOD/SOS	2 (9%)
TA-TMA	2 (9%)
Refractory GvHD	2 (9%)

of aGvHD (grade 2 or higher) was 34 ± 7%. The median time to aGvHD was 45 days (range, 10–90 days). Fourteen patients developed cGvHD (mild in six cases, moderate in six cases, and severe in six cases), with a probability of 25 ± 6%. The median time to cGvHD was 110 days (range, 76–306 days). We found no variables associated to develop neither acute nor chronic GvHD.

Non-relapse Mortality

With a median follow-up of 28 months (range; 4–72) the cumulative incidence of NRM was 23 ± 5%. Seven patients died by day +100 and 5 at a later time with a median time to death of 86 days (range, 27–216). Univariate analysis revealed that NRM was influenced by ethnicity (white caucasians 14 ± 5% vs. non-caucasians 58 ± 15%; $p < 0.001$); presence of SOS (yes 85 ± 10% vs. no 14 ± 5%; $p = 0.0003$); development of severe aGvHD (yes 66 ± 12% vs. no 5 ± 3%; $p = 0.0002$); and $CD56^{dim}/CD56^{bright}$ NK cell ratio on day +30 (low 37 ± 9% vs. high 7 ± 5%). However, multivariate analysis showed that presence of SOS was the only variable associated with NRM (HR: 95% CI; 6.6; 1.1–40.24 $p = 0.04$). The primary causes of death are shown in **Table 4**.

Subset Analysis of Infection Complications Infection Complications

In all, 72 infectious episodes were diagnosed using microbiological and/or clinical criteria (**Table 5**). All patients experienced at least one infection, with a median of 1 events/patients (1–3) and a median time onset to first infection episode of 13 days (6–45). Median time to first infection was 13 days for bacterial, 25 days for viral, and 20 days for fungal infections. Early immune reconstitution (on day +30) was associated with severe infection defined as sepsis, viral disease, and/or invasive fungal infection. So, patients who developed severe infection had lower counts of NK cells and γδ T-cells (median 122, range; 1–332; and 11, range; 1–77, respectively) compared to those patients who did not develop severe infection episode (median 230, range; 1–1,962; $p = 0.018$ and 34, range; 1–573; $p = 0.02$). However, on day +60, severe infection was associated to low counts of $CD8^+$ T-cells (median 23, range 0–51) compared to patients with no severe infection (median 56, range 0–759) ($p = 0.009$).

Detailed data regarding bacterial, viral, and fungal infections are provided in **Table 5**.

Relapse Incidence

With a median follow-up for survivors of 28 months (range; 4–72 months), 15 patients relapsed, with a median time to relapse of 130 days (range; 37–265 days), and a cumulative incidence of 27 ± 6%. The following variables influenced the probability of relapse in the univariate analysis: disease phase at transplantation (early phase, 5 ± 5%; vs. advanced phase, 37 ± 8%; $p = 0.01$), high $CD56^{dim}/CD56^{bright}$ NK cell ratio on day +30 (≥3.5; 15 ± 7% vs. <3.5; 37 ± 9%; $p = 0.04$) and type of acute leukemia (myeloid 11 ± 6% vs. lymphoid 42 ± 9%; $p = 0.006$). Multivariate analysis (MVA) showed that relapse was influenced by type of malignancy and $CD56^{dim}/CD56^{bright}$ NK cell ratio on day +30 after transplantation **Table 6**.

TABLE 5 | Infectious complications.

Total number of infection episodes	72
Bacterial infections	33 (46%)
Gram-negative bacilli	8 (24%)
Enterobacteriaceae species	5 (62%)
Pseudomonadaceae species	2 (25%)
Campylobacteraceae species	1 (13%)
Gram-positive bacilli	8 (24%)
Clostridium species	8 (100%)
Gram-positive cocci	17 (51%)
Staphylococcus species	14 (82%)
Streptococcus species	2 (12%)
Enterococcus species	1 (6%)
Viral infections	32 (44%)
CMV	5 (16%)
Adenovirus	4 (12%)
HHV-6	6 (19%)
VZV	3 (9%)
Rinovirus	3 (9%)
Coronavirus	2 (6%)
SRV	3 (9%)
Other viruses	6 (19%)
Fungal infections	7 (10%)
Candida species	4 (57%)
Aspergillus species	3 (43%)

Disease-Free Survival (DFS)

The probability of DFS was 52 ± 6% for the whole group. The following variables influenced DFS in the univariate analysis: Ethnicity (caucasians 59 ± 7% vs. non-caucasians 15 ± 11%, $p = 0.006$), disease phase at transplantation; early vs. intermediate vs. advanced (81 ± 9% vs. 41 ± 11% vs. 29 ± 11%, respectively; $p < 0.001$), MRD status (negative 68 ± 8% vs. positive 32 ± 11%; $p = 0.03$), type of malignancy; myeloid vs. lymphoid (65 ± 9% vs. 36 ± 9%, respectively; $p = 0.035$), presence of VOD/SOS (no 56 ± 7% vs. yes 14 ± 12%; $p = 0.004$), severe aGvHD (no 62 ± 8% vs. yes 28 ± 11%; $p = 0.03$), chronic GvHD (no 41 ± 8% vs. yes 79 ± 11%; $p = 0.015$), and $CD56^{dim}/CD56^{bright}$ NK cell ratio on day +30 (≥3.5; 77 ± 8% vs. <3.5; 28 ± 5%; $p = 0.001$). Variables associated to DFS in MVA are given in **Table 7**. DFS curves according to MVA are shown in **Figure 1**.

TABLE 6 | Multivariate analysis of relapse.

Factors	HR (95% CI)	*p*-value
Type of malignancy		
Myeloid	Reference	
Lymphoid	8.8 (2.39–32.45)	0.001
$CD56^{dim/bright}$ NK cell ratio		
High	Reference	
Low	3.75 (1.17–12.03)	0.02

TABLE 7 | Multivariate analysis of DFS.

Factors	HR (95% CI)	*p*-value
Type of malignancy		
Lymphoid	Reference	
Myeloid	32.37 (4.25–121.07)	0.0001
EMR status pre-alloHCT		
Positive	Reference	
Negative	5.03 (1.20–21.03)	0.025
Chronic GvHD		
No	Reference	
Yes	20.73 (3.16–136.19)	0.002
$CD56^{dim/bright}$ NK cell ratio		
Low	Reference	
High	6.02 (1.62–22.44)	0.007

DISCUSSION

Allogeneic hematopoietic stem cell transplantation from haploidentical donors is a suitable transplant option for patients lacking a HLA-identical donor. Nowadays, several haploidentical transplant platforms have gained clinical relevance using "*ex-vivo*" T-cell depletion (TCD) (19). In TCD haploidentical setting, different techniques of graft manipulation have been developed over last 15 years to avoid or minimize undesirable GvHD (19–21). These techniques have evolved from the initial almost total T and B cells depletion by means of $CD34^+$ isolation and $CD3^+/CD19^+$ depletion to more recently graft manipulation techniques such as $\alpha\beta^+$ TCR/$CD19^+$ or $CD45RA^+$ depletions that resulted in partial TCD maintaining NK cells in an attempt to improve immune reconstitution following transplantation (22, 23). An approach to circumvent delayed immune recovery represented by a more sophisticated method of graft manipulation that has been recently developed and implemented is based on selective depletion of $\alpha\beta^+$ T-cells and B-cells (10). This strategy allows not only the infusion of donor hematopoietic stem cells, but also committed hematopoietic progenitors, mature NK cells, and $\gamma\delta^+$ T-cells. Recently, haploidentical transplant after $\alpha\beta^+$ T-cell/B-cell depletion has shown to be effective in single-center studies (10). In these studies, myeloablative total body irradiation (TBI) conditioning regimens combined with serotherapy have been used. Other reported studies used chemotherapy-based conditioning regimens even with no serotherapy as part of conditioning (7–9, 11). Children with acute leukemia receiving haploidentical transplant using $\alpha\beta^+$ T-cell/B-cell depletion had a relatively lower risk for acute and chronic GVHD even than those transplanted from an unrelated donor. However, major challenges were relapsing disease and delayed immune reconstitution leading infectious morbidity and mortality (11). So, immune reconstitution studies and risk factors for transplant outcome analysis using this TCD haploidentical platform are needed. Herein, we reported the risk factors analysis and a detailed analysis of immune reconstitution and its impact on transplant outcomes in a prospective study using haploidentical transplant with $\alpha\beta^+$ T-cell/B-cell depletion after chemotherapy-based conditioning regimen with no serotherapy.

The main findings of present study are commented below.

First, engraftment kinetics were fast, probably due to the large number of infused progenitors (**Tables 1**, **2**), as has been recently published (8, 10, 11, 24). As other authors (10, 20, 21) have noted, grafts so depleted, contain high numbers of graft-facilitating cells and $CD34^+$ cells, and result on enhanced engraftment and immune recovery. Graft failure was lower than reported (8) with other previous graft manipulation procedures using the same conditioning regimen and very similar to other published studies using TBI-based conditioning with or without serotherapy as part of preparatory regimen. Non-radiation-based preparative regimens have be used in pediatric patients with high-risk acute leukemia and busulfan-based myeloablative conditioning regimens have been reported safe and effective in hematopoietic transplantation (11). An important observation in this study was the fact that the conditioning regimen was well-tolerated (**Table 3**), with a low incidence of severe complications, similar to that of recently published in adults (25) and children (7–9). However, we have observed a high incidence of severe VOD/SOS in non-Caucasian population not previously observed and reported. This complication was associated with high mortality in the mentioned population. Non-Caucasian (mainly white Hispanic) patients are being increasingly included in the transplant program at our institution. It is possible that there is an interethnic variability in genetic polymorphisms frequencies for metabolizing enzymes that probably influence toxicity incidence such as VOD/SOS. Based in our findings, ethnicity should be considered as potential risk factor for further prospective VOD/SOS studies.

The incidence of ES is remarkable (**Table 3**). ES is an inflammatory condition that occurs after HSCT and it is characterized by a non-infectious fever and skin rash. We have previously reported (8) this complication in pediatric haploidentical transplantation setting, using other TCD platform. ES has been considered as a herald of GvHD and both complications might be related with not using serotherapy as part of preparatory regimen. However, this complication had no impact on clinical outcomes in our series (8).

Second, immune reconstitution was preceded by a rapid and sustained appearance of NK-cells (**Supplemental Table 2**). Immune reconstitution after allogeneic transplantation depends on multiple factors such as stem cell source and dose (26), graft manipulation procedure (6–9), donor age (13), conditioning regimen, and post-transplant immunosuppression

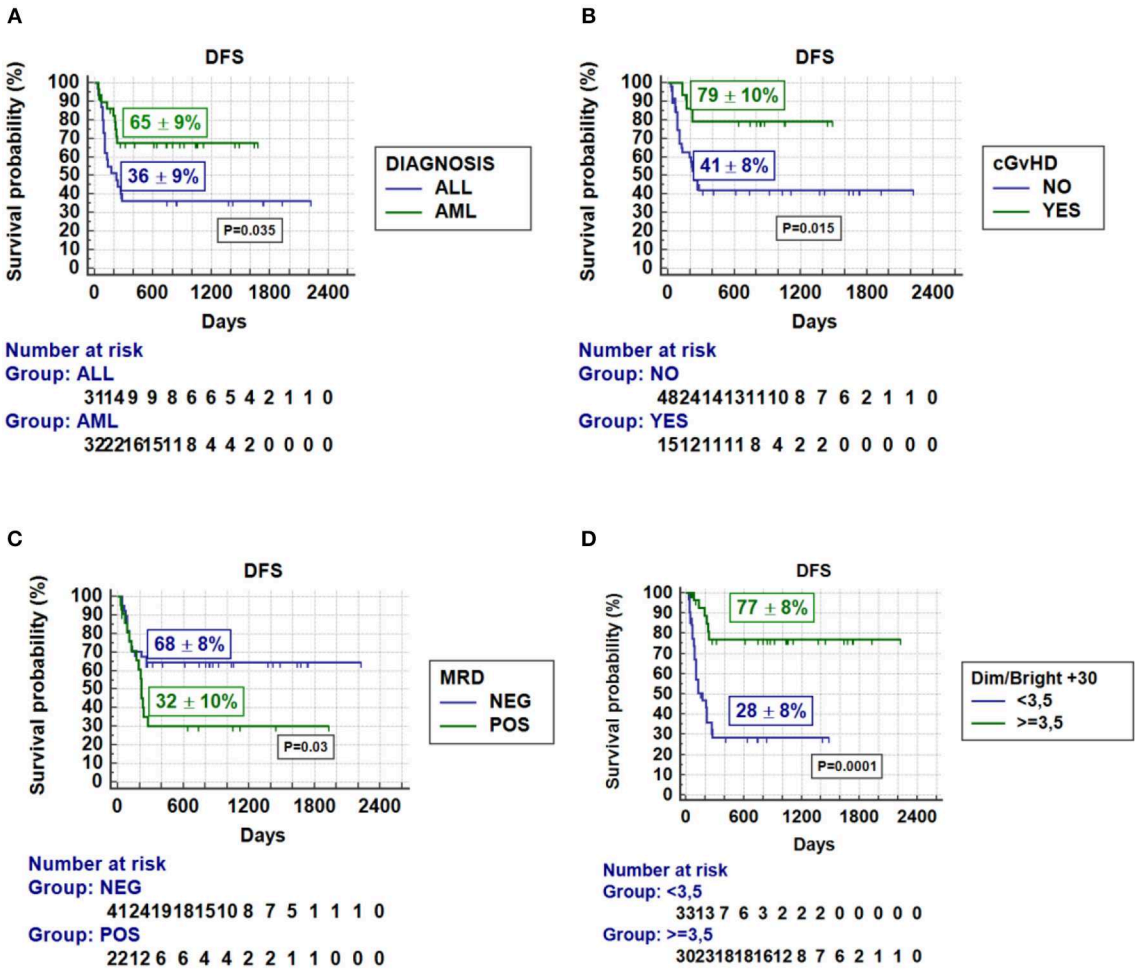

FIGURE 1 | Probability of disease-free survival according to multivariate analysis. **(A)** diagnosis; **(B)** chronic GVHD; **(C)** minimal residual disease status; **(D)** $NK^{dim/bright}$ ratio on day +30.

used. We (7, 8) and others (6, 24, 26) previously reported that immune reconstitution in children receiving TCD haploidentical transplant was characterized by early and fast NK-cell recovery constituting major lymphocyte subpopulation in the early phase following transplantation. NK-cells in the graft and fast NK-cell recovery have been considered of importance in allogeneic transplant especially on haploidentical transplant. After conditioning, blood high level of interleukin-15 provides a favorable environment not only for mature donor NK-cell infused but also for NK-cells maturing from engrafted HSCs (27). NK-cell reconstitution and its function following haploidentical transplant is clearly influenced by GvHD prophylaxis, especially by post-cyclophosphamide that eliminate most mature donor NK cells including alloreactive NK cells as it has been recently published (27).

We previously observed that the higher NK-cells numbers early post-transplant the lower incidence of relapse (8) resulted in better outcome using $CD3^+/CD19^+$ depleted grafts. We now analyzed the potential impact of $CD56^{dim}$ and $CD56^{bright}$ NK cells subpopulation on relapse incidence and we observed that the higher $CD56^{dim}/CD56^{bright}$ NK cell ratio, the lower relapse incidence, resulting in better DFS.

A relevant role of NK cells infused in the graft in TCD allogeneic transplant setting has been previously published (28). The number of NK cells in the graft was a powerful determinant of relapse risk. Analysis of NK cell subset clearly showed that this GvL effect was mediated by $CD56^{dim}$ population and high-level expression of activatory receptor DNAM on $CD56^{dim}$ population cells was also strongly protective (28). However, a similar analysis has not been published in the TCD haploidentical setting. A recent study in pediatric patients, receiving $\alpha\beta^+$ T-cell depletion haploidentical transplant, showed an overshooting NK cell recovery on day +14 comparable to their donor level (29). Despite a rapid expansion of $CD56^{bright}$ NK cell subset, $CD56^{dim}$ NK cells were predominant during the study time period (29). However, they did not report any correlation between $CD56^{dim}$ or $CD56^{bright}$ recovery and relapse incidence. Our previous data indicate that the number of total NK cells early after

transplantation was a strong determinant of GvL effect (8). More specifically, $CD56^{dim}/CD56^{bright}$ NK cell ratio was predictive of disease relapse and had a relevant impact on DFS.

Usually, $CD56^{dim}$ NK cell constitute around 90% of peripheral blood NK cells in healthy donors. NK cells have a powerful activity against virus transformed cells and cancers cells specially AML cells (30, 31). This activity against cancer cells is mainly supported by $CD56^{dim}$ cytotoxic activity (32–34). $CD56^{bright}$ population has a different cytokine profile resulting in lower cytotoxicity against cancer cells. So, the higher $CD56^{dim}/CD56^{bright}$ NK cell ratio might result in more cytotoxicity exerted by $CD56^{dim}$ subset and in lower risk of relapse. Unfortunately, we do not have data on cytotoxicity in our series to support this explanation. Alternatively, NK cells could act as a boost for T-cell activation that might increase the T-cell based GvL effect. However, our data clearly shows that T-cell recovery is delayed for a longer period of time following transplant especially $\alpha\beta^+$ T-cells.

IL-15 is a cytokine necessary not only for NK-cell homeostasis and development but also for $\gamma\delta^+$ T-cells. As during early phase following transplantation there is high levels of IL-15 in recipient, this provides a favorable environment for NK cell and γδ T-cell proliferation, maturation and function. Early reconstitution of NK and $\gamma\delta^+$ T-cells comparable to their healthy donors, has been observed after allogeneic match sibling and match unrelated donors (35). In fact, we observed an association between early immune reconstitution and risk of severe infection. However, and despite rapid innate immune reconstitution there was a relevant incidence of viral reactivation as previously reported (8, 10, 11, 24). After the early post-transplant phase, $CD8^+$ T-cells leaded response to infections.

T-cell recovery was delayed compared to that of NK cells, especially that of $\alpha\beta^+$ T-cells. However, we observed even early after transplantation that $\alpha\beta^+$ T-cells were the predominant T-cell population despite low numbers of such T-cells infused. The slow T-cell reconstitution was clearly related to the risk of opportunistic and largely viral infections. Furthermore, infections were the leading cause of NRM and the second leading cause of death after relapsing disease (**Table 4**).

Third, we consider our results in DFS for the whole group as acceptable taking into account the stages of disease of the study population. As expected, it was particularly good for the patients transplant in first CR. A more important result is that a relevant proportion of patients who underwent transplantation in more advanced phase of disease or even with active disease were rescued by haploidentical transplantation and still continue in CR.

The incidence of cGvHD was lower than observed using $CD3^+/CD19^+$ depletion (8). Although most patients developed mild or moderate cGvHD, near 10% of them required protracted treatment. However, cGvHD was associated with better DFS, primarily due to better control of disease relapse. Although the median follow-up is not long enough to consider the results as mature, we believe they are encouraging and support the idea of using haploidentical donors as early as possible whenever a patient needs an HSCT.

Several conclusions can be drawn. First, haploidentical transplantation using $\alpha\beta^+$ TCR/$CD19^+$ depletion yields encouraging results, especially in patients undergoing transplantation in early phase of disease and in complete remission. Second, the conditioning regimen used is well-tolerated. Third, ethnicity may predispose to development of severe liver toxicities what implies increased risk of NRM.

Fourth, viral infection/disease still continues being an unresolved problem clearly associated to delayed T-cell recovery that impacts on cause of death after transplant. A need for rebuilding T-cell reconstitution exits. Last but not least, rapid and sustained "mature" NK cell reconstitution results in better disease control and better transplant outcome that would support the use of fresh or IL-15 stimulated NK cell infusion early after transplant to enhance GvL with no risk of GvHD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MD, JZ, BM, and MG-V designed the study, analyzed the data, and wrote the manuscript. JR, JV, JS, MR, LA, AC, EG, and ES designed the study, provide and revised data, and data analysis. All authors approved final manuscript version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02504/full#supplementary-material

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REVIEW
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CAR-Engineered NK Cells for the Treatment of Glioblastoma: Turning Innate Effectors Into Precision Tools for Cancer Immunotherapy

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Glioblastoma (GB) is the most common and aggressive primary brain tumor in adults and currently incurable. Despite multimodal treatment regimens, median survival in unselected patient cohorts is <1 year, and recurrence remains almost inevitable. Escape from immune surveillance is thought to contribute to the development and progression of GB. While GB tumors are frequently infiltrated by natural killer (NK) cells, these are actively suppressed by the GB cells and the GB tumor microenvironment. Nevertheless, *ex vivo* activation with cytokines can restore cytolytic activity of NK cells against GB, indicating that NK cells have potential for adoptive immunotherapy of GB if potent cytotoxicity can be maintained *in vivo*. NK cells contribute to cancer immune surveillance not only by their direct natural cytotoxicity which is triggered rapidly upon stimulation through germline-encoded cell surface receptors, but also by modulating T-cell mediated antitumor immune responses through maintaining the quality of dendritic cells and enhancing the presentation of tumor antigens. Furthermore, similar to T cells, specific recognition and elimination of cancer cells by NK cells can be markedly enhanced through expression of chimeric antigen receptors (CARs), which provides an opportunity to generate NK-cell therapeutics of defined specificity for cancer immunotherapy. Here, we discuss effects of the GB tumor microenvironment on NK-cell functionality, summarize early treatment attempts with *ex vivo* activated NK cells, and describe relevant CAR target antigens validated with CAR-T cells. We then outline preclinical approaches that employ CAR-NK cells for GB immunotherapy, and give an overview on the ongoing clinical development of ErbB2 (HER2)-specific CAR-NK cells currently applied in a phase I clinical trial in glioblastoma patients.

Keywords: natural killer cells, NK-92, chimeric antigen receptor, adoptive cancer immunotherapy, glioblastoma

INTRODUCTION

Glioblastoma (GB) is the most frequent malignant primary brain tumor in adults, without any curative treatment options available at present. Patients diagnosed with GB have a dismal prognosis, and typically succumb to the disease within 3 months if untreated. Present standard of care for most patients includes surgical resection followed by radio- and chemotherapy. Despite this aggressive treatment, median survival of glioblastoma patients is only about 15 months, and recurrence remains almost inevitable (1, 2). Still <5% of patients diagnosed with GB survive more than 5 years (3). In contrast to other cancers such as adenocarcinomas of the lung or melanoma, primary brain tumors like GB and low grade gliomas (LGG) are known as rather immunologically "cold" tumors, typically with low numbers of tumor-infiltrating lymphocytes (TILs) (4), and the mere amount of TILs is not associated with patient survival (5). Nevertheless, the composition of the immunologic tumor microenvironment undergoes changes upon radiotherapy, chemotherapy, or even after anti-angiogenic therapy (6). Hence, intensifying efforts to overcome the general resistance of glioblastoma to immunotherapy appears highly warranted (7–9).

Recent clinical trials with chimeric antigen receptor (CAR)-engineered T cells demonstrated the feasibility and safety of this approach for the treatment of recurrent glioblastoma, with signs of clinical activity and transient responses observed in some of the patients (10–12). Efforts are underway to further refine these strategies, which includes evaluation of NK cells as alternative CAR-engineered effectors. The critical role of NK cells in cancer immune surveillance is increasingly being recognized (13, 14). NK cells do not only contribute to antitumor immunity by directly eliminating malignant cells, but also by regulating tumor-specific adaptive immune responses through crosstalk with dendritic cells (DCs) (15). NK cells thereby regulate DC maturation, and so determine the effectiveness of subsequent DC-mediated T-cell activation (16). Conversely, DCs enhance the direct antitumor activity of NK cells (17), which is also relevant for glioblastoma as shown in a recent phase II clinical trial with a dendritic cell vaccine (18). However, in cancer patients NK cells are often functionally compromised due to the immunosuppressive activity of the tumor. NK cells do not carry a T-cell receptor restricted to a particular peptide epitope presented by major histocompatibility complex (MHC) molecules, but recognize stress ligands on cancer cells via germ-line encoded activating receptors, which are counter-balanced by inhibitory receptors that are triggered by self-MHC class I. Hence, for adoptive cancer immunotherapy HLA-mismatched NK cells from healthy donors are preferred, which do not recognize tumor cells as "self," thereby bypassing inhibitory signals. Since NK cells do not carry a high risk of inducing graft-vs.-host-disease (GvHD), this approach is generally considered to be safe (19–21). The better understanding of NK-cell biology, together with the development of strategies to enhance NK-cell activity by blocking inhibitory receptor pathways or redirect NK cells to tumors using bispecific antibodies or genetic modification with CARs, have paved the way for many therapeutic approaches that are now actively pursued in a clinical setting. These extend from the treatment of different types of leukemia to various solid tumors (22–24). Such refined approaches also hold enormous potential to improve immunotherapy of glioblastoma (25), assigning to NK cells a dual role as targeted killers and modulators of innate and adaptive immunity in the GB tumor microenvironment.

NK CELLS IN THE TUMOR MICROENVIRONMENT OF GLIOBLASTOMA

Studies that evaluated infiltration of malignant gliomas by NK cells have reported different findings, ranging from the presence of insignificant NK cell numbers to large NK cell infiltrates in up to 89% of glioblastomas (26, 27). Another study described less infiltration of high-risk gliomas with NK cells and M1-like macrophages than lower grade gliomas (28). Therefore, differences in the subtype and stage of the disease, treatment history and permeability of the blood-brain barrier (BBB) appear to affect the likeliness of infiltration by NK cells. The presence of NK cells and their repertoire of activating killer cell immunoglobulin-like receptors (KIRs) can have an impact on disease progression. This was shown by Dominguez-Valentin et al. who analyzed 108 glioblastoma patients and 454 healthy individuals for HLA-A, -B, -C, NK-cell KIRs, and CMV-specific antibodies, and correlated the results with clinical parameters. The KIR allele KIR2DS4*00101 was thereby identified as an independent prognostic parameter of prolonged survival (29). Notably, all patients carrying KIR2DS4*00101 were CMV seropositive, and showed an increase in NK-cell subpopulations that expressed the cytotoxicity receptors CD16, NKG2D, and CD94/NKG2C. Healthy controls had a reduced risk to develop glioblastoma if they harbored two KIR2DS4*00101 alleles. Likewise, Gras Navarro et al. identified a subpopulation in donor-derived NK cells expressing KIR2DS2, which had a functional advantage in killing GB cells (30). Compared to KIR2DS2-negative NK cells, KIR2DS2-positive NK cells showed higher cytotoxicity, at least in part also mediated through expression of NKG2D ligands by the tumor cells. NKG2D-mediated control of glioblastoma by NK cells has also been demonstrated in experimental GB models (31).

Escape from immune surveillance is thought to contribute to the development and progression of GB (32, 33). GB tumors which are infiltrated by NK cells actively suppress NK-cell function via expression of factors such as transforming growth factor (TGF)-β, which is a main contributor to the immunosuppressive GB microenvironment (34, 35). TGF-β impairs NK cells by downregulating activating NK receptors such as NKG2D and NKp30 (36), or by repressing the mTOR pathway (37). This may be circumvented by making NK cells resistant to TGF-β, as recently achieved by expression of a dominant-negative TGF-β receptor in cord blood NK cells (38). Nevertheless, most GB cells also express high levels of MHC class I molecules, which inhibit autologous NK cells via inhibitory KIRs. Thus, blockade of such KIRs may favor a less tumor-promoting microenvironment and enhanced NK-cell mediated killing (25). GB cells can also affect NK-cell activity through expression of inhibitory molecules like regeneration

and tolerance factor (RTF) and lectin-like transcript (LLT)-1 (33, 39), or indirectly through myeloid cells (40). Resident microglial cells in the brain are induced by glioma-derived TGF-β to acquire an immunosuppressive phenotype (41). Although the concept of M1 and M2 polarization of microglia in GB has been challenged as both subtypes are present in GB and prognostic relevance remains under debate (42), it is generally accepted that glioma-associated macrophages can reinforce an immunosuppressive tumor microenvironment (43, 44). Nevertheless, while actively suppressed in glioblastoma tumors, even patient-derived autologous NK cells can recover their cytolytic activity upon *ex vivo* culture with IL-2 or IL-15, in particular directed to GB stem-like cells (45, 46).

CLINICAL UTILITY OF NK CELLS FOR THE TREATMENT OF GLIOBLASTOMA

Combinations of donor-derived NK cells with an antibody recognizing a glioblastoma-restricted surface antigen or a histone deacetylase inhibitor (HDACi) that induced upregulation of NKG2D ligand expression by GB cells have been shown in preclinical models to overcome immunosuppressive effects of brain tumors (47, 48). Also pre-treatment with the proteasome inhibitor bortezomib sensitized GB cells toward NKG2D- or TRAIL-mediated NK-cell lysis and enhanced survival in animal models (49). Available clinical data, however, are so far still restricted to earlier approaches based on *ex vivo* activated autologous immune cells. Ishikawa et al. performed a phase I clinical trial with autologous NK cells combined with systemic low-dose interferon (IFN)-β in nine patients with recurrent malignant glioma, including three patients with glioblastoma (50). NK cells were expanded from peripheral blood mononuclear cells (PBMCs) using irradiated feeder cells and IL-2. Repeated doses of NK cells were either only applied intravenously, or both, intravenously and directly into the tumor cavity through an Ommaya reservoir. The NK cell therapy proved to be safe and partially effective, with two patients experiencing a partial response (PR), two patients a mixed response (MR) and three patients stable disease (SD) during different courses of treatment (50).

The majority of early studies evaluating adoptive cell therapy, however, were performed with autologous lymphokine-activated killer (LAK) cells in combination with IL-2 injected into the resection cavity of recurrent or progressive malignant gliomas, with the cells usually applied through a reservoir. LAK cells are a mixture of T and NK cells derived by *ex vivo* culture of peripheral blood lymphocytes in IL-2-containing medium (51). Thereby the main lytic activity of LAK cells is mediated by $CD3^{-}CD56^{+}$ NK cells, while the contribution of $CD3^{+}CD56^{-}$ T cells is rather limited (52). The studies with LAK cells in glioma patients reported disease stabilization and partial or even complete responses in some of the patients, without encountering dose-limiting toxicities (53–64). Despite activation with IL-2, LAK cells can still be inhibited by immunosuppressive molecules secreted or presented by GB cells (65), which may explain why, despite the observed clinical activity in some cases, responses after therapy with autologous LAK cells were not durable. These reports are nevertheless encouraging and important for ongoing and future studies with enhanced NK-cell products such as CAR-NK cells, since they document feasibility and overall safety and tolerability of repeated intracavitary or intralesional injection of large numbers of lymphocytes together with IL-2, in some cases reaching up to 10^{10} cells per dose (55).

THE CONCEPT OF CHIMERIC ANTIGEN RECEPTORS

Chimeric antigen receptors were initially developed as a means for T cells to bypass MHC restriction of the T-cell receptor (TCR) and instead acquire TCR-independent, predetermined specificity for a defined cell surface antigen expressed by the target cell of interest (66–68). Since the first description of the basic CAR design encompassing a single chain fragment variable (scFv) antibody for target recognition linked to CD3ζ or FcεRIγ chains for signaling (first-generation CARs) (66), this approach has continuously been refined to enhance effector cell activity, and improve engraftment and persistence in the host upon adoptive transfer. Accordingly, receptors currently employed for CAR-T cell products approved for the treatment of malignancies of B-cell origin or undergoing clinical testing for various hematologic or solid tumor indications include in addition to CD3ζ one or more costimulatory protein domains, typically derived from CD28 and CD137 (4-1BB) (referred to as second- or third-generation CARs) (69, 70).

Also NK cells can be genetically engineered to express chimeric antigen receptors, thereby acquiring in addition to their natural cytotoxicity built-in ADCC-like activity, similar to FcγRIIIa (CD16) activation by target-specific IgG molecules (**Figure 1**). This was first demonstrated for a CAR-like CD4-CD3ζ fusion receptor in human NK3.3 cells (72), and a scFv-CD3ζ-based first-generation CAR in NK-92 cells (73). Subsequently, peripheral blood NK cells from healthy donors, cord blood derived NK cells, and NK cells generated from induced pluripotent stem cells (iPSCs) have successfully been used for the generation of CAR-NK cells in preclinical studies (74–76). While conventional T-cell CARs with CD3ζ and CD28 and/or CD137 domains are functional in NK cells (74–80), several groups have reported improved activity if one or more signaling domains derived from CD244 (2B4), NKG2D, DAP10 or DAP12 were included in the receptors (75, 81, 82). It is presently unknown whether a particular CAR design would thereby be preferable for all CAR-NK cells irrespective of the nature of the cell binding domain, the target antigen, and the source and differentiation state of the effector cells. So far no CAR-NK cell product has received marketing authorization, but several early phase clinical trials in different cancer indications are ongoing, mainly based on genetically engineered NK-92 and cord blood NK cells (15, 23, 83).

GLIOBLASTOMA ANTIGENS TARGETED IN CLINICAL TRIALS WITH CAR-T CELLS

Most studies on CAR-engineered effector cells in glioblastoma have so far focused on autologous CAR-T cells. These approaches

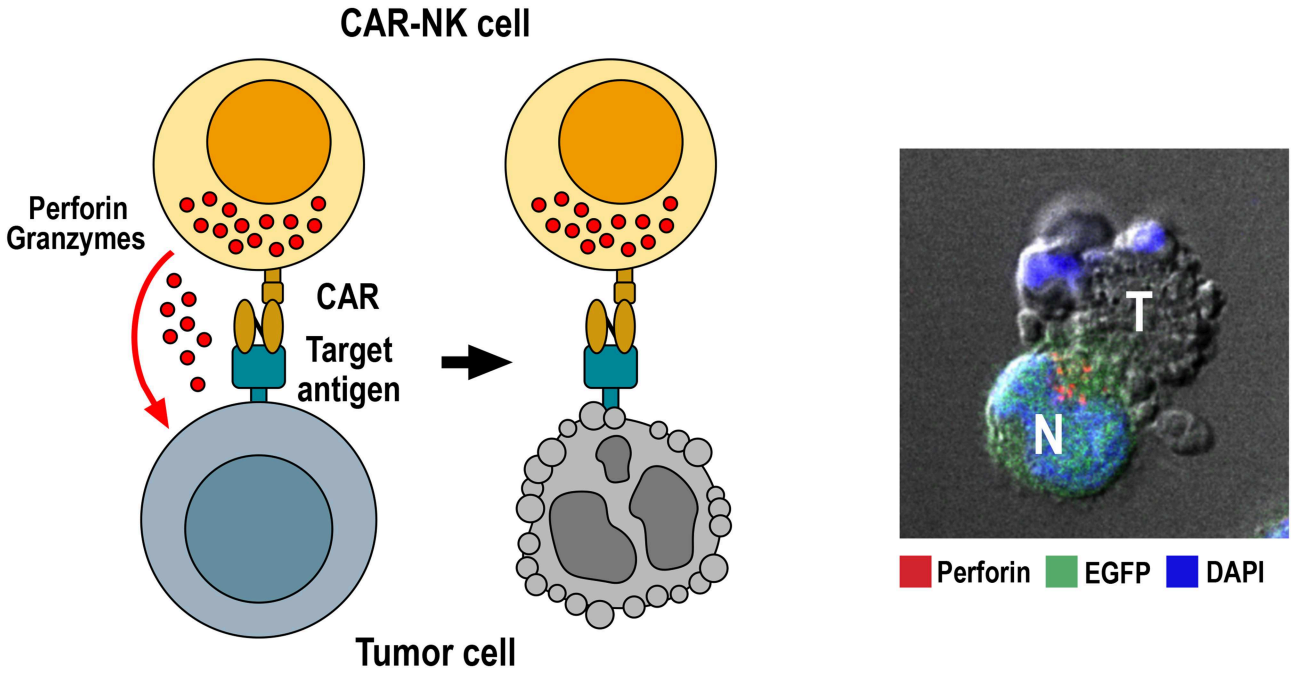

FIGURE 1 | Killing of tumor cells by CAR-NK cells. Specific binding of the chimeric antigen receptor to its target antigen on the tumor-cell surface triggers CAR activation. This results in re-orientation of cytotoxic granules toward the immunological synapse formed between NK and target cell, followed by release of perforin and granzymes from cytotoxic granules into the synaptic cleft. Perforin and granzymes taken up by the target cell then trigger apoptotic cell death indicated by membrane blebbing and disintegration of the nucleus. The confocal microscopy image to the right shows conjugate formation between human GB cells co-expressing EGFR and EGFRvIII with NK-92/225.28.z CAR-NK cells that recognize both target antigens (71). Tumor (T) and EGFP-positive CAR-NK cells (N, green) were co-incubated for 1 h, fixed, permeabilized and stained for perforin (red) to identify cytotoxic granules. Cell nuclei were labeled with DAPI (blue).

have been reviewed extensively in recent years (84–88). Nevertheless, due to their relevance for NK-cell based therapies, some of their key findings are summarized below, concentrating in particular on tumor-associated antigens that have already been targeted in clinical trials.

IL-13Rα2

Interleukin-13 receptor α 2 (IL-13Rα2) has first been described as a therapeutic target for GB two decades ago (89). It has been found overexpressed in >50% of high-grade gliomas, but is undetectable in normal brain tissue. IL-13Rα2 expression alone can induce invasiveness of GB cells without affecting cell growth, but together with the epidermal growth factor receptor variant EGFRvIII promotes GB cell proliferation (90). Initial CAR-T cell approaches targeting IL-13Rα2 employed IL-13 muteins for cell recognition in first-generation CARs (termed zetakines) to reduce binding to IL-13Rα1, which is not restricted to GB but broadly expressed in other tissues (91, 92). Later work demonstrated improved activity of such CAR-T cells *in vitro* and in orthotopic GB xenograft models if costimulatory protein domains were included in the CARs (93–96). To achieve exclusive binding to the target antigen, also scFv-based IL-13Rα2-specific CARs were developed, which eliminated remaining cross-reactivity to IL-13Rα1 (97–100). Safety and feasibility of local treatment of glioblastoma patients by repeated application of up to 10^8 $CD8^+$ T cells expressing a first-generation IL-13 zetakine into the resection cavity through a catheter and reservoir was demonstrated in a pilot trial at the City of Hope National Medical Center with three subjects, with signs of transient clinical activity observed in two patients. For one patient, tumor material before and after therapy was available, which indicated reduced IL-13Rα2 expression in the tumor after treatment (101). More recently, the same group reported on a patient with recurrent multifocal glioblastoma from an ongoing phase I clinical trial with autologous CAR-T cells carrying an improved second-generation CAR with an IL-13 mutein and CD137 and CD3ζ signaling domains (NCT02208362, clinicaltrials.gov) (10). The patient was treated with repeated infusions of up to 10^7 CAR-T cells per dose into the resection cavity and the ventricular system. This resulted in the regression of intracranial and spinal tumors, with the clinical response continuing for 7.5 months after initiation of treatment. No dose-limiting toxicities were observed.

EGFRVIII

Epidermal growth factor receptor (EGFR) is a well-established therapeutic target in glioblastoma (102). *EGFR* gene amplification and EGFR protein overexpression is present in 40 to 60% of GB tumors (103), while the receptor is not or only minimally expressed in normal brain tissue (104). Glioblastoma cells with *EGFR* gene amplification often co-express a constitutively active EGFR mutant form (EGFRvIII)

(105, 106), which drives tumorigenicity and mediates radio- and chemoresistance (107, 108). *EGFR* gene amplification and expression of EGFRvIII are both correlated with poor prognosis and shorter survival of GB patients (109). *EGFRvIII* harbors an in-frame deletion of exons 2–7 of the wildtype *EGFR* gene. This generates a tumor-specific neo-epitope at the N-terminus of the receptor which can be targeted by specific immunotherapy. Consequently, several groups investigated EGFRvIII-targeted CAR-T cells that employed second- or third-generation CARs with scFv antibody domains specifically recognizing the EGFRvIII neo-epitope. These CAR-T cells demonstrated selective cytotoxicity against EGFRvIII-positive GB cells *in vitro* and potent activity against subcutaneous or orthotopic human GB xenografts in mice upon intravenous or intratumoral injection (110–115). In a phase I clinical trial at the University of Pennsylvania with 10 patients suffering from recurrent glioblastoma, intravenous application of a single dose of up to 5×10^8 autologous EGFRvIII-specific CAR-T cells proved to be safe without evidence of off-tumor toxicity or cytokine release syndrome (12). One patient showed stable disease for over 18 months of follow-up. Transient expansion of CAR-T cells in the peripheral blood was found in all patients, and presence of CAR-T cells in GB tissue in five of seven patients where post-treatment tissue was available. While the level of *EGFR* gene amplification did not change, EGFRvIII expression had declined or was undetectable in the majority of such patients, which may have been due to the selective pressure exerted by the CAR-T cells (12). In a dose-escalation trial at the National Cancer Institute, 18 patients with recurrent EGFRvIII-positive glioblastoma were treated by intravenous infusion of up to $\geq 10^{10}$ autologous T cells carrying an EGFRvIII-specific third-generation CAR together with IL-2 after conditioning by lymphodepleting chemotherapy (116). One patient developed fatal and another patient serious respiratory symptoms shortly after cell infusion at the highest dose levels, which was attributed to congestion of pulmonary vasculature by activated T cells. No objective responses were observed, but one patient was still free from disease progression 6 months after therapy (116).

ERBB2 (HER2)

Like EGFR, ErbB2 (HER2) is a member of the family of EGFR-related receptor tyrosine kinases. Activating mutations of ErbB2 appear less common than in the case of EGFR (117), but overexpression of the receptor is frequently found in breast carcinomas and many other epithelial cancers. Due to its ability to form signaling-competent heterodimers with all other members of the EGFR family, overexpression of ErbB2 strongly contributes to malignant transformation (118). While absent in the adult central nervous system (119), ErbB2 protein expression was found in up to 80% of GB tumors and was correlated with impaired survival (120–124). In a recent study with 56 primary GB tumors, immunohistochemical analysis revealed high ErbB2 expression in 21.5%, more moderate expression in another 19.6%, and no or low expression in 58.9% of the samples. Thereby, in the majority of cases ErbB2 expression in relapsed tumors was comparable to or higher than that of primary tumors from the same patients (125). In a preclinical study Ahmed et al. evaluated patient-derived CAR-T cells transduced with a retroviral vector encoding an ErbB2-specific CAR with CD28 and CD3ζ signaling domains. These CAR-T cells exhibited remarkable antitumor activity against ErbB2-positive autologous GB cells *in vitro* including CD133-positive stem-like cells derived from primary GB tumors, and in orthotopic GB xenograft models in mice (126). In a subsequent phase I dose-escalation trial at Baylor College of Medicine, the same group treated 17 patients with progressive ErbB2-positive glioblastoma by systemic infusion of one or more doses of up to $10^8/m^2$ autologous ErbB2-specific CAR-T cells. The infusions were well-tolerated, without encountering dose-limiting toxicities. Of 16 evaluable patients, one had a partial response lasting 9 months, seven had stable disease, and eight progressed after CAR-T cell infusion. At the time of publication, three patients with stable disease were alive without evidence of progression 24 and 29 months after treatment (11).

EPHA2, CSPG4, CD133, AND CD70

Other antigens that were proposed as targets for CAR-T cell approaches in glioblastoma are EphA2, CSPG4, CD133, and CD70. The receptor tyrosine kinase erythropoietin-producing hepatocellular carcinoma A2 (EphA2) is overexpressed in GB and contributes to its malignancy (127, 128). CAR-T cells carrying EphA2-specific second- or third-generation CARs displayed potent activity against glioma-initiating cells growing as neurospheres and orthotopic GB xenografts in murine models (129, 130). Chondroitin sulfate proteoglycan 4 (CSPG4) is highly expressed in different solid tumors including glioblastoma, where CSPG4 expression is associated with more aggressive disease. Of 46 tumor samples tested in a recent study, 67% were positive for CSPG4 (131). CAR-T cells with CSPG4-specific second- or third-generation CARs, among other cancer entities, also lysed CSPG4-positive glioblastoma cells, including GB stem-like cells (131–133). The CAR-T cells also effectively controlled the growth of orthotopic GB xenografts upon intracranial injection in mice, without selection of antigen-loss tumor cell variants. Interestingly, TNF-α released by tumor-associated microglia further induced CSPG4 expression in the GB cells (131). CD133 (prominin-1) is a stem cell marker, also expressed by cancer stem cells of many tumor entities including glioblastoma (134). Despite concerns of on-target/off-tumor toxicity against hematopoietic stem cells, the feasibility of systemic application of CD133-specific CAR-T cells with manageable toxicity was recently shown in a phase I clinical trial in patients with hepatocellular carcinoma and different epithelial cancers (135). In preclinical models, CAR-T cells expressing CD133-specific third-generation CARs with CD28, CD137, and CD3ζ signaling domains displayed specific cytotoxicity against patient-derived GB stem cells *in vitro* and in orthotopic GB mouse models (136, 137). CD70 is a member of the tumor necrosis factor (TNF) family and the ligand for CD27. While CD70 expression is typically restricted to highly activated T- and B-lymphocytes and

a subset of mature DCs, certain hematologic malignancies and solid tumors, including gliomas, can constitutively overexpress CD70 (138, 139). Thereby, CD70 expression in GB cells directly facilitates immune evasion by selectively inducing $CD8^+$ T-cell death (140). T cells expressing CD70-specific first- and second-generation CARs which utilized the CD70-binding domain of CD27 for target-cell recognition specifically lysed CD70-positive primary GB cells *in vitro*, and inhibited growth or induced complete tumor regression in xenograft and syngeneic GB models in mice (141). To reduce the risk of immune escape due to inhomogeneous target antigen expression, also CAR-T cell products were developed which simultaneously target two or three GB antigens. Strategies validated in GB xenograft models include the combination of individual CAR-T cells targeting ErbB2 or IL-13Rα2, or co-expression of ErbB2- and IL-13Rα2-specific CARs, or receptors targeting ErbB2, IL-13Rα2 and EphA2 in the same T cells (142, 143). Alternatively, an ErbB2-specific scFv antibody and an IL-13 mutein were directly combined in a single bispecific tandem CAR (TanCAR) (144).

ADVANTAGES OF CAR-NK CELLS AS OFF-THE-SHELF THERAPEUTICS

Since autologous CAR-T cells are made from the cancer patients' own peripheral blood lymphocytes, yield, transduction efficiency, T-cell subtype distribution, and activation state can vary, affecting overall product composition and quality. In contrast, NK cells can safely be administered to an HLA-mismatched recipient. Hence, donor-derived peripheral blood and cord blood NK cells provide readily available resources not only for donor lymphocyte infusions of unmodified effector cells, but also for the generation of genetically engineered NK cells that may be provided as cost-effective off-the-shelf products (21, 76, 145). Furthermore, NK cells carry natural cytotoxicity receptors (NCRs) and the C-type lectin-like receptor NKG2D which are triggered rapidly by engagement of ligands selectively expressed by stressed and transformed cells (146). This natural cytotoxicity of NK cells can complement CAR-mediated cell killing, and may allow CAR-NK cells to also attack tumors with heterogeneous expression of the CAR target antigen (15). Nevertheless, because of the limited life span and expansion potential of primary NK cells, *ex vivo* culture for the generation of the required cell numbers can be demanding (147). Consequently, also human NK cell lines that continuously expand in the presence of IL-2 are being evaluated as an alternative source for the generation of well-defined clinical grade NK-cell products (148–150).

So far most of such efforts have focused on the NK-92 cell line, which was initially isolated from a non-Hodgkin lymphoma patient (151). NK-92 cells display features of activated primary NK cells and express many activating NK-cell receptors such as NKp30, NKp46, and NKG2D as well as high levels of granzymes A and B, but lack inhibitory NK-cell receptors except for KIR2DL4, Ig-like transcript 2 (ILT-2) and NKG2A/CD94 (151–155). General safety of repeated infusions of irradiated NK-92 cells at doses up to 10^{10} cells/m^2 has been established in phase I clinical trials in patients with advanced cancers, with durable responses observed in some of the treated subjects (156–159). NK-92 cells were also instrumental to demonstrate that it is feasible to generate tumor-targeted CAR-NK cells for cancer immunotherapy. Since the initial description of this concept (73), the number of preclinical studies evaluating CAR-engineered NK-92 cells has steadily increased, demonstrating markedly enhanced antitumor activity of the cells if targeted to surface molecules expressed by different hematologic malignancies and solid tumors (15, 83), including tumor-associated antigens such as EGFR, EGFRvIII and ErbB2 that are relevant for the development of immunotherapies for glioblastoma (71, 125, 160) (outlined in the following section). In a recent phase I clinical trial with CD33-specific CAR NK-92 cells for the treatment of acute myeloid leukemia (AML), no dose-limiting toxicities were encountered upon repeated intravenous infusions of up to 5×10^9 irradiated cells per dose (161), suggesting that the safety profile of these CAR NK-92 cells is similar to that of unmodified NK-92. Several other early phase clinical trials with CAR-engineered NK-92 cells are presently ongoing in Europe, China and the US (15, 83), including the CAR2BRAIN phase I clinical study which investigates a clonal ErbB2-specific CAR NK-92 product in glioblastoma patients and is described in more detail in a subsequent section (125, 162).

ACTIVITY OF EGFRVIII-SPECIFIC CAR-NK CELLS IN PRECLINICAL GLIOBLASTOMA MODELS

The therapeutic utility of CAR-engineered NK cells for the treatment of glioblastoma has so far mainly been investigated in preclinical studies with effector cells targeting EGFRvIII, EGFR or ErbB2 (**Table 1**). Müller et al. generated a CAR based on a scFv fragment of EGFRvIII-specific antibody MR1-1 (166), which was fused to an intracellular DNAX-activating protein 12 (DAP12) domain for signaling (82). DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM), and like CD3ζ which harbors three ITAMs, it transmits signals from activating NK-cell receptors (167). The CAR was expressed in the established human NK cell line YTS, with the resulting effector cells showing enhanced lysis of GB cells transfected with an EGFRvIII construct or endogenously expressing the target antigen. Intravenous injection into mice carrying subcutaneous EGFRvIII-positive GB xenografts resulted in inhibition of tumor growth and extended survival, which was further enhanced by co-expressing the chemokine receptor CXCR4 in the CAR-NK cells for improved tumor homing (82). Enhanced cytotoxicity against EGFRvIII-expressing GB cells was also found in *in vitro* cell culture assays with established human KHYG-1 NK cells expressing an EGFRvIII-specific CAR (163). In a study with CAR-engineered NK-92 cells, a second-generation chimeric antigen receptor was used that employed EGFRvIII-specific antibody MR1-1 as a cell binding domain and CD28 and CD3ζ domains for signaling (71). Upon contact formation, the respective CAR-NK cells re-oriented their cytotoxic granules toward EGFRvIII-positive but not EGFRvIII-negative GB cells, resulting in rapid and selective tumor cell killing. High and specific cytotoxicity of

TABLE 1 | Preclinical studies with CAR-NK cells in brain cancer models.

Target	Antibody	Hinge	TM	Signaling	Effector cells	Gene transfer	Cancer type	*In vivo* model	Treatment	Reference
EGFRvIII	MR1-1	Myc-tag	DAP12	DAP12	YTS	Lentivirus	GB	s.c. xenografts in NMRI nude mice	i.v. injection	(82)
EGFRvIII	MR1-1	CD8α	CD28	CD28- CD3ζ	NK-92	Lentivirus	GB	orthotopic xenografts in NSG mice	i.t. injection	(71)
EGFRvIII	3C10	CD8α	CD28	CD28-CD137-CD3ζ	KHYG-1	Lentivirus	GB	–	–	(163)
EGFR	R1	CD8α	CD28	CD28- CD3ζ	NK-92	Lentivirus	GB	orthotopic xenografts in NSG mice	i.t. injection	(71)
EGFRvIII and EGFR	528	n.s.	CD28	CD28- CD3ζ	NK-92 NKL	Lentivirus	GB	orthotopic xenografts in NSG mice	i.t. injection	(160)
EGFRvIII and EGFR	Cetuximab (225)	CD8α	CD28	CD28- CD3ζ	NK-92	Lentivirus	GB	orthotopic xenografts in NSG mice	i.t. injection	(71)
ErbB2 (HER2)	FRP5	CD8α	CD3ζ	CD3ζ	NK-92	Retrovirus	Breast ca. brain metastasis	orthotopic xenografts in athymic nude rats	i.v. injection with FUS	(164, 165)
ErbB2 (HER2)	FRP5	CD8α	CD28	CD28- CD3ζ	NK-92	Lentivirus	GB	orthotopic xenografts in NSG mice	i.t. injection	(125)
ErbB2 (HER2)	FRP5	CD8α	CD28	CD28- CD3ζ	NK-92	Lentivirus	GB	syngeneic orthotopic tumors in C57BL/6 mice	i.t. injection	(15, 125)

TM, transmembrane domain; s.c., subcutaneous; i.v., intravenous; i.t., intratumoral; n.s., not specified; GB, glioblastoma; Breast ca., breast carcinoma; FUS, MRI-guided focused ultrasound.

these CAR-NK cells was also demonstrated with targets growing in a tissue-like environment using patient-derived primary colon cancer organoids transduced with an EGFRvIII-encoding vector as a model (168). Repeated stereotactic injection of the EGFRvIII-specific NK-92 cells into orthotopic EGFRvIII-positive GB xenografts in immunodeficient NSG mice delayed tumor growth and markedly improved symptom-free survival (71). However, treatment of mixed tumors which similar to the clinical situation consisted of EGFR and EGFRvIII double-positive, and EGFR-expressing but EGFRvIII-negative GB cells, resulted in a less pronounced survival benefit and selective outgrowth of EGFRvIII-negative tumors still expressing the wildtype receptor. This is reminiscent of EGFRvIII-negative tumor recurrence observed in some GB patients after treatment with EGFRvIII-specific CAR-T cells (12).

CAR-NK CELLS SIMULTANEOUSLY TARGETING EGFR AND EGFRVIII

To circumvent the problem of heterogeneous EGFRvIII expression in GB tumors, two groups generated CAR-NK cells which simultaneously target wildtype EGFR and the receptor variant. Han et al. employed as a cell binding domain of a second-generation CAR with CD28 and CD3ζ signaling domains a scFv fragment of EGFR-specific antibody 528, which recognizes an epitope conserved in EGFRvIII (160). Established human NK-92 and NKL cells expressing this CAR displayed enhanced cytotoxicity and IFN-γ secretion when cocultured with GB cell lines or patient-derived GB stem cells expressing EGFR or EGFRvIII. Treatment of NSG mice carrying orthotopic GB xenografts expressing wildtype EGFR or EGFRvIII by repeated intracranial injection of CAR-engineered NK-92 cells inhibited tumor growth more strongly than treatment with NK-92 control cells and improved overall survival of the animals. In an independent study, Genßler et al. used a scFv fragment derived from the clinically applied antibody cetuximab as a cell binding domain for a dual-specific second-generation CAR with CD28 and CD3ζ signaling domains (71). Like antibody 528, chimeric antibody cetuximab and its murine parent 225 recognize an epitope common to EGFR and EGFRvIII (169, 170). The MR1-1-based CAR outlined above which is specific for the EGFRvIII neo-epitope and a similar CAR based on antibody R1 that recognizes an N-terminal epitope only present in wildtype EGFR were included in this study for comparison. *In vitro* analysis of NK-92 cells carrying these CARs revealed high and specific cytotoxicity of EGFR-targeted effectors against established and primary human GB cells, which was dependent on EGFR expression and CAR signaling. Cytotoxicity of EGFRvIII-targeted NK-92 was restricted to EGFRvIII-positive GB cells, while dual-specific NK cells were active against tumor cells positive for EGFR and/or EGFRvIII (**Figure 1**). Importantly, in NSG mice carrying orthotopic GB xenografts xenografts either expressing EGFR, EGFRvIII or both

receptors, repeated local treatment with the dual-specific NK cells was superior to treatment with either one or a mixture of the corresponding monospecific CAR-NK cells. This led to a marked extension of survival without inducing the rapid immune escape of antigen-loss variants that was observed upon therapy with monospecific CAR-NK cells (71). In later work, Jiang et al. followed a similar strategy to co-target EGFR and EGFRvIII with CAR-T cells recognizing a shared epitope of the receptors (171). Co-targeting of EGFR and EGFRvIII has also been achieved by expressing a secreted EGFR-specific T-cell engager in EGFRvIII CAR-T cells (172). Nevertheless, due to the more limited life span and expansion potential of NK cells when compared to T cells, utilizing CAR-NK cells may be a safer approach for targeting a surface antigen such as wildtype EGFR which is highly expressed also by vital normal tissues.

PRECLINICAL EVALUATION OF CAR-NK CELLS SPECIFIC FOR ERBB2 (HER2)

In addition to EGFRvIII and EGFR, the receptor tyrosine kinase ErbB2 has been targeted with CAR-engineered NK-92 cells. The respective NK-92/5.28.z cells represent a molecularly and functionally well-defined single cell clone isolated upon transduction of NK-92 cells under GMP-compliant conditions with a lentiviral vector encoding a second-generation CAR very similar to the one used by Ahmed et al. for CAR-T cells (11), based on ErbB2-specific antibody FRP5 and a composite CD28-CD3ζ signaling domain (78, 173). In preclinical studies, these cells displayed high and selective cytotoxicity against ErbB2-positive target cells of different solid tumor origins including established GB cell lines, and primary GB stem cell cultures (78, 125). Importantly, specific cytotoxicity of NK-92/5.28.z against GB cells was retained under hypoxic conditions and in the presence of high concentrations of immunosuppressive TGF-β, indicating that the cells remain functional in an environment similar to that of a GB tumor. Systemic application in NSG mice resulted in selective enrichment of NK-92/5.28.z cells in orthotopic breast carcinoma xenografts and a reduction of pulmonary tumor nodules in an experimental renal cell carcinoma metastasis model (78). In orthotopic GB xenograft models, repeated stereotactic injection of the cells into the tumor area effectively inhibited tumor progression, and led to a marked extension of survival (125). Furthermore, in an immunocompetent GB mouse model, NK-92/5.28.z cells displayed strong immunomodulatory activity and enhanced endogenous antitumor immunity upon intratumoral injection, resulting in tumor rejection in the majority of mice carrying syngeneic intracranial GL261/ErbB2 glioblastomas. In contrast, unmodified parental NK-92 cells were unable to inhibit tumor progression (15, 125). Without further treatment, those mice that were cured from their initial tumors also rejected a rechallenge with the GB cells injected into the other brain hemisphere more than 120 days after initial therapy, indicating induction of a long-lasting protective immune response induced in the animals by treatment with NK-92/5.28.z. IgG antibodies in the sera of these mice were broadly directed against the GB cells and not limited to the CAR target antigen. Protective immunity induced by initial treatment with NK-92/5.28.z cells was also dependent on T-cell memory, since depletion of $CD4^+$ and $CD8^+$ T cells before rechallenge prevented tumor rejection in some of the animals (15). Because of these promising preclinical data, the ErbB2-specific NK-92/5.28.z CAR-NK cells were chosen for further development toward clinical application in glioblastoma patients (described in the following sections).

CAR-NK CELLS TARGETING CD133 AND GD_2

Also NK-92 cells engineered to express a CAR specific for the tumor-associated antigen and GB target CD133 have been investigated, but preclinical data are so far limited to *in vitro* assays with CD133-positive established and primary ovarian carcinoma cells, which were eliminated by the CAR-NK cells when combined with cisplatin (174). In addition, the disialoganglioside GD_2 is considered a potential target for glioblastoma therapy (175, 176). While GD_2-specific CAR-NK cells have so far not been tested in GB models, both, primary donor-derived NK cells and NK-92 cells expressing GD_2-specific first- or second-generation CARs demonstrated selective antitumor activity in preclinical *in vitro* and *in vivo* models of neuroblastoma, melanoma, breast carcinoma and Ewing sarcoma (81, 177–179).

MANUFACTURING OF CAR-NK-92 CELLS FOR CLINICAL APPLICATION

As outlined above, the use of an NK cell line such as NK-92 offers certain advantages over primary NK cells which can overcome hurdles like variable transduction efficiency of viral vectors and limited expansion potential, that can complicate clinical translation of donor-derived NK cells (15). On the downside, due to their origin from a non-Hodgkin lymphoma, there may be a risk of secondary lymphoma formation in treated patients, which is currently addressed by including γ-irradiation of NK-92 before transfusion into a recipient (156–159, 161). Preclinical titration experiments demonstrated that an irradiation dose of 10 Gy is sufficient to inhibit proliferation of unmodified NK-92 cells as well as CAR-engineered NK-92/5.28.z, without affecting their *in vitro* cytotoxicity for at least 48 h or reducing *in vivo* antitumor activity in murine models (78, 125, 148, 180). This has of course implications for the manufacturing strategy. Autologous CAR-T cell products can be frozen after transduction and will expand *in vivo* upon injection of relatively small amounts of the cells. In contrast, NK cells in general and especially NK cell lines need to be expanded to a therapeutic dose before infusion. Irradiation will prevent any *in vivo* expansion, which mandates repeated therapeutic dosages for continued control of cancer growth. Hence, it would be ideal to produce cryopreserved cell doses in advance that can be thawed before transfusion. However, NK cells represent large granular lymphocytes which are more sensitive to cryopreservation than T cells, usually displaying impaired viability and long lag phases after thawing (181). While

efforts are made to improve cryopreservation of NK cells, and some NK-92 cell products under commercial development are provided in cryopreserved form (154, 182, 183), we decided to use fresh NK-92/5.28.z cells in exponential growth phase for application in glioblastoma patients.

Adapting the strategy developed for unmodified NK-92 cells (148, 157), first a qualified Master Cell Bank (MCB) of CAR-engineered NK-92/5.28.z cells was generated, providing a reliable source for subsequent production of patient doses for clinical trials (180). To bridge the lag phase of cell proliferation after thawing of vials from the MCB and to have a therapeutic dose of NK-92/5.28.z cells readily available for a patient within <1 week, a process was established which relies on a maintenance culture of the CAR NK-92 cells. From this maintenance culture therapeutic dosages can be expanded in batch culture within 5 to 6 days upon seeding the cells at an initial density of 5×10^4 cells/mL in 2 L of medium in gas permeable cell culture bags. After approximately 3.5 doublings (doubling time of NK-92/5.28.z: 32–36 h), the culture yields a total cell number of 1×10^9, which is sufficient to supply several cell dosages comprising 1×10^8 cells, which is the highest dose level currently planned for GB patients (180). Quality control performed on the maintenance culture involves monthly in-process controls for sterility, mycoplasma, endotoxin, absence of replication-competent lentivirus (p24 antigen ELISA), viability, identity, and phenotype ($CD56^+$, $CD16^-$, CAR^+), as well as potency of the NK-92/5.28z cells (lysis of ErbB2-positive target cells) (180). Release criteria of the irradiated finished cell product comprise the same tests, but with the results of sterility testing available only after injection due to the required time period. Special care was taken to choose an excipient used as injection solution for intracranial injection of CAR-NK cells that would not elicit adverse reactions due to calcium or electrolyte imbalances. Accordingly, the NK-92/5.28.z cells are resuspended in human serum derived from a single blood donor. Using this approach, a shelf life of up to 22 h at $4 \pm 2°C$ was established, which allows preparation and release of the investigational medicinal product 1 day before scheduled injection into a patient. The irradiated cell product is transferred to the clinical site in a bag comprising cells at a density of 5×10^7 cells/mL. The neurosurgeon then transfers the required amount of this cell suspension into a syringe at the site of operation to dilute the CAR-NK cells depending on the dose level and to perform the intracranial injections. Our experience shows that this approach is feasible and provides a ready-to-use dosage of CAR-NK cells to the application site without the need for local processing (thawing, washing, cell counting), which reduces the risk for secondary contaminations. Nevertheless, the limited shelf life demands an orchestrated and well-organized process involving all partners.

THE CAR2BRAIN PHASE I CLINICAL TRIAL

The phase I clinical trial CAR2BRAIN (NCT03383978, clinicaltrials.gov) is an investigator-initiated, prospective, open-label study carried out at the Center for Neurology and Neurosurgery at the University Hospital Frankfurt, Frankfurt am Main, Germany in patients with recurrent or refractory ErbB2-positive glioblastoma with a scheduled relapse surgery. Initially conducted as a single-center trial, it is now planned to include several other participating study centers in Germany. Main objective of the trial is to evaluate safety and tolerability of ErbB2-specific NK-92/5.28.z CAR-NK cells (78, 180) and to determine the maximum tolerated dose (MTD) or maximum feasible dose (MFD). In the dose-escalation cohort of the CAR2BRAIN study, NK-92/5.28.z cells are injected into the wall of the resection cavity during relapse surgery (**Figure 2**). The CAR-NK cells are subjected to γ-irradiation at a dose of 10 Gy prior to injection as part of the manufacturing process (see above). The dose levels explored are 1×10^7, 3×10^7, and 1×10^8 NK-92/5.28.z cells used as a single dose application in a total injection volume of 2 mL, distributed by the neurosurgeon with multiple injections. Patients are enrolled in cohorts of three to six patients per dose level in a 3 + 3 dose escalation scheme. Up to now we concluded the first two dose levels, with evaluation of the third and last dose level ongoing. So far, no dose-limiting toxicities were encountered.

Direct injection of the CAR-NK cells into the wall of the resection cavity was chosen to allow for high effector cell density in the tumor and the infiltration zone. Unlike ErbB2-specific autologous CAR-T cells that were applied systemically in GB patients in a recent phase I dose-escalation trial (11), irradiated NK-92/5.28.z CAR-NK cells cannot engraft permanently or expand *in vivo*, considerably limiting the number of viable cells that may reach the brain tissue from the circulation. Systemically applied NK-92/5.28.z cells homed to orthotopically implanted breast carcinoma xenografts in mice and were effective against experimental metastasis irrespective of γ-irradiation (78). However, intravenously injected CAR NK-92 cells had no therapeutic effect against intracranial tumors in preclinical studies, suggesting that at least in rodent models they cannot cross the blood-brain barrier in sufficient numbers without applying measures such as focused ultrasound (164, 165). This may be different in GB patients where the BBB can be severely disturbed in large parts of the tumor (184), and apparently allows systemically applied CAR-T cells access to the cancer cells (11, 12). Nevertheless, encouraging data on the successful treatment of a glioblastoma patient from a phase I clinical trial by local injection of IL-13Rα2-specific CAR-T cells suggest that intracranial delivery of effector cells may indeed be the preferred route of administration (10).

While in the case of B-cell malignancies a single dose of CAR-T cells is often sufficient to achieve durable responses (69, 70), repeated application can be more beneficial for the therapy of solid tumors, and will likely be mandatory to induce a lasting response if treatment consists of irradiated effector cells such as NK-92 (15, 150). Accordingly, after concluding dose escalation in the CAR2BRAIN trial, it is planned to include additional patients in an expansion cohort, scheduled for multiple injections of the CAR-NK cells at the recommended safe dose established during dose escalation (**Figure 2**). In these patients, intraoperative injection of NK-92/5.28.z cells is followed by implantation of a Rickham reservoir, with the catheter of the reservoir placed in the resection cavity. Beginning 1 week

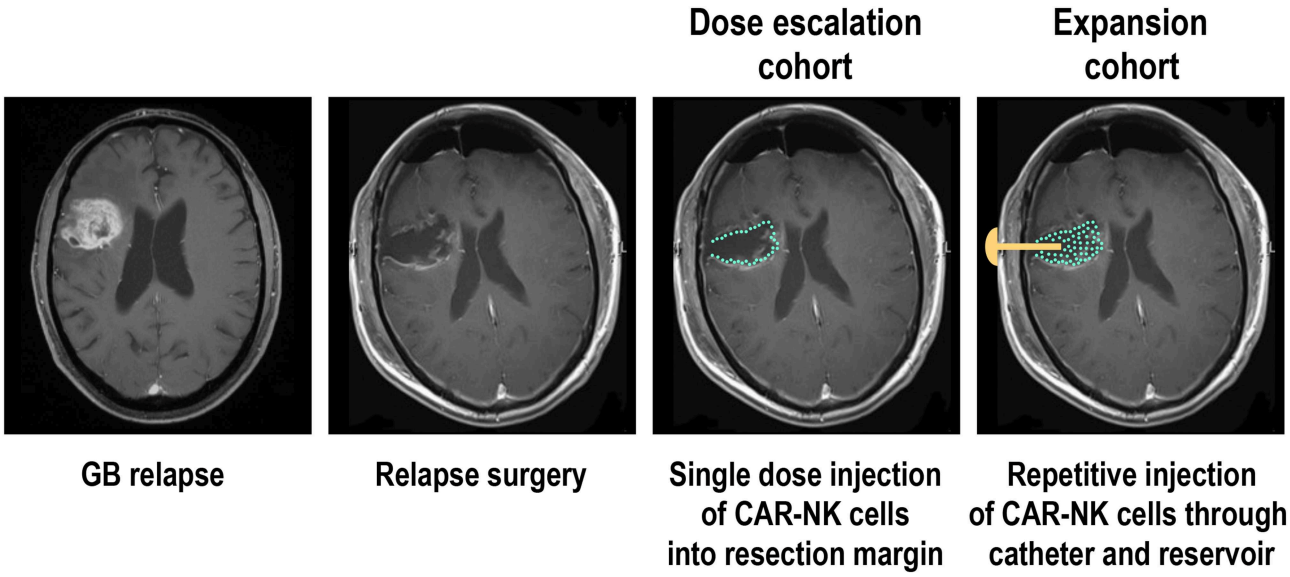

FIGURE 2 | Phase I clinical trial with ErbB2-specific CAR NK-92 cells in glioblastoma patients. Patients with recurrent ErbB2-positive glioblastoma in the dose escalation cohort of the CAR2BRAIN phase I clinical trial (NCT03383978, clinicaltrials.gov) receive a single treatment with irradiated ErbB2-specific NK-92/5.28.z cells injected into the resection margin during relapse surgery. In the subsequent expansion cohort, patients are scheduled to receive in addition up to 12 weekly treatments applied into the resection cavity through an implanted catheter and reservoir. MRI images are for illustration only and do not show an actual patient treated in the study.

after surgery and intra-operative treatment with CAR-NK cells, patients will receive up to 12 additional injections of NK-92/5.28.z into the resection cavity, applied through the reservoir and catheter. With these treatments spaced 1 week apart, resident GB cells can be exposed to NK-92/5.28.z for a prolonged time period while no potentially problematic dose accumulation of the irradiated effector cells is expected. In addition to the analysis of peripheral blood scheduled at different time points—if possible—also cerebrospinal fluid (CSF) will be collected through the catheter before each treatment for analysis of soluble factors and cells contained therein, expected to provide insights on possible effects of the CAR-NK cells on endogenous immune cells over the course of therapy. Patients will also be tested for potential immune responses to the allogeneic NK-92 cells and the CAR construct. In different phase I clinical trials with parental NK-92 cells, an anti-HLA antibody response to NK-92 was reported in one out of two, one out of seven, and six out of twelve patients evaluated (156, 157, 159). It is presently unknown to what extent such responses can affect the activity of NK-92 cells during repeated dosing.

CONCLUSIONS AND FUTURE PERSPECTIVES

NK-cell based adoptive immunotherapy of cancer is a rapidly expanding field. Recent advances such as the identification and effective blocking of distinct NK-cell immune checkpoints, and tumor-specific redirection of the exquisite lytic capacity of these innate lymphocytes using bispecific killer-cell engagers or chimeric antigen receptors can be expected to benefit patients suffering from many hematologic malignancies and solid tumors. Thereby glioblastoma, due to its location in the brain, its aggressiveness and highly immunosuppressive tumor microenvironment represents a particular challenge. Following similar approaches with CAR-T cells, CAR-NK cells have now entered clinical development for the treatment of malignant glioma. In addition to ongoing work with CAR-engineered NK-92 cells, other allogeneic off-the-shelf therapeutics based on donor-derived peripheral blood or cord blood NK cells, or NK cells differentiated from iPSCs may be tested for their effectiveness against brain tumors in the near future. However, overcoming inhibitory mechanisms in GB, and addressing immune escape due to inhomogeneous expression of CAR target antigens remain significant obstacles. While currently limited to a few cases, analysis of glioblastoma tissues from patients before and after treatment with CAR-T cells suggests that high effector cell activity can result in rapid selection of antigen-loss variants (12, 101). A similar problem may be encountered with CAR-NK cells. Nevertheless, NK cells naturally exhibit broad cytotoxicity triggered by interaction of their activating receptors with stress ligands expressed by tumor cells, which is retained by CAR-NK cells and may help to eliminate glioblastoma cells with low or absent expression of the CAR target antigen. Stem-like GB cells in particular appear sensitive to this natural cytotoxicity of allogeneic NK cells (185). Also preclinical data with NK-92 cells point in this direction, where ErbB2-specific CAR NK-92 cells lysed ErbB2-positive stem-like GB cells growing as neurospheres quite rapidly, but after prolonged exposure, the target cells were also eliminated by parental NK-92 cells lacking the CAR (125). The shorter life span and limited *in vivo* expansion of NK cells, while providing increased safety when compared to CAR-T cells, will likely restrict their long-term effectiveness and require repeated treatment. Ectopic expression of pro-inflammatory cytokines such as IL-15 together with the CAR may not only increase persistence of the CAR-NK cells themselves

(76, 77), but also support T-cell infiltration and activation (186). Induction of endogenous immunological memory by CAR-NK cell treatment has been identified as the basis for cures in immunocompetent animal models of glioblastoma (15, 125). In GB patients, avoidance of prophylactic dexamethasone administration (187), and rational combination of adoptive NK-cell transfer with radiotherapy, immune checkpoint inhibitors, inhibitors of angiogenesis or modulators of the myeloid cell compartment may all be effective strategies to fully realize the cytotoxic potential of CAR-NK cells against glioblastoma (6, 49, 188, 189), and further enhance their ability to modulate innate and adaptive immune cells in the tumor microenvironment.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception, design and writing of this review article, critically evaluated the cited literature, revised the manuscript, and approved the final version of this study. All authors agreed to be accountable for the content of this work.

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Polyoxidonium® Activates Cytotoxic Lymphocyte Responses Through Dendritic Cell Maturation: Clinical Effects in Breast Cancer

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Immunotherapy, which is seen as a major tool for cancer treatment, requires, in some cases, the presence of several agents to maximize its effects. Adjuvants can enhance the effect of other agents. However, despite their long-time use, only a few adjuvants are licensed today, and their use in cancer treatment is rare. Azoximer bromide, marketed under the trade name Polyoxidonium® (PO), is a copolymer of N-oxidized 1,4-ethylenepiperazine and (N-carboxyethyl)-1,4-ethylene piperazinium bromide. It has been described as an immune adjuvant and immunomodulator that is clinically used with excellent tolerance. PO is used in the treatment and prophylaxis of diseases connected with damage to the immune system, and there is interest in testing it in antitumor therapy. We show here that PO treatment for 1 week induced positive pathological changes in 6 out of 20 patients with breast cancer, including complete response in a triple-negative patient. This correlated with an increased tumor $CD4^+$ T-lymphocyte infiltration. The immune effects of PO are associated with myeloid cell activation, and little is known about the action of PO on lymphocyte lineages, such as natural killer (NK) and T cells. We reveal that PO increases T-cell proliferation *in vitro* without negative effects on any activation marker. PO does not affect dendritic cell (DC) viability and increases the expansion of immature DC (iDC) and mature DC (mDC) at 100 μg/ml, and it stimulates expression of several DC co-stimulatory molecules, inducing the proliferation of allogeneic T cells. In contrast, PO decreases DC viability when added at day 5 post-expansion. PO is not toxic for NK cells at doses up to 100 μM and does not affect their activation, maturation, and cytotoxicity but tends to increase degranulation. This could be beneficial against target cells that show low sensitivity to NK cells, e.g., solid tumor cells. Finally, we have found great variability in PO response between donors. In summary, our *in vitro* results show that PO increases the number of costimulatory molecules on DC that prime T cells, favoring the production of effector T cells. This may support the future clinical development of PO in cancer treatment.

Keywords: polyoxidinidum, dendritic cells, cytotoxic lymphocyte, breast cancer, aliphatic polyamines, natural killer cells, T cell

INTRODUCTION

Immunotherapy is now seen as the new frontier for cancer treatment, with several impressive successes (1). In several cases, cancer immunotherapy requires that immune responses be targeted toward specific antigens, and there is usually a lack of efficient stimulation of the immune system. In this sense adjuvants, agents that modify the effect of other agents, boost the immune response and have been largely used in vaccination (2), thus minimizing the dose of antigen needed. Despite their long-time use, only a few adjuvants are licensed today to generate an adaptive immune response to vaccines, and their use in cancer treatment is rare (3).

Polyoxidonium (PO) is a physiologically active compound from a new class of heterochain aliphatic polyamines that are attracting clinical interest (4). Chemically, PO is a copolymer of N-oxidized 1,4-ethylenepiperazine and (N-carboxyethyl)-1,4-ethylene piperazinium bromide, which is soluble in water and biodegradable and has a molecular weight of 60–100 kD (5). PO is approved in Russia as a vaccine adjuvant drug that stimulates antibody production (http://petrovax.com/medication/catalog/polyoxydonium/). The copolymer chains are cleaved and easily released from the body (5), which explains its low renal toxicity and good safety profile, as demonstrated in an extensive post-marketing study in Slovakia (6). Correspondingly, PO complexed with antigens in commercial influenza vaccine has also demonstrated high safety according to an analysis of about 50 million recipients (5).

PO binds to human peripheral blood monocytes and neutrophils, and, to a lesser extent, to lymphocytes (7). It is used as an immune adjuvant, particularly for vaccines, and as an immune modulator for the treatment of acute and chronic bacterial, viral, or fungal infectious diseases (4). PO has several immunogeneic properties. First, it stimulates the production of IL-6 (7). Second, it increases the bactericidal activity of leukocytes (8). Third, PO induces H_2O_2 production and improves the capacity of neutrophils and macrophages to capture and process different infectious agents, including bacteria, e.g., staphylococci, by about 40–60% (7, 8). This can explain PO's ability to enhance resistance to infections.

The effect of PO on the lymphocytic compartment is less known, although its immune-modulatory functions could partially involve improved antigen presentation resulting in effective antibody production (4). First, PO was covalently conjugated to antigenic components of the influenza vaccine: hemagglutinin and neuraminidase (5). Data from about 50 million recipients indicated that the vaccine was safe and effective (5). Second, PO was evaluated with trivalent live attenuated measles, mumps and rubella vaccine (9). Healthy children did not need PO to produce a high level of specific antibodies. In contrast, children with abnormal T cell counts could benefit from the use of PO (9).

Due to the excellent clinical safety profile of PO, we decided to try it in breast cancer patients prior to surgery. We observed a positive clinical outcome in 30% of the patients, which correlated with increased tumor infiltration by $CD4^+$ T cells. The results prompted us to investigate the effects of PO *in vitro* to identify cell targets on three different immune lineages playing important roles in tumor immune surveillance, namely dendritic cells (DC), T-cells, and NK cells (10). We found, however, that several immunomodulatory properties of PO varied between donors. Hence, there is a real need for a better understanding of the immune effects of PO to support new clinical developments.

PATIENTS, MATERIALS, AND METHODS

Compounds

PO was provided by NPO Petrovax (Moscow, Russia). Recombinant human (rh) IL-15 obtained from Miltenyi and rhIL-2 from PeproTech. Recombinant human GM-CSF and rhIL-4 were obtained from R&D systems and LPS from Sigma. All other products are described below.

Breast Cancer Patients

PO is authorized in Russia and in other countries as an immune adjuvant. Patients were treated in the department of surgery at the N.N. Blokhin National Oncology Research Center in Moscow according to the internationally approved guidelines and regulations used by the local Ethics Committee. Pathologists morphologically verified the presence of cancer by staining with hematoxylin-eosin before PO treatment. Twenty patients with histologically confirmed breast adenocarcinoma without metastasis received neoadjuvant PO at a dose of 12 mg by intramuscular injection at days 1, 2, 3, 5, and 7. Staging was determined using the TNM classification (11). **Table 1** describes the patients' stages; according to this classification, T describes the size of the original (primary) tumor and whether it has invaded nearby tissue, N describes nearby (regional) lymph nodes that are involved, and M describes distant metastasis. We also analyzed Her2/neu, the estrogen and progesterone receptors,

TABLE 1 | Clinical characteristics of the 20 breast cancer patients treated with PO.

Number of patients	20
Median age (range)	53.5 years (32–78)
TNM	
T1	1
T2	19
N0	6
N1	7
N2	7
N3	0
M0	20
Histological pattern	
Infiltrative ductal carcinoma	14
Infiltrative lobular	4
Tubular	1
Medullar	1

The table describes patient stages; according to the TNM classification, T describes the size of the original (primary) tumor and whether it has invaded nearby tissue, N describes nearby (regional) lymph nodes that are involved, and M describes distant metastasis. Histological pattern and the pathological response are also shown.

and Ki-67 as a proliferative index. Patients had subsequent surgery at day 8. Pre- and post-surgery pathological samples were compared according to a pathomorphosis scoring system that defines the pathological changes observed between samples performed before and after a specific therapy, as previously described (12, 13). Briefly, pathomorphosis degree 1 corresponds to mild modification, degrees 2 and 3 correspond to low to moderate reduction of tumor cell infiltrate, and degree 4 indicates complete disappearance of the tumor cell infiltrate. We also studied the subsets of leucocytes infiltrating the tumor and, moreover, we analyzed the changes in lymphocytes in blood and in bone marrow aspirates at Day 0 and Day 8 in nine patients. Cell suspensions were analyzed for CD4/CD3/CD25/CD45 and CD8/CD3/CD56/CD45 using Flow Cytometry and the FCS3 program (Becton Dickinson, Bioline BD Biosciences, St. Petersburg Russia).

Healthy Donor Samples

Data were obtained from three individual donors of the "Etablissement Français du Sang" (*EFS*). We prepared three biological samples from each buffy coat for each of the following experiments: PO concentration and cell type. This work benefited from umbilical cord blood units (UCBs) and the expertise of Prof. John De Vos, in charge of the Biological Resource Center Collection of the University Hospital of Montpellier–http://www.chu-montpellier.fr/en/platforms (BIOBANQUES Identifier–BB-0033-00031).

In vitro Dendritic Cell (DC) Expansion/Differentiation/Maturation

After Ficoll purification, PBMCs were plated in RPMI 1640 Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1% glutamine (RP10), and 2 h later, non-adherent cells were removed. Adherent cells were used as a starting population and cultured in RP10 medium supplemented with rhGM-CSF (100 ng/ml) and rhIL-4 (25 ng/ml) for 7 days.

PO was dissolved in water and added to cells growing in RP10 media at several final concentrations (1, 10, or 100 μg/ml). PO was added from day 0 to day 7 (immature DC, iDC D0). We added 1 ml of fresh RP10 medium supplemented with GM-CSF and IL-4 at day 2. At day 5, some iDCs (iDC D5) were treated with various concentrations of PO (1, 10, and 100 μg/ml) to examine its effect on iDC maturation. As a positive control, we used LPS (50 ng/ml) to induce DC maturation (mDC), and we also investigated the effects of PO on the LPS-induced maturation. Experiments were performed in triplicate, and the results are expressed as mean ± SEM. The expression of cell surface molecules was analyzed after 7 days of culture. Cell staining was performed using fluorescent-conjugated monoclonal antibodies. The antibodies PE-Cy™5-conjugated anti CD1a (clone HI149), BV421-conjugated anti CD83 (clone HB15e), BV605-conjugated anti CD80 (clone L307.4), BV650-conjugated anti CD14 (clone M5E2), BV711-conjugated anti CD40 (clone 5C3), PE-Cy™7-conjugated anti CD86 (clone 2331), Alexa Fluor®647-conjugated anti CCR1 (clone 53504), and Fc-Block were obtained from BD Biosciences. The APC-Alexa Fluor®750-conjugated anti-HLA-DR antibody (clone Immu-357) was purchased from Beckman Coulter. The Human CCR7 Fluorescein-conjugated Antibody (clone 150503) was purchased from R&D Systems, and phycoerythrin (PE)-conjugated anti CD1c (clone AD5-8E7) was purchased from Miltenyi Biotech. Samples were acquired on a BD-LSR Fortessa (Becton Dickinson), and all data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). iDCs were identified by expression of CD1a and CD1c and loss of CD14 expression, and DC maturation was monitored by expression of CD40, CD80, CD83, and CD86 in the HLA-DR+ cells.

In vitro T-Cell Expansion/Activation

Cells were treated with PO all through the expansion/activation protocol. Several concentrations were used: PO 1, 10, 100, and 500 μg/ml. After Ficoll, we used the EasySep™ CD3 positive selection kit (StemCell Technologies) according to the manufacturer's protocol. Purified $CD3^+$ cells were resuspended in RPMI-Glutamax, 10% FBS. Cells were activated by human T-activator CD3/CD28 Dynabeads (Life Technologies) according to the manufacturer's protocol. At day 5, we analyzed T-cell proliferation and different T-cell markers. Some samples were treated with PMA (50 ng/ml)/Ionomycin (1 μg/ml). Intracellular staining was performed by adding BD GolgiPlug, a protein transport inhibitor containing Brefeldin A (BD Biosciences).

The expression of cell surface molecules was analyzed after 7 days of culture. Cell staining was performed using fluorescent conjugated murine monoclonal antibodies. V500-conjugated anti CD4 (clone L200), PerCP-Cy5.5-conjugated anti CD25 (clone MA251), V450-conjugated anti Foxp3 (clone 259D/C7), PE-conjugated anti CD127 (clone HIL7RM21), PE-Cy7-conjugated anti IFN-g (clone B27), and PerCP-Cy5.5-conjugated anti IL-17a antibodies were obtained from BD Biosciences. PB-conjugated anti CD4 (clone 13B8.2) and APC-conjugated anti CD8 (clone B9.11) were purchased from Beckman Coulter. Stained samples were analyzed on a Gallios flow cytometer (Beckman Coulter) using Kaluza software. The experiment was performed in triplicate, and the results are expressed as mean ± SEM.

Expansion and Activation of Human NK Cells

This was performed as previously described (14). Briefly, blood units were depleted of T cells by using an EasySep™ CD3 Positive Selection Kit (STEMCELL Technologies). Cells were cultured for 20 days with γ-irradiated PLH cells at a 1:1 NK cell:accessory cell ratio in the presence of IL-2 (100 U/mL) and IL-15 (5 ng/mL). PLH cells were added every 4 days and fresh cytokines every second day. We did not add PLH for the last 4 days, and, at the end of the process, NK cell purity ($CD56^+/CD3^-$) was always higher than 90% with no living PLH cells remaining.

For phenotype analysis, cells were stained with 7AAD (Beckman Coulter) to identify viable cells and antibodies

against surface markers. FITC-conjugated anti CD25 (clone B1.49.9), anti CD45RO (clone UCHL1), PE-conjugated anti CD69 (clone TP1.55.3), anti CD62L (clone DREG56), anti CD19 (clone J3-119), anti CD3 (clone UCHT1), ECD-conjugated anti CD19 (clone J3-119), PacificBlue-conjugated anti CD16 (clone 3G8), anti CD57 (clone NC1), APC-AlexaFluor750-conjugated anti CD45 (clone J33), anti CD45RA (clone 2H4LDH11LDB9), KromeOrange-conjugated anti CD45 (clone J33), and anti CD16 (clone3G8) were obtained from Beckman Coulter. FITC-conjugated anti CD158b (clone CH-L), PE-conjugated anti CD158a (clone HP-3E4), and V450-conjugated anti CD107a (clone H4A3) were provided by BD Biosciences. APC-conjugated anti CD56 (clone REA196), anti CD3 (clone AC146), and Vioblue-conjugated anti CD158e (clone DX9) were purchased from Miltenyi. PECy7-conjugated anti CD56 (clone HCD56) was obtained from BioLegend. 1×10^5-3×10^5 cells were incubated for 20–30 min at 4°C with different antibodies in PBS containing 2.5% FBS. Cells were then washed and suspended in 200–250 μL of the same media. Stained samples were analyzed on a Gallios flow cytometer (Beckman Coulter) using Kaluza software. Viable lymphocytes were gated using FSC/SSC and 7AAD staining. B cells ($CD19^+$), T cells ($CD3^+CD56^-$), and NK cells ($CD56^+CD3^-$) were distinguished using CD19, CD3, and CD56 antibodies, respectively.

NK Cell-Mediated Cytotoxicity

This was performed as previously described (14, 15). NK cells were labeled with 3 μM of CellTracker™ Violet BMQC Dye (Life Technologies) and incubated overnight with target cells at different E:T ratios. Subsequently, phosphatidylserine (PS) translocation and membrane damage were analyzed in the violet fluorescence-negative target cell population by flow cytometry using Annexin V-FITC (Immunostep) and 7AAD (BD Biosciences) or propidium iodide (PI) as previously described (16, 17). We consider all cells positive for annexin-V and/or PI (or 7-ADD) as dead (or dying).

NK Degranulation Assay

This was done as previously described (15). Briefly, 50×10^3 target cells per well were placed in RPMI, 10% FBS, IL-2 100 U/mL with monensin (BD Biosciences) in a 96-well V-bottom plate. NK and target cells were incubated overnight at 37°C in 5% CO_2, and living cells were counted using a Muse cytometer (Millipore) with a count and viability kit (Millipore). As a control, NK cells were incubated without target cells. $CD107a^+$ NK cells were analyzed on a Gallios flow cytometer (Beckman Coulter) using 7AAD, CD45RO-FITC, CD19-PE, CD56-PECy7, CD3-APC, CD45RA-APCAlexaFluor750, CD16-KromeOrange, and CD107a-HV500 (BD Biosciences). The results were analyzed using Kaluza software.

Statistical Analysis

Experimental values were processed and statistical analysis was performed using GraphPad Prism (v6.0) software. All statistical data are provided as $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, and $^{****}p < 0.0001$. Mean values are expressed as mean plus or minus the standard error of the mean (SEM).

Results were obtained from three individual donors of the "Etablissement français du sang" (*EFS*). We prepared three biological samples from each buffy coat for each of the following experiments: PO concentration and cell type.

RESULTS

Effects of PO in Patients With Breast Cancer

We selected a cohort of 20 female patients with a mean age of 53.5 years (range 32–78). The first biopsy at day O showed that they had diverse TNM scores and that the majority had infiltrative ductal carcinoma (**Table 1**). We treated patients intramuscularly with PO for 1 week and obtained a second biopsy at day 8. Among the patients, six demonstrated pathological changes after surgery compared to results before surgery (**Table 2**). Responding patients had diverse TNM scores, estrogen and progesterone receptors, Her2/neu staining, and proliferative indexes (**Table 2**). One patient had the particularly aggressive triple-negative phenotype, i.e., negative for the receptors for estrogen, progesterone, and Human Epidermal Growth Factor 2 (HER2), and had a high proliferative index as measured by a high proliferation ratio based on Ki-67 staining (**Figure 1**). She received 1 week of PO treatment and received a radical mastectomy of the left breast with saving of breast muscles. We observed pathomorphosis degree 4 in the post-surgery material

TABLE 2 | Clinical characterization of patients with pathomorphosis after PO treatment (up) and receptor status and proliferative index of their tumors (down).

Pt number	Stage	pT	N	Pathomorphosis degree
1	IIb	2	1	4
2	IIIa	2	2	1
3	IIIa	2	1	1
4	IIb	2	0	2
5	I	1	0	1
6	IIIa	2	2	2

Pt number	Estrogen receptors	Progesterone receptors	Her2/neu	Ki-67
1	0	0	0	75
2	7	5	1	28
3	3	5	0	18
4	7	0	3	31
5	7	6	0	26
6	0	0	2	20

The table describes patient stages; according to the TNM classification, T describes the size of the original (primary) tumor and whether it has invaded nearby tissue and N describes nearby (regional) lymph nodes that are involved. Numbers under estrogen and progesterone receptors mean the standard score of receptor positivity in points. Her2/neu is given as a score, which is in points from 0 (negative) to 3 (positive). Ki-67 is a proliferative index in percentage of positive cells.

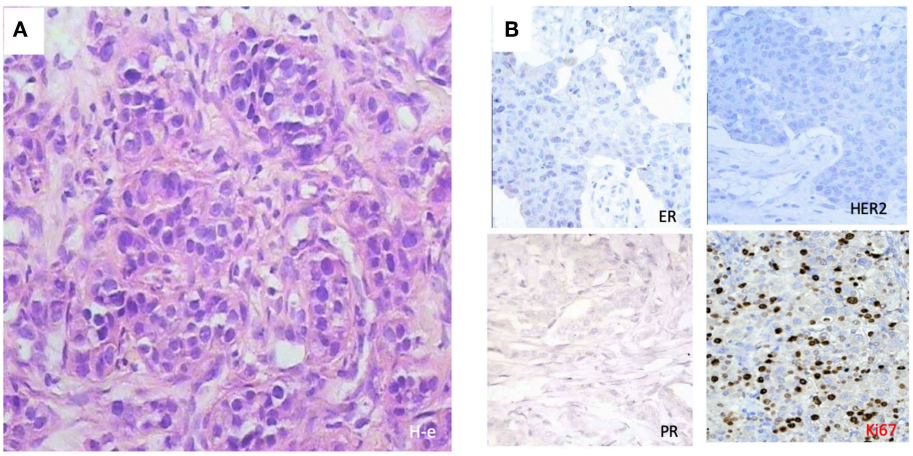

FIGURE 1 | Histological patterns at diagnosis of a 32-year-old patient with triple negativity that reached a complete pathological response after PO treatment. **(A)** Morphological features with hematoxylin-eosin (H-e) staining (x50) showed ductal tumor infiltrate. **(B)** Immunohistochemical stainings showed triple negativity for progesterone receptor (PR), estrogen receptor (ER), and human estrogen growth receptor 2 (HER2), with a high Ki-67 index.

(**Figures 2A,B**). This corresponds to a total disappearance of the tumor cell infiltrate, meaning complete pathological response. We also observed an inflammatory infiltrate in the lodge of the tumor (**Figure 2C**), which was composed of cells of both cellular and humoral immunity with an almost equivalent ratio of T and B cells (**Figure 2C**). Macrophages were composed of separate histiocytes, clustered as granulomas, and showed the appearance of a giant cell reaction (**Figure 2C**). Finally, around 15–20% of infiltrating cells were granulocytes, mainly neutrophils (data not shown). Hence, the changes detected after PO treatment in this patient corresponded to active chronic inflammation with granuloma formation.

In addition, minor changes (degree 1) were observed in three patients, and a reduction of the tumor infiltrate (degree 2) was found in two patients. We also obtained cell suspensions and studied the subsets of leucocytes infiltrating the tumor. We found a significant difference in the $CD4^+$ population on the tumor cell infiltrate between the six patients with pathological changes and those without pathological changes, respectively $50.91 \pm 2.05\%$ vs. $40.89 \pm 2.26\%$ ($P = 0.006$). In addition, the CD8/CD4 ratio was 0.79 ± 0.09 vs. 1.17 ± 0.13 ($P = 0.03$). We also analyzed the CD4 and CD8 subsets in blood and in bone marrow aspirates of nine of our patients at day 0 and at day 8 after PO treatment and did not find any significative difference (data not shown).

Effects of PO in T Cells

The previous results concerning T-cell recruitment prompted us to investigate direct effects of PO on T cells. We stimulated purified T cells from three healthy donors (HD) with anti-CD3/CD28 antibodies. This protocol induced efficient activation, as measured by CD127 downregulation and CD25 upregulation (**Supplementary Figure 1A**) and CD45RA downregulation (data not shown). To partially mimic the relatively long-term PO treatment in breast cancer patients, we stimulated T cells and treated them with different PO concentrations. T cell counts statistically increased by day 5 at 100 and at 500 μg/ml (**Figure 3**). In contrast, PO did not change the percentage of $CD4^+$ cells expressing CD127, CD25, and CD45 nor their level of expression (**Supplementary Figure 2**). Similarly, PO did not affect $CD8^+$ activation, except that decreased CD25 levels were induced by higher PO doses (**Supplementary Figure 3**). T-cell restimulation at day 5 with PMA/ionomycin induced IFNγ production in both T-cell compartments. This response was not affected by PO (**Supplementary Figure 3**). In summary, *in vitro* chronic PO treatment had no toxic effect and, in fact, increased T-cell proliferation without negative effects on any of the activation markers tested.

Effects of PO on Dendritic Cells (DCs)

DCs are key regulators of immune responses, capable of priming naive resting T cells and initiating primary T-cell responses. Hence, the effects of PO on T cells in breast cancer patients could be mediated by DC modulation. We treated PBMC adherent cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 5 days to generate immature DCs (iDCs) and promoted their maturation (mDCs) by an additional 2-day incubation with lipopolysaccharide (LPS). We analyzed the effect of PO on DC generation and maturation using three protocols. Firstly, we added several PO concentrations from day 0 to 7. This indicates the capacity of PO to influence the generation/expansion of iDCs. Secondly, we treated iDCs with PO at day 5 (D5). This allowed us to monitor PO ability to induce iDC maturation *per se*, therefore indicating if PO favors the formation of a humoral and/or cell-mediated immune response. Thirdly, we maturated iDCs with LPS while at the same time treating cells with several PO concentrations. This examines the ability of PO to affect LPS-induced DC maturation. iDCs were identified by CD1a and CD1c expression and loss of CD14 expression. DC maturation was monitored by expression of the co-stimulatory molecules CD40, CD80, CD83, and CD86

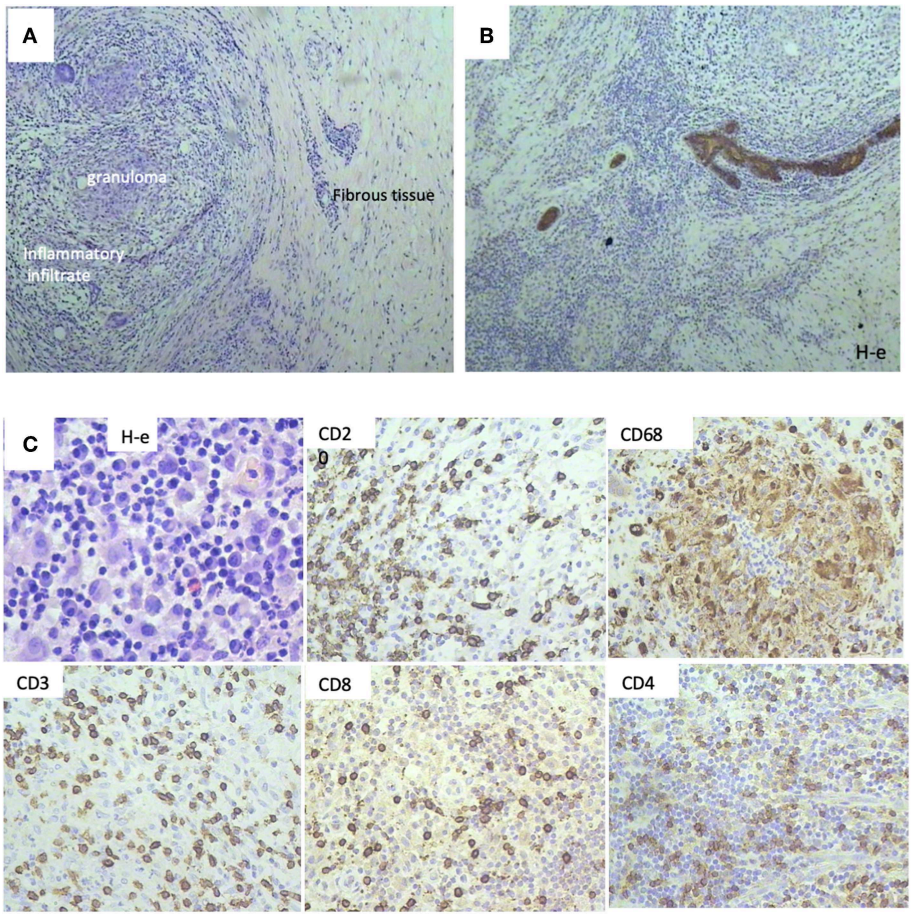

FIGURE 2 | Histological patterns after Polyoxidonium (PO) administration of a 32-year-old patient with triple negativity that reached a complete pathological response after treatment. **(A)** Morphological aspects, with the presence of granuloma, inflammatory infiltrate, and tissue fibrosis, with no tumor cell infiltrate (H-e; x20). **(B)** Immunohistochemical study with pancytokeratin antibodies (AE1/AE3) followed by Mayer haematoxilin staining. Positivity was only limited to breast ducts with no tumor cell infiltrate, corresponding to a complete pathological response. **(C)** Immune infiltrate was observed in the biopsy sections, with immunohistochemical staining on post-surgical material. T-lymphocytes (CD3, CD4, CD8 positive cells), B-lymphocytes (CD20 positive cells), and macrophages (CD68 positive cells) were observed.

in HLA-DR+ cells. The percentage of iDCs that expressed the costimulatory molecules CD80, CD86, CD40, and CD83 was relatively low and increased significantly in LPS-induced mDCs (**Supplementary Figure 4**).

PO did not affect iDC and mDC viability and increased iDC and mDC generation when added at 100 μg/ml at day 0 (**Figure 4**). In contrast, when added at day 5 at 1 and 10 μg/ml, it decreased DC viability slightly (for HD3) and, consequently, the final count (**Figure 4**). We observed variability between donors, but PO was consistently well tolerated in terms of DC viability and expansion (**Supplementary Figure 5**). Taken together, these results suggested that PO could improve the expansion of mDCs. Hence, in sites of inflammation sites where DC maturation occurs, PO would facilitate the production of mDCs.

Next, we performed phenotypic analyses at D7 to evaluate the level of expression of HLA-class II and the co-stimulatory molecules CD40, CD80, CD83, and CD86 (**Supplementary Figure 4**). The expression of these surface markers increases during DC maturation and could be revealing regarding the immunogenic properties of PO. PO did not affect the expression of any of these markers during iDC generation in terms of the percentage of positive cells (**Figure 5A**) or the expression levels (**Figure 5B**). The addition of 10 μg/ml PO at day 5 increased the percentage of iDCs expressing some costimulatory molecules in two out of three donors (**Figure 5A**). Moreover, the same PO concentration increased the expression levels of some costimulatory molecules in these patients (**Figure 5B**). This shows that PO has immunogenic properties. PO did not change LPS-induced expression of costimulatory molecules (**Supplementary Figures 4**, **6**). In fact, the LPS-induced increase was already very high, suggesting that PO is not capable of increasing it further.

Finally, to validate the PO-induced DC maturation, PO-treated DCs were co-cultured with CFSE-labeled T cells to evaluate their ability to induce allogeneic CD4 and CD8 T-cell proliferation. We used immature and mature DCs as controls. Firstly, it is important to note that both CD4 and CD8 T-cell

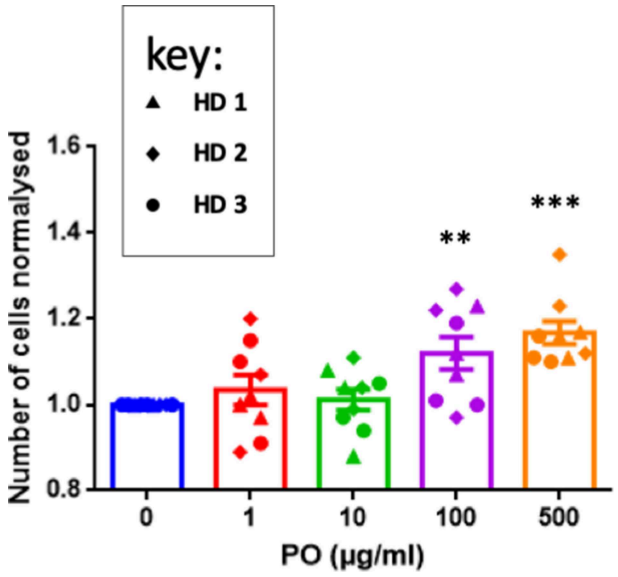

FIGURE 3 | PO increases T-cell proliferation. Purified T cells from three individual healthy donors were activated by anti-CD3/CD28 costimulation in triplicate. The indicated concentrations of PO were added at Day 0. The number of cells was quantified. Control non-treated cell average count was standardized to 1 to decrease the variability between donors. Graphs represent means ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA test was used for comparison to non-treated cells.

proliferation was induced in all conditions tested in the three independent biological experiments for two individual donors.

Higher proliferating T cell counts were observed following incubation with mDCs in both donors. Incubation with PO-treated iDCs increased the proliferation of allogeneic CD4 and CD8 T cells compared with control iDCs (**Figure 6**). Although these results varied between donors, they demonstrated the good immunogenicity of PO-treated DCs and their potential to induce CD4 and CD8 T-cell proliferation.

In conclusion, PO does not affect DC differentiation but may promote their maturation and the expression of co-stimulatory molecules leading to good DC immunogenicity reflected by T-cell proliferation. However, there is variability, suggesting that the effects of PO could depend on patient/donor immune status.

Effects of PO in NK Cells

NK cells are parts of the innate immune system and have natural cytotoxicity. NK cells predominantly target cells lacking MHC-I, including transformed or virus-infected cells, which downregulate MHC-I expression to avoid recognition by CTLs. Therefore, the "missing self" hypothesis proposes that NK cells discriminate target cells from other healthy "self" cells based on MHC-I expression. However, it is now clear that NK-cell activation depends on a complex signaling process mediated by activating and inhibitory receptors. The outcome depends on the strength of the various activating and inhibitory signals. The inhibitory receptors mainly recognize MHC-I (HLA in humans) molecules, and activating receptors can recognize stress ligands in target cells. Therefore, NK cells also eliminate "stressed" cells even if they express normal MHC-I levels.

As NK cells provide anticancer defense (18, 19), we analyzed the effects of PO on NK-cell activation and expansion. $CD3^+$-depleted PBMC cells were incubated at different PO concentrations, and the cell number was analyzed at day 7, 14, and 21. We observed decreased counts only at 500 µg/ml concentrations, but the effect was not statistically different at any concentration (**Supplementary Figure 7**). In fact, the raw data were very heterogeneous due to differences in the initial NK cell numbers in the blood bags and the response to the activation/expansion protocol. This was expected, given the variable percentages of NK cells in healthy humans (roughly 5–20%) and the NK cell responses to different stimuli (14). To counteract this issue, we calculated the average cell counts for each donor and produced a normalized value. We used this to normalize values in PO-treated cells. This showed that PO at 500 µg/ml decreased NK cell proliferation (**Figure 7**). In contrast, lower concentrations did not produce any effect. Therefore, PO does not affect NK-cell viability and proliferation at concentrations below 100 µg/ml and up to 21 days of stimulation, suggesting that these concentrations should not negatively affect NK-cell viability and proliferation in patients. In this context, it should be noted that peripheral NK cells have a short lifespan, 1 week on average (20), so it is unlikely that most NK cells will be in contact with PO for longer periods.

We next investigated the effects of PO on three well-known NK-cell activation markers, CD69, CD25, and the transferrin receptor CD71, at day 21 post-activation (**Supplementary Figure 8A**). CD71 is upregulated in cells with high metabolism. CD25 is the high affinity IL-2 receptor. CD69 is an early activation NK marker. All these markers were increased after NK-cell activation, and their expression was higher in NK with high anticancer activity (21, 22). None of the PO concentrations affected the percentage of NK cells expressing CD69 and CD71 or their expression levels as measured by MFI intensities. We observed a trend toward an increased percentage of CD25 positive cells and higher CD25 levels at PO 10 µg/ml. However, generally, PO did not change the expression of these activation markers.

During NK-cell maturation, $CD56^{bright}$ cells become $CD56^{dim}CD62L^+CD57^-$ cells, which produce perforin while maintaining high IFN-γ production in response to cytokines (23, 24). $CD56^{dim}CD62L^-CD57^+$ cells then show low response to cytokines and higher cytotoxic capacity and are considered fully mature NK cells (23, 25). *In vitro* stimuli for periods of up to 20 days do not induce CD57 (14). PO at 10–100 µg/ml showed a tendency to decrease the expression of both markers (**Supplementary Figure 8B**). Finally, fully mature, cytotoxic, NK cells express inhibitory Killer-cell immunoglobulin-like receptors (KIRs) and CD16 (26). **Supplementary Figure 8B** shows a trend toward an increase in KIR expression and no changes in CD16 in PO-treated cells (up to 100 µM).

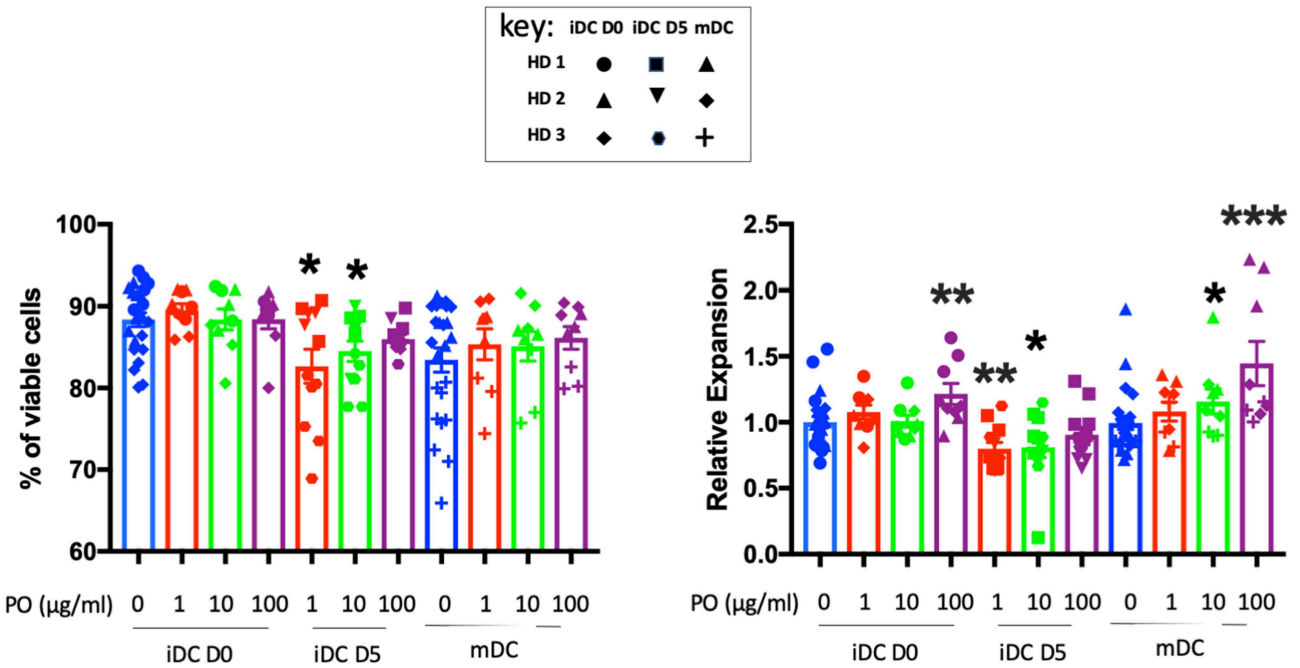

FIGURE 4 | Effects of PO on DC viability and expansion. Number of cells (right) and their viability (left) obtained at Day 7 from three individual healthy donors. Various concentrations of PO (1, 10, and 100 µg/ml) were added either at Day 0 (iDCs D0) or Day 5 (iDCs D5). DC maturation (mDCs) was induced by the addition of LPS (50 ng/ml) in the absence or presence of various PO concentrations. Due to inter-donor variability, we normalized expansion, giving the value of 1 to the iDC expansion and adjusting other values to that number. Graphs represent means ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Mann–Whitney U was used for comparison to non-treated cells.

Cytotoxic NK-cell function is mediated by activating receptors, e.g., NKG2D, which recognizes stress ligands in target cells. Their engagement induces natural cytotoxicity. In addition, NK cells recognize Fc domains in mAb-opsonized targets by the FcγRIIIa (CD16a). The engagement induces antibody-dependent cell-mediated cytotoxicity (ADCC). PO at doses below 500 µM did not affect the expression of these essential receptors (**Supplementary Figure 8B**).

CD45 is a protein, tyrosine phosphatase, that is specifically expressed in leucocytes (27). The largest isoform, CD45RA, is expressed on naïve T cells. Activated and memory T lymphocytes express the shortest CD45 isoform, CD45RO, which lacks RA, RB, and RC exons. This shortest isoform facilitates T-cell activation. The expression of CD45 isoforms gives NK cells different functional properties (22). NK cells coexpressing the long (CD45RA) together with the short (CD45RO) isoforms show higher antitumor activity in hematological cancer patients (21). After *in vitro* stimulation, CD45RA+ are resting cells, CD45RO+ are activated, CD45RARO show higher cytotoxicity, and CD45RAdim are cells in the process of activation. PO did not significantly change these *in vitro*-generated populations (**Supplementary Figure 9**), although there was a trend toward decreased expression of CD45RA and toward increased CD45RO, which could represent increased cytolytic activity (22).

We next analyzed the effects of PO on NK-cell-mediated cytotoxic function by investigating natural cytotoxicity and degranulation. We used two effector:target (E:T) ratios of 1:1 and 1:3. Because more than 50% of NK cells express KIRs, we used as targets primary cells expressing MHC-I molecules that can inhibit KIR-expressing NK cells. Analysis of raw data did not show changes in any of these parameters (**Supplementary Figure 10**). In fact, there was large heterogeneity between donors. Hence, we used a similar approach as in **Figure 7**, measuring the average cytotoxicity in control cells for each donor and producing respective normalized values. We used these to calculate normalized values for PO-treated cells. We did not find any significant changes in cytotoxicity (**Figure 8**). When we analyzed degranulation, we observed a trend toward increased response at PO 10 µM at both E:T ratios. In conclusion, PO did not affect cytotoxicity, although it could facilitate NK degranulation.

DISCUSSION

Stimulation of the antitumor activity of the immune system is becoming a major clinical approach. Recent advances in oncology suggest that the use of clinical molecules that stimulate the immune system against infectious diseases of various origins is an alternative to traditional chemotherapy (1, 4). Foreign natural polyelectrolytes (proteins, polysaccharides, nucleic acids) and their structural analogs (polypeptides, polynucleotides) have antigenic properties and may serve as immune stimulants (5).

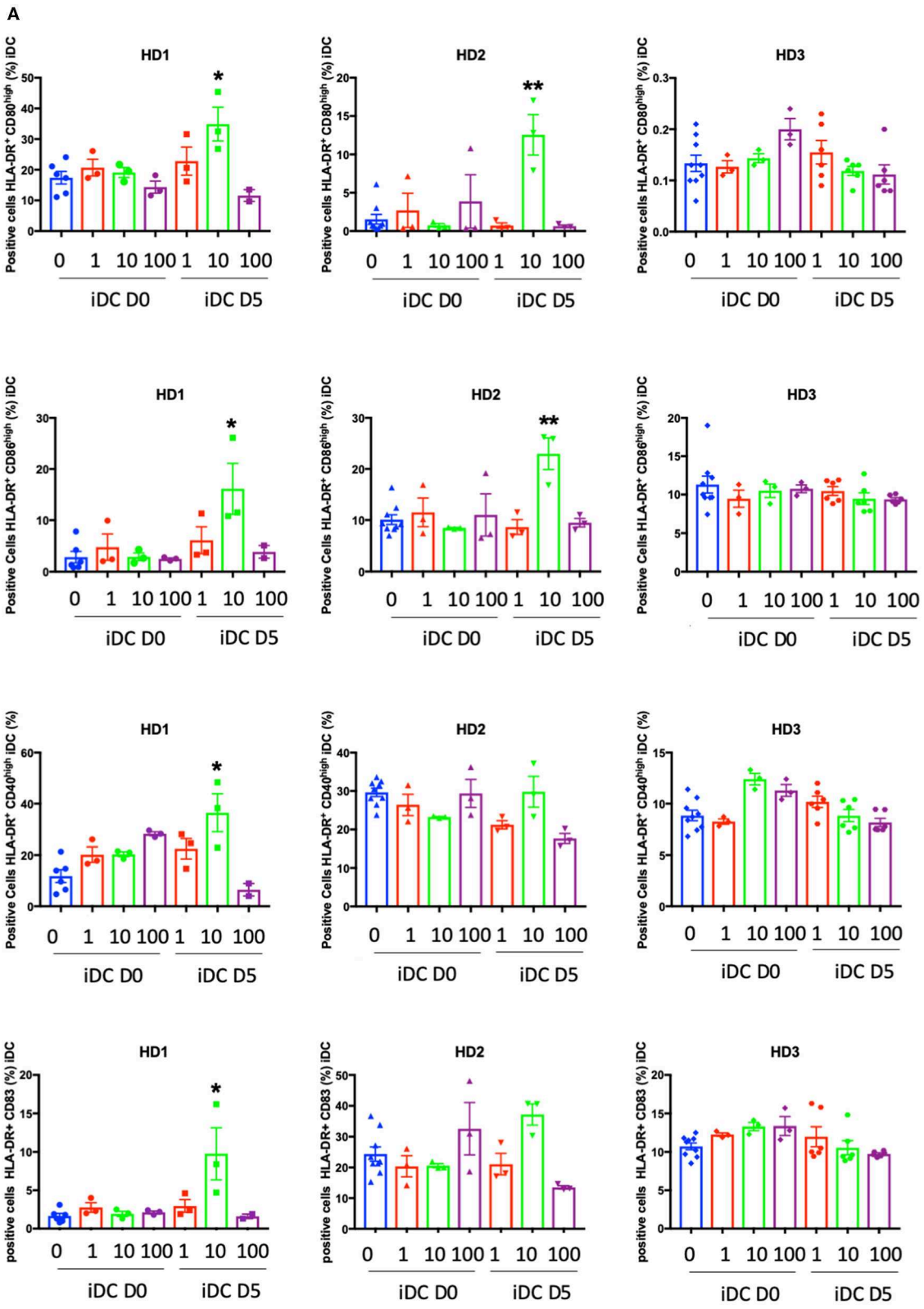

FIGURE 5 | Continued

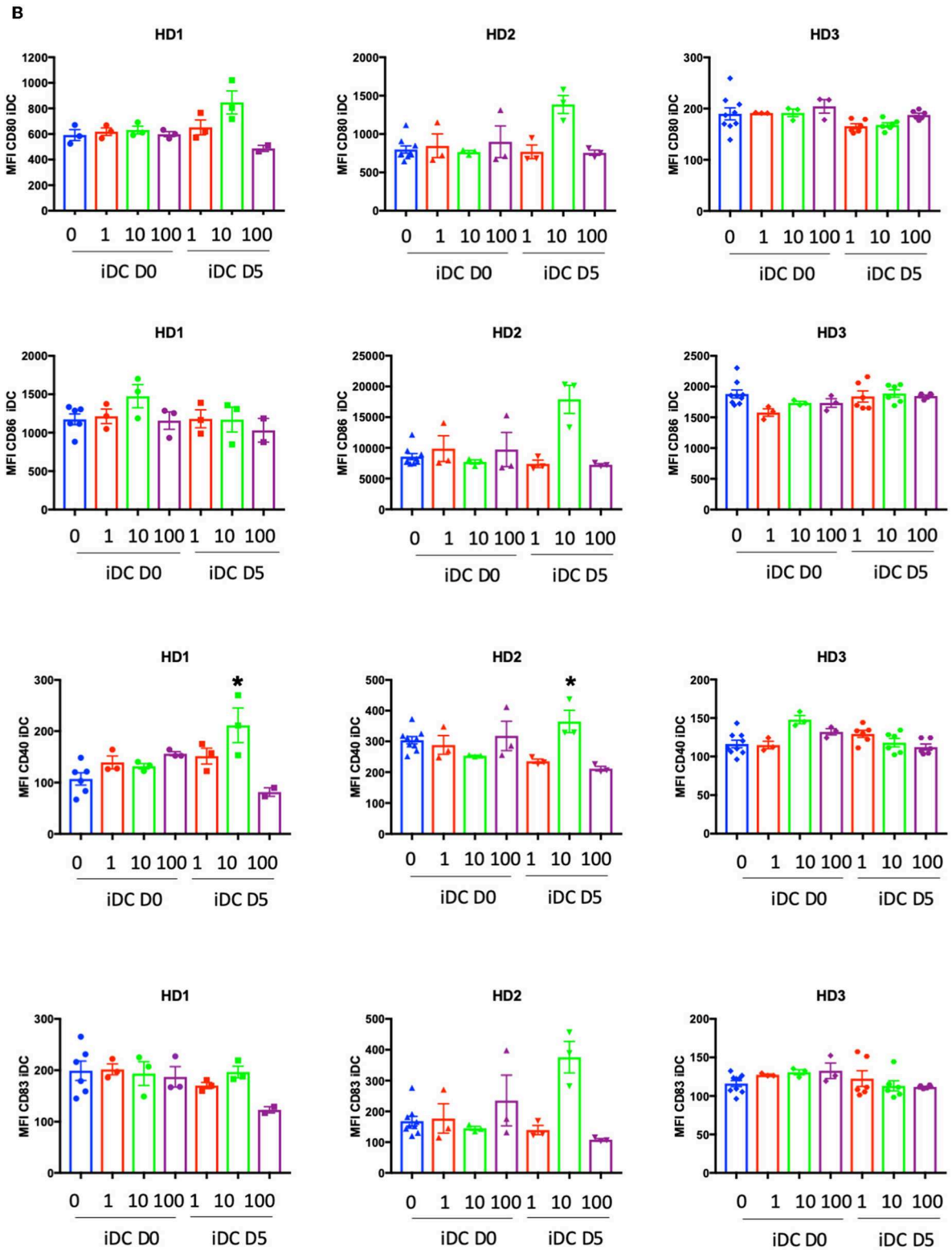

FIGURE 5 | Effect of PO on the percentage of DCs expressing DC maturation markers. iDCs were induced as described in the caption of **Figure 4**. **(A)** Percentage of DCs expressing various markers. **(B)** The expression levels (MFI) of DC maturation markers were analyzed. Graphs represent means ± SEM of each individual healthy donor with biological experiments performed in biological triplicates. $^{*}p < 0.05$, $^{**}p < 0.01$; Mann–Whitney U test was used for comparison to non-treated cells.

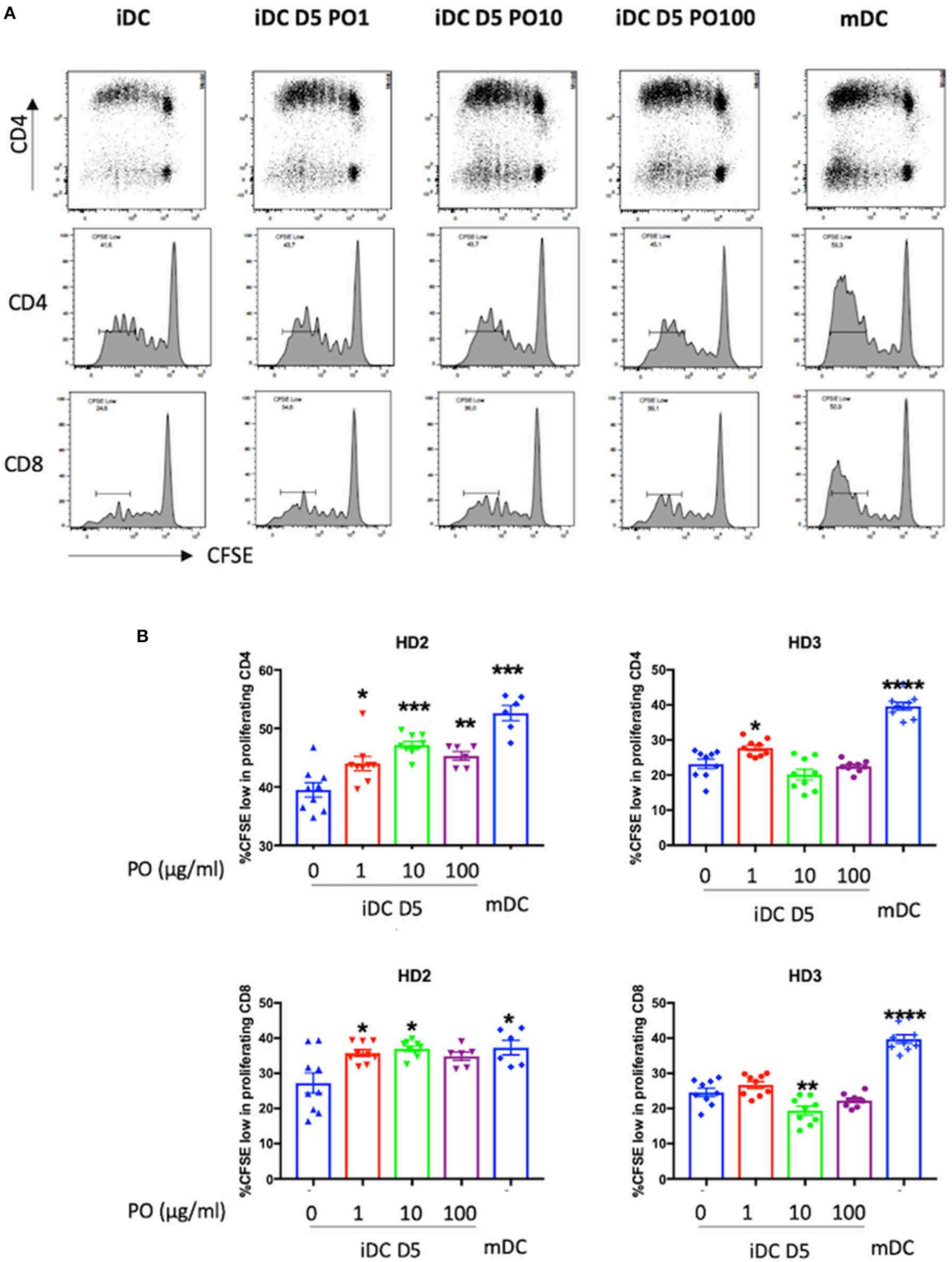

FIGURE 6 | PO increased iDC potential to stimulate mixed lymphocyte reaction (MLR). iDCs treated at D5 with different concentrations of PO (0, 1, 10, 100 µg/ml) or mDCs, as described in the caption of **Figure 4**, were used to stimulate CFSE-labeled allogeneic T-cells at a DC:T-cell ratio of 1:40. **(A)** Proliferation of T cells was analyzed at day 5. All conditions were tested at least in triplicate. Figure presents representative dot plots (top) and histograms depicting the proliferation of allogeneic CFSE-labeled $CD4^+$ (middle) and CFSE-labeled $CD8^+$ cells (bottom). **(B)** Percentage of highly proliferating CD4 (top) and CD8 cells (bottom). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0005$; Mann–Whitney *U* was used for comparison to non-treated cells.

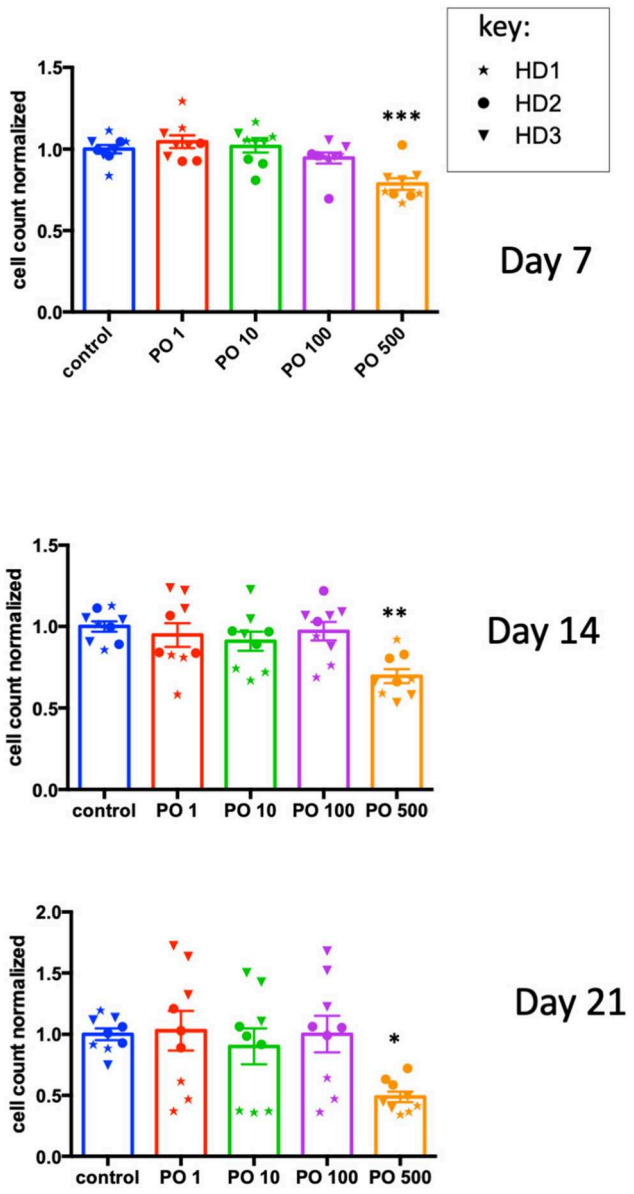

FIGURE 7 | High PO concentration decreases NK cell proliferation. NK cells from three individual healthy donors were activated by costimulation with target cells and low doses of cytokines for different times periods. Various concentrations of PO were added at day 0, and the number of cells quantified. The average control values were standardized to 1 to decrease the variability between donors. Graphs represent means ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; ANOVA test was used for comparison to non-treated cells.

Synthetic polyelectrolytes (SPEs) present the advantage of not being immunogenic, and the N-oxide groups of PO decrease the inherent toxicity of polyamines. This has been probed in multiple clinical settings (5, 6). Hence, PO is a clear candidate for being tested in cancer therapy. This was our first goal, and the results are encouraging, with 6 out of 20 patients responding to treatment, mainly by recruiting $CD4^+$ cells to the tumor site. However, it is important to understand the mechanism of action of new drugs, and the effect of PO on lymphocytes is basically unknown. We performed an exploratory study to try to identify the molecular basis of its clinical effect.

Although PO preferentially binds to myeloid cells, it also binds to lymphocytes, although with lower affinity (7). Therefore, we studied the effects of PO *in vitro* in three immune cell subsets involved in antitumor immune responses (10). Because PO pharmacology was mainly unknown, we used multiple concentrations. Moreover, because we did not know in which functions PO could be involved, we investigated its activity in several contexts, i.e., activation, maturation, and proliferation, for each cell type analyzed, i.e., NK, T, and DC.

Although we analyzed only three HDs, we revealed that PO was immunogeneic and observed large variability: one gave a relatively strong response, one a moderate response, and the third was mainly unresponsive. This resembled our observation in breast cancer patients, with ∼30% of responders. Hence, although the objective of this study was not to reveal the percentage of patients that respond to PO treatment, we believe that our results show that the effect of PO is patient/donor-dependent. This relatively low percentage of responding patients is found in most immunotherapies (28) and does not preclude its use, mainly in view of its low toxicity. New drugs usually fail in clinics due to low effect and/or high toxicity (29). The percentage of "responding" patients to a treatment is highly variable. This is more remarkable in immunotherapy because the target cell can modulate or be modulated by others or by the environment (28). For example, antibodies blocking PD-1/PD-L1 interaction are considered one of the biggest advances in cancer treatment in the last 20 years. However, this therapy improves the prognosis in only the 50% of patients with the best responder tumors. For the low responding tumors, the percentage decreases below 5%, even if patients express PD-L1 (28). Regardless of the mechanism of action and the direct effect of PO on lymphocytes, it is remarkable that this drug is barely toxic for these cells at concentrations up to 100 μg/ml, or even 500 μg/ml for T cells. This was observed even if lymphocytes were treated for several weeks. Moreover, it does not affect lymphocyte activation *in vitro*. Hence, our results *in vitro* and *in vivo* suggest that PO is a safe product.

Our results suggest that PO does not have "big" effects in the cell types and mechanisms we have investigated. This is usually seen when adjuvants are used alone (30), and although the effects of PO are small, they are coherent. For example, we observed efficient activation of several DC activation markers at certain PO concentrations (**Figure 5**), which correlated with the best activation of allogeneic T cells (**Figure 6**). Hence, DCs are clearly activated by the best immunogenic PO concentrations. Second, we observed that PO significantly increased T-cell expansion *in vitro* at the two higher concentrations, whereas the expansion of another lymphocyte lineage, i.e., NK, was not affected or decreased. Remarkably, this correlated with $CD4^+$ T-cell recruitment to the tumor site in breast cancer patients.

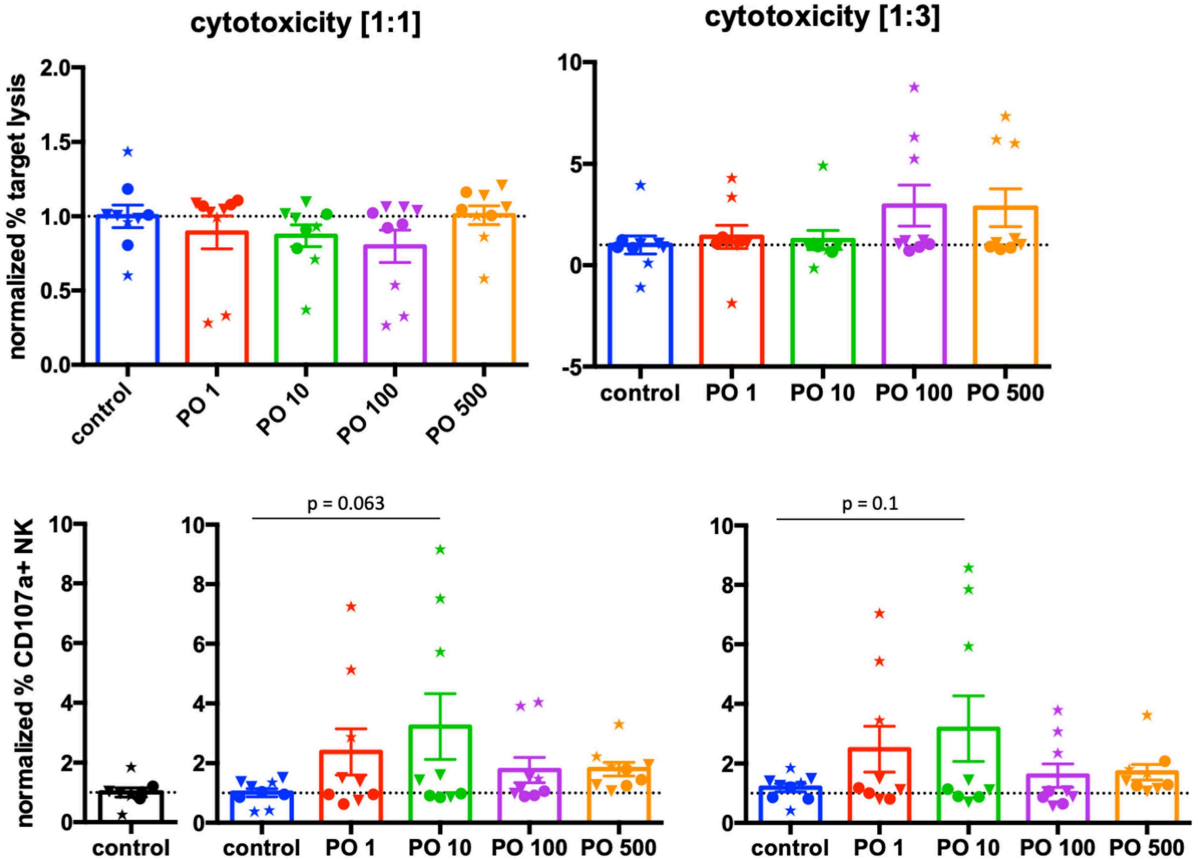

FIGURE 8 | PO does not affect NK-cell cytolytic function. NK cells from three individual healthy donors were activated by costimulation with target cells and low doses of cytokines for 21 days. Various concentrations of PO were added at day 0. Upper graphs represent toxicity against tumor cells from a B cell lymphoma patient at two different effector:target (E:T) ratios. Lower graphs represent the percentage of $CD107^+$ cells. The averages of the control values were standardized to 1 to decrease the variability between donors. Graphs represent means ± SEM; ANOVA test was used for comparison to non-treated cells.

The effects of PO were not dose-dependent. This forced us to utilize multiple concentrations under multiple conditions to unveil those conditions showing immunogenicity. In fact, the lack of a dose-dependent effect is not unusual in the immune system, where excessively strong immune activation can lead to cell inhibition. In lymphocytes, biphasic responses rely on the phosphatase CD45, which dephosphorylates the inhibitory residues of Src-kinases and leads to lymphocyte expansion and activation. However, strong CD45 activation leads to dephosphorylation of the Src-kinases activating residues, which inhibits T-cell activation (31). Hence, excessively strong activating signals can effectively lead to impaired lymphocyte activation. In addition, naïve and activated lymphocytes express different CD45 isoforms, which have different activities (22, 32). Hence, activated and naïve lymphocytes do not respond similarly to the same stimuli.

Hence, the PO cell targets that we have unveiled here could explain the variable response to this polyamine. Thus, although PO has shown its clinical value in several situations, future work should clearly establish which cancer patients can benefit from PO treatment to improve its clinical use.

This is the first study demonstrating both clinical and biological activity of PO in the domain of immune therapy for cancers. In this context, it is very interesting that two patients who responded better to PO, a complete pathological response and a partial response, suffered from triple-negative breast cancer.

This type has a poor prognosis (33), and the use of PO could improve it.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by N.N. Blokhin Russian Cancer Research Center in Moscow. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CA, MC, PL-P, D-NV, YE, ED-L, and Z-YL perform the *in vitro* experiments. PL-P, JH, J-FR, and MVi design the *in vitro* experiments and wrote the manuscript. FS, OC, MVa, IV, YV, and NT perform the clinical part including collection of samples and analysis of *ex vivo* samples from Breast cancer patients.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02693/full#supplementary-material

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REVIEW
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Recalling the Biological Significance of Immune Checkpoints on NK Cells: A Chance to Overcome LAG3, PD1, and CTLA4 Inhibitory Pathways by Adoptive NK Cell Transfer?

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Immune checkpoint receptors (IC) positively or negatively regulate the activation of the host immune response, preventing unwanted reactions against self-healthy tissues. In recent years the term IC has been mainly used for the inhibitory ICs, which are critical to control Natural Killer (NK) and Cytotoxic $CD8^+$ T cells due to its high cytotoxic potential. Due to the different nature of the signals that regulate T and NK cell activation, specific ICs have been described that mainly regulate either NK cell or T cell activity. Thus, strategies to modulate NK cell activity are raising as promising tools to treat tumors that do not respond to T cell-based immunotherapies. NK cell activation is mainly regulated by ICs and receptors from the KIR, NKG2 and NCRs families and the contribution of T cell-related ICs is less clear. Recently, NK cells have emerged as contributors to the effect of inhibitors of T cell-related ICs like CTLA4, LAG3 or the PD1/PD-L1 axes in cancer patients, suggesting that these ICs also regulate the activity of NK cells under pathological conditions. Strikingly, in contrast to NK cells from cancer patients, the level of expression of these ICs is low on most subsets of freshly isolated and *in vitro* activated NK cells from healthy patients, suggesting that they do not control NK cell tolerance and thus, do not act as conventional ICs under non-pathological conditions. The low level of expression of T cell-related ICs in "healthy" NK cells suggest that they should not be restricted to the detrimental effects of these inhibitory mechanisms in the cancer microenvironment. After a brief introduction of the regulatory mechanisms that control NK cell anti-tumoral activity and the conventional ICs controlling NK cell tolerance, we will critically discuss the potential role of T cell-related ICs in the control of NK cell activity under both physiological and pathological (cancer) conditions. This discussion will allow to comprehensively describe the chances and potential limitations of using allogeneic NK cells isolated from a healthy environment to overcome immune subversion by T cell-related ICs and to improve the efficacy of IC inhibitors (ICIs) in a safer way.

Keywords: NK cell, adoptive cell therapy, T cell, immune check point, cancer

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INTRODUCTION

Natural Killer (NK) cells are a class of innate lymphocytes that have evolved to eliminate rapidly infected and tumor cells. In humans there are two main subsets, phenotypically and functionally different, classified phenotypically according to the level of membrane expression of CD56 and CD16 and functionally according to their cytotoxic potential (1, 2). The $CD56^{dim}CD16^{pos}$ NK cell subset is eminently cytotoxic and expresses high levels of perforin and granzyme B. They share similar immunosurveillance function and killing mechanisms with the other main cell population responsible for killing infected or tumor cells, cytotoxic $CD8^{+}$ T (Tc) cells. However, mature NK cells can exert their cytotoxic function without previous activation and independently of the presence of non-self antigens presented by MHC molecules. Despite the ability of NK cells to eliminate target cells without previous sensitization, it is now well-known that previous activation enhances NK cell activity by regulating the expression of both cytotoxic mediators and several receptors as explained below (**Figure 1**) (3, 4). Moreover, a previous exposition to specific antigens (haptens), viral infection (CMV) or cytokines (IL12, IL15, and IL18) generates a type of NK cells known as adaptive NK cells that possess immunological memory features (5). However, the mechanisms that regulates the generation of adaptive NK cells are not clear yet and, specifically, it is not known if the HLA-self peptide complexes play any role in this process (6). However, previous results suggest that, at least in mouse models, this is not the case since mature NK cells are able to proliferate when transferred into MHC-I deficient recipients (7).

Since the activity of mature educated NK cells does not depend on neoantigens presented by MHC, they might be the perfect candidates to develop therapeutic strategies to eliminate tumors that are not visible for T cells either because they have downregulated MHC/HLA-I or because they do not present "good" HLA-I associated antigens. However, as it will be discussed below, since the key steps involved in the regulation of T and NK cells are different, it is very difficult to extrapolate the wide knowledge acquired on T cell regulation to NK cells, including the function of some inhibitory immune checkpoints (ICs) like CTLA-4 and PD1. Indeed, the development of specific immunotherapy protocols based on NK cells to treat cancer has been dampened by the complexity of the mechanisms that regulate NK cell function and elimination of target cells. Luckily, times are changing and, at present, in the era of cancer heterogeneity and immunotherapy, NK cells are emerging as the golden boys to eliminate non-antigenic tumor cell clones. A perfect duet in the symphony of destruction, Tc and NK cells destroying immune "visible" and "invisible" cancer cells to overcome immunogenic tumor heterogeneity.

However, to fully exploit the potency of this consortium in a safe way, it is required to understand in more detail the role of ICs in the regulation of Tc and/or NK cells during physiological immune responses as well as in the cancer microenvironment. As indicated in the summary, conceptually IC could be defined as any molecule involved in the regulation of the immune response, either in a positive or negative way. However, for reasons of clarity, in this review the term IC will be used to refer to the inhibitory ICs, unless the contrary is indicated.

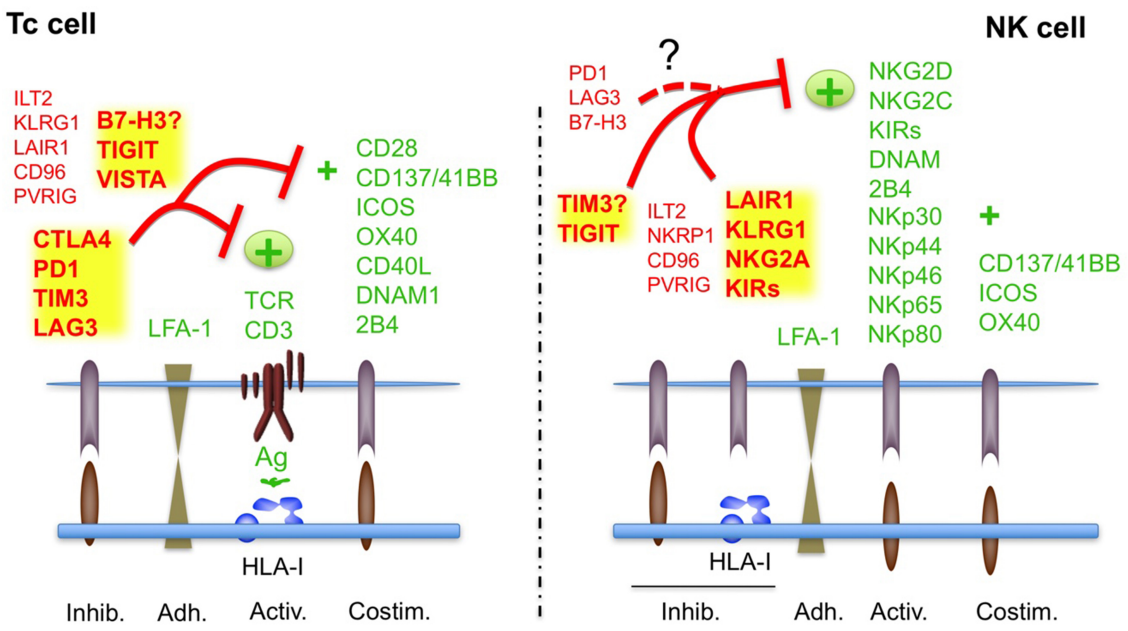

FIGURE 1 | Regulation of Tc and NK cell activity by ICs in physiological conditions. The canonical ICs, involved in tolerance are indicated in bold. See also **Tables 1**, **2**. Receptors with no clear functions are indicated with?. It is not clear if B7H3 plays an inhibitory or activating role in T cell tolerance; TIM has been shown to inhibit or activate NK cells.

TABLE 1 | Canonical human NK cell checkpoints.

Receptor	CD	Effect	Other cell types	Ligand	Tolerance[a]	Induced/Constitutive
KIR-L	CD158	Inh	T, Tγδ, Tm	HLA- I	Yes	C
CD94/NKG2A	CD159a	Inh	T $CD8^+$, Tγδ	HLA-E	Yes	C
ILT2	CD85j	Inh	T, B, Tm	HLA-A, B, C, HLA- G UL18	?	C
KLRG-1	-	Inh	ILC2, T	Cadherins	Yes[b]	C
LAIR-1	CD305	Inh	T, B, myeloid	Collagen	Yes[b]	C
NKRP1A	CD161	Inh	T, NKT, ILC	LLT1	?	C
Fc?RIII	CD16	Act	T, myeloid	IgG-Fc	Yes	C
KIR-S	CD158	Act	T, Tγδ, Tm	HLA-I	Yes	C
NKp30	CD337	Act	ILC2, T, Tγδ c	B7H6, BAG6/BAT3	Yes	C
NKp44	CD336	Act	ILC3, myeloid	MLL5 Nidogen-1 HLA-DP	?	I
NKp46	CD335	Act	-	Properdin, HA, HN	Yes	C
NKp65	-	Act	-	KACL	?	C
NKp80	-	Act	T $CD8^+$, Tγδ	AICL	?	C
NKG2D	CD314	Act	T $CD8^+$, Tγδ	MICA/B, ULBPs	Yes	C
CD94/NKG2C CD94/NKG2E	CD159c CD159e	Act	T $CD8^+$, Tγδ	HLA-E	Yes	C
2B4	CD244	Act/Inh[c]	T, Tγδ, granulocyte	CD48	Yes	C
DNAM-1	CD226	Act	T, B, granulocyte	CD112 (Nectin-2), CD155 (PVR)	No	C
41BB	CD137	Act	T, myeloid, endothelial, tumor	CD137L	No	I
ICOS	CD278	Act	T	ICOS-L B7RP-1	No	I
OX40	CD134	Act	T, NKT granulocyte	OX40-L (CD252)	No	I

HA, Hemagglutinin; HN, Hemaglutinin-Neuraminidase; LFA, Lymphocyte function-associated antigen 1; MICA/B, Major histocompatibility complex (MHC) class I chain-related protein A/B; MLL5, Mixed lineage leukemia 5; Tm, Memory T cell; PVR, Poliovirus receptor; ULBP, UL16-binding protein; C, constitutive; I, induced.

[a] *Regulation of human NK cell education and/or tolerance.*

[b] *Role in NK cell tolerance is controversial.*

[c] *2B4 is an activating receptor in human mature NK cells but an inhibitory receptor in mouse NK cells, human NK cell precursors during NK cell differentiation and in X-linked lymphoproliferative disease (XLP) patients.*

? No reported function on NK cell tolerance.

THE CANONICAL IMMUNE CHECKPOINTS OF NK CELLS (NK CELL-ICs)

The mechanisms involved in the regulation of NK cell self-tolerance and immunosurveillance against damaged cells has been by far much less understood that those regulating Tc cell activity (8). However, the knowledge accumulated during the last two decades has allowed to reveal a plethora of receptors involved in the regulation of NK cell activation and elimination of tumor cells. These receptors are known as ICs of NK cells (NK cell-ICs), since they are involved in the regulation of NK cell tolerance against healthy tissues, while ensuring an efficient response against damaged cells (**Table 1**). Thus, according to the physiological function of ICs, they could be considered as canonical ICs. Among others, NKG2A, KIRs and 2B4 are considered as canonical NK cell-ICs and have been proposed to regulate NK cell self-tolerance, also known as NK cell education (9, 10). It should be noted here that 2B4 can act either as an inhibitory or activating NK cell receptor (11). For example, inhibitory functions have been reported in mouse NK cells (12), human NK cell progenitors (9), and X-linked lymphoproliferative syndrome patients (13). In mature human NK cells, 2B4 mainly acts as an activating receptor (**Figure 1**). NK cell education is also regulated by the activating NK cell receptors SLAM, NKG2C, NKG2D, and NKp46 (9, 10).

In contrast, the role of other ICs, which are known to regulate T cell-tolerance and autoimmunity like PD1, CTLA4, or LAG3 (T cell-ICs, **Figure 1**), in the control of NK cell tolerance is less clear, and, thus, they should not be considered as canonical ICs of NK cells, at least under non-pathological healthy conditions. Indeed, the expression of most of them is very low or absent in most NK cells from healthy patients (14, 15). A notable exception is the expression of PD1 that has been identified in a subset of mature NK cells which share features of adaptive memory-like NK cells (16). This subset is produced by individuals (around 25% of healthy people) previously exposed to CMV infection

(17). The role of these non-canonical NK cell-ICs in the function of healthy NK cells will be discussed in more detail below.

Regulation of NK Cell Activity by NK Cell-ICs

The net balance between inhibitory and activating signals transduced by the respective receptors will dictate if a NK cell kills or respect a target cell (**Figure 1**) (3, 4).

The inhibitory receptors/ICs mostly interact with HLA-I molecules and on this way they can sense transformed cells that have downregulated HLA-I, which makes them invisible for T cells (**Figure 1**). The main NK cell-ICs are members of the KIR (Killer-cell immunoglobulin-like receptor) family, NKG2A and ILT2 that recognizes HLA-A/B/C, HLA-E/G, and HLA-G haplotypes, respectively (18–20) (**Table 1**). As indicated in **Table 1** and **Figure 1**, other inhibitory receptors modulate NK cell activity, albeit they have not been considered as ICs, since its role in NK cell tolerance and education is not clear.

Concerning activating receptors (**Table 1**), the family of Natural Cytotoxicity Receptors (NCRs), mainly NKp30, NKp44, and NKp46 (21) recognize different ligands and NKG2D recognizes stress ligands of the MIC family (22). Some members of the KIR family act as activating receptors when binding to specific HLA-C and HLA-A proteins and to HLA-G (23). NKG2C is an activating receptor for HLA-E (19, 20). NK cells also express the activating low affinity IgG receptor (FcγRIII), CD16 that mediates Antibody Dependent Cellular Cytotoxicity (ADCC) and the activating receptor DNAM-1 that binds to different ligands (**Table 1**). Finally, the C-type lectin receptors NKp65 and NKp80 also participates in NK cell activation after interacting with their respective ligands of the CLEC family, KACL, and AICL (**Table 1**) (24). NK cells also require adhesion and co-stimulatory proteins, like LFA-1 or 2B4 to eliminate cancer cells (25).

The only activating receptor that has not been found in non-activated NK cells is NKp44 that is rapidly upregulated after *in vitro* cytokine-mediated activation (26). Although NKp44 has been found to be constitutively expressed in a tissue-specific fashion on type 3 innate lymphoid cells and a subset of DCs (27), the role of this receptor in tumor immunosurveillance is not clear since it has not been detected yet in circulating or tumor infiltrated NK cells *in vivo*. Thus, in contrast to most NK cell-ICs and activating receptors, NKp44 has not been tested yet as a candidate for immunotherapy (28). It should be noted that an isoform of NKp44 can act as inhibitory receptor after interacting with the Proliferating Cell Nuclear Antigen (PCNA) (29). Indeed, it has been recently shown that an antibody against PCNA enhances the anti-tumoral effect of NK cells in a mouse model (30), suggesting that NKp44 acts as an IC in cancer. However, it is not known yet whether this isoform is a canonical NK cell-IC, regulating NK cell function in healthy individuals.

It has been recently described that some of these receptors like NKG2A, NKG2D, or NKp30 are also expressed by some T-cell subsets (31–33), although their expression in T cells from healthy individuals seems to be less frequent and they do not regulate T cell peripheral tolerance. Thus, its action might be restricted to pathological conditions like infection or cancer. Indeed, NKG2A has been recently shown to regulate both NK- and Tc cell-mediated anti-tumoral immunity (34, 35).

Reciprocally, as indicated above, it could be speculated that the regulatory activity of some ICs involved in the regulation of T cells like PD1, LAG3, CTLA4, or B7H3 is less pronounced in NK cells than in T cells (**Figure 1**). Supporting this hypothesis several studies have found that, in healthy individuals, CTLA4 is only expressed at very low levels in the cytosol of NK cells and PD1 or LAG3 could be restricted to specific memory-like subsets of individuals previously exposed to CMV infection (16, 36).

Other inhibitory receptors like ILT2, TIGIT, TIM-3, PVRIG, and TACTILE (CD96) might share regulatory functions in both NK and T cells, behaving as ICs in both cell types, although in this case their function in regulating NK cell tolerance is less clear (37, 38).

The contribution of these non-canonical emerging ICs to the regulation of healthy NK cell anti-tumoral activity will be discussed in more detail in section Allogeneic NK Cells Beyond KIR-Ligand Mismatch-Driven Alloreactivity: The Emerging Inhibitory NK-ICs.

Modulation of Canonical ICs for Cancer Immunotherapy

In light of the biological relevance of ICs, regulation of self-tolerance vs. elimination of infected or transformed cells, it could be possible to differentiate between Tc cell- and NK cell-ICs, at least under physiological conditions (**Figure 1**). Thus, pharmacological or biological manipulation of NK cell-ICs has been mainly focused on low immunogenic tumor types that do not respond to T cell-based immunotherapy and for which the immunomodulatory role of some Tc cell-ICs like PD1 might be less pronounced (28). Especially on hematological cancers, where NK cells occupy the same niches as tumor cells (39).

As indicated above, the increased knowledge about the receptors regulating NK cell activity and cancer immunosurveillance is allowing the development of therapeutic approaches to increase NK cell activity against cancer cells. These protocols involve mono- and bi-specific antibodies against inhibitory or activating NK cell-ICs or against membrane tumor antigens (14, 28) as well as adoptive cell therapy using autologous or allogeneic NK cells (28).

Monospecific antibodies against membrane tumor antigens promote ADCC by engaging the activating receptor CD16 on NK cells. Among all the activating receptors it has been shown CD16 engagement is one of the most potent signals to activate NK cells and eliminate tumor target cells (40).

Antibodies against inhibitory KIRs were developed and its efficacy validated in mouse xenograft lymphoma models (41, 42). Subsequently they have entered clinical phase in acute myeloid leukemia, lymphoma, and some solid tumors. Pending of finishing the clinical trials, it seems that the anti-tumoral activity of these antibodies as monotherapy seems to be very

low. However, the combination with chemotherapy or anti-PD1 antibodies might be more promising (43, 44).

Other antibodies that have been developed include anti-NKG2A (35) or bi- and tri-specific antibodies engaging NKp46 and/or CD16 and a tumor antigen (45, 46).

Only anti-NKG2A is currently being tested in clinical trials as monotherapy or in combination with anti-EGFR or anti-PD1 antibodies. Although the trial is not finished yet the results combining anti-NKG2A and anti-EGFR seem to be promising (35).

Albeit most clinical trials testing the antibodies against NK cell-ICs are still ongoing, the preliminary data indicate that they are not as efficient as the inhibitors of T cell-ICs, PD1, and CTLA4, neither against hematological nor against solid cancer (39). Regarding hematological cancer, this is likely due to that engaging a single IC is not enough to overcome the unregulated balance of signals received by NK cells on cancer patients. This is not easy to overcome since simultaneous engagement of several NK-ICs might lead to break down self-tolerance and induce toxicity. Among the potential consequences of targeting NK cell-ICs, it should be taken into account the implications of the findings by Carlsten et al. showing that inhibition of KIR2D by IPH2101 antibody impacts NK cell education (47). The authors showed a rapid decline in the function of NK cells from myeloma patients treated with IPH2101, which was related to the reduction of KIR2D expression on NK cell surface through monocyte trogocytosis. This treatment would affect the generation of educated NK cells with anti-tumoral activity due to the absence of interaction between KIR2D and its HLA-ligand (48). Thus, it might have a negative impact on other treatments designed to enhance NK cell activity by regulating ICs.

In the context of solid tumors, in addition to those problems, the lack of infiltration of NK cells and/or the immunosuppressive components of the tumor microenvironment might also contribute to the low efficacy of antibodies against NK cell-ICs (49).

ADOPTIVE ALLOGENIC NK CELL THERAPY: KIR-KIR LIGAND MISMATCH AND NK CELL ALLOREACTIVITY

An ingenious way to overcome the limitations of antibodies against NK cell-ICs is the use of allogeneic NK cells: NK cells isolated from healthy haploidentical donors, which are transferred to patients un-manipulated or after *in vitro* activation and expansion. The question that allogeneic NK cells could efficiently kill tumor cells was addressed by Velardi et al., soon after discovery of the HLA-I inhibitory ligands of the KIR family. This finding indicated that NK cells are able to sense and response against missing-self or missing-HLA-I (50), due the loss of inhibitory signals transduced by inhibitory KIRs (51). Thus, it was found that NK cells generated in the host after haploidentical bone marrow transplantation presented alloreactivity against recipient leukemic cells (52), a process known as KIR-ligand mismatch. The clinical benefit of this alloreactivity was subsequently confirmed in acute leukemia patients undergoing allogenic bone marrow transplantation. Specifically, those patients that received a transplant from an haploidential donor and, thus, presented NK cell alloreactivity, prevented leukemia relapse (53). This finding was further confirmed by Miller's group (54).

Subsequently, different protocols to activate and expand allogenic NK cells from healthy haploidentical donors were developed and infusion of purified NK cells was tested in leukemia, lymphoma, and myeloma patients as well in solid tumors with different results (55, 56). In general, these clinical trials confirm a benefit of KIR-ligand mismatch in acute myeloid leukemia patients, yet there are number of factors affecting the effectivity of this protocol which have not been completely clarified. Among them, it is noteworthy to mention the selection of donors expressing specific KIR-ligand mismatched combination and the functional expression of KIRs on the membrane of NK cells. In addition, it is becoming evident the importance of selecting an adequate conditioning protocol, not only to prepare the recipient of the transplant, but also during the preparations of NK cells to be infused in the patients. For example, development of protocols that remove specific cell populations that inhibit NK cell activity like T regulatory cells (55, 57–59).

ALLOGENEIC NK CELLS BEYOND KIR-LIGAND MISMATCH-DRIVEN ALLOREACTIVITY: THE EMERGING INHIBITORY NK-ICs

Biological Significance of T Cell-Related ICs: the Emerging NK Cell-ICs

Despite the unsolved questions in the clinical application of adoptive NK cell therapy, allogeneic NK cells might present several advantages over therapeutic manipulation of host NK cells. These advantages go beyond alloreactivity due to missing HLA-I inhibitory ligands. Specially, it should be stressed that allogeneic NK cells are selected from a healthy host and are not under the negative influx of cancer manipulation of host immunity. Thus, it is tempting to speculate that its action might not be restricted by ICs employed by cancer cells to overcome host Tc and NK cell anti-tumoral activity (28).

ICs were discovered in T cells by its ability to regulate T cell tolerance against self-antigens. Thus, both ICs and co-stimulatory signals have been shown to be involved in the regulation of T cell tolerance and prevention of autoimmunity (**Figure 1**) (15, 60). Subsequently, it was hypothesized that cancer cells might take advantage of the regulatory function of ICs to overcome T cell-immunosurveillance and, thus, pharmacological manipulation of ICs could be used as an effective cancer immunotherapy. The best example of this evolution is CTLA-4 and PD1 ICs. Both regulate T cell tolerance, preventing self-damage (61–64), and its inhibition by specific antibodies can reverse T cell inactivation (65) and is effective against specific cancer types (66, 67).

In contrast to NK cells, the regulation of T cell activity is restricted by their ability to differentiate self from non-self antigens and, thus, control of tolerance by inhibitory and co-stimulatory signals should be different in T and NK cells

(**Figure 1**) (15, 60–64). In this context, meanwhile NK cells mainly counterbalance the activating signals by the presence of KIRs and other inhibitory ligands recognizing HLA-I (9, 10), T cells require other inhibitory signals to prevent TCR signaling by HLA-I-associated self-antigens and/or co-stimulation. Here the presence of specific T cell-ICs would contribute to self-tolerance, avoiding autoimmune reactions.

Expression and Function of Emerging NK Cell-ICs in Cancer and Healthy Individuals

As indicated above this picture might be different under pathological conditions like cancer, and in those circumstances, some ICs traditionally associated to T cell function might promote NK cell immune-evasion by cancer cells (**Table 2**). Thus, although NK cells might be important players that contribute to the anti-tumor activity of IC inhibitors (ICIs) like antibodies against CTLA-4, LAG3, and the PD1/PD-L1 axes in cancer patients (68), this should not overestimate the function of some T cell-ICs in healthy NK cells. Indeed, most of Tc-ICs are not expressed by NK cells from healthy individuals even after activation and the level of expression of those that have been found is much lower than in T cells. These findings, key to develop efficient protocols and to exploit all the potential of adoptive allogeneic NK cell therapy, have passed unnoticed in the NK cell field. Indeed, most studies analyzing the expression of Tc-ICs in healthy NK cells are restricted to the use of healthy NK cell controls when analyzing expression of ICs in NK cells from cancer patients.

Notably, a few studies have analyzed the expression of the main Tc-ICs, PD1, CTLA-4, TIM-3, LAG3, TIGIT, and VISTA in freshly isolated and *in vitro* activated/expanded NK cells in humans. From these studies it has been consistently reported that both TIM-3 and TIGIT are expressed by naïve as well as activated NK cells (69–72). Notably, TIM-3 and TIGIT has been found to regulate NK cell tolerance *in vivo* suggesting that they might work as a canonical IC in NK cells (15). Indeed, TIM3 regulates NK cell-tolerance during pregnancy (73) and TIGIT regulates liver regeneration (74). In contrast, it has not been reported yet if other Tc cell-ICs, like VISTA, PD1, CTLA4, B7-H3/H4, or LAG3, contribute to NK cell tolerance and would act as canonical ICs of NK cells (**Figure 1**).

VISTA has not been found in healthy human NK cells even after activation (75) and the expression of CTLA4 in human NK cells is not clear. It was found CTLA-4 in activated mouse NK cells (76) and later on another group reported that human NK cells expressed intracellular CTLA4 (77). However, the relevance of this finding is not clear since it was previously shown that human NK cells do not express CTLA4 and are not co-stimulated by the CD28/CTLA4 pathway (78). In agreement with the later study, we have not found CTLA4 in the membrane of cytokine-activated human NK cells (Lanuza et al., in preparation). Regarding other i ICs like B7-H3 and B7-H4, only B7-H3 has been found in activated healthy NK cells (79). However, although B7-H3 seems to be a negative regulator of both Tc and NK cell activity in mouse cancer models (80), in contrast to T cells, it does not seem to be involved in the regulation of NK cell tolerance (79). Finally, inhibitory LAG3 is expressed by activated NK cells (81) although it has not been found to contribute to the regulation of NK cell tolerance (15) and/or NK cell cytotoxic activity (82). A recent work found out that LAG3 was expressed at very low levels in activated NK cells, but its expression was substantially increased in the adaptive NK cell subset chronically exposed to NKG2C ligands like antibodies or CMV-infected cells (36). Importantly, these cells presented low activity against tumor cells, suggesting that memory-like NK

TABLE 2 | Emerging human NK cell checkpoints.

Receptor	CD	Effect	Other cell types	Ligand	Tolerance[a]	Induced/Constitutive
B7H3	CD276	Inh	T, B, Treg, myeloid, non-immune cells, tumor	-	No	I
TIGIT	CD226	Inh	T, NKT	PVR (CD155) Nectin-2/-3 (CD112, 113)	Yes	C
TIM3	CD366	Act/ Inh	T, NKT, myeloid	Gal-9, HMGB1, PS, CEACAM1	Yes	C
LAG3	CD223	Inh	T, NKT, Treg, B	HLA-II, L-sectin, FGL1	No	I
PD1	CD279	Inh	ILC-2, T, B, NKT, myeloid	PD-L1, PD-L2	No	I
CTLA4	CD152	Inh	T, Treg	B7-1, B7-2	No	Unknown
PVRIG	CD112R	Inh	T	PVRL2 Nectin-2 (CD112)	?	C
TACTILE	CD96	Act/Inh?	T, NKT	PVR (CD155) Nectin-1 (CD111)	?[b]	C

CEACAM1, Carcinoembryonic antigen-related cell adhesion molecule 1; FGL1, Fibrinogen-like protein 1; ILC, Innate Lymphoid cell; Gal-9, Galectin 9; HMGB1, High mobility group box 1; PS, Phosphatidylserine; Treg, regulatory T cell; C: constitutive, I: induced.

[a] *Regulation of human NK cell education and/or tolerance.*

[b] *The role of CD96 in NK cell education/tolerance is not clear. It has been found that NK cells from mice deficient for CD96 are hyperactivated after LPS challenge. Both inhibitory and activating functions have been reported for CD96.*

cells from healthy donors previously infected with CMV, might become exhausted in case of CMV reactivation or after exposure to a new CMV infection in patients transferred with this NK cell subset. Notably, all experiments were performed in the presence of IL15 suggesting that chronically activation of adaptive NK cells in the presence of NKG2C ligands might require IL15 for induction of PD1 and LAG3 expression. However, the specific role of IL15 in this process is not completely clarified yet since the analyses of chronical activation with other cytokines like IL21 was not performed.

This issue is of special interest in light of the different protocols to expand allogenic NK cells that commonly rely on IL15 presence and the recent findings suggesting that continuous stimulation by IL15 exhausts NK cells (83). Since this study did not analyse the expression of PD1 and LAG3 on NK cells, it will be important to find out whether long term expansion of NK cells in the presence of IL15 could render NK cell populations whose anti-tumoral activity might be restricted by PD1 and/or LAG3.

Different Function of the PD1/PD-L1 Inhibitory Axes in the Regulation of NK Cells in Cancer vs. Healthy Individuals

It seems that only TIM3 and TIGIT are clearly expressed and act as conventional ICs in NK cells (15, 69–72) and, thus, they might contribute to the regulation of NK cell anti-tumoral activity during adoptive allogeneic NK cell therapy. But, what about the golden boy of ICs in cancer immunotherapy, the PD1/PD-L1 inhibitory axes?

Recently, it has been shown that NK cells contribute to the efficacy of antibodies against the PD1/PD-L1 axes *in vivo* (84). This finding confirms that PD1 is not only inhibiting T cells in the cancer microenvironment but, in addition, it prevents NK cell anti-tumoral activity. Studies in humans have also suggested such a function since NK cells from cancer patients express increased levels of PD1, which correlates with a lower anti-tumoral activity (85–87).

However, and in contrast to NK cells isolated from cancer patients, the role of PD1/PD-L1 inhibitory axes in the anti-tumoral activity of NK cells from healthy donors is unclear. Indeed, its contribution to the anti-tumoral activity of allogeneic NK cells has not been clarified yet. In a recent study we have analyzed the role of PD /L1 inhibitory axes in the anti-tumoral activity of NK cells against colorectal cancer cells (88). Using colorectal cell lines expressing different levels of PDL1 expression we found that activated allogeneic NK cells can kill colorectal cancer cells irrespectively of PDL1 expression. This finding indicates that allogeneic healthy NK cells can overcome the PD1/PD-L1 inhibitory axes. However, since we did not analyse the effect of PD1/PD-L1 inhibitors, it is still possible that blocking of the PD1/PD-L1 axes increases the anti-tumoral activity of healthy allogeneic NK cells. If true, activated allogeneic NK cells should express PD1, an issue that still requires further clarification (89).

It should be noted here that in contrast to T cells, PD1 seems not to be involved in the regulation of NK cell tolerance, indicating that like in the case of CTLA4, LAG3, VISTA, or B7-H3/4, PD1 is not a conventional IC in NK cells. This is not surprising since the level of expression of PD1 in the membrane of naïve and activated NK cells from most healthy patients is generally very low in comparison with activated T cells or with NK cells from cancer patients (85–87, 90). In line with these findings, it has been recently found that a pool of intracellular PD1 is expressed at low level by both naïve and activated NK cells from healthy patients. Notably, the expression of this pool of cytosolic PD1 did not increase after *in vitro* cytokine activation and neither naïve nor activated NK cells expressed membrane PD1 (91). As indicated previously, a notable exception was recently reported by Pesce et al. who found that PD1 could be expressed at high levels in a subset of NK cells corresponding to fully mature cells, detectable in peripheral blood from around 25% healthy individuals who were serologically positive for cytomegalovirus (CMV) (16). This subset corresponded to CD56dim NK cells characterized by the absence of NKG2A and a high expression of CD57 and KIR, indicating an adaptive memory-like NK cell phenotype. This finding was confirmed later on by Merino et al., who reported a significant increase of PD1 (together with LAG3 as mentioned above) in the adaptive NK cell subset chronically activated by the NKG2C receptor, involved in CMV recognition (36).

Although it is not clear yet the significance of this finding, it merits special attention due to the special characteristics of adaptive memory-like NK cells. It has been found that in the context of viral infections adaptive NK cells specifically respond against the specific virus for which they were originally activated (5, 6). Thus, somehow it could be said that they are restricted by specific antigens/signals, although this restriction does seem to involve HLA-mediated antigen presentation (7). Thus, it could be speculated that similarly to T cells, the expression of PD1 and LAG3 in adaptive NK cells might be related to the regulation of antigen specificity, albeit this hypothesis will require experimental validation. In the context of tumor development, the role of antigen specificity in the generation and response of adaptive NK cells is not known.

Anyway, these findings suggest that like in the case of LAG3, PD1 expression could be upregulated in NK cells that have responded to CMV infection and thus, healthy NK cells from these individuals might be partially regulated by the PD1/PD-L1 inhibitory axes. A question that should be taken into account when designing protocols for adoptive NK cell therapy.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The emergence of ICIs like antibodies against the PD1/PD-L1 axes and CTLA4, or more recently against TIM3, LAG3, TIGIT, or PVRIG, has supposed a great advance in the treatment of very aggressive cancers like melanoma and lung carcinoma (92). These treatments mainly rely on the generation of robust cytotoxic T (Tc) cell responses against mutations/neoantigens present in cancer cells. Indeed, cancer has learnt to use the main T cell-related ICs, naturally evolved to control T cell activity and avoid autoimmunity, to overcome T cell-mediated recognition and destruction. However, several tumor types

present low immunogenicity and do not respond to these therapies, remaining incurable. Even immunogenic cancers that initially respond to ICIs, frequently relapse due to the selection of poorly immunogenic cell clones and/or the apparition of alternative resistance mechanisms (92, 93). The combination of different ICIs like PD1/PD-L1 and CTLA4 inhibitors to overcome these limitations often increases toxicity and treatment has to be discontinued (92).

As an alternative, the use of NK cells isolated from haploidentical healthy patients and activated *in vitro*, allogeneic NK cells, presents several advantages that might help to overcome the limitations of ICI immunotherapy and to treat patients that do not respond to ICIs. First, alloreactivity due to KIR-HLA-I mismatch overcome the main NK cell-ICs, KIRs, and thus the inhibitory signals by HLA-I expressing tumor cells (52–54). Second, NK cell tolerance and NK cell-mediated cancer and infection immunosurveillance are mainly regulated by ICs different from T cell-related ICs (3, 4, 8) (**Figure 1**). Thus, NK cells developed in a healthy environment and activated *in vitro* should not be affected by cancer immune evasion based on ICs of T cells. Canonical inhibitory NK cell-ICs include the KIR family, NKG2A and ILT2 and among other ICs, only TIM3 and TIGIT have been clearly found to be involved in NK cell function in healthy individuals (3, 4, 8). Thus, the most potent ICs found in cancer, CTLA4, LAG3, and the PD1/PD-L1 axes should not affect the cytotoxic anti-tumoral activity of allogeneic NK cells. Finally, in contrast to Tc cells, which strictly depends on the activating signals transduced by the TCR after recognizing antigens presented by HLA-I, NK cells present a vast array of receptors that transduce activating signals like activating KIRs, NCRs of NKG2D/C. Thus, reduction of the inhibitory signals in the tumor microenvironment employing healthy allogeneic NK cells that express few T cell-ICs should unmask cancer cells to be efficiently eliminated by transferred activated NK cells.

However, in order to develop efficient protocols based on allogeneic NK cells to treat solid and hematological tumors and generate activated NK cells resistant to ICs, some limitations and open questions needs to be solved. Among them, we propose some key questions that, from our point of view, are important and deserve further experimental validation.

1. It will be important to find out if healthy *in vitro* activated allogeneic NK cells are induced to express other T cell-ICs like PD1, CTLA4, VISTA or LAG3 by cytokines present in the tumor microenvironment. On this regard it was recently found that PD1 was induced by TGF β in T cells (94, 95). It will be interesting to analyse if a similar effect is found in healthy activated NK cells.
2. It will be interesting to find out if specific cytokines or other stimulus are able to regulate IC expression on NK cells activated/expanded *in vitro*. In this regard IL15 was recently found to exhaust NK cells in long term *in vitro* cultures, although the expression of ICs like PD1 or LAG3 was not analyzed (83). Thus, in order to predict its efficacy on a personalized way, it should be required to analyse the profile of T cell-IC expression on *in vitro* expanded NK cells used for adoptive cell transfer. Depending on the results specific protocols for NK cell activation and expansion might be developed to minimize the expression of ICs on NK cells without affecting the expansion rate and cytotoxic potential.
3. Since it has been found increased PD1 and LAG3 expression in adaptive NK cells from CMV positive healthy individuals, it would be interesting to analyse if other T cell-ICs are also upregulated in these individuals. This finding also suggests that selection of CMV negative NK cell donors might help to generate PD1/LAG3 insensitive NK cells.
4. Combination of antibodies against tumor antigens or against soluble ligands that inhibits activating NK cell receptors with allogeneic NK cells should augment the activating signals on allogeneic NK cells and help to treat cancers that express high levels of ligands for emerging inhibitory NK-ICs like TIM3 or TIGIT.
5. As indicated, it has been found that low levels of CTLA4 and PD1 are expressed in activated human healthy NK cell intracellularly. Thus, it will be important to find out the stimulus that might mobilize PD1 and/or CTLA4 to the cell membrane and if this low level of expression is enough to inhibit NK cell-mediated elimination of cancer cells in the presence of the respective inhibitory ligands. This will be important to predict if intracellular PD1 will be expressed on the cell membrane of NK cells once they reach the tumor microenvironment.
6. It will be important to clarify if the contribution of host NK cells to immunotherapy with ICIs is due to a direct effect of ICI to NK cell activation or to a synergic effect between NK cells and Tc cells released from the IC breaks. On this way more effective protocols combining allogeneic NK cells and ICIs could be developed.
7. It will be important to find out the role of specific NK cell-ICs in tolerance regulation. On this way genetic manipulation of ICs in NK cells or combination of allogeneic NK cells with ICIs could be rationally developed to increase the efficacy of adoptive allogeneic NK cell transfer in a safe way.

Pending of validate experimentally all these hypotheses and in light of the available experimental evidences, it could be concluded that the regulation of the anti-tumoral activity of most subsets of allogeneic activated NK cells by T cell-ICs like CTLA4, PD1, VISTA, LAG3, or B7H3/4 is low. This might be an advantage to use adoptive allogeneic NK cell therapy to treat cancer types that despite presenting high immunogenicity and/or high levels of ICs do not respond to ICIs. Thus, efforts should be focused in solving other questions that might affect the efficacy of allogeneic NK cell therapy like migration and infiltration into solid tumors or the presence of other immunosuppressive factors in the tumor microenvironment like immunosuppressive cytokines or metabolic negative regulators.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing and editing of the manuscript. JP, AR-L, and PL designed the review. JP designed the figures.

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REVIEW
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Non-Genetically Improving the Natural Cytotoxicity of Natural Killer (NK) Cells

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The innate lymphocyte lineage natural killer (NK) is now the target of multiple clinical applications, although none has received an agreement from any regulatory agency yet. Transplant of naïve NK cells has not proven efficient enough in the vast majority of clinical trials. Hence, new protocols wish to improve their medical use by producing them from stem cells and/or modifying them by genetic engineering. These techniques have given interesting results but these improvements often hide that natural killers are mainly that: natural. We discuss here different ways to take advantage of NK physiology to improve their clinical activity without the need of additional modifications except for *in vitro* activation and expansion and allograft in patients. Some of these tactics include combination with monoclonal antibodies (mAb), drugs that change metabolism and engraftment of specific NK subsets with particular activity. Finally, we propose to use specific NK cell subsets found in certain patients that show increase activity against a specific disease, including the use of NK cells derived from patients.

Keywords: NK cells, microenvironment, monoclonal antibodies (mAbs), antibody-dependent cell cytotoxicity (ADCC), autoimmune diseases, CD45RARO

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INTRODUCTION

Innate lymphoid cells (ILCs) play a main role in immune-related disorders and are divided into three groups: ILC1s, ILC2s, and ILC3s (1). Natural killer (NK) cells, which belongs to the ILC1 group, are bone marrow derived cytotoxic lymphocytes (CL) that are well-equipped for the destruction of target cells without the need for prior antigen stimulation. In peripheral blood, human NK cells are mostly $CD3^{-}CD56^{dim}$ cells with high cytotoxic activity, while $CD3^{-}CD56^{brigth}$ cells excel in cytokine production (2). Additional markers can be used to identify specific subsets within these NK cell populations (2–4). *In vitro* evidence indicates that $CD56^{bright}$ NK cells are precursors of $CD56^{dim}$ NK cells and this might also be the case *in vivo* (3). In contrast to T cells, grafted NK cells show short live, low expansion and low alloreactivity such as graft-versus-host (GVH) in humans. Hence, NK can provide a potential source of allogeneic "off-the-shelf" cellular therapy and mediate major anti-target effects without inducing potentially lethal alloreactivity. Given the multiple unique advantages of NK cells, researchers are now exploring different ways to expand and/or activate them for clinical purposes.

NK CELLS IN CLINICS: THE PROBLEMS

Researchers working on the clinical use of NK cells have found numerous challenges. First, this cell lineage represents a low percentage of lymphocytes, usually estimated to 5–15%. In addition this changes during human development (4), making the transfer of sufficient allogeneic cells from a single donor to a patient challenging.

Second, NK cells have low lifespans, in average 1 week (5), suggesting that allogenic cells will shortly survive after engraftment. However, these results should be taken with caution. Lifetime studies were performed using deuterium incorporation, and only actively dividing cells incorporate it. Hence, this technique may not account for long-lived, non-dividing cells. Moreover, researchers normally focus on peripheral blood, hence NK cells mainly homing in lymph nodes such as $CD56^{bright}$ cells are not taken into account in their real weight (5). But, studies in blood are valid considering that allogeneic NK cells for engraftment are obtained from peripheral blood. Moreover, *in vitro* stimulated NK cells normally gain a mature phenotype despite high CD56 expression (6). Therefore, the previous estimates are a reasonable proxy for the amount of time NK cells will be active after allogenic engraftment. In agreement, the persistence of *ex vivo* haploidentical IL-2-activated and -expanded NK cells ranges between 7 and 10 days in patients with AML, NHL, and ovarian cancer (7).

The third challenge is that NK cells show doubling times of 1.25 days after activation (8). This is significantly longer than T cell doubling time during the initial expansion phase, which are 8 and 11 h for $CD8^+$ and $CD4^+$ T cells, respectively (9). Moreover, after allogeneic engraftment most clinical results failed to show significant expansion of donor NK cells (6, 7, 10–13). Perhaps the high renew and short lifespan account for these poor *in vivo* expansions because NK cells have already strongly expanded during their maturation and they are prone to "effector-like" phenotype, at least in the blood population.

Fourth, naïve NK cells possess a relatively low activity compare to activated cells (6, 14). This could be responsible of the low efficacy of NK cell-mediated therapies (11–13).

Fifth, there are several attempts to activate endogenous NK cells, e.g., by blocking NK cell inhibitory receptors. This led to the development of IPH2101, a killer inhibitory receptors (KIRs)/KIRL blocking antibody (Ab) (15), or monalizumab, a humanized anti-NKG2A Ab (16). This approach has the inconvenience that in cancer patients NK cells are hyporeactive (11, 12, 17). Moreover, new therapies such as NK cell-based therapies are usually tested on patients with advance clinical stages, which correlate with enhance NK cell dysfunction, at least in multiple myeloma (18). This suggests that endogenous NK could be unable to eliminate tumor cells even after releasing KIR inhibition. Interestingly, recent clinical data also in myeloma suggest that such antibodies can modify the endogenous NK repertoire and make them further hyporeactive (19). Other clinical attempts to activate endogenous NK cells include the use of lenalidomide [LEN; (20, 21)]. Biological results from the Phase Ib/II clinical trial GALEN suggest that LEN could facilitate obinutuzumab (OBZ)-mediated NK cell activation (21), as was observed with rituximab (RTX) (22). In fact cancer patients, at least those with hematological cancers, already possess NK cells, which recognize and kill tumor cells, but are unable to control the disease (21, 23, 24). Why only a fraction of NK cells is fighting against the tumor is unknown. Which is known is that blood-born cancer cells use different mechanisms for immune escape (25, 26), e.g., by inducing NK cell dysfunction (27). This mechanism has also been observed in a variety of solid tumor patients (17).

Due to all these adverse points recent clinical approaches target *in vitro* expanded and activated NK cells and hence the use of allogeneic NK cells.

MECHANISMS OF NK CELL EXPANSION

In this context, clinical-grade production of allogeneic NK cells is efficient (28) and NK cell–mediated therapy, including the use of *in vitro* expanded allogeneic NK cells, seems safe (11, 13, 28–31). This review does not focus on NK cell expansion, but in how we can "naturally" increase NK activity. There are recent reviews regarding NK cell expansion, e.x. (32).

But, it is important to note that choosing the correct donor can improve the killing activity of NK cells. There are different possibilities to choose the "best" donor including selection od donors with HLA/KIR mismatch with the patient (33), donors with a group B KIR haplotype (these donors have 1 or more of the B-specific genes: KIR2DS1, 2, 3, 5, KIR2DL2, and KIR2DL5) (34) or even donors with $KIR2DS2^+$ immunogenotype (35).

New attempts try developing disease-specific cytokine cocktails to activate *in vitro* patient NK cells (36–38). This is pertinent because *in vitro* the effects of these cocktails are different between patients and healthy donors. (39). However, despite the strong cytolytic potential of expanded NK cells against different tumors *in vitro*, clinical results have been very limited (11–13), e.g., NK are considered highly cytotoxic against AML tumor cells, but their efficacy as monotherapy in the clinic is low (11–13). Moreover, the results using NK cell therapy in animal models of solid tumors or in clinical trials are disappointing, even if NK cells can eliminate the engrafted cell type or the primary tumor cells *in vitro* (11–13). In this context, it should be noted that different culture media affect tumor recognition by NK cells (40). In summary, there is not any expansion protocol that produces allogeneic NK cells able to efficiently eliminate solid tumor cells *in vivo*. Why NK cells destroy most targets *in vitro* but not *in vivo* is unknown. Tumor cells strongly modify the expression of ligands, which are

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AML, acute myeloid leukemia; B-CLL, B-cell chronic lymphocytic leukemia; B-NHL, B-cell non-Hodgkin's lymphoma; BCL, B-cell lymphoma; DLBCL, Diffuse large B-cell lymphoma; EBV, Epstein–Barr virus; EGFR, epidermal growth factor receptor; e-NK, expanded NK cells; FL, follicular lymphoma; GMP, good manufacturing practices; GvHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; LEN, lenalidomide; mAbs, monoclonal antibodies; NCRs, natural cytotoxicity receptors; NK cells, natural killer cells; OBZ, obinutuzumab; PFS, progression-free survival; RTX, rituximab; UCB, umbilical cord blood; UCBT, umbilical cord blood transplantation.

recognized by NK cell activating or inhibiting receptors when cultured *in vitro* (40). This could lead to the mistrust that those specific tumor cells would be NK sensitive or resistant *in vivo*. Allogeneic NK cells survive for several days in patient's body (see above), hence their initial survival is probably not the blocking step for their efficacy *in vivo*. Impaired tumor infiltration and/or low cytolytic activity in the immunosuppressive tumor environment are usually pointed out as responsible of their low function *in vivo*. Hence, researchers have focused on protocols to activate them enough to bypass these clinical obstacles.

There are many protocols to expand and activate *in vitro* NK cells (6, 11, 13, 28–31). For many clinical uses, the manufactured cells should express the FcγRIIIa, also called CD16. The probably exception is those protocols wishing to generate chimeric antigen receptor (CAR) NK. We have produced umbilical cord blood (UCB)-derived NK cells because they are rapidly available, present low risk of viral transmission and have less strict requirements for HLA matching and lower risk of GvH disease (GvHD) (11). Expansion was driven by Epstein–Barr virus (EBV)-transformed lymphoblastoid B cell lines as accessory cells, which induce a unique NK cell genetic reprogramming (14), generating effectors that overcome the anti-apoptotic mechanism of leukemic cells (41) and that are able to eliminate tumor cells from patients with poor prognosis (42). NK cells obtained with this protocol perform antibody-dependent cell cytotoxicity (ADCC) *in vitro* and *in vivo* with different therapeutic antibodies and against diverse target cells (6).

NK cell expansion is extremely challenging from an industrial point of view (43, 44), partly due to the problems described in the previous section. In addition, NK cell production should be easily scaled up and developed with good manufacturing practices (GMP). Several biotech companies are now producing NK cell-based products that could reach the clinic in the future (44). We will discuss now mechanisms to naturally improve NK cytotoxicity. We will not discuss about lympho-depleting chemotherapy, e.g., cyclophosphamide followed by daily fludarabine, which is already largely use in clinics prior to NK cell infusion (45).

CYTOKINES MEDIATE NK ACTIVATION

Generally, when NK will reach the target microenvironment they will receive a burst of cytokines from other cells, e.g., those immune cells that have already infiltrated the tumor. These cytokines affect NK cell behavior and activation and has extensively been reviewed elsewhere (46). Hence, we will only briefly describe some few uses. IL-2 and IL-15 are strong NK cell activators, but their clinical use *in vivo* is challenging due to their toxicity (44). Moreover, IL-2 expands and mobilizes regulatory T cells, which dampen the activity of several effector cells including NK (44). IL-15, although less toxic than IL-2, is limited by its short half-life leading to poor functional activity *in vivo*. However, *in vitro* both cytokines are very efficient stimuli to activate and expand NK cells (6, 14). In fact, membrane-bound IL-15 is currently the best activating cytokine (47), although membrane-bound IL-21 is becoming an interesting challenger (48, 49). In any case, long-term cytokine treatment can lead to NK cell exhaustion, which will inhibit NK activity (50).

MODIFYING THE TARGET MICROENVIRONMENT

Tumor cells, directly or by controlling non-transformed cells, modify the environment to make it immunosuppressive and avoid destruction by effector immune cells (25, 26, 50). We will discuss here some approaches that can reverse this "negative" microenvironment. We will not discuss drugs that *per se* sensitize target cells to NK cells. Tumor-induced modifications include metabolic changes with the production of metabolites that negatively affect NK cell cytotoxicity, e.g., lactate (26, 50). This is the classical metabolite produced by tumor cells under the Warburg effect: cells perform glycolysis even in the presence of ample oxygen (26). To recover the reducing power of NAD^+, which has been reduced to $NADH^+$-H^+ during glycolysis, cells reduce pyruvate creating lactate. This mechanism recovers the cell reducing power and allows the glycolysis to proceed. During the Warburg effect, the products that are not oxidized, i.e., that are not consumed to produce CO_2, serve to create new intermediate metabolites that are used for anabolism. But in addition, tumor cells release lactate to the external medium. This acidifies the environment and inhibits the antitumor response of CLs because the killing activity of these cells is extremely sensitive to the decrease in pH (50, 51). There are some compounds such as dichloroacetate (DCA) or metformin that inhibit the Warburg effect and block lactate production (26, 52, 53). It is hence conceivable that such drugs could increase the cytolytic activity of NK, or other CLs, *in vivo* (**Figure 1**).

In contrast, during the killing of yeast cells or cryptococcoma, NK cells appeared to profit from the acidic pH of the microenvironment by displaying enhanced perforin degranulation and killing capacity (51). Therefore, an interesting possibility would be to modify the NK-tumor environment to match that of NK-yeast cells/cryptococcoma in order to increase NK cytotoxicity even at low pH.

Another way to increase NK activity would be to decrease adenosine concentration in tumor environment. This nucleotide is found as much as 100-fold higher in tumors than in normal tissues and contributes to immune evasion by inhibiting for example NK cell cytolytic activity (50). The ectonucleotidases CD39 and CD73 produce large amounts of adenosine, hence their inhibition decreases tumor growth and metastasis. This type of treatment has reached the clinic with the anti-CD73 antibody Oleclumab (50).

Several vitamins, e.g., A, C, and E, induce changes in NK cell markers associated to activation (60). Vitamin A/retinoic acid increases target expression of natural-killer group 2, member D (NKG2D) ligands in mouse, RAE-1 (60), and humans, MICA/B (61, 62). However, it can activate (60, 62) or inhibit (14, 60, 63) NK activity depending on the cellular context. Hence, their use in clinics must be carefully studied.

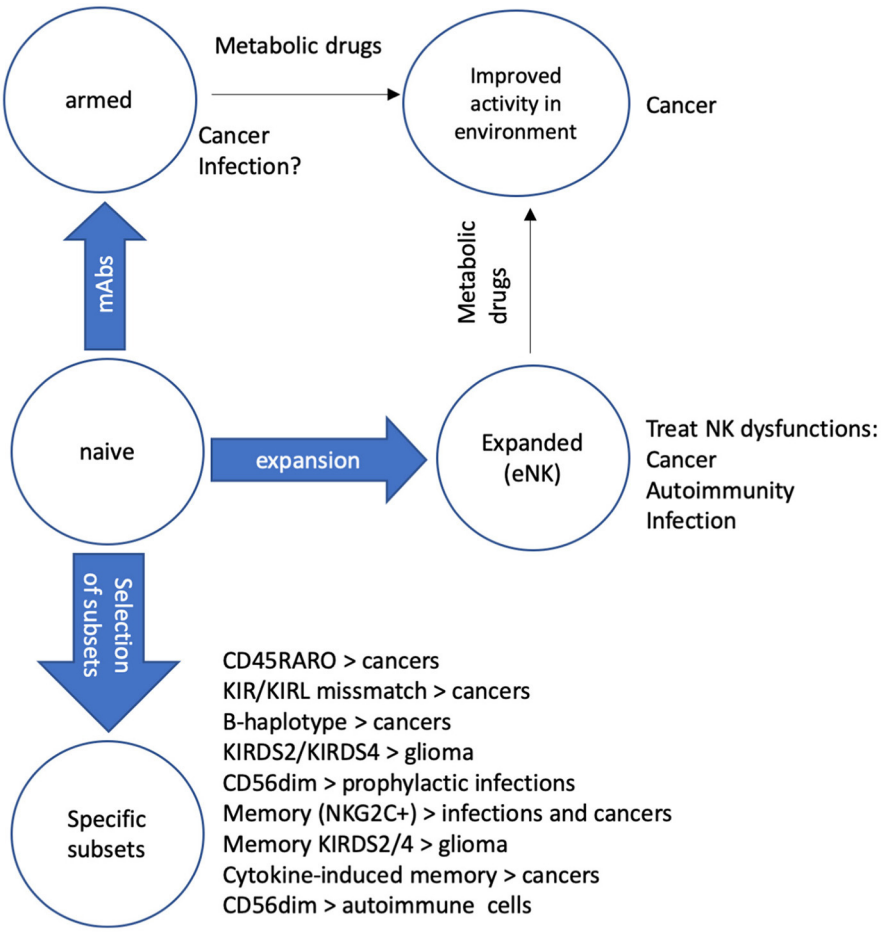

FIGURE 1 | Protocols to recover/improve NK function. We describe several mechanisms to improve NK activity in patients. Naïve NK cells can be "armed" with mAbs that recognize tumor antigens (Ags) to improve their cytolytic activity against cancer cells (6). If specific mAbs against Ags of different pathogens are available, they can be used to arm NK cells to fight infections, mainly in immune compromised patients (54, 55). NK cells can be expanded (eNK) to recover NK cell functions in several diseases such as cancer, autoimmune diseases and infections (32). Treatment of patients with metabolic drugs that modify the microenvironment of the target can increase the function of both "armed" NK and eNK (25, 53). We also believe that it is possible transfer specific NK cell subsets to treat different diseases such as cancers (11–13), including glioblastoma (56) that has a poor prognosis. Some NK subsets, e.x. memory NK cells could also fight infections (57) when engrafted in patients. Finally, in autoimmune diseases could be clinically relevant to replace immature $CD56^{bright}$ NK, which are mostly proinflammatory with mature $CD56^{dim}$ NK, which eliminate activated immune cells. These two NK subsets differentially express various chemokine receptors, which attract them to distinct organs (58, 59). Hence, locally playing with different chemokines should naturally facilitating the recruitment of a specific subset.

ADCC IS NATURAL: NK CELLS AND mAbs

Cell-mediated immune defense includes ADCC. NK only harbor the activating Fcγ receptors CD16a and FcγRIIc, also known as CD32c. This gives NK a preponderant role in ADCC in humans (64). Although not include in the so-called "natural NK cytotoxicity," ADCC is totally a natural physiological process mediated, at least in large part, by NK cells, but involving coordination and crosstalk of different immune cells (64). Through ADCC NK cells can modulate the adaptive immune response and generate long term protection (65).

Differential response to therapeutic mAbs has been reported to correlate with a specific polymorphism in *CD16* (V158F) (66). This polymorphism is associated with differential affinity for mAbs (64). Indeed FcγRs variant play an important role in determining prognosis of monoclonal IgG antibodies (mAbs) therapy (67). Hence, an obvious possibility is using NK cell from donors with the 158V polymorphism, which shows increase affinity for Fc and better prognosis to mAb treatment (64, 67). This engrafted NK should show improved activity after transplantation, mainly when associated to mAb cotherapy. Conversely, different approaches modify the antibody Fc region to increase patient NK cell activity. For example, obinutuzumab, an anti-CD20 mAb, is afucosylated to increase CD16 binding and thereby enhance its ADCC activity (68).

ARMING NK CELLS

As previously described NK cells recognize antibody-opsonized target cells and hence take advantage of the exquisite selectivity of mAb to generate a discriminatory immune response against

target cells. An interesting possibility of increasing NK function is loading mAbs into the NK CD16 Fc receptor, giving them an exogenous selectivity against target cells (**Figure 1**). Recent data show that expanded NK retain RTX on their CD16 at least overnight (6). Moreover, RTX-armed NK show improved cytolytic activity compared to non-armed NK cells. In fact, *in vitro* results using RTX and $CD20^+$ tumor cells deerived from chronic lymphocyte leukemia (CLL) patients do not show any differences on NK cell-mediated ADCC between opsonizing targets or "arming" NK (6).

There are other possibilities to "arm" expanded NK cells, e.g., (i) with activating receptors that enhance their natural anti-tumor capacity; (ii) with chimeric antigen receptors (CAR) that can redirect them toward specific tumor targets (45); or (iii) with death receptor ligands such as a glycosylated form of TNF-related apoptosis-inducing ligand (TRAIL) fusion protein (69). These armed NK cells show improved antitumor function, but these approaches require genetic modification of NK cells, and we do not consider them "natural."

NATURALLY OCCURRING ANTITUMOR NK CELLS: TROGOCYTOSIS AND THE CD45RARO PARADIGM

The NK cell population with antitumor activity has recently been identified (21, 23, 24). In multiple hematological cancer patients there is a population of highly activated $CD56^{dim}CD16^+$ NK cells that have recently degranulated, evidence of killing activity. These cells generally expressed NKp46, NKG2D, and KIRs, whereas expression of NKG2A and CD94 is diminished. They are also characterized by a high metabolic activity and active proliferation. Notably, these NK cells carry, non-NK, tumor cell antigens on their surface, evidence of trogocytosis during tumor cell killing, i.e., they carry CD19 in B cell-derived cancers and CD14 in myeloid-derived cancers (21, 23, 24). The antitumor NK cells are distinguished by their $CD45RA^+RO^+$ phenotype, as opposed to non-activated cells in patients or in healthy donors displaying a $CD45RA^+RO^-$ phenotype (21, 23, 24). Therefore, antitumor NK cells exist (23). Hence, there is the possibility of selectively expand this population. However, *in vitro* expansion does not really produce similar phenotypes to those found in cancer patients. Moreover, NK cell markers change *in vitro* (23, 24). Another possibility would be to exchange the antitumor population of two cancer patients. Notably, CD45RARO cells show strong activity against a different tumor cell (23). This is reminiscent with previous *in vitro* studies showing that NK cells exhibited enhanced cytotoxicity after a prior co-culture with some tumor cells (70, 71). But the *in vivo* interest of using patient CD45RARO cells to treat other patients goes further that this possible "priming" effect. It is known that tumor cells have been immune sculpted by the host immune system (72). This allows them to immune escape and generate cancers. However, the mechanisms of tumor immune escape are usually different between host/tumor pairs. This suggests that tumors will be better recognized by antitumor NK cells of another patient, supporting the exchange of NK cells between patients (**Figure 1**). Obviously, the national health agencies should carefully examine this possibility.

Another possibility is transferring NK cell genotypes that show higher activity against a specific cancer, such as the B-haplotype in AML (34) or KIR2DS2 immunogenotype in glioblastoma (35) (**Figure 1**).

NK AND INFECTIOUS DISEASES

In contrast to cancer patients, CD45RARO populations have not been described in patients with viral infections yet (23, 24). In view of the safety of allogeneic NK in different tumor treatments described above, their use in infectious diseases is clinically relevant. However, if we usually consider "cancer" as a complex disease, what to say about pathogens so diverse as virus, bacteria, and fungi. Remarkably, a growing body of evidences show that NK cells play a major role in the immunity against all these pathogens, not only by their direct killing of pathogens or infected cells, but also by producing cytokines that activates other immune cells (73). In several pathological conditions leading to immunodeficient patients, allogeneic NK cells could support the recovery of enough protection to decrease infectious complication (54). An example of immunodeficiency occurs during hematopoietic stem cell transplantation (HSCT). The recipient's immune system is usually destroyed with radiation or chemotherapy before the stem cell transfer. Hence, infection is a major complication. Currently, clinicians are trying to use certain immune cell types such as granulocytes or infectious-specific T cells to control infection. Although randomized studies failed to demonstrate a significant survival benefit of granulocyte transfusions after HSCT (73). It is known that rapid reconstitution of NK population protects from both infection (54) and tumor relapse (11, 13, 73). Hence, prophylactic engraftment of NK cells in HSCT patients could protect from infection and decrease relapse, with the advantage that NK cells, and not T cells, target a broad range of pathogens (**Figure 1**).

If the use of allogeneic NK cells in patients at risk, i.e., immunocompromised patients as those described above, clinically sounds, this is not the case of immunocompetent individuals. Obviously clinical results of allogeneic NK cell engraftment in these patients are lacking. Despite this, we want to discuss certain medical situations in which it could be useful. Again, with the assumption that the transplant would not be toxic to the patient.

First, the severity of influenza can be associated with transient T and NK cell deficiency (55) and with specific haplotypes of killer-immunoglobulin-like receptors (KIRs) (74). Engraftment of NK cells from donors with such haplotypes can improve the prognostic of humans that barely respond to influenza vaccine. A similar approach, i.e., engraftment of allogeneic NK cells, could be clinically relevant to treat patients infected with flavivirus, which includes viruses such as West Nile, yellow fever, dengue, Zika, and Chikungunya. Flavivirus-induced diseases are currently generating major health problems and, interestingly, NK cells play a central role in controlling these viruses (75). In other deadly viral infection such as Ebola, which also decreases peripheral NK cell numbers, the transplant of NK cells could

be inefficient because Ebola virus uses specific NK evasion mechanism (76) and can modulate NK function to increase viral pathogenicity (77). The possible use of virus-specific memory NK cells will be discussed below.

Second, NK cells, through release of perforin and granulysin, kill a variety of bacteria including *Mycobacterium tuberculosis*, *Bacillus anthracis*, *Escherichia coli*, *Salmonella typhi,* and *Trypanosoma congolense* (73, 78). NK cells can also eliminate host cells infected with intracellular bacterial pathogens by engagement of target cell death receptors, such as Fas- FasL and TNF-related apoptosis-inducing ligand (TRAIL) (73). The transfer of allogeneic, expanded, NK cells in patient infected with those bacteria and with a bad prognostic could have an obvious clinical benefit.

Third, allogeneic NK cells could also be useful for fungal infections of poor prognosis due to their direct effect against a number of pathogenic fungi including mucormycetes, *Aspergillus fumigatus*, *Cryptococcus neoformans,* and *Candida albicans*. In addition, NK cells produce a number of cytokines that activate the antifungal activity of other immune cells (73).

MEMORY NK CELLS

Viral-infected patients have NK subsets that are associated to antiviral immunity and could be used for clinical purposes. Human cytomegalovirus (HCMV) infection promotes expansion of NKG2C$^+$ NK cells with memory-like properties (79, 80). Furthermore, NK cells expressing high levels of NKG2C and CD57 are associated with prior HCMV infection. Certain cytokines such as IL-12, which is produced by CD4$^+$ monocytes, are mandatory for NKG2C$^+$ cell expansion (81). However, there is a lack of evidence concerning their specific effect against HCMV itself or if there is a recall response to HCMV reactivation (82).

These HCMV-specific NK cells can originate from CD16-induced memory-like NK cells and hence they can be waked up by HCMV antibodies (57). Once activated, these cells could not only attack HCMV-infected cells, but also other NK cell targets such as transformed cells. Subsequently, they could be transferred to patients lacking them to generate the desired immunity (**Figure 1**). Direct transfer of anti-HCMV antibodies would probably not work because these antibodies presumably do not mediate in the initial generation of NKG2C$^+$ "adaptive" NK cells in HCMV-seronegative individuals (83).

Most glioblastoma express HCMV proteins and HCMV infection imprint NK cells. In addition, KIR2DS2+ and KIR2DS4+ are more potent killers that bulk NK cells in glioblastoma cells (35). Remarkably, CMV impacts disease progression in glioblastoma and the KIR allele KIR2DS4*00101 is an independently prognostic of prolonged survival (56). Hence, the transfer of KIR2DS4*00101 NK cells could specifically improve prognosis of glioma patients.

Another possibility is generating "memory"-like NK cells by incubation with different cytokine cocktails, e.g., IL-2/IL-15/IL-18 (57, 82). Some of these cytokines are already part of the current cocktails to amplify and activate human NK cells *in vitro* as described earlier. In fact, several of these protocols also used accessory, target, cells to drive NK cell expansion and/or activation. The target cell contact-dependent priming signals to enhance NK cell activation has already been described, although the priming stimulus is unknown (57). This has not stopped their clinical test in clinics (12).

In summary, exploiting NK cells with memory-like properties might increase the efficacy of these cells and help their clinical development. However, it is uncertain if current protocols to produce *in vitro* expanded NK cells are not really generating "memory-like" NK cells, and hence, the use of "memory-like" NK cell is perhaps already used in clinical studies.

RECOVERING NK ACTIVITY IN AUTOIMMUNE DISEASES BY REPLACING ENDOGENOUS NK CELLS

NK cells from patients of several autoimmune diseases present populations that can contribute to disease progression. In other cases, endogenous NK cells are defective, e.g., in cytotoxicity, due to genetic or environmental facts. Hence, engrafting NK subsets with proper activity could rescue NK activity and improve prognosis. Below we discuss some specific diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and systemic lupus erythematosus (SLE). However, similar approaches could also target type I diabetes (T1D) and Sjögren's syndrome (58).

RA patients accumulate immature NK cells in damaged joints. Sinovial fluid (SF) NK (sfNK) cells derived from these patients are enriched in the CD56^{bright} population (84). Moreover, sfNK produce more IFNγ and TNFα after interleukin-15 activation (84, 85). IL-15, which is present in the SF of RA patients, correlates with disease severity and is important in disease progression (59). Hence all this may contribute to the production of proinflammatory cytokines and long-term inflammation (58).The sfNK cell subset, high CD56, CD94/NKG2A, CD69, and NKp44 and low CD16, is unlike any population documented in any other organ and is enriched in patients with erosive deformative RA (DRA) (84, 85). The percentage of total NK cells was doubled in the peripheral blood and tripled in SF of DRA, as compared to non-deformative RA (NDRA), patients (85). Other characteristics of these sfNK in RA are almost absent KIR expression, low CD57 and high natural killer cell p46-related protein (NKp46) (85). Probably the chemokine receptors specifically expressed by immature NK cells facilitate their infiltration into the damaged joints and favor RA damage exacerbation (85). Interestingly, the sfNK CD56^{bright} population express CD16, something that is unique, although its functionality was not investigated (85). Since sfNK may play an important role in destruction of joints, which should implicate their IFNγ and TNFα production, it would be interesting to replace the immature sfNK with mature CD56^{dim} cells (**Figure 1**). An interesting possibility is using those protocols to produce *in vitro* expanded NK cells described earlier. Although these NK cells present high CD56 levels, they possess all characteristics of mature and activated NK such as KIR and NKG2D expression [e.g., (6)]. The engraft of these

cells in damaged joints could reverse the damaging effect of the autologous $CD56^{bright}$ cells.

MS is an autoimmune inflammatory disease affecting the central nervous system (CNS). Autoreactive CD4 T cells targeting myelin components are critical mediators. NK cells can control inflammation by killing activated, autoimmune, T cells (58). Activated T cells increase expression of the death-receptor Fas. In patients in remission, NK highly express Fas ligand (FasL), which can eliminate autoreactive T cells through Fas/FasL interactions (86).

During relapse the $FasL^{high}$ NK population is lost (86). The site of autoimmunity, i.e., the cerebrospinal fluid, is enriched in immature $CD56^{bright}$ NK subset, whereas this population is reduced in peripheral blood (87). Daclizumab, an anti-IL-2Rα antibody, ameliorates CNS lesions with a decrease in blood CD4 T cells and increase in blood $CD56^{bright}$ NK (88). Hence, current knowledge on the biology of MS suggest that engraftment of a mature, cytolytic, $CD56^{dim}$ subset could facilitate elimination of autoreactive T cells (**Figure 1**). Although a possible negative effect cannot be ruled out due to the presence of NKG2D ligands in oligodendrocytes, astrocytes and microglia (58).

SLE is an autoimmune disorder characterized by production of autoantibodies against DNA and nuclear proteins. Like in RA, there is a polyclonal B-cell activation and expansion. NK cell deficiency correlates with SLE in humans and in mouse models of the disease (58). Again, SLE patients show an increase in the proportion of blood $CD56^{bright}$ NK cells (89). In addition, NK-dependent cytotoxicity decreases (58). Interestingly in pediatric patients, who show the same NK defects (90), the impaired activity is observed at diagnosis (90). Like in previous described autoimmune diseases reconstitution of a mature $CD56^{dim}$ population in SLE patients could improve their prognosis. These approaches requiring the engraftment of "missing" NK cell subsets need proper allogeneic NK recruitment into the target organ. An obvious solution is local engraftment. Another possibility is using a chemokine cocktail. The two NK cell subsets, i.e., $CD56^{bright}$ and $CD56^{dim}$, differentially express various chemokine receptors, which attract them to distinct organs (58, 59). Hence, locally playing with different chemokines should naturally facilitating the recruitment of a specific subset. In anyway, reconstitution of NK cell activity in periphery should improve patient prognosis in these diseases heavily dependent on NK cell function.

NK CELL LINES: ARE THEY NATURAL?

The difficulties for purify, isolate, expand, and transduce primary NK cells for therapeutic applications led researchers to also focus on NK-cell lines such as NK-92 (NK-92® ATCC® CRL-2407™ and NK-92® MI ATCC® CRL-2408™). There are other NK cell lines, but their antitumor cytotoxicity is questioned (91). In any case, our discussion here on NK-92 cells should be valid for new NK cell lines that could reach the clinic. NK-92 phenotype is $CD3^{-}CD56^{+}CD16^{-}$ and display cytotoxicity against a wide range of human primary leukemias, e.g., B-ALL and CML and leukemic cell lines *in vitro* and in SCID mouse models (92). Stable expression of mouse and human CD16 gives ADCC to NK-92 cells and generates the cell lines NK-92 ^{mCD16} and NK-92 ^{hCD16}, respectively (93). In addition, they are a renewable resource to generate CAR-NK-92 cells. In line with our previous comments we will not discuss about these transduced cells. In contrast, non-modified NK cell lines show therapeutic effect without the need of genetic modifications (91). However, transformed cell lines present worries, such as uncontrolled growth, which require irradiation before infusion into patients. This suppress cell proliferation while, hopefully, maintaining enough cell cytotoxic activity. NK-92 cells have completed phase I trials in cancer patients, e.g., NCT00900809 and NCT00990717. Results show that irradiated NK-92 cells are safe even at very high doses with minimal toxicity in patients with refractory blood cancers (94). In addition, they show clinical benefits with 2 out of 12 patients showing complete response (94).

CONCLUSION

In a challenging clinical environment with the arrival of "new" cell-therapy products, NK present several advantages and inconveniences. Their clinical improvement by "natural" means that can easily be accepted by natural agencies will greatly favor their use.

AUTHOR CONTRIBUTIONS

All authors were involved in preparing and writing the manuscript.

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Indirect Impact of PD-1/PD-L1 Blockade on a Murine Model of NK Cell Exhaustion

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The induction of exhaustion on effector immune cells is an important limiting factor for cancer immunotherapy efficacy as these cells undergo a hierarchical loss of proliferation and cytolytic activity due to chronic stimulation. Targeting PD-1 has shown unprecedented clinical benefits for many cancers, which have been attributed to the prevention of immune suppression and exhaustion with enhanced anti-tumor responses. In this study, we sought to evaluate the role of the PD-1/PD-L1 pathway in murine natural killer (NK) cell activation, function, and exhaustion. In an *in vivo* IL-2-dependent exhaustion mouse model, neutralization of the PD-1/PD-L1 pathway improved NK cell activation after chronic stimulation when compared to control-treated mice. These cells displayed higher proliferative capabilities and enhanced granzyme B production. However, the blockade of these molecules during long-term *in vitro* IL-2 stimulation did not alter the progression of NK cell exhaustion (NCE), suggesting an indirect involvement of PD-1/PD-L1 on NCE. Given the expansion of CD8 T cells and regulatory T cells (Tregs) observed upon acute and chronic stimulation with IL-2, either of these two populations could influence NK cell homeostasis after PD-L1/PD-1 therapy. Importantly, CD8 T cell activation and functional phenotype were indeed enhanced by PD-1/PD-L1 therapy, particularly with anti-PD-1 treatment that resulted in the highest upregulation of CD25 during chronic stimulation and granted an advantage for IL-2 over NK cells. These results indicate a competition for resources between NK and CD8 T cells that arguably delays the onset of NCE rather than improving its activation during chronic stimulation. Supporting this notion, the depletion of CD8 T cells reversed the benefits of PD-1 therapy on chronically stimulated NK cells. These data suggest a bystander effect of anti-PD1 on NK cells, resulting from the global competition that exists between NK and CD8 T cells for IL-2 as a key regulator of these cells' activation. Thus, achieving an equilibrium between these immune cells might be important to accomplish long-term efficacy during anti-PD-1/IL-2 therapy.

Keywords: NK, exhaustion, chronic stimulation, PD-1/PD-L1 pathway, CD8

INTRODUCTION

Natural killer (NK) cells are a subset of innate lymphocytes that have the property of destroying target cells without prior immune sensitization in an MHC unrestricted manner (1, 2). There is strong evidence for the importance of NK cells in the eradication of cancer cells, and thus, NK cell-based therapy has been explored in a number of cancers (3–5). Indeed, the adoptive transfer of NK cells after *ex vivo* activation has proven to be safe and well-tolerated in many cancers (4). Unfortunately, clinical benefits have not been observed in all cases (2, 6). Therefore, new therapeutic strategies to fully exploit NK cell cytotoxic potential are needed.

Impaired NK cell function due to the presence of immunosuppressive cells [regulatory T cells (Tregs) or myeloid-derived suppressor cells] or cytokines (TGFβ, IL-10), downregulation of activating receptors, or increase of inhibitory receptors accounts for the limitations of NK cell-based therapy (1, 7, 8). Furthermore, NK cell exhaustion (NCE) has been identified as a self-regulatory mechanism responsible for the induction of a dysfunctional phenotype to prevent exacerbated immune responses under chronic stimulatory conditions (9). Importantly, exhaustion, described in both NK and T cells, represents a gradual process that causes a reduction in the proliferative and functional capacities of immune cells that can ultimately culminate in the elimination of the effector cells. Thus, this phenomenon has become a crucial component in the immune evasion mechanisms used by tumor and viruses to circumvent immune responses, as exhausted NK and T cells have been described after tumor exposure and chronic viral infections (7, 9–11).

An exhausted NK cell has been defined as a NK cell incapable of responding to further stimuli with downregulation of the activating transcription factors eomesodermin (Eomes) and T-box transcription factor TBX21 (T-bet), along with lower expression of activating receptors while also showing an upregulation of inhibitory receptors (7, 9, 10, 12, 13). We have recently demonstrated that the induction of the ataxia-telangiectasia mutated (ATM) DNA repair damage pathway during prolonged NK cell proliferation played a critical role in the exhaustion process (9). NKG2D downregulation, likely caused by internalization due to its binding to the stress molecule MULT1, which is upregulated upon NK activation, had a partial role in NCE as well (9). Felices et al. also showed metabolic defects in human exhausted NK cells, which were characterized by a reduction in the mitochondrial respiration profile dependent on fatty acid oxidation. This effect was prevented by mechanistic target of rapamycin (mTOR) signaling inhibition (10).

Currently, therapeutic strategies that exploit the ability of immune cells to target cancer cells have become a promising and effective approach, such as with immunomodulatory monoclonal antibodies (mAbs). Among them, mAbs that neutralize the action of checkpoint inhibitors, including PD-1 and CTLA-4 among others, have become quite popular given their tremendous success, alone or combined with other strategies, in many types of cancers (14–18). The mechanisms of action for blocking checkpoint inhibitors are mainly attributed to an increase in effector immune cells with potent antitumor responses due to a reduction of immunoregulation (14, 19).

The role of the PD-1/PD-L1 axis in the regulation of NCE, unlike in T cells (14, 20), is poorly understood, particularly in mouse NK cells (21, 22). Many studies have shown that human NK cells do, in fact, express PD-1 (23–25), but in some cases, this expression has been correlated with poor prognosis (26) and an exhausted phenotype (27, 28), whereas other studies have suggested that PD-1^+ NK cells present a higher activation phenotype that is only suppressed by PD-L1-expressing NK cells (29). In mouse, however, the expression of PD-1 on NK cells is more restrictive, despite the fact that some studies have shown a contribution of the NK cell compartment in PD-1 blockade therapy (22, 30). According to Hsu et al., PD-1^+ NK cells were mainly limited to the tumor microenvironment (22). Tumor-infiltrating NK cells were highly susceptible to suppression by PD-$L1^+$-expressing tumor cells (22). During chronic cytomegalovirus infection in mice, we also observed an upregulation of PD-1 on NK cells, particularly in the salivary gland (9). Nevertheless, anti-PD-1 and anti-PD-L1 therapy have shown potential benefits in rescuing and/or improving NK cell functional capacities and improving anti-tumoral and anti-viral responses (26, 28, 29, 31–34), although the precise mechanisms are incompletely understood.

In this study, we aim to evaluate the role of the PD-1/PD-L1 pathway in the induction of NCE. Using an *in vivo* chronic stimulation exhaustion murine model, we found that PD-1 blockade indirectly impacts *in vivo* IL-2-induced NCE by modulating CD8 T cell expansion.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 (H-2^b) mice were purchased from Jackson Laboratories (Sacramento, CA) or Harlan Laboratories (Barcelona, Spain). C57BL/6 FoxP3 mutant mice expressing diphtheria toxin receptor (FoxP3 DTR) were a kind gift from Dr. Rudensky and bred in the Stanford animal facility. Female mice were used at 8–12 weeks of age and housed under specific pathogen-free conditions. All animal protocols were approved by the IACUC at Stanford University and University of Navarra.

In vivo NK Cell Stimulation Model

Mice were treated with high doses (0.5–1 million IU) of recombinant human IL-2 (National Cancer Institute repository, Frederick, MD) or PBS as previously described (9) (**Figure 1A**). In some experiments, 200 μg of anti-PD-1 (clone RMP1-14, BioXcell, Lebanon, NH), anti-PD-L1 (clone 10F.9G2), anti-CD8β (clone Lyt 3.2, BioXcell), or control rat gamma globulin (rIgG; Jackson ImmunoResearch) was given a day prior to the beginning of IL-2 and 5 days after the first mAb treatment dose.

In vitro NK Cell Stimulation Model

Single-cell suspensions from bone marrow (BM) and spleens of mice were T-cell-depleted using CD90-positive selection kit (StemCell Technology, Vancouver) according to the manufacturer's instructions and cultured in RPMI

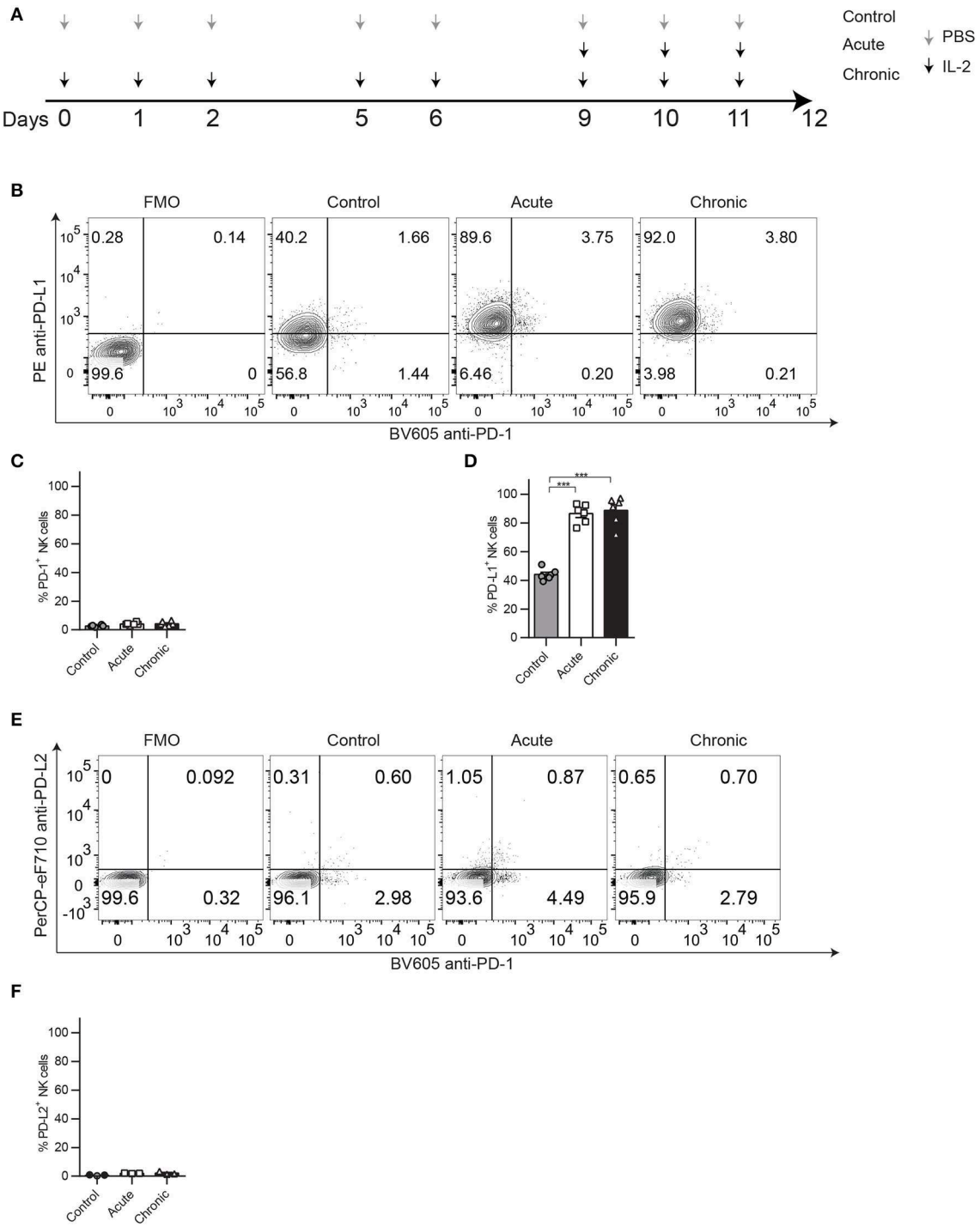

FIGURE 1 | PD-1 and PD-L1 expression patterns on NK cells after chronic IL-2 stimulation. C57BL/6 were treated acutely or chronically with IL-2 or PBS (control) following the regimen dose explained in **(A)** and spleens were collected 24 h after the last treatment to analyze NK cell phenotype and function by flow cytometry. **(A)** IL-2 NCE mouse model regimen dose. **(B)** Representative dot plots on gated NK cells ($CD45^+TCR\beta^-NK1.1^+$) of PD-1 and PD-L1 are shown. **(C,D)** Total percentage of PD-1 **(C)** and PD-L1 **(D)** is shown for gated NK cells. **(E)** Representative dot plots on gated NK cells ($CD45^+TCR\beta^-NK1.1^+$) of PD-1 and PD-L2 are shown. **(F)** Total percentage of PD-L2 is shown for gated NK cells. Data are representative of five independent experiments with 3–4 mice per group (mean ± SEM). One-way ANOVA was used to assess significance. Significant differences are displayed for comparisons with the acute group ***$p < 0.001$).

complete media at 37°C with 5% CO_2 and with 1000 IU/ml of IL-2. On day 3 of culture, cells were treated with 20 μg/ml of anti-PD-1, anti-PD-L1 (BioXcell), or controls when indicated. Activated lymphocyte adherent killer cells, which represent 90% of purified NK cells (35), were collected at multiple time points (day 0: control, day 4: acute; days 7 and 9: chronic) to analyze NK cell activation and function. When indicated, total cells (no T cell depletion was performed) were cultured under the same conditions.

Analysis of NK Cell Phenotype and Function

For the *in vivo* model, spleens were collected 24 h after the last IL-2 treatment and processed as previously described (9, 36). For the *in vitro* model, NK cell phenotype and function were analyzed at the indicated time points. Cell suspensions were analyzed for activating transcription factors (Eomes and T-bet), NK cell activating (NKG2D, Thy1.2, Ly49G2, DNAM-1) and inhibitory (TIGIT, CD96, PD-1, PDL-1) markers, as well as proliferation marker Ki67 as previously described (9, 36). The T cell compartment was also analyzed. Foxp3/TF staining buffer kit (eBioscience, San Diego, CA) was used according to the manufacturer's instructions. For a detailed description of the mAbs used, refer to **Supplemental Table 1**.

Intracellular staining was performed to detect Granzyme B (GranB) and IFNγ production after NK cell stimulation for 4 h with 10 μg/ml plate-bound anti-NK1.1 as previously described (9).

Stained cells were analyzed with an LSRII cytometer (Becton Dickinson, San Jose, CA) or Cytoflex LX (Beckmann Coulter, Indianapolis, IN). Fluorescence minus one (FMO) or biological comparison controls were used for cell analysis.

Data Analysis

Principal Component Analysis

PCA was performed using the RStudio ggfortify package on the values obtained by flow cytometry. The two dominant principal components were plotted against one another to assess the relationships between the different treatments, and the PCA loading vectors (eigenvectors) were also represented.

T-SNE Analysis

t-Distributed Stochastic Neighbor Embedding (tSNE) dimensionality reduction algorithm was performed to analyze multiparameter flow cytometry data using Cytobank (Santa Clara, CA). Populations of T cells, MDSC, B cells, and NK cells were plotted, and the expression for Eomes and Ki67 was shown for these populations using this method.

Statistical Analysis

Each experiment was performed at least two times with 3–4 mice per group. Student's two-tailed *t*-test, one-way ANOVA (Bonferroni post-test analysis), or two-way ANOVA (Bonferroni post-test analysis) was used when appropriate to determine statistical significance (Graphpad Prism 6, La Jolla, CA). *p*-values were considered statistically significant when $p < 0.05$.

RESULTS

Impact of PD-1/PD-L1 Neutralization on NCE After Chronic IL-2 Stimulation

In a recent publication, we have demonstrated that chronic stimulation of NK cells leads to a phenotype characteristic of exhaustion defined by impaired function and downregulation of markers associated with activation and upregulation of inhibitory receptors. We showed in different models that exhaustion could be detected by the phenotypic analysis of Eomes, NKG2D, and KLRG1 expression, which was linked to reduced proliferative and functional capacities of NK cells (9). Upon IL-2-induced chronic stimulation (**Figure 1A**), despite not observing relevant expression of PD-1 (**Figures 1B,C**), an inhibitory receptor frequently associated with both NK and T cell exhaustion, we did consistently observe an upregulation of its ligand, PD-L1, compared to control-treated groups (**Figures 1B,D**). This phenomenon was also induced by chronic stimulation with IL-15 (**Supplemental Figure 1A**) or poly I:C (**Supplemental Figure 1B**). In contrast, PD-L2, another ligand for PD-1, was barely detected on NK cells (**Figures 1E,F**). Chronically stimulated NK cells also displayed reduced proliferative capacities measured by Ki67 expression (**Supplemental Figure 2A**) and reduced abilities to respond to NK1.1 stimulation, exemplified by lower production of granzyme b (GranB) (**Supplemental Figure 2B**) and IFNγ (**Supplemental Figure 2C**).

Another important characteristic during NCE, that we have previously observed, was a drastic reduction in the total numbers of NK cells recovered after chronic stimulation when compared to acute stimulation (9). Given the immunoregulatory role of PD-1, as well as the high levels of PD-L1 surface expression on NK cells, we next evaluated the impact of the neutralization of the PD-1/PD-L1 pathway in the induction of NCE during chronic stimulation. Mice were treated with neutralizing mAbs against PD-1 or PD-L1 before IL-2 treatment, and the phenotypic and functional analysis of NK cells was determined by flow cytometry (**Figure 2**). A principal component analysis (PCA) of all the parameters studied revealed a clear differentiation between control and acutely stimulated NK cells driven by the expression of activating and inhibitory markers according to the PC1 and PC2 such as Eomes, T-bet, KLRG1, Thy1.2, and GranB among others with no major differences between rIgG-, anti-PD-L1-, and anti-PD-1-treated NK cells (**Figure 2A**, **Supplemental Figures 3A,B**). rIgG-treated chronically stimulated NK cells clustered closer to unstimulated NK cells as previously described (9) (**Figure 2A**). However, anti-PD-L1- or anti-PD-1-treated chronically stimulated NK cells were highly influenced by the parameters involved in PC1 (Thy1.2 and GranB), and their location was somewhat intermediate between chronic and acute stimulated NK cells (**Figure 2A**, **Supplemental Figures 3A,B**). These differences in the phenotype of NK cells could explain the slight increase in the percentage and the significant increase in the total number of NK cells obtained from spleens of IL-2 chronically stimulated mice after PD-1/PD-L1 blockade (**Figures 2B,C**), which hints toward a reduction in exhaustion.

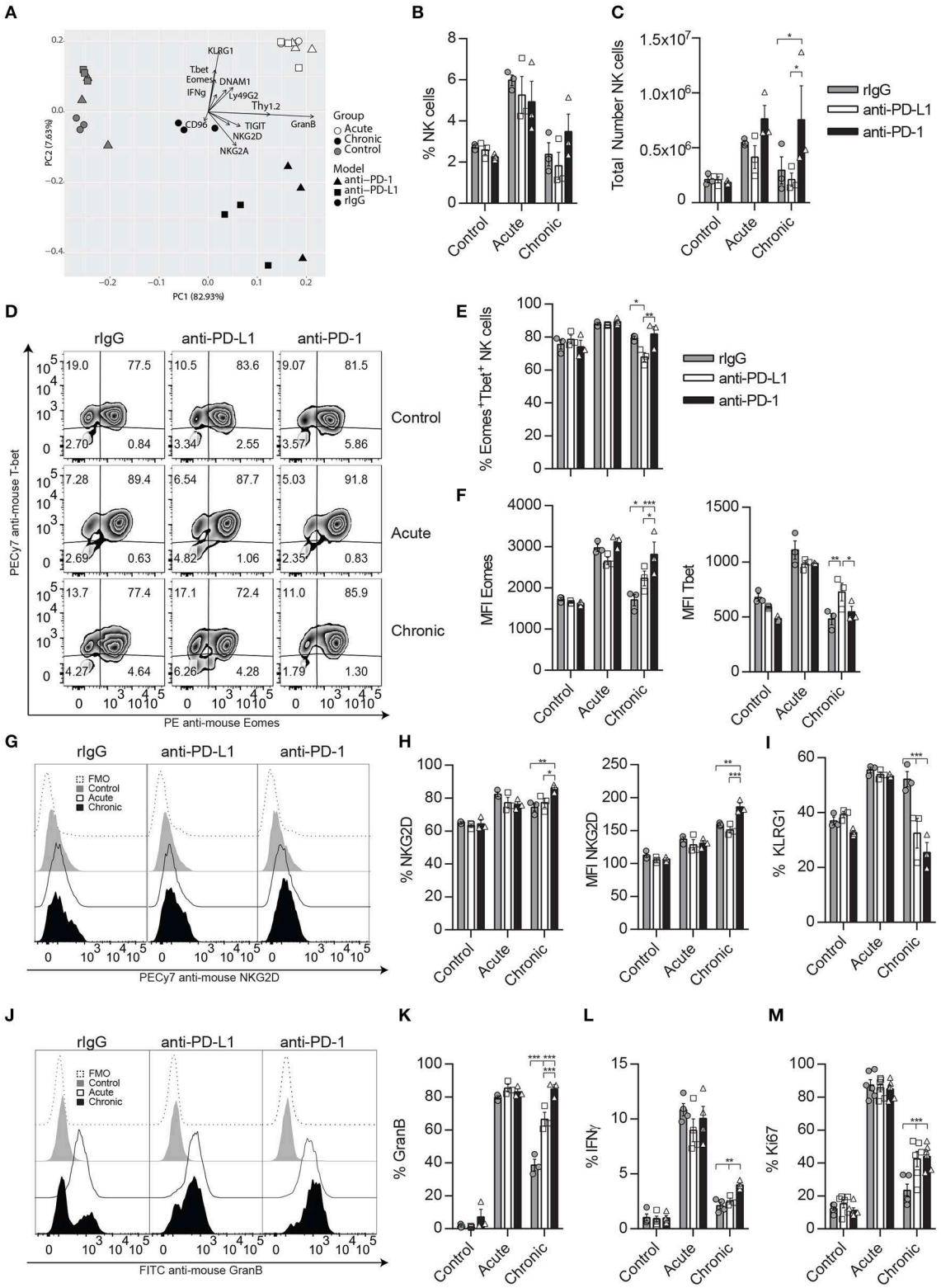

FIGURE 2 | Neutralization of the PD-1/PD-L1 axis ameliorates NK cell exhaustion phenotype after chronic IL-2 stimulation. C57BL/6 mice were given two doses of anti-PD-1, anti-PD-1, or rIgG a day prior to starting IL-2 regimen dosage (**Figure 1A**) and 5 days after the initial mAb doses. Spleens were again collected 24 h after the last IL-2 treatment as previously explained. **(A)** Principal component analysis (PCA) of rIgG-treated (circle), anti-PD-L1-treated (square) and anti-PD-1-treated

(Continued)

FIGURE 2 | (triangle) NK cells after control (gray), acute (white), or chronic (black) IL-2 stimulation is shown. The data represent the weight that the flow cytometer analyzed NK cell parameters (loading vectors: eigenvectors) have on the PCA distribution. **(B,C)** Total percentage **(B)** and the total number **(C)** of NK cells ($CD45^+TCR\beta^-NK1.1^+$) is shown after IL-2 stimulation in the spleen. **(D–F)** Representative dot plots **(D)** and MFI **(E,F)** expression for Eomes and T-bet are shown on gated $Eomes^+$ or $T\text{-}bet^+$ NK cells, respectively. **(G–I)** Representative dot plots **(G)**, total percentage **(H)**, and MFI **(I)** are shown for NKG2D on gated NK cells. **(I)** Total percentage of $KLRG1^+$ NK cells is shown on gated NK cells **(J–L)** Representative histograms **(J)** and the total percentage of GranB **(K)** as well as IFNγ **(L)** are shown for gated NK cells ($CD3^-CD49b^+$) after NK1.1 stimulation. **(M)** Ki67 expression is shown for gated NK cells. Data are representative of three independent experiments with 3–4 mice per group (mean ± SEM). Two-way ANOVA was used to assess significance. Significant differences are displayed for comparisons with the rIgG-treated group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

A detailed examination of the transcription factors influencing NCE (Eomes and T-bet) showed that the percentage and median fluorescence intensity (MFI) of Eomes were significantly increased in anti-PD-L1- and/or anti-PD-1-treated NK cells (**Figures 2D–F**). Similarly, NKG2D percentage and MFI were also enhanced after anti-PD-1 treatment in chronically stimulated NK cells when compared to rIgG-treated NK cells (**Figures 2G,H**), while the percentage of KLRG1 was diminished in both anti-PD-L1- and anti-PD-1-treated chronically stimulated NK cells when compared to rIgG (**Figure 2I**). The expression of the inhibitory receptor Ly49G2, previously associated with an activation phenotype (37) was also significantly upregulated after chronic stimulation of anti-PD-1-treated NK cells compared to rIgG-treated NK cells (**Supplemental Figures 3C,D**). In agreement with a superior activation phenotype, the activation markers Thy1.2 and DNAM1 were significantly upregulated as well, particularly in the case of anti-PD-1 treatment (**Supplemental Figures 3C–F**). In contrast, the expression of NK cell inhibitory receptors (NKG2A, TIGIT, and CD96) was not altered by PD-1/PD-L1 inhibition (**Supplemental Figures 3G–I**).

Notably, *ex vivo* NK cell re-stimulation with anti-NK1.1 induced an increase of GranB production by chronically stimulated NK cells treated with anti-PD-L1 or anti-PD-1 compared to rIgG (**Figures 2J,K**). Indeed, GranB production in the case of anti-PD-1-treated NK cells reached levels similar to those observed during acute stimulation (**Figures 2J,K**), results that correlated with the lytic capacities of anti-PD-1 treated NK cells (**Supplemental Figure 3J**). IFNγ production was just mildly increased in anti-PD-1-treated chronically stimulated NK cells (**Figure 2L**, **Supplemental Figure 3K**). Interestingly, the phenotypic and functional changes in chronically stimulated NK cells treated with anti-PD-L1/PD-1 suggested a reduction of exhaustion, also signified by a retention in their proliferative capacities measured by an increase of Ki67 expression when compared to chronically stimulated rIgG-treated NK cells (**Figure 2M**).

To determine if the implication of PD-1 therapy on NCE was exerted directly, we next evaluated the impact of PD-1 neutralization during long-term *in vitro* IL-2 stimulation. Correlated with the lower expression of PD-1 detected by a non-competitive binding antibody (**Figure 3A**), no significant differences were observed in GranB and IFNγ production upon NK1.1 stimulation (**Figures 3B–D**). Accordingly, except for a small decrease on the MFI of T-bet and Eomes at the peak of activation on day 4 (**Figures 3E–G**), no differences on T-bet, Eomes, NKG2D, KLRG1, or Ki67 were detected after anti-PD-1 treatment during long-term *in vitro* IL-2 NK cell activation (**Figures 3E–J**), unlike what has previously been demonstrated with the inhibition of the ATM DNA repair damage pathway (9). NK cell lytic capacities were not altered by anti-PD-1 treatment as well (**Figure 3K**). Similarly, blockade of PD-L1 did not cause any changes on NK cell phenotype and function (**Supplemental Figure 4**). These results suggest that PD-1 does not directly affect NCE despite observing a mild reduction of exhaustion when administered *in vivo*.

Depletion of Treg Boost CD8 T Cell Expansion and Activation

In order to determine if a crosstalk between immune cell populations exists during PD-L1/PD-1 therapy that might influence NK cells, the T cell compartment was also evaluated. As expected, the percentage of CD8 and Tregs ($TCR^+CD4^+Foxp3^+$) was increased during acute and chronic IL-2 stimulation, but no significant differences were detected between treated groups within each stimulatory condition (**Figure 4A**). When the total number of T cells was analyzed, an increase of CD8 T cells was observed during acute IL-2 stimulation, and these numbers were maintained after chronic stimulation (**Figure 4B**), unlike conventional CD4 T cells, whose numbers did not suffer big changes during IL-2 stimulation (**Figure 4C**). Similar to CD8 T cells, and equally expected, Tregs underwent a strong expansion upon IL-2 stimulation when compared to unstimulated control mice (**Figure 4D**). However, anti-PD-1/PD-L1 therapy did not seem to majorly alter the number of either CD8 T cells or Tregs during IL-2 treatment. When different Treg activation markers were analyzed, we observed that PD-1/PD-L1 and/or IL-2 therapy did not affect the PD-1 and CD69 expression on Tregs (**Figures 4E–G**).

Many studies have demonstrated that in addition to preventing CD8 T cell exhaustion, anti-PD-1/PD-L1 therapy works through the suppression of the PD-1 dependent inhibitory properties of Tregs, resulting in an increase of CD8 T cell activation and function (11, 19, 38–40). In NK cells, Tregs have also shown to suppress NK cells through a variety of mechanisms (36, 41, 42).

In our IL-2 model, Treg expansion is likely benefited by the constitutive expression of the high-affinity IL-2Rα CD25, thus exerting stronger suppression toward CD8 T cells and NK cells. In order to explore how Tregs could affect NK and CD8 T cell distribution and activation status and see if these results correlate with the ones obtained during PD-L1/PD-1 therapy where Tregs immunosuppression efficacy might be diminished, we depleted Tregs prior to acute IL-2 stimulation using Foxp-3-expressing

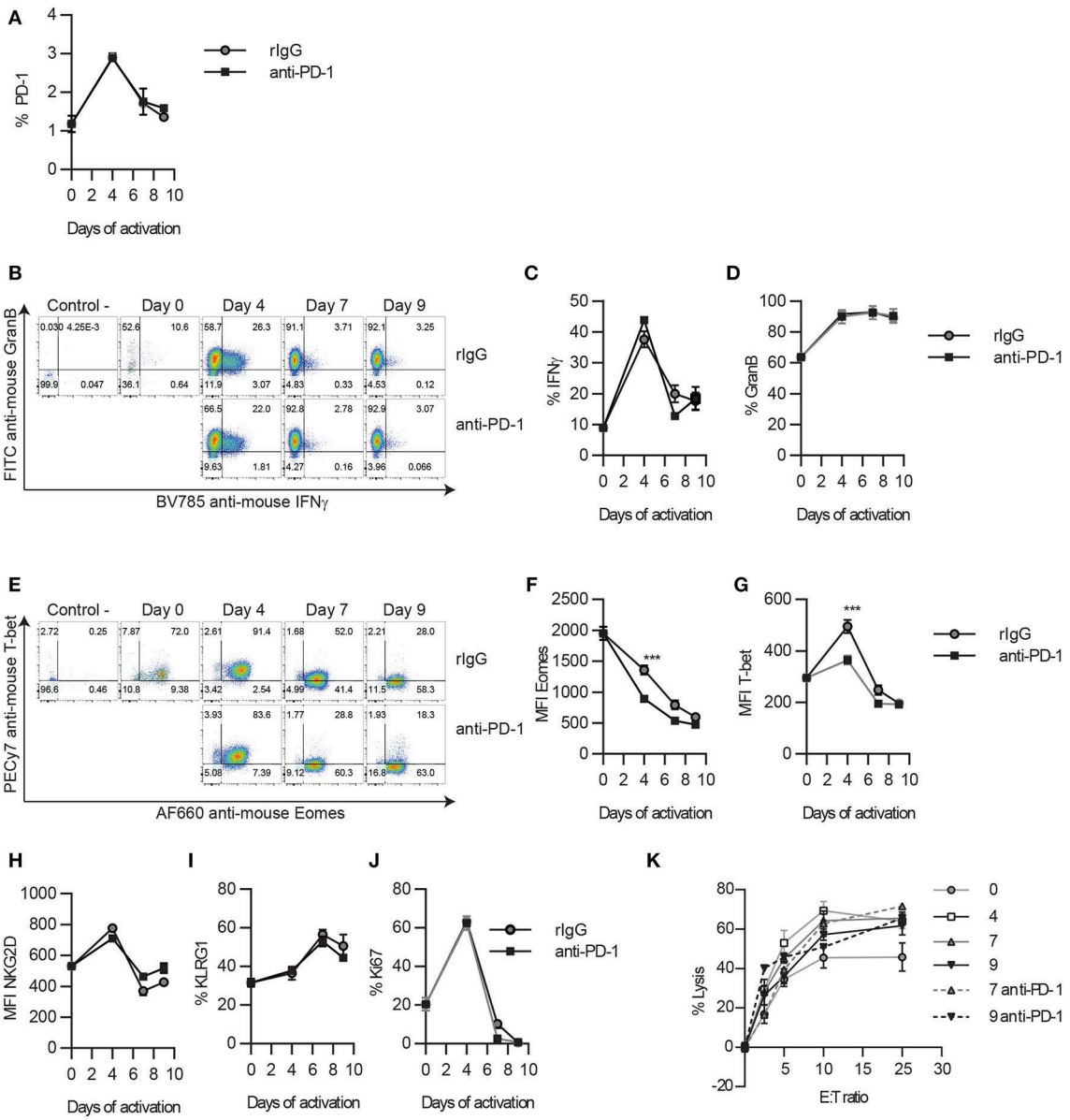

FIGURE 3 | PD-1 blockade does not alter the timeline for NK cell exhaustion during *in vitro* long-term stimulation. Thy1.2$^-$ NK cells were cultured with IL-2 in the presence of anti-PD-1 or isotype control, as explained in the Materials and Methods section. Adherent NK cells were collected at different time points and analyzed by flow cytometry. **(A)** The expression of PD-1 on NK cells is shown. **(B–D)** Representative dot plots **(B)**, and the total percentage of GranB **(C)** and IFNγ **(D)** producing NK cells after NK1.1 stimulation are shown. **(E)** Representative dot plots of Eomes and T-bet are shown for gated NK cells. **(F,G)** The MFI of Eomes **(F)** and T-bet **(G)** on Eomes$^+$ or T-bet$^+$ NK cells is shown, respectively. **(H–J)** The total percentage of the other hallmarks of NCE (NKG2D, KLRG1, and Ki67) are shown on gated NK cells. **(K)** The percentage of lysis of CFSE-labeled Yac1 cells is shown at different effector:target (E:T) ratios. Data are representative of three independent experiments done in triplicate (mean ± SEM). Two-way ANOVA was used to assess significance. Significant differences are displayed for comparisons with the rIgG-treated group (***$p < 0.001$).

diphtheria toxin (DT) receptor transgenic mice, a mouse model that allows *in vivo* Treg depletion (**Figures 5A–C**). Under these conditions, the total numbers of both NK and CD8 T cells were dramatically augmented when IL-2 was given in the absence of Tregs (**Figures 5D,E**). Surprisingly, the MFI of Eomes was significantly reduced on NK cells (**Figures 5F,G**), whereas the proportion of CD8 T cells double-positive for Eomes and T-bet was highly enhanced (**Figures 5F,H**). According to an exhaustion phenotype, the percentage of NKG2D was decreased while the expression of KLRG1 was maintained at high levels on IL-2-treated NK cells stimulated in the absence of Tregs (**Supplemental Figure 5**). A reduction of Ki67 in NK cells (**Figure 5I**) but not in CD8 T cells (**Figure 5J**), along with an increased number of CD8 T cells expressing NKG2D MFI

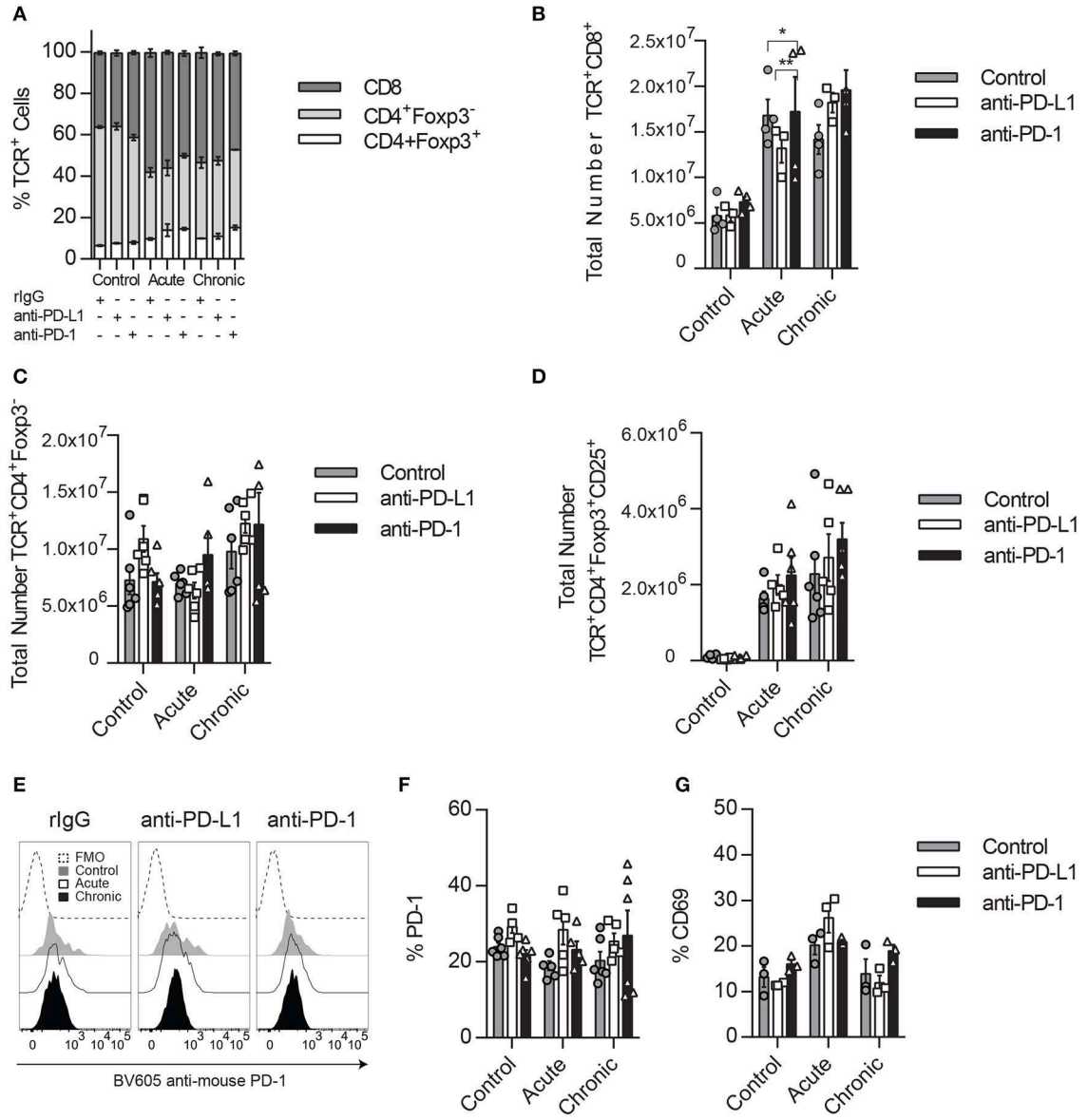

FIGURE 4 | Impact of PD1/PD-L1 neutralization in the Tregs compartment. **(A)** Splenic percentage distribution of CD8 T cells ($CD45^+TCR\beta^+CD8\alpha^+$), CD4 T cells ($CD45^+TCR\beta^+CD4^+Foxp3^-$), and Tregs ($CD45^+TCR\beta^+CD4^+Foxp3^+$) is shown for $TCR\beta^+$ cells ($CD45^+TCR\beta^+$). **(B–D)** Total number of CD8 T cells **(B)**, conventional CD4 T cells **(C)**, and Tregs **(D)** collected from the spleen after IL-2 stimulation. **(E,F)** Representative histograms **(E)** and total percentage **(F)** of PD-1 expression on gated Tregs. **(G)** Total percentage of CD69 is shown on gated Tregs. Data represent one or two experiments of a total of three independent experiments with 3–4 mice per group (mean ± SEM). Two-way ANOVA was used to assess significance. Significant differences are displayed for comparisons with the rIgG-treated group ($^*p < 0.05$, $^{**}p < 0.01$).

(**Figure 5K**), also indicated a higher activation status of CD8 T cells when Tregs were not present. More importantly, the lack of response to stimuli by NK cells shown by a lower production of IFNγ (**Figure 5L**) contrasted with the strong increase of GranB production by CD8 T cells during acute treatment in the absence of Tregs (**Figure 5M**). These data suggest that situations of elevated CD8 T cell activation result in a negative feedback on NK cells due to a reduction of the NK cell activation threshold, thus delaying the onset for exhaustion.

The Inhibition of the PD-1/PD-L1 Pathway Augments CD8 T Cell Activation

Because in the absence of Tregs we have seen an improvement in the activation and functional properties of CD8 T cells, likely due to the competition for cytokines between NK and CD8 T cells, we argue that PD-1/PD-L1 blockade might result in a similar phenomenon during chronic IL-2 treatment. Therefore, the CD8 T cell activation and functional status were exhaustively evaluated after IL-2 administration in anti-PD-1-

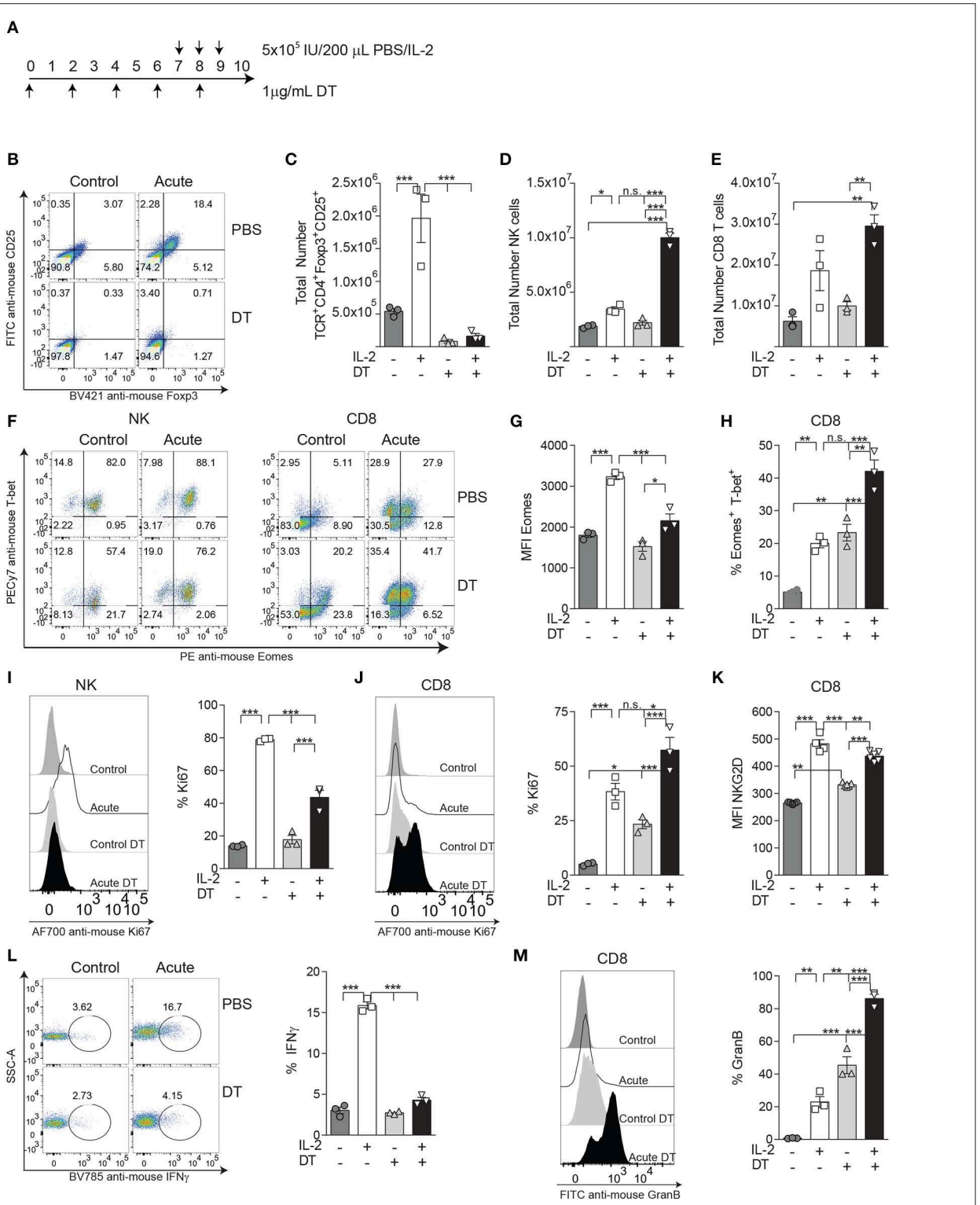

FIGURE 5 | The activation status on CD8 T cells, but not on NK cells, is enhanced during IL-2 stimulation by the absence of Tregs. **(A)** The experimental regimen used to deplete Tregs on DTR-Foxp3 transgenic mice with diphtheria toxic (DT) treatment on acutely IL-2 stimulated mice. **(B)** Representative dot plots of CD25 and Foxp3 on TCRβ$^+$CD4$^+$ T cells after DT treatment after IL-2 stimulation. **(C–E)** Total number of Tregs (TCRβ$^+$CD4$^+$Foxp3$^+$CD25$^+$), NK cells (TCRβ$^-$NK1.1$^+$), and CD8 T cells (TCRβ$^+$CD4$^-$CD8$^+$) obtained from the spleen of treated mice. **(F)** Distribution of Eomes and T-bet is shown for gated NK (left panel) and CD8 T cells (right panel). **(G)** MFI expression of Eomes on gated Eomes$^+$ NK cells after acute IL-2 stimulation. **(H)** The percentage of the activating population Eomes$^+$T-bet$^+$ on CD8 T cells is

(Continued)

FIGURE 5 | shown. **(I,J)** The total percentage of Ki67 for NK cells **(I)** and CD8 T cells **(J)** is shown. **(K)** The MFI of NKG2D gated on NKG2D$^+$ CD8 T cells is shown. **(L)** The ability of IL-2-treated NK cells to respond to NK1.1 stimulation, assessed by IFNγ production, is shown. **(M)** GranB production by IL-2-treated CD8 T cells is shown. Data are representative of two independent experiments with three mice per group (mean ± SEM). Two-way ANOVA was used to assess significance (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$).

or anti-PD-L1-treated mice. Interestingly, chronic stimulation prompted a significant increase of the MFI of Eomes in CD8 T cells, as well as an increase of the total percentage of CD8 T cells expressing both Eomes and T-bet, which suggests a superior activation level at this time point in anti-PD-1-treated cells (**Figures 6A–D**). Despite no statistically significant differences observed in the proportion of Ki67-positive expressing CD8 T cells after PD-L1/PD-1 therapy during chronic stimulation when compared to control-treated mice, a less severe reduction of this proliferative marker between acute and chronic stimulation was observed when anti-PD-1 was administered (**Figure 6E**). The improvement on Eomes and T-bet expression was not associated with an increase of the central memory (CD44$^+$CD62L$^+$) or effector memory (CD44$^+$CD62$^-$) CD8 T cell populations as no significant differences were found between rIgG- and anti-PD-1-treated chronically stimulated CD8 T cells (**Supplemental Figure 6A**). In agreement with the results suggesting a higher activation status, an increase of a CD8 T cell population that expresses PD1 but not Tim-3 was also observed after PD-1 or PD-L1 inhibition during acute and chronic stimulation (**Figures 6F,G**). Furthermore, and also correlated with a higher activation status, chronically stimulated CD8 T cells treated with anti-PD-L1 or anti-PD-1 expressed significantly higher levels of the activating receptor NKG2D when compared to rIgG (**Figures 6H,I**). It has been previously shown that IL-2 can expand non-antigen-specific bystander memory CD8 T cells within the CD44$^+$ CD8 T cell compartment that express NKG2D but lack expression of CD25, the high-affinity receptor for IL-2 (IL2R) (36, 43). However, during chronic stimulation, a decrease in this population was still observed independently of PD-1 treatment (**Supplemental Figure 6B**). Interestingly, when CD25 expression was evaluated on CD8 T cells, the highest increase of CD25 after IL-2 administration was observed in anti-PD-1-treated mice (**Figure 6J**). This phenomenon could provide a homeostatic advantage to anti-PD-1-treated CD8 T cells over NK cells in the presence of IL-2.

Notably, PD-1/PD-L1 pathway neutralization did have a functional benefit on CD8 T cells, as they expressed higher levels of GranB when treated with anti-PD-L1 or anti-PD-1 (**Figures 6K,L**). These results suggest that anti-PD-1 treatment does not seem to favor a specific CD8 T cell subset but rather improves overall activation and functional parameters of CD8 T cells.

The Onset of NCE Is Restored in the Absence of CD8 T Cells

Next, CD8 depletion studies were done in order to discern the impact of PD-1 between this immune cell population and NK cells as well as to determine if, in the absence of CD8 T cells, the IL-2-dependent induction of NCE follows the patterns previously observed (**Figure 7A**). This study was performed because of the negative feedback observed between NK and CD8 T cells during IL-2 stimulation in a Treg-deficient mouse model (**Figure 5**) and the higher activation status of CD8 T cells after PD-1/PD-L1 therapy. Therefore, a similar interaction might be driving the effect of PD-L1/PD-1 therapy on chronically stimulated NK cells, altering NK cell homeostasis and the course of NCE.

A t-SNE analysis of the flow cytometer data was used to better visualize the different immune populations affected by PD-1 blockade and CD8 T cell depletion and the changes in expression of Eomes and Ki67. After CD8 T cell depletion (**Figure 7B**, **Supplemental Figure 7**), the percentage and number of NK cells were significantly increased during both acute and chronic stimulation (**Figures 7B–D**). When the exhaustion parameters were studied on NK cells after chronic stimulation, a reduction of the MFI for Eomes as well as in the proportion of Eomes$^+$T-bet$^+$ NK cells was noted in anti-PD-1/CD8-treated mice compared to anti-PD-1 treatment (**Figures 7E–H**). Curiously, there were no differences between rIgG and anti-PD-1/CD8-treated NK cells in these parameters (**Figures 7E–H**). Similar trends were obtained for NKG2D and KLRG1, with the expected reduction of NKG2D MFI and increase of KLRG1 on NK cells, phenomena typical of exhaustion, when comparing anti-PD-1/CD8- and anti-PD-1-treated mice (**Figures 7I–K**). Additionally, the delay observed after anti-PD-1 treatment in the reduction of the proliferative capacities of NK cells during chronic stimulation when compared to rIgG was lost in the absence of CD8 T cells (**Figures 7L,M**). Furthermore, IFNγ production was mildly, although not significantly, reduced upon NK1.1 stimulation with levels of anti-PD-1/CD8-treated NK cells similar to those of rIgG-treated NK cells (**Figure 7N**).

The *in vitro* culture of NK cells in the presence of the T cell compartment also caused a delay on NK cell activation after long-term IL-2 stimulation with lower IFNγ and granzyme B production after NK1.1 stimulation, and lower Eomes, T-bet, and NKG2D expression at day 4 (**Supplemental Figures 8A–G**). Although no major differences were observed at later time points between NK cells growth with (no T cell depl) or without T cells (T cell depl), a delay in the drop of proliferation was observed in NK cells cultured in the presence of T cells (**Supplemental Figure 8H**), which recapitulates the data observed *in vivo*. These changes in NK cell phenotype correlated with the degree of activation of CD8 T cells during the course of the culture (**Supplemental Figures 8I–N**), limiting the level of NK cell expansion (**Supplemental Figure 8O**). Altogether, our findings indicate that anti-PD1 treatment during sustained IL-2 stimulation causes expansion and activation of CD8 T cells that compete with NK cells for the use of stimulating cytokines, therefore delaying the activation and consequently the induction of exhaustion on these innate cells.

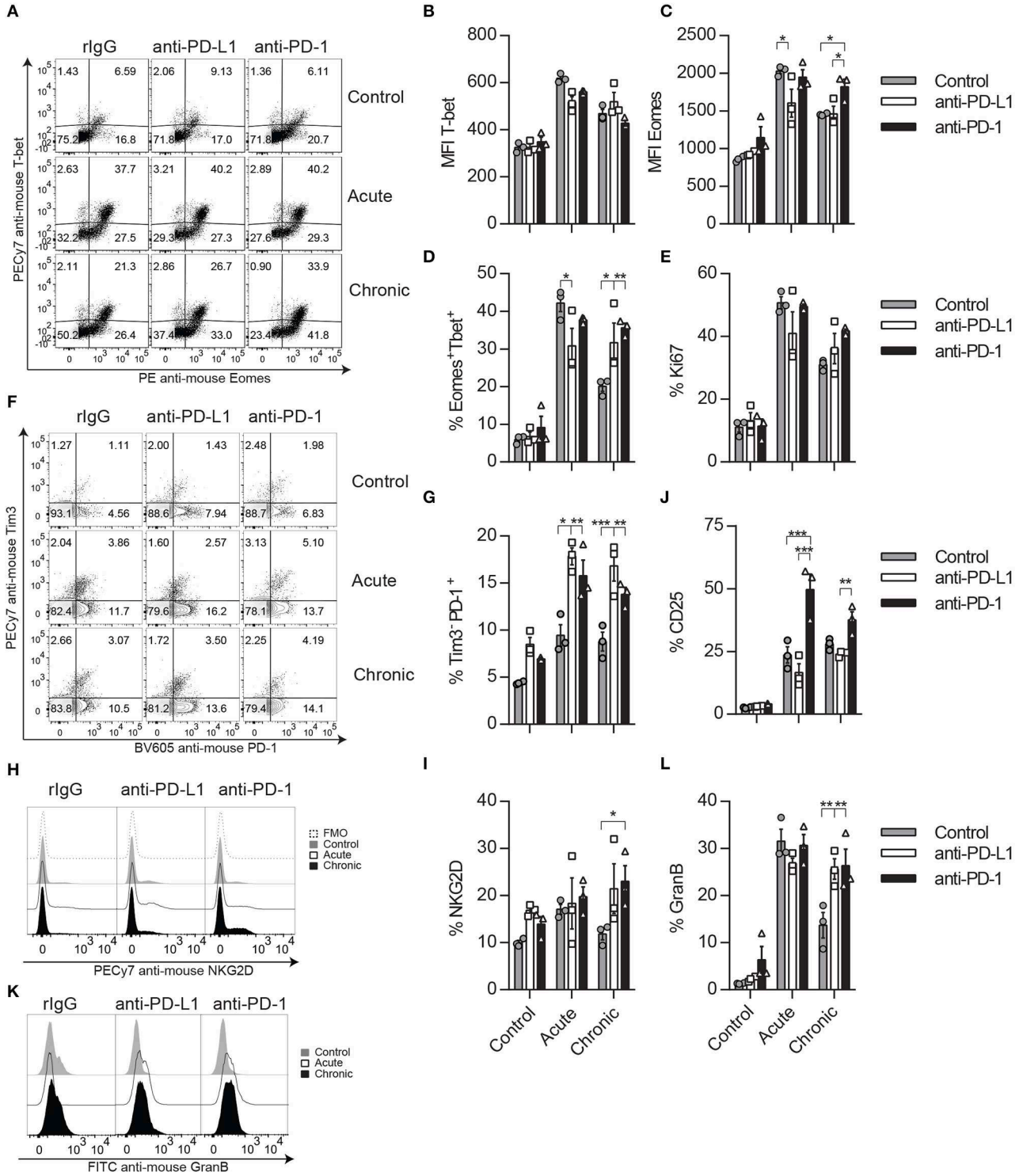

FIGURE 6 | CD8 T cell activation phenotype is improved after anti-PD-L1 or anti-PD-1 treatment during chronic IL-2 stimulation. **(A–C)** Representative dot plots **(A)** and MFI **(B,C)** expression for Eomes and T-bet are shown on gated CD8 T cells ($CD45^+TCR\beta^+CD4^-CD8\alpha^+$). **(D)** Total percentage of Eomes and T-bet-positive CD8 T cells is shown. **(E)** Proliferative potential of CD8 T cells assessed by Ki67 expression is shown. **(F)** Representative dot plots of Tim-3 and PD-1 are shown on gated CD8 T cells. **(G)** Total percentage of Tim-3^-PD1^+ cells is shown on gated CD8 T cells. **(H,I)** Representative histograms **(H)** and total percentage **(I)** of NKG2D are shown for gated CD8 T cells. **(J)** Total percentage of CD25 is shown on gated CD8 T cells. **(K,L)** Representative histograms **(K)** and total **(L)** GranB production of CD8 T cells are shown. Data are representative of three independent experiments with 3–4 mice per group (mean ± SEM). Two-way ANOVA was used to assess significance. Significant differences are displayed for comparisons with the rIgG-treated group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

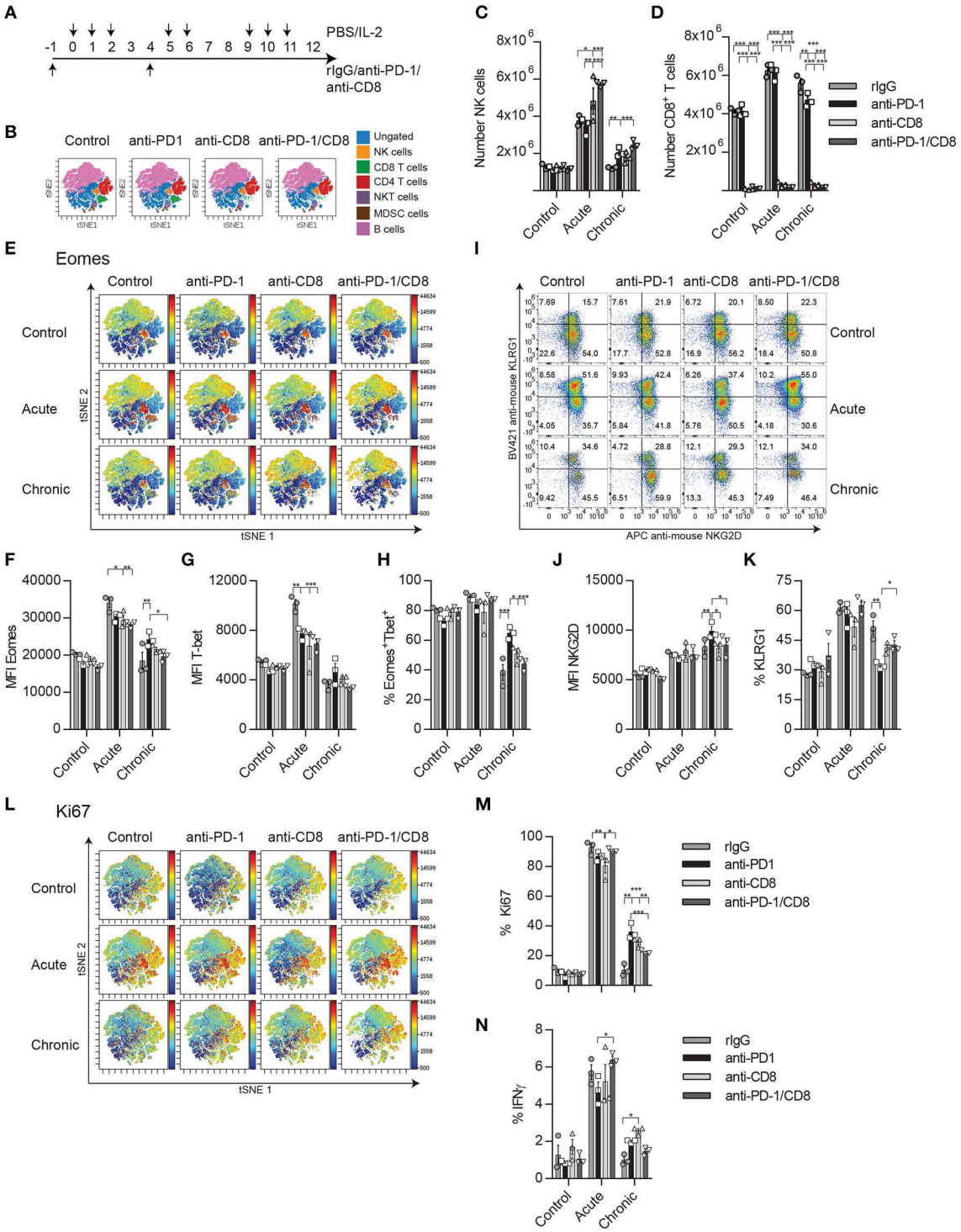

FIGURE 7 | CD8 T cell depletion reverses the bystander effect of PD-1 blockade on NK cell activation during chronic IL-2 stimulation. **(A)** Regimen dose schedule followed for CD8 T cell depletion experiments. **(B)** t-SNE analysis is shown displaying the distribution of immune cell populations (ungated: blue; $CD19^-Ly6G^-CD3^-CD4^-CD8^-NK1.1^+$ NK cells: yellow; $CD19^-NK1.1^-CD3^+CD4^-CD8$ T cells: green; $CD19^-NK1.1^-CD3^+CD8^-$ CD4 T cells: red; $CD19^-CD3^+CD8^-NK1.1^+CD4^+$ NKT cells: purple; $CD19^-CD3^-Ly6G^+CD11b^+$MDSC: brown; and $CD3^-NK1.1^-Ly6G^-CD19^+$ B cells: pink) after CD8 T cell depletion in unstimulated mice. **(C,D)** The total number of NK cells **(C)** and CD8 T cells **(D)** collected from the spleen after IL-2 stimulation is shown. **(E)** t-SNE analysis shows the Eomes expression of the different immune populations after IL-2 stimulation. **(F,G)** MFI expression of Eomes and the total percentage of the activating $Eomes^+$T-bet^+ NK cell population is shown on gated $Eomes^+$ or T-bet^+ NK cells ($CD19^-Ly6G^-CD3^-CD4^-CD8^-NK1.1^+$). **(H,I)** NKG2D percentage **(H)** and MFI

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FIGURE 7 | (I) are shown. **(J)** The total percentage of KLRG1 on gated NK cells is shown. **(K)** t-SNE analysis shows the Ki67 expression of the different immune populations after IL-2 stimulation. **(L)** The percentage of Ki67 is shown for gated NK cells. **(M)** The percentage of IFNγ is shown on gated NK cells after NK1.1 stimulation. Data are representative of two independent experiments with three mice per group (mean ± SEM). Two-way ANOVA was used to assess significance. Significant differences are displayed for comparisons with the PD-1-treated group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

DISCUSSION

Our results suggest that the phenotypical and functional benefits observed after anti-PD-1 treatment on chronically stimulated NK cells are mediated by an indirect effect of anti-PD-1/PD-L1 on CD8 T cells. The activation and expansion of CD8 T cells limit the amount of stimulating cytokines available for NK cells, resulting in a delay of NK cell activation and consequently the induction of exhaustion during prolonged stimulation. These results, therefore, suggest the existence of a delicate balance between CD8 T cells, Tregs, and NK cells, regulated in part by their ability to respond to cytokines. A disruption of this equilibrium influences the homeostatic response to stimuli of each other.

Despite the fact that there was barely any expression of PD-1 in mouse NK cells during IL-2 *in vivo* stimulation, we initially hypothesized that the decrease of the number of NK cells after chronic stimulation could be the consequence of a preferential death of a PD-1-expressing population. PD-1 blockade therapy resulted in a mild improvement of the NK cell activation phenotype after chronic stimulation, but no effect was observed *in vitro*, suggesting that other mechanisms might contribute to the improvement observed after anti-PD-1 *in vivo* administration. This result disagrees with many others that have suggested a role for PD-1 on NCE, a correlation that we could not find in our model (17, 22, 23, 26, 27, 44). Many of these studies were done in human NK cells or infection models that might be more efficient in inducing a PD-1-dependent effect (26, 27, 29, 34). Additionally, Hsu et al. showed an improvement of PD-1/PD-L1 blockade only on tumor-infiltrating NK cells, where higher expression of PD-1 was observed (22). Like our study, NK cells collected from the spleen of the tumor-bearing mouse had low levels of PD-1 (22).

A recently published study has shown in an *in vivo* mouse tumor model that PD-L1 blockade enhances NK cell function and prevents NCE by directly targeting PD-L1$^+$ NK cells, identifying a mechanism of NK cell regulation independent of PD-1 (45). We could not identify a direct effect of PD-L1 blockade on NK cells as anti-PD-L1 treatment failed to induce any phenotypical changes on NK cells cultured with IL-2. However, like in this study, we did observe improvement on NK cell activation *in vivo*, which in our case was a process mediated by CD8 T cells. The CD8 T cell compartment was not analyzed in Dong's article and therefore its contribution on the regulation of NCE in the mouse model studied in this paper could not be fully excluded (45).

IL-2 is a pleiotropic cytokine that activates T cells, NK cells, and DC cells. IL-2 binds to IL-2Rs, which display different affinities for the cytokine. CD25 (IL-2Rα) has the highest IL-2 affinity compared to CD32 (IL-2Rγc) and CD122 (IL-2Rβ). CD25 is constitutively expressed on Tregs and thus IL-2 therapy results in an expansion of these cells, which mediates NK and CD8 T cell inhibition through a variety of mechanisms (36, 46–48). Some include the release of immunosuppressive cytokines (IL-10 and TGFβ) upon activation, whereas others require cell-to-cell interactions and act through the activation of checkpoint inhibitors (CTLA-4 and PD-1) causing apoptosis (36). Additionally, Tregs can further contribute to regulate IL-2-mediated immune responses by sequestering IL-2, making it less available to effector cells. Thus, it does not come as a surprise that the depletion of Tregs or the neutralization of IL-10 or TGFβ causes an enhanced expansion of CD8 T cells and NK cells, as previously reported (36, 46, 47). However, the extent to which Tregs depletion affected the activation and expansion of CD8 T cells, while NK cell activation phenotype was somewhat restricted, was interesting. The upregulation of CD25 observed on CD8 T cells upon activation (43) (**Supplemental Figure 4E**) can indeed provide an advantage over NK cells for IL-2 and explain the differences in the level of activation between both cell types. Similar competition between immune cells has been observed for IL-15 (49).

The existence of regulation between NK and CD8 T cells has been previously reported in many infection models (50–55) and an antigen-independent IL-2 model (36). The results obtained from this study, where a low dose of IL-2 was administered for a short period, indicated a bi-directional regulation between CD8 T and NK cells; yet, NK cells seemed more prone to exert a stronger control on CD8 T cells in a FasL-dependent manner (36). This regulation of NK cells toward CD8 T cells has been previously described as a mechanism to prevent exacerbated CD8 T cell-dependent immune responses and to be critical in the control of immunopathology during mouse cytomegalovirus (MCMV) and lymphocytic choriomeningitis virus (LCMV) infections due to toxic levels of circulating cytokines produced by effector CD8 T cells (52, 53, 56). The control of the infection was implemented by directly eliminating effector cells in an IL-10 and/or perforin-dependent manner (52, 53). A mechanism dependent on NKG2D has also been suggested as a means to eliminate effector CD8 T cells due to upregulation of NKG2D ligands shortly after activation (51, 57, 58). NK cells were also reported to indirectly regulate CD8 T cells by controlling CD4 T cells and DCs (50, 55). Similarly, high levels of PD-L1 on NK cells from tumor-bearing mice limited DC-dependent cross-priming, reducing tumor-specific CD8 T cell priming (59).

CD8 T cells, along with other immune cells such as DCs, can also exert an immune suppressor effect on NK cells due to the upregulation of the NKG2A ligand on CD8 T cells during activation that inhibits NK cells after engaging with NKG2A (54). Notably, the presence of tumor-infiltrated NK cells at the draining lymph nodes of breast cancer patients that express PD-1 and NKG2A has been described (25).

However, targeting NK cells has also shown beneficial effects in the control of persistent infection by facilitating the expansion and activation of T cells. Importantly, to obtain these effects, the timing of NK depletion was crucial (52). Waggoner et al. showed that early NK cell depletion during LCMV infection caused T cell-mediated immunopathological effects, as reported by many others (50–53). In contrast, if NK cell depletion was postponed 2–3 weeks after infection, then an improvement in the control of viral load and in the presence of effector CD8 T cells was seen (52). In this model, the presence of CD4 T cells was important to increase IFNγ^+ CD8 T cells (52). It was suggested that rescuing dysfunctional CD8 T cells was the mechanism by which a better control of viral load was achieved (52). Additionally, it was also possible that the availability of more stimulating cytokines in the absence of NK cells at that specific time point could promote naïve CD8 T cells to differentiate toward effector cells and contribute to better management of the infection.

In our current and previous studies, we have seen that mouse NK cell dysfunction is observed after ∼12 days of prolonged high doses of IL-2, reaching the peak of function and activation shortly after stimulation is started (9). The initial expansion of NK cells after acute IL-2 stimulation is accompanied by a reduction of NK cell numbers during chronic stimulation, a phenomenon that is not observed in Tregs and CD8 T cells. It is important to note that there is a possibility of a conversion of NK cells toward a less functional innate lymphocyte type 1 (ILC1) subset during chronic stimulation as suggested by Gao et al. (35). We believe this is not the case because the analysis is mainly focused on splenic NK cells where the proportion of ILC1 is minimal (1–3% over the total Lin$^-$CD49b$^+$NKp46$^+$ cells) in resting mice when compared to other organs such as the liver or small intestine (60), but we cannot fully exclude this possibility without further analysis.

Nevertheless, in the present study, we show that this NCE phenotype is ameliorated by PD-1/PD-L1 neutralization. Interestingly, the inhibition of PD-1/PD-L1 resulted in enhanced expansion, activation, and function of CD8 T cells. Indeed, CD8 T cells displayed features characteristic of better activation and function such as upregulation of Eomes, T-bet, and NKG2D. Upregulation of a PD1$^+$Tim-3$^-$ CD8 T cell subset was observed as well during chronic stimulation in anti-PD-1- or anti-PD-L1-treated mice. The fact that CD8 T cells upregulates PD-1 and CD25 upon IL-2 stimulation suggests that PD-1/PD-L1 inhibition might provide an advantage for CD8 T cells over NK cells in the use of IL-2 and other stimulating cytokines.

Some studies have suggested that the Eomes^{high}PD-1^{high} CD8 T cell subset, along with the co-expression of other inhibitory checkpoint molecules (BTLA, CD160, and Lag3), represents a subset with a severe state of exhaustion (20, 61). On the contrary, the T-bet^{high}PD-1^{int} CD8 T cell population is more responsive and shows enhanced cytokine secretion potential (20, 61). Indeed, T-bet expression, but not Eomes, on tumor-reactive T cells was shown to be expanded after combinatorial checkpoint blockade therapy (62). In accordance with this study, upregulation of T-bet on CD8 T cells was also observed after PD-1 therapy and chronic IL-2 stimulation. However, in our model, this upregulation was accompanied by Eomes expression as well. This subset has shown to be capable of producing higher levels of GranB after anti-PD-1 therapy as described by other studies (20).

The expression of PD-1 on CD8 T cells has also been highly associated with their activation status. Supporting this concept, it has been shown that the adoptive transfer of tumor-infiltrated PD-1$^+$ CD8 T cells was capable of containing tumor progression (63). Similarly, an increase of antigen-specific CD8 T cells that express PD-1, but not Tim-3, has been found in the tumor microenvironment after PD-1/CTLA-4 checkpoint blockade therapy (9, 19). In agreement with these studies, an increase of a Tim-3$^-$PD-1$^+$ CD8 T cell subset was indeed observed after acute and chronic stimulation after neutralization of the PD-1/PD-L1 pathway.

Additionally, similar to NKG2D on NK cells (9, 64, 65), several studies have suggested an important role of NKG2D$^+$ CD8$^+$ T cells in the battle against tumor and infections (58, 66). Indeed, a recent immunotherapeutic approach that triggers IL-2 signaling in only NKG2D$^+$ cells has provided a superior antitumor response (67). Hu et al. showed that NKG2D expression of CD8 T cells was regulated by STAT3 phosphorylation upon CD28 activation (66). This CD28/PD-L1 costimulatory pathway was also shown to be critical for PD-1 therapy success (38), which could be correlated to the enhanced levels of NKG2D on CD8 T cells observed in PD-1-treated mice after chronic stimulation.

Taking these studies into account, our results indicate that PD-1 therapy might provide an activating advantage on CD8 T cells to compete for IL-2, preventing NK cell access of this cytokine. The depletion of CD8 T cells during IL-2 stimulation does, in fact, regress any benefit that PD-1 therapy could have on NK cells after chronic stimulation supporting this hypothesis.

Interestingly, a study has recently shown that IL-2 therapy combined with anti-PD-1 therapy causes an increase of CD8 T cells, but not of NK cells (68). In this study, the authors used an IL-2/anti-IL-2 immune complex (IL-2Cx) with a longer half-life that favors interactions with CD32 and CD122, making IL-2 more readily accessible to CD8 T cells and NK cells (68). When combined with anti-PD-1 therapy in tumor mouse models, the improved anti-tumor efficacy was mediated specifically by increasing the proportion of tumor-infiltrating CD8 T cells, but not tumor-infiltrating NK cells as only the depletion of CD8 T cells resulted in the loss of anti-PD-1/IL2Cx anti-tumoral benefits. Surprisingly, in order to reveal the role of NK cells in the anti-tumor response, Tregs needed to be controlled by anti-CTLA-4 therapy (68).

In spite of the dual regulation described between both CD8 T cells and NK cells, caused by the competition for space and resources, direct or indirect lysis, and functional inhibition, the reality is that both of these populations can also work together to mount a stronger response. Therapeutic approaches involving neutralization of checkpoint inhibitors that requires both CD8 and NK cell responses support this idea of immune-self regulation (21, 22, 69). Moynihan et al. also demonstrated the necessity of both immune populations to eradicate large established tumors treated with combined neutralization of checkpoint inhibitors CTLA-4 and PD-1 (70). Additionally, a recent report has shown the importance of DC and NK cell crosstalk in the CD8 T cell anti-tumor response (71).

Current therapies are searching for the magical weapon that results in a prolonged and stronger anti-tumor response, focused on enhancing the function and presence of effector cells. Preventing immune cell exhaustion has recently become an important target in this search for this perfect immunotherapy. However, a delicate balance between immune cells exists and disruption of this balance might result in unknown effects in the form of toxicities, suppression, competition, or compensatory mechanisms. The results obtained from this study suggest a delay of NCE due to direct competition with CD8 T cells, revealing a positive bystander effect of anti-PD-1 therapy in NK cells despite the absence of a relevant expression of PD-1 in this cell type. This study does also show the importance of evaluating the short- and long-term impact of immunotherapy on any single immune cell component in order to truly understand the implications and exploit the beneficial effects of any immunotherapeutic approach.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC at Stanford University and University of Navarra.

AUTHOR CONTRIBUTIONS

MA designed and performed research, analyzed data, and wrote the manuscript. FS, JB, AW, and AM contributed in conducting experiments. FS, AP, and PB provided scientific input and assisted with the preparation of the manuscript. RN provided overall scientific guidance and helped write 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/fimmu.2020.00007/full#supplementary-material

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Natural-Killer-Derived Extracellular Vesicles: Immune Sensors and Interactors

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Natural killer (NK) cells contribute to immunosurveillance and first-line defense in the control of tumor growth and metastasis diffusion. NK-cell-derived extracellular vesicles (NKEVs) are constitutively secreted and biologically active. They reflect the protein and genetic repertoire of originating cells, and exert antitumor activity *in vitro* and *in vivo*. Cancer can compromise NK cell functions, a status potentially reflected by their extracellular vesicles. Hence, NKEVs could, on the one hand, contribute to improve cancer therapy by interacting with tumor and/or immune cells and on the other hand, sense the actual NK cell status in cancer patients. Here, we investigated the composition of healthy donors' NKEVs, including NK microvesicles and exosomes, and their interaction with uncompromised cells of the immune system. To sense the systemic NK cell status in cancer patients, we developed an immune enzymatic test (NKExoELISA) that measures plasma NK-cell-derived exosomes, captured as $tsg101^{+}CD56^{+}$ nanovesicles. NKEV mass spectrometry and cytokine analysis showed the expression of NK cell markers, i.e., NKG2D and CD94, perforin, granzymes, CD40L, and other molecules involved in cytotoxicity, homing, cell adhesion, and immune activation, together with EV markers tsg101, CD81, CD63, and CD9 in both NK-derived exosomes and microvesicles. Data are available via Proteome Xchange with identifier PXD014894. Immunomodulation studies revealed that NKEVs displayed main stimulatory functions in peripheral blood mononuclear cells (PBMCs), inducing the expression of human leukocyte antigen DR isotype (HLA-DR) and costimulatory molecules on monocytes and CD25 expression on T cells, which was maintained in the presence of lipopolysaccharide (LPS) and interleukin (IL)-10/transforming growth factor beta (TGFβ), respectively. Furthermore, NKEVs increased the $CD56^{+}$ NK cell fraction, suggesting that effects mediated by NKEVs might be potentially exploited in support of cancer therapy. The measurement of circulating NK exosomes in the plasma of melanoma patients and healthy donors

evidenced lower levels of tsg101^{+}CD56^{+} exosomes in patients with respect to donors. Likewise, we detected lower frequencies of NK cells in PBMCs of these patients. These data highlight the potential of NKExoELISA to sense alterations of the NK cell immune status.

Keywords: natural killer cells, microvesicles, exosomes, extracellular vesicles, immunosurveillance, healthy donors, melanoma patients

INTRODUCTION

Natural killer (NK) cells belong to the innate immunity and represent the first-line defense of the immune system in the control of pathogens, tumor growth, and metastasis diffusion. NK cells constitute a population of large, granular lymphocytes that are located in the blood where they comprise 10–15% of lymphocytes, in lymphoid organs, i.e., thymus and spleen, and in non-lymphoid organs, such as the liver and uterus, as well as in tissues, i.e., skin (1, 2). NK cells are recognized as the most effective immune cells involved in immunosurveillance, as they can clear infected or transformed cells without the need for priming and are not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules (3). Human peripheral blood NK cells can be identified by their expression of CD56 in the CD3^{neg} lymphocyte gate. They can be further divided into CD56^{bright}CD16^{+} NK cells, which produce interferon gamma (IFNγ) and represent 10% of NK cell population, and into cytotoxic CD56^{dim}CD16^{bright} NK cells, which represent 90% of the NK cell population under physiological conditions (4). NK cells constitutively express a lytic machinery able to kill target cells independently from any previous activation and can be induced to migrate toward inflammation sites and infected or transformed cells, by different chemoattractants (5). Previous studies have shown that a low activity of NK cells in peripheral blood is associated with increased cancer risk (6). A role of NK cells in tumor immunosurveillance has also been reported in models of spontaneous and induced tumors (7). In cancer patients, NK cells exhibit profound defects in the ability to degranulate (8). The therapeutic use of NK cells has been explored over the years, but the results have not been very encouraging. Considerable efforts are being made to achieve better activation, proliferation, and viability of NK cells, to be used in cancer immunotherapy (9).

Like other innate immune cells, NK cells communicate with dendritic cells and/or T and B cells and regulate innate and adaptive immune responses. NK cells do not act via antigen-specific mechanism but their activity is mediated by alternative receptors. The resulting inhibitory or activating signal depends on the ligand-expressing cells (10). This cell-to-cell communication is supported by the release of a broad range of soluble mediators, including cyto- and chemokines (11). Cells can also release soluble factors via encapsulation in extracellular vesicles (EVs) (12, 13). These nanometer-sized vesicles mainly comprise microvesicles (MV), stemming from the cell membrane with a diameter ranging from 150–1,000 nm and exosomes deriving from the endosomal compartment of the cells with a size ranging from 30 to 150 nm and apoptotic bodies, characterized by a dimension ranging from 1,000 to 5,000 nm (14). Among the different EV types, exosomes have captured the main interest from the scientific community for many years. Exosomes are important mediators for cell-to-cell communication in physiological (15–17) and pathological conditions such as cancer, where they play a major role in immune responses, cancer progression, and metastasis (18). Preclinical studies have shown that immune cells can release EVs with both stimulatory and tolerogenic properties into the extracellular microenvironment, a finding that supports their future application in cancer therapies (16, 19, 20). Moreover, there is a growing interest in the use of EVs, especially those deriving from cancer, as biomarkers, thanks to their simple detection in biological fluids, as recently shown for PD-L1^{+} exosomes in melanoma patients' plasma (21).

We were the first to report that NK cells release exosomes (NKExo), independently from the cells' activation status. NKExo express typical NK and EV markers together with killer proteins and are endowed with cytotoxic activity against tumor cells (22). In the years following their discovery, NKExo attracted major interest, and their antitumor activity was confirmed *in vitro* and *in vivo*, expanding the knowledge about these vesicles (10). Their therapeutic effect was also studied using NK cell lines as exosome producers to facilitate cell expansion for vesicle retrieval. Exosomes from the NK-92MI cell line showed remarkable cytolytic activity against melanoma (23) and glioblastoma (24), while EVs from *in vitro* expanded NK cells cocultured with K562.mbIL21 cells displayed antitumor activities against acute lymphoblastic leukemia and breast carcinoma (25). Apart from a consistent equipment of molecules aimed at target killing, NKEVs were also found to contain miR-186, a tumor suppressor micro-RNA that contributes to their cytostatic effects in neuroblastoma and prevents the inhibitory effects on NK cells of transforming growth factor beta (TGFβ) (26).

Here, we investigated the morphology and proteome of NK-cell-derived microvesicles (NKMV) and NKExo produced by *ex vivo* expanded NK cells from healthy donors and their effects on peripheral blood mononuclear cells (PBMCs) of healthy donors to uncover potential stimulatory activity on T cells, monocytes, and NK cells. Experiments recapitulating an immunosuppressed condition were performed in the presence of TGFβ/interleukin (IL)-10, tolerogenic conditioning of monocytes with lipopolysaccharide (LPS). In addition, we developed a method, the NKExoELISA, to sense alterations at EV level that could inform about the systemic NK cell immune status of cancer patients. Taken together, our data suggest that NKEVs

could cover a promising role in the support of NK-mediated immunosurveillance to sustain cancer therapies, at the same time representing a sensor for systemic NK cell alterations.

MATERIALS AND METHODS

NK Cell Expansion and PBMC Isolation

Blood of 20 healthy donors and 20 melanoma patients was provided by Centro Trasfusionale Universitario and Clinica Dermatologica of Azienda Policlinico Umberto I, University Sapienza, Rome, Italy. The study was approved by the ethical committee of Azienda Policlinico Umberto I, and subjects gave written informed consent to participate. Human PBMC were isolated with Ficoll-Histopaque 1077 gradient (Sigma-Aldrich, St. Louis, MO, United States). *Ex vivo* expanded human NK cells were obtained as previously described (22). Briefly, PBMCs from buffy coats were cocultured with cobalt-irradiated B lymphoblastoid Roswell Park Memorial Institute (RPMI) 8866 cells. On day 7, cells were incubated with human rIL-2 (100 U/ml; Hoffman-La Roche, Nutley, NJ, United States) for 3 days. The resulting NK cell population was >80% $CD56^+$, $CD3^-$, and $CD14^-$ as assessed by flow cytometry analyses (cell viability, >90%). Using this culture method, an average of 30–40-fold increase in activated NK cell number was obtained. The supernatant of NK cell culture was properly frozen at −80°C for NKEVs isolation.

Isolation of NKEVs

The culture supernatants of e*x vivo* expanded human NK cells were subjected to differential centrifugation as previously described (22). Briefly, conditioned cell culture medium was centrifuged for 5 min at 300 × *g* and 20 min at 1,200 × *g* to remove cells and debris; NKMVs were pelleted for 30 min at 10,000 × *g* and washed in phosphate-buffered saline (PBS), while NKExo were collected by ultracentrifugation at 100,000 × *g* for 90 min at 10°C using a Sorvall WX Ultra Series centrifuge in an F50L-2461.5 rotor (Thermo Scientific, Germany). The resulting pellet was washed in PBS and again ultracentrifuged at 100,000 × *g* for 60 min. MV or/and Exo was resuspended in PBS and RPMI 1640 medium or dissolved in lysis buffer for further analyses. To obtain plasma-derived exosomes, the plasma was centrifuged for 30 min at 500 × *g* and 45 min at 12,000 × *g* to collect microvesicles, filtered through a 0.22-μm filter (Sartorius, Germany), and ultracentrifuged for 2 h at 110,000 × *g* at 10°C to collect exosomes. The resulting pellet was washed in PBS, ultracentrifuged at 110,000 × *g* for 90 min, and properly preserved for subsequent analyses.

Nanoparticle Tracking Analysis

The number and size of the isolated NK-derived EVs were assessed by nanoparticle tracking analysis (NTA) (NanoSight Model NS300, Malvern Instruments, NanoSight Ltd., Salisbury, United Kingdom). The parameters for NTA capture setting were as follows: camera type (sCMOS), Laser type Blue488, capture level 15, threshold 5, slider gain (366), and capture duration (60 s). Five videos of typically 60 s duration were taken. Data were analyzed by NTA 3.0 software (Malvern Instruments), which was optimized to first identify and then track each particle on a frame-by-frame basis.

Microscopy Analysis

Phase Contrast Microscopy

Images were acquired with a Nikon Eclipse T100 inverted microscope (Nikon Instruments Inc., Melville, NY, United States) equipped with an LWD 10X 0.40 N.A. phase contrast objective, a Nikon DS-Fi1 color camera, and the NIS-Elements F v3.0 software (Nikon Instruments Inc.).

Electron Microscopy

For scanning electron microscopy (SEM), purified exosomes and microvesicles from NK cell supernatants ($>10^9$/ml) were suspended in PBS and left to adhere to polylisine-treated round glass coverslips (Ø10 mm). Samples were fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer and processed for SEM as previously described with slight modifications (27). Briefly, samples were postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer and were dehydrated through a graded series of ethanol solutions (from 30 to 100%). Then, absolute ethanol was gradually substituted by a 1:1 solution of hexamethyldisilazane (HMDS)/absolute ethanol and successively by pure HMDS. The final drying process was concluded, removing completely the HMDS and leaving to evaporate all the liquid phase on air and in a desiccator. Dried samples were mounted on stubs, coated with gold (10 nm), and analyzed in an FE-SEM Quanta Inspect F (FEI-Thermo Fisher Scientific). For transmission electron microscopy (TEM), for negative staining, a pellet of extracellular vesicle purified from NK cell supernatants was suspended in PBS and were deposited on carbon-coated grids for electron microscopy. Phosphotungstic acid 2% was added on grids to give sample contrast. Samples were air dried and observed with a PHILIPS EM208S transmission electron microscope (FEI-Thermo Fisher) (28).

Immunoelectron Microscopy

Either purified exosomes and microvesicles from healthy donor NK cells were suspended in PBS and adsorbed on carbon-coated grids for electron microscopy, accordingly to Thery group's protocol (28) with slight modifications. Samples were air dried, and all the successive passages were carried out by floating the grids on drops of different solutions. First, the grids were floated on anti-CD81 (mAb B11, Santa Cruz Biotechnology, Heidelberg, Germany) or anti-CD56 (mAb 123C3, Santa Cruz) monoclonal antibodies, rinsed on buffer and incubated on 10 nm gold-conjugated goat antimouse immunoglobulin G (IgG) serum (Sigma-Aldrich, St. Louis, MO). Then, samples were rinsed and incubated on 2% paraformaldehyde. Finally, after rinsing on water, the samples were contrasted with 2% ammonium molybdate ("positive-negative" contrast) and dried with a filter paper. Samples were observed with a PHILIPS EM208S transmission electron microscope (FEI-Thermo Fisher).

Proteomics Analysis

Proteins obtained from three pools of total NK cell extracts, NKExo, and NKMV derived from three healthy donors each

were loaded (15 μg for each sample), separated on a 1D-gel NuPAGE 4–12% (Novex, Invitrogen, CA, United States), and stained with Coomassie blue (Colloidal Blue Staining kit, Invitrogen). Each lane was cut in six contiguous slices that were treated with dithiothreitol (DTT) and iodoaceatamide and finally digested with trypsin (Promega Corporation, WI, United States) as previously described (29). Peptides were analyzed by liquid chromatography–MS/MS (LC-MS/MS) on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, CA, United States) equipped with an Ultimate 3000 UHPLC (Dionex, Thermo Fisher Scientific). Peptides were desalted on a trap column (Acclaim PepMap 100 C18, Thermo Fisher Scientific) and then separated on a 20-cm-long silica capillary (Silica Tips FS 360-75-8, New Objective, MA, United States), packed in-house with a C18, 5 μm, 100 Å resin (Michrom BioResources, CA, United States).

The analytical separation was run for 91 min using a gradient of buffer A (95% water, 5% acetonitrile, and 0.1% formic acid) and buffer B (95% acetonitrile, 5% water, and 0.1% formic acid).

The gradient was run as follows: buffer A was fixed at 5% for the first 5 min, then was linearly increased to 30% in 60 min and subsequently to 80% in 10 min, then remained at 80% for 5 min, and finally decreased at 5% for a 10-min long column re-equilibration step.

Full-scan MS data were acquired in the 350–1,550 m/z range in the Orbitrap at 60 k resolution. Data-dependent acquisition was performed using top-speed mode (3 s long maximum total cycle): the most intense precursors were selected through a monoisotopic precursor selection (MIPS) filter and with charge >1, quadrupole isolated and fragmented by higher-energy collision dissociation (HCD) (32 collision energy). Fragment ions were analyzed in the ion trap with rapid scan rate. The automatic gain control (AGC) target value was set to 4e5 (FT) for MS1 and 2e3 (IT) for MS2. Maximum injection times of 50 and 100 ms were used for MS1 and MS2, respectively. Raw data were analyzed by Proteome Discoverer 2.3 (Thermo Fisher Scientific) using the human database from UniProtKB/Swiss-Prot database (Release 25 October 2017; 42253 sequences). Spectral matches were filtered using Percolator node, based on q values, with 1% false discovery rate (FDR). Only master proteins were taken into account and considered identified with at least two peptides with specific trypsin cleavages with two miscleavages admitted. Cysteine carbamydomethylation was set as static modification, while methionine oxidation and N-acetylation on protein terminus were set as variable modifications.

Quantification was based on precursor intensity using only unique peptides and normalizing abundances on total peptide amount. The sample group abundance was calculated as the median abundance of biological replicates. Protein abundance ratios for each couple of sample groups NKExo/NKTotExtr and NKMV/NKTotExtr were calculated as a pairwise protein ratio: no imputation was applied, and t-test and P-values corrected for multiple testing using the Benjamini–Hochberg procedure were adopted to evaluate significant differences in protein abundances. Proteins with an abundance ratio >1.5 and $P < 0.05$ were considered differentially expressed. Proteome Discoverer was also applied to get information about correlation among the data: normalized abundances were used for principal component analysis (PCA), and hierarchical cluster analysis of the grouped abundances was obtained.

All the identified proteins were analyzed to recognize species unique or shared among sample groups (http://bioinformatics.psb.ugent.be/webtools/Venn/) and compared with Exocarta (http://www.exocarta.org/) (30) and Vesiclepedia (http://www.microvesicles.org) (31) database. Overexpressed proteins were evaluated for protein–protein interaction analysis using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (https://string-db.org/) considering high confidence data with minimum required interaction score of 0.7; cellular component and Reactome pathway enrichment were also evaluated with FDR < 1E−3 (32). Gene Ontology (GO) terms relative to cellular component or biological process were also analyzed separately in the proteins enriched in NKExo (proteins overexpressed in NKExo and those identified in NKExo or NKExo plus NKMV) and proteins enriched in NKMV (proteins overexpressed in NKMV and those identified in NKMV or NKMV plus NKExo) using DAVID version 6.8 (https://david.ncifcrf.gov/), taking into account GO terms with Benjamini $P < 1E{-}3$ and FDR < 1E−3 (33) and considering all the proteins identified in the NK total cell extract as our background. All the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (34) partner repository with the dataset identifier PXD014894.

Cytokine Bead Array

NK-derived EVs (15 μg NKMV and 30 μg NKExo) and supernatants of coculture experiments were assayed for the presence of cyto-chemokines by cytokine bead array (CBA, Becton Dickinson, San Diego, CA, United States) according to manufacturer's instructions. Samples were acquired with a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed by the FCAP Array software (Becton Dickinson).

Coculture Experiments

All experiments were performed using RPMI 1640 medium (Lonza Verviers, Belgium), supplemented with 10% bovine EV-depleted fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin (Lonza), and 2 mmol/L glutamine in a 5% CO_2 environment at 37°C. All cell lines were negative for mycoplasma contamination, as routinely tested by a PCR mycoplasma detection kit (Venor GeM; Minerva Biolabs, Berlin, Germany). Before coculture experiments with CD3/CD28 stimulation, PBMCs were labeled with carboxyfluorescein diacetate succimidyl ester (CFSE; Molecular Probes, Invitrogen Technologies, MA, United States). To study the activity of NKEVs on monocytes, 10^5 PBMCs or purified $CD14^+$ cells from healthy donors were incubated with 15 μg isolated NKMV or 30 μg NKExo for 24 h. PBMC experiments included conditions in the presence or absence of LPS (250 ng/ml). Effects of NKMV or NKExo were also investigated on CFSE-labeled PBMCs or CD14-depleted PBMCs stimulated or not for 72 h with anti-CD3 (plate bound, 1 μg/ml; OKT3 Orthoclone, Janssen Biotech, Inc. PA, United States) and anti-CD28 (1 μg/ml; Becton Dickinson, NJ,

United States). To mimic an immunosuppressive environment, experiments with CFSE-labeled PBMC were also performed in the presence of recombinant IL-10 (10 ng/ml; Peprotech, NJ, United States) and recombinant TGFβ (10 ng/ml; Peprotech). At the end of the incubation, cells were harvested and incubated with fluorochrome-conjugated Abs to allow phenotyping of monocytes, NK cells, and T cells. Supernatants were collected from coculture experiments for cyto-chemokine detection by cytokine bead array. To test the stimulatory activity of NKEV-conditioned monocytes on activated T cells, $CD14^+$ cells, isolated from PBMCs with anti-$CD14^+$ beads (MACS, Miltenyi Biotec, Germany) according to the manufacturer's instructions, were cultured in the presence of NKEVs (15 μg NKMVs or 30 μg NKExo) for 24 h. After a washing step to eliminate excess NKEVs, monocytes were added to the autologous CD14-negative fraction, previously labeled with CFSE, in the presence of T-cell stimuli anti-CD3/antiCD28, as described above. At the end of the incubation (72 h), cells were harvested and evaluated for proliferation and CD25 expression by flow cytometry.

Flow Cytometry

Acquisitions were performed using a Beckman Coulter Cytoflex flow cytometer, and data were analyzed by the Kaluza software (Beckman Coulter, Milan, Italy). Cytoflex Daily QC Fluorospheres (Beckman Coulter) were used to calibrate the flow cytometer. The following monoclonal fluorochrome-conjugated antibodies were used for cell labeling of $CD14^+$ cells from NKEV-monocyte cultures and PBMCs harvested from LPS-stimulated NKEV-PBMC cocultures: CD3-KO (Beckman Coulter), CD14-Alexa 750 (Beckman Coulter), CD16-BV650 (Biolegend), CD80-86-PE (Becton Dickinson), and HLA-DR-APC (Beckman Coulter).

The following antibodies were used for cell labeling of peripheral blood lymphocytes (PBL), NK cells and PBMCs harvested from CD3/CD28-stimulated or unstimulated NKEV-PBMC cocultures, in the presence or absence of TGFβ/IL-10: CFSE (CellTrace, Thermo Fisher Scientific), CD3-ECD (Beckman Coulter), CD4-Alexa700 (Beckman Coulter), CD8-BV605 (Biolegend), CD56-BV510 (Becton Dickinson), CD16-BV650 (Biolegend), PD-1-PC7 (Beckman Coulter), CD25-PercpCy5.5 (Becton Dickinson), HLA-DR-APC (Beckman Coulter), and CD14-Alexa750 (Beckman Coulter).

Samples were incubated with Fc blocking reagent (Miltenyi Biotec, Germany) for 10 min at room temperature before the addition of monoclonal antibodies for 40 min at 4°C. Thereafter, samples were washed and fixed.

NKExoELISA

To measure NK exosomes isolated from the plasma of healthy donors and melanoma patients, a homemade specific immune-enzymatic test, the NKExoELISA, was conducted. Briefly, 96-well plates (Nunc, Milan, Italy) were coated with 4 μg/ml rabbit polyclonal anti-tsg101 antibody (ab70974, Abcam, Cambridge, United Kingdom) and incubated overnight at 4°C. After 1× PBS washes, a blocking solution [0.5% bovine serum albumin (BSA) in 1× PBS] was added at room temperature for 1 h. Following PBS washes, nanovesicles isolated by differential centrifugation of 1.0 ml of plasma were incubated overnight at 37°C. After PBS washes, 4 μg/ml of monoclonal anti-CD9 (ab2215, Abcam) or monoclonal anti-CD56 (123C3, Santa Cruz) was added for 1 h at 37°C. After three washes with PBS, the plate was incubated with horseradish peroxidase (HRP)-conjugated antimouse antibody (Pierce, Milan, Italy) for 1 h at room temperature, and the reaction was developed with Blue POD for 15 min (Roche Applied Science, Milan, Italy) and blocked with 1 N H_2SO_4 stop solution, and optical densities were recorded at 450 nm.

Statistical Analysis

Data are expressed as mean ± SEM or mean ± SD, as indicated, using GraphPad Prism, and *P*-values of 0.05 or less were considered to be significant. The statistical analysis was performed by paired and unpaired Student's *t*-test, as indicated.

RESULTS

Characterization of NKEVs: Microvesicles and Exosomes

MV and Exo produced by *in vitro*-amplified NK cells from healthy donors were isolated by differential centrifugation and characterized. NTA revealed bigger dimensions and major heterogeneity of NKMV as compared to NKExo (315.2 vs. 124.8 nm mean value, respectively). NKExo appeared more concentrated than NKMV (5.4×10^{10} vs. 1.51×10^9/ml, respectively) (**Figures 1A,B**). We calculated that a single NK cell produced 0.6×10^3 MVs and 2.1×10^4 Exo in our cell culture condition.

Scanning electron microscopy analysis (**Figures 1C,D**) showed an overview of NKMV and NKExo and confirmed a high density of both types of EVs in the 10,000 × *g* fraction (**Figure 1C**), while that one purified at 100,000 × *g* consisted prevalently of vesicles with dimensions smaller than 150 nm, which were accordant with an exosome population (**Figure 1D**). Transmission electron microscopy analysis confirmed the membranous nature of both kinds of EVs (**Figures 1E,F**) and revealed once again the quite exclusive presence of exosome-like vesicles in the 100,000 × *g* pellet (**Figure 1F**). Immunoelectron microscopy combined with positive/negative contrast method revealed the presence of CD81 EV marker (**Figures 1G,I**) and CD56 NK cell marker (**Figures 1H,J**) in both kinds of EVs.

Proteomic Profiles of NKEVs

To characterize healthy donor circulating NKMV and NKExo, we performed a proteomic analysis by LC-MS/MS of a biological triplicate constituted by three pools, derived from three different healthy donors each, in parallel to their parental *ex vivo*-expanded NK total cell extracts. Relative protein abundances were similar in all samples (**Figure S1**), and a total of 4,698 proteins were identified (**Figure 2A**; **Table S1**). Overall, 3,830 proteins were comprehensively identified in NKExo and NKMV: some of these proteins were also detected in the total cell extracts, while some others were specifically identified in NKExo (125 proteins) or NKMV (50 proteins) or in both (211 proteins) preparations. The majority of NKExo proteins as well as most of the NKMV proteins were present in the Vesiclepedia (95% for both) or Exocarta (81 and 73%, respectively) databases

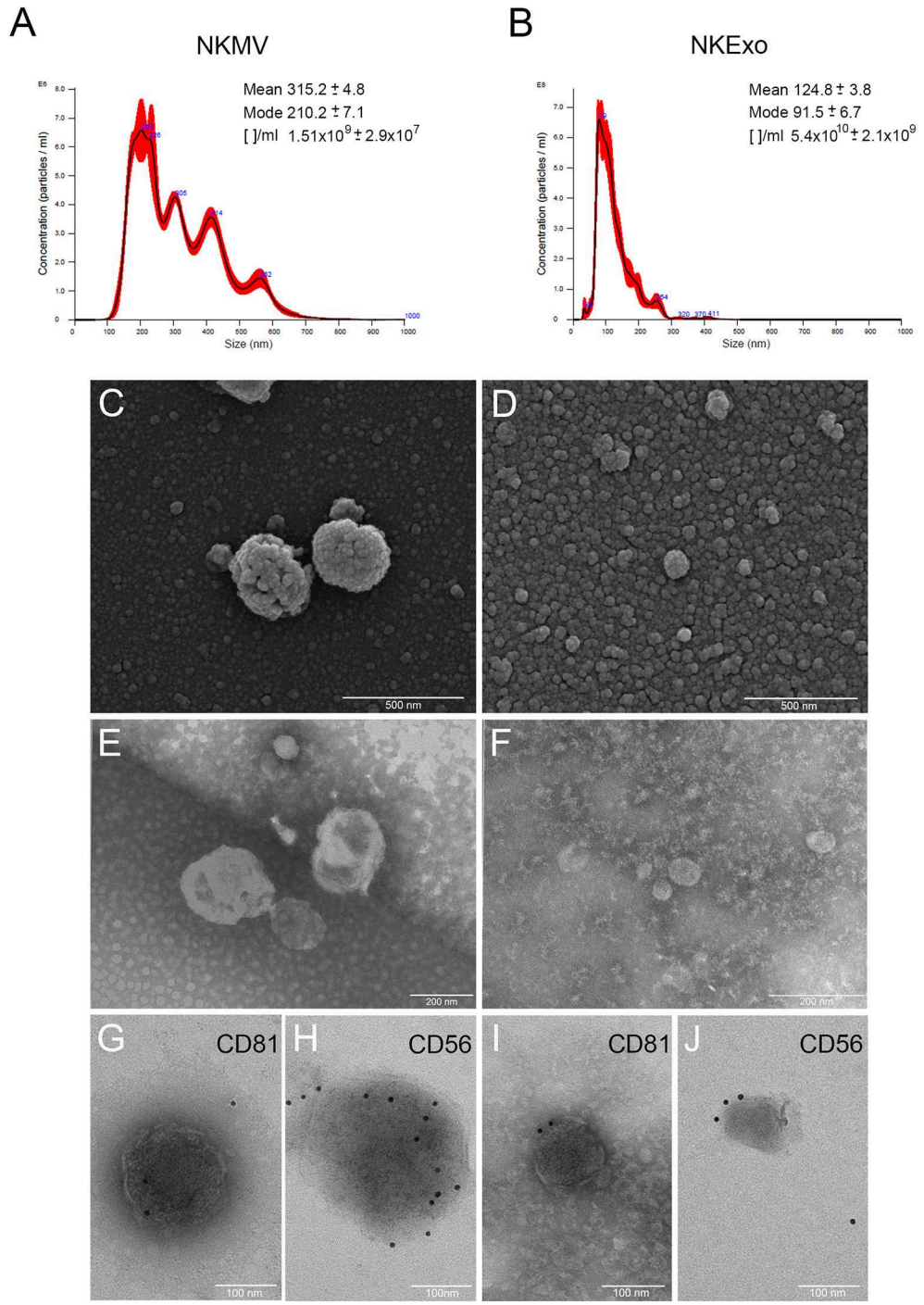

FIGURE 1 | Morphological characterization of natural killer cell-derived extracellular vesicles (NKEVs). **(A,B)** Nanoparticle tracking analysis (NTA) analysis of NK-cell-derived microvesicle (NKMV) and NKExo. Representative spectra are shown, and mean, mode, and particles number/ml ([]/ml) are reported. **(C,D)** Scanning electron microscopy analysis of NKMV **(C)** and NKExo **(D).** Bars, 500 nm. **(E,F)** Transmission electron microscopy analysis of NKMV **(E)** and NKExo **(F).** Bars, 200 nm. **(G–J)** Immunoelectron microscopy combined with positive/negative contrast method of NKMV **(G,H)** and NKExo **(I,J)** revealed the presence of CD81 **(G,I)** and CD56 **(H,J).** Bars, 100 nm.

(**Figures 2B,D**). In addition, 97 and 94% of the Top 100 proteins reported in the Exocarta database were also detectable in our preparations of NKExo and NKMV, respectively (**Figure 2C**), confirming a high enrichment of proteins previously identified in vesicles and/or exosomes. In particular, we have examined the relative amount of proteins expected to be enriched or not in

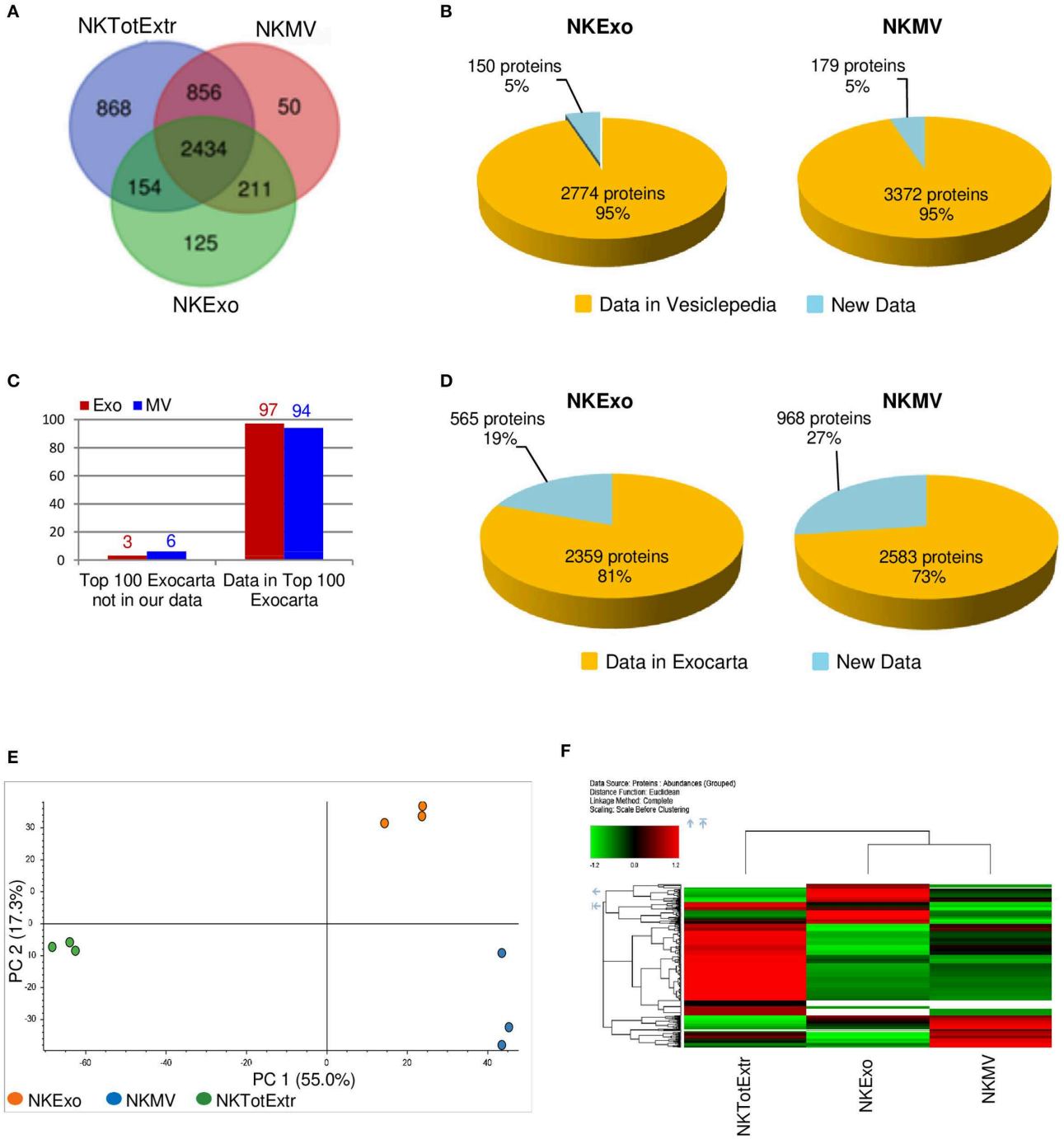

FIGURE 2 | Proteomic analysis of proteins identified in natural killer (NK) cell total extract, NK-cell-derived microvesicle (NKMV) and NKExo. **(A)** Venn diagram of all identified proteins in NK cell total extract (TotExtr), NKMV, and NKExo. **(B)** Proteins identified in NKExo and NKMV samples compared with those present in Vesiclepedia database. **(C,D)** Proteins identified in NKExo (Exo) and NKMV (MV) samples compared with those present in the top 100 Exocarta **(C)** and in the whole Exocarta database **(D). (E)** Principal component analysis (PCA) of NKExo, NKMV, and TotExtr normalized protein abundances. **(F)** Hierarchical cluster analysis of the grouped abundances of NKExo, NKMV, and TotExtr scaled before clustering and using the euclidean function for the distance.

the vesicles (both in NKExo and NKMV) according to categories 1–3 described by Lötvall and coworkers (35) (**Figures S2–S6**). As expected, we detected an enrichment of several proteins belonging to category 1, including tetraspanins (CD9, CD81, and CD82), integrins (integrin alpha-6, beta-3), flotilins (Flotilin-1 and Flotilin-2), and cell adhesion proteins (ICAM1 and

VCAM1), and category 2 (Syntenin-1, ALIX, and Annexin A3) both in NKExo and NKMV. Conversely, proteins expected not to be enriched in EVs, defined as category 3, including endoplasmic reticulum associated proteins (ERAP1, ERMP1, ERP29, and Calnexin), mitochondrial proteins (mitochondrial 2-oxoglutarate/malate carrier protein, mitochondrial dicarboxylate carrier, mitochondrial glutamate carrier, and mitochondrial proton/calcium exchanger protein), and the protein Ago1 were enriched in the parental cell extracts with respect to their vesicles. This analysis showed that the protein characteristics of our NKEVs are in line with previous definitions of EVs (35).

Principal component analysis was carried out on proteins identified in each individual biological replicate for each sample to evaluate the similarity among the different data sets (**Figure 2E**): the first and second principal component (PC1 and PC2) together account for ∼72% of the total sample variance and indicated low variance between biological replicates and a distinct separation among NKExo, NKMV, and cells. In particular, the predominant variance (55%) in the proteome content derived from differences between particles (NKMV and NKExo) and total cell extracts, while the second component (17%) evidenced the low level of similarity between NKMV and NKExo, as distinct from parental NK cell profiles. Differences in protein abundance profiles in NKMV, NKExo, and total cell extracts were compared using hierarchical clustering (**Figure 2F**): this relative quantitative analysis confirmed that the variance among the biological replicates was lower than that observed among sample groups (data not shown) and highlighted that NKExo and NKMV protein profiles shared a low level of similarity and were together highly different from that of total NK cell extract.

Differential protein expression among samples was examined by label-free approach: proteins with abundance ratio >1.5 and $P < 0.05$ were considered as significantly differentially expressed. A total number of 413 proteins were overexpressed in NKExo and/or NKMV in comparison with total cell extract, 48% of which was increased in both sets, while 24 and 28% were uniquely overexpressed in NKExo and NKMV, respectively (**Figure 3A**, **Table S2**). STRING analysis showed enrichment in protein–protein interactions (PPI enrichment, $P < 1.0E{-}16$) and in the EV cellular component (with 115 proteins belonging to extracellular region GO term 0005576 with FDR of 3.7E−28). Term enrichment in Reactome pathway knowledge revealed a relevant enrichment in proteins involved in the immune system followed by those implicated in protein metabolism and signal transduction, extracellular microenvironment, trafficking, and lipid metabolism (**Figure 3B**).

We then analyzed GO terms relative to cellular component and biological process separately in proteins enriched in NKExo (assembling proteins overexpressed in NKExo and those identified in NKExo and in NKExo plus NKMV) and proteins enriched in NKMV (assembling proteins overexpressed in NKMV and those identified in NKMV and in NKMV plus NKExo) (**Figure 4**). Cellular component terms were very similar between NKExo- and NKMV-enriched proteins, with the most important enrichment in extracellular region and extracellular exosome, as expected. NKExo and NKMV also shared several GO terms belonging to biological process annotation, but in some cases with different degrees of significance, as in the case of complement activation, immune response, and proteolysis, which resulted specifically significant among proteins enriched in NKExo. On the other side, retinoid and lipoprotein metabolic processes resulted in a significant unique enrichment in NKMV. These differences probably reflected a slightly different nature and function between NKExo and NKMV.

Among all the identified proteins in NKEVs, we selected 165 proteins playing key roles in the biology of NK cells and belonging to different clusters of cellular processes such as immune response, cell adhesion, complement activation, and blood coagulation (**Table 1**). As shown in **Figure 5**, a large amount of proteins were overexpressed by NKEVs compared to NK total extracts. These proteins belong to multiple categories with different biological roles, comprising cell adhesion, immune response, coagulation, and vesicle trafficking. Some proteins such as CD276, interferon-induced transmembrane protein 2, and tsg-101 were upregulated in NKExo as compared to that in NKMV. Other proteins, such as CD40 ligand, lysosomal-associated membrane protein 1 (LAMP-1), and coagulation factor IX were upregulated in NKMV, with respect to NKExo. These data suggest that even though NKMV and NKExo express the same proteins they might have different roles in the same pathways. Additional cyto- and chemokine profiling was performed by cytokine bead array (CBA) of NKMV (15 μg) and NKExo (30 μg) isolated from the same starting volume of NK cell culture supernatant. This was the condition we chose for coculture experiments based on our previous work with NK exosomes (22), where we used 30 μg of exosomes to obtain a biological effect. In the case of MV, the amount of protein that we recovered was much less compared to exosomes; thus, based on 30 μg NKExo, we used the maximum amount possible of MV, isolated from the same volume of supernatant as exosomes. CBA results confirmed that both types of vesicles carry remarkable amounts of different soluble factors. A similar distribution in MV and Exo could be measured for IL-8, regulated upon activation, normal T cell expressed and secreted (RANTES), and CD62L, while IL-6, IL-2, IFNγ, IL-12/IL23p40, FasL, and macrophage inflammatory protein 1α (MIP1α) appeared to concentrate in the MV and CCL2 in the Exo fraction. Considering both populations, we could detect higher concentrations of IL-6, FasL, CD62L, RANTES, MIP1α, and CCL2 with respect to IL-12/IL-23p40, IFNγ, and IL-2 (**Figure 6A**). Moreover, we could detect both in NKMV and NKExo a large amount of granzyme B, known to be a strong cytokine modulator as well as a potent cytotoxicity inducer (**Figure 6B**).

Effects of NKEVs on T Cells

To investigate the outcome of NKEVs and T-cell interactions, we isolated PBMCs from peripheral blood of healthy donors and incubated them for 72 h with NKMV (15 μg) or NKExo (30 μg) in the presence or absence of soluble IL-10 and TGFβ to mimic an immune suppressed environment. We could measure a statistically significant T-cell activation detectable as CD25 upregulation in $CD3^+$ cells, which was more pronounced in the presence of NKMV than in the presence of NKExo (**Figure 7A**),

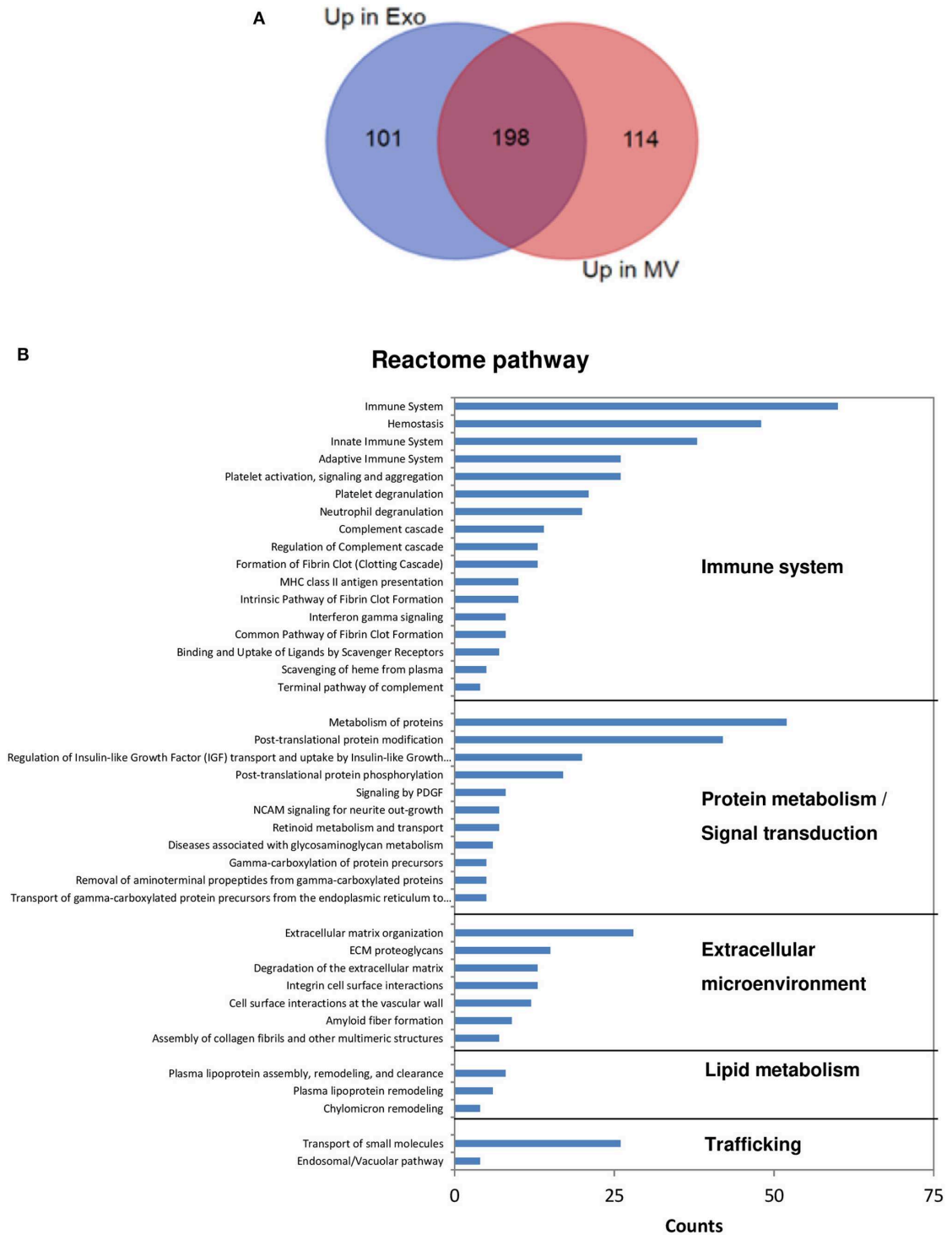

FIGURE 3 | Analysis of proteins overexpressed in NKExo and/or NK-cell-derived microvesicle (NKMV). **(A)** Venn diagram displaying unique and shared overexpressed proteins in NKExo (Up in Exo) and NKMV (Up in MV). **(B)** Reactome pathway annotation of proteins overexpressed in NKExo and/or NKMV obtained by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). The number of proteins (counts) involved in each Gene Ontology (GO) term is displayed.

confirming our hypothesis of an immune stimulatory role of NKEVs. NKEV-induced T-cell activation was still detectable in the presence of IL-10/TGFβ, even though at lower levels (**Figure 7A**), and was accompanied by a release of tumor necrosis factor alpha (TNFα), IFNγ, IL-12/IL-23p40, and RANTES (**Figure 7B**). The production of these cytokines was substantially

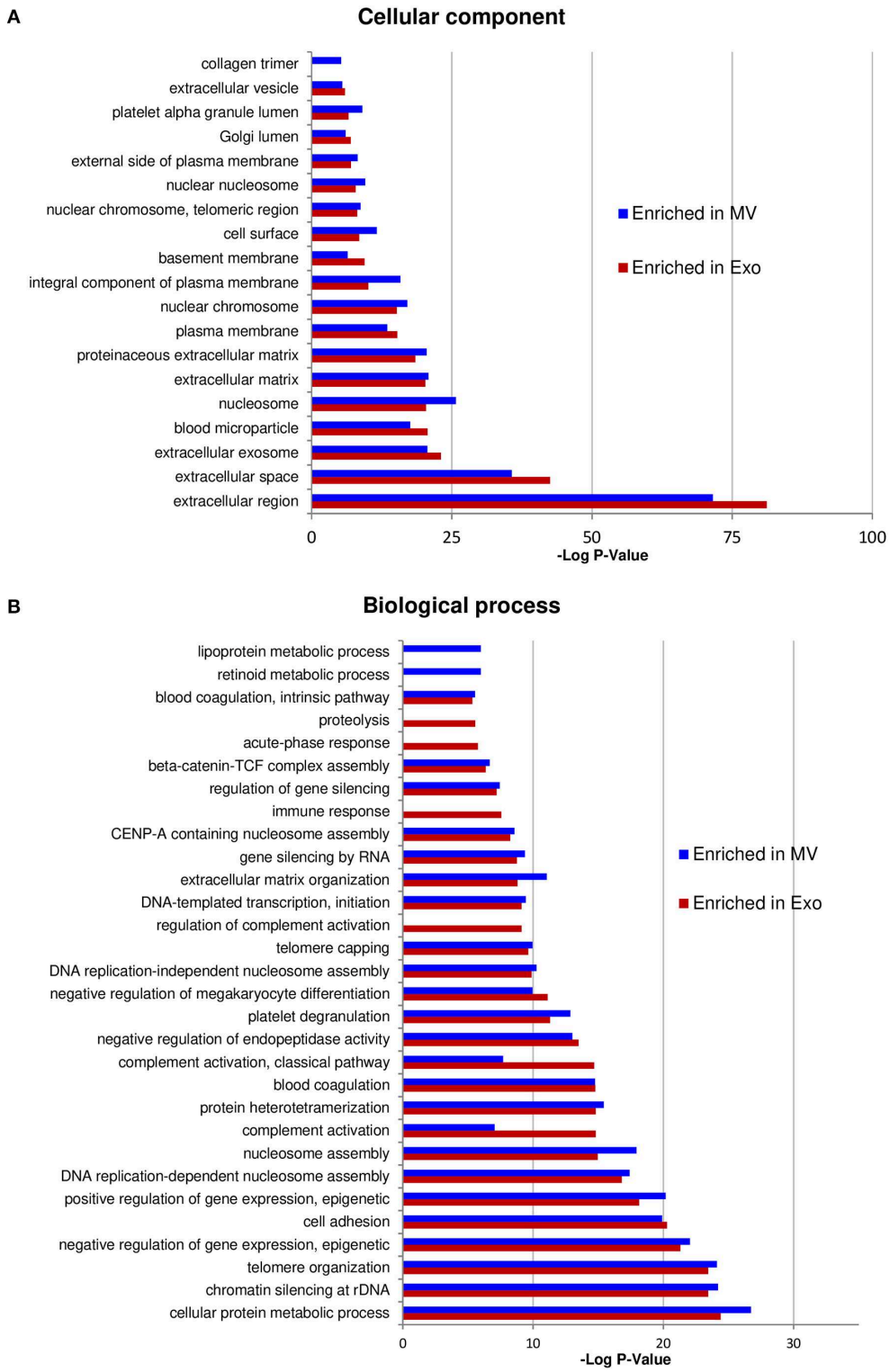

FIGURE 4 | Analysis of proteins enriched in NKExo and/or NK-cell-derived microvesicles (NKMV). Gene Ontology (GO) annotation of proteins overexpressed in NKExo and identified in NKExo or NKExo plus NKMV (enriched in Exo) and proteins overexpressed in NKMV and identified in NKMV or in NKMV plus NKExo (enriched in MV). The significance [$-\log_{10}(P\text{-value})$] for each GO term relative to cellular component **(A)** and biological process **(B)** is reported.

TABLE 1 | Selected proteins identified in natural killer cell-derived extracellular vesicles (NKEVs).

Category	Accession	Description	Entrez Gene ID	Up in MV[a]	Up in Exo[b]
Cell adhesion	P22303-1	Acetylcholinesterase	43	Up	Up
	Q9UHX3	Adhesion G protein-coupled receptor E2	30817	Up	Up
	Q9NVD7	Alpha-parvin	55742	Up	Up
	Q9HBI1-1	Beta-parvin	29780	Up	Up
	Q99828	Calcium and integrin-binding protein 1	10519	/	Up
	P49747	Cartilage oligomeric matrix protein	1311	Up	Up
	P48960-1	CD97 antigen	976	/	/
	P02452	Collagen alpha-1(I) chain	1277	/	/
	P20908	Collagen alpha-1(V) chain	1289	/	Up
	P12109	Collagen alpha-1(VI) chain	1291	Up	Up
	P39059	Collagen alpha-1(XV) chain	1306	Up	Up
	P12110	Collagen alpha-2(VI) chain	1292	Up	Up
	P12111	Collagen alpha-3(VI) chain	1293	Up	Up
	A6NMZ7-1	Collagen alpha-6(VI) chain	131873	Up	Up
	P29279-1	Connective tissue growth factor	1490	Up	Up
	Q12860	Contactin-1	1272	/	Up
	Q14574-1	Desmocollin-3	1825	/	/
	O43854-1	EGF-like repeat and discoidin I-like domain-containing protein 3	10085	/	/
	P17813	Endoglin	2022	Up	Up
	P02751	Fibronectin	2335	Up	Up
	Q14520-1	Hyaluronan-binding protein 2	3026	Up	Up
	Q13308-1	Inactive tyrosine-protein kinase 7	5754	/	Up
	P35858	Insulin-like growth factor-binding protein complex acid labile subunit	3483	/	Up
	P56199	Integrin alpha-1	3672	/	/
	P17301	Integrin alpha-2	3673	/	/
	P13612	Integrin alpha-4	3676	/	/
	P08648	Integrin alpha-5	3678	/	/
	P23229-1	Integrin alpha-6	3655	Up	Up
	P08514-1	Integrin alpha-IIb	3674	/	/
	P20701-1	Integrin alpha-L	3683	/	/
	P11215-1	Integrin alpha-M	3684	/	/
	P06756	Integrin alpha-V	3685	/	/
	P20702	Integrin alpha-X	3687	/	/
	P05556-1	Integrin beta-1	3688	/	/
	P05107	Integrin beta-2	3689	/	/
	P05106	Integrin beta-3	3690	Up	Up
	P26010-1	Integrin beta-7	3695	/	/
	Q08431	Lactadherin	4240	/	Up
	P07942	Laminin subunit beta-1	3912	/	/
	P55083	Microfibril-associated glycoprotein 4	4239	Up	Up
	P35580	Myosin-10	4628	Up	Up
	Q92859	Neogenin	4756	/	/
	Q14112-1	Nidogen-2	22795	/	Up
	Q15063-1	Periostin	10631	Up	Up
	P13224	Platelet glycoprotein Ib beta chain	2812	Up	Up
	P14770	Platelet glycoprotein IX	2815	Up	Up
	Q12884-1	Prolyl endopeptidase FAP	2191	Up	Up
	Q14517	Protocadherin Fat 1	2195	Up	/
	Q9UN70-1	Protocadherin gamma-C3	5098	Up	Up
	P16109	P-selectin	6403	Up	Up

(Continued)

TABLE 1 | Continued

Category	Accession	Description	Entrez Gene ID	Up in MV[a]	Up in Exo[b]
	Q13332	Receptor-type tyrosine-protein phosphatase S	5802	/	Up
	P78509	Reelin	5649	/	Up
	P05026	Sodium/potassium-transporting ATPase subunit beta-1	481	Up	/
	P24821	Tenascin	3371	Up	/
	P22105	Tenascin-X	7148	Up	Up
	P16591-1	Tyrosine-protein kinase Fer	2241	/	/
	P19320-1	Vascular cell adhesion protein 1	7412	/	/
Cell adhesion/immune response	Q13740-1	CD166 antigen	214	/	/
	P24001	Interleukin-32	9235	/	Up
	P16671	Platelet glycoprotein 4 CD 36	948	/	/
	P07996	Thrombospondin-1	7057	Up	Up
	P35442	Thrombospondin-2	7058	/	/
	P49746	Thrombospondin-3	7059	/	/
	P35443	Thrombospondin-4	7060	Up	Up
	O43294-1	Transforming growth factor beta-1-induced transcript 1 protein	7041	Up	Up
	Q15582	Transforming growth factor-beta-induced protein ig-h3	7045	Up	Up
	P04004	Vitronectin	7448	Up	Up
	P14151	L-Selectin	6402	/	/
Immune response	P13727	Bone marrow proteoglycan	5553	/	/
	P32248	C–C chemokine receptor type 7	1236	Up	/
	Q5ZPR3	CD276 antigen	80381	Up	Up
	P29965	CD40 ligand	959	Up	Up
	P09326	CD48 antigen	962	/	/
	P32970	CD70 antigen	970	Up	/
	P49682-1	C–X–C chemokine receptor type 3	2833	/	/
	P61073	C–X–C chemokine receptor type 4	7852	/	/
	Q86WI1	Fibrocystin-L	93035	/	/
	Q9Y6W8-1	Inducible T-cell costimulator	29851	Up	/
	P13164	Interferon-induced transmembrane protein 1	8519	/	/
	Q01629	Interferon-induced transmembrane protein 2	10581	/	/
	Q01628	Interferon-induced transmembrane protein 3	10410	/	Up
	Q9NPH3	Interleukin-1 receptor accessory protein	3556	/	Up
	Q13478	Interleukin-18 receptor 1	8809	/	/
	P14784	Interleukin-2 receptor subunit beta	3560	/	/
	Q9BZW8-2	Isoform 2 of Natural killer cell receptor 2B4	51744	/	/
	P43627	Killer cell immunoglobulin-like receptor 2DL2	3803	/	/
	P43628-1	Killer cell immunoglobulin-like receptor 2DL3	3804	/	/
	O43561	Linker for activation of T-cells family member 1	27040	Up	Up
	Q9GZY6	Linker for activation of T-cells family member 2	7462	/	/
	O14931-1	Natural cytotoxicity triggering receptor 3	259197	/	/
	Q13241	Natural killer cells antigen CD94	3824	/	/
	P26715	NKG2-A/NKG2-B type II integral membrane protein	3821	/	/
	P26718-1	NKG2-D type II integral membrane protein	22914	/	/
	Q92626	Peroxidasin homolog	7837	/	/
	P42081	T-lymphocyte activation antigen CD86	942	/	/
	Q03167	Transforming growth factor beta receptor type 3	7049	/	/
	P50591-1	Tumor necrosis factor ligand superfamily member 10	8743	/	/
	P19438-1	Tumor necrosis factor receptor superfamily member 1A	7132	/	/
	P43489	Tumor necrosis factor receptor superfamily member 4	7293	Up	/

(Continued)

TABLE 1 | Continued

Category	Accession	Description	Entrez Gene ID	Up in MV[a]	Up in Exo[b]
	P25942-1	Tumor necrosis factor receptor superfamily member 5	958	Up	/
	P25445-1	Tumor necrosis factor receptor superfamily member 6	355	/	/
	Q15628	Tumor necrosis factor receptor type 1-associated DEATH domain protein	8717	/	/
	P22749	Granulysin	10578	Up	/
	P12544-1	Granzyme A	3001	/	/
	P10144	Granzyme B	3002	/	/
	Q14761	Protein tyrosine phosphatase receptor type C-associated protein	5790	/	/
	P30455	HLA class I histocompatibility antigen, A-36 alpha chain	3105	Up	/
	P30479	HLA class I histocompatibility antigen, B-41 alpha chain	3106	Up	/
	P01889	HLA class I histocompatibility antigen, B-7 alpha chain	3106	Up	Up
	Q07000	HLA class I histocompatibility antigen, Cw-15 alpha chain	3107	Up	Up
	P30501	HLA class I histocompatibility antigen, Cw-2 alpha chain	3107	/	Up
	P01906	HLA class II histocompatibility antigen, DQ alpha 2 chain	3118	Up	/
	P79483	HLA class II histocompatibility antigen, DR beta 3 chain	3125	Up	Up
	Q30154	HLA class II histocompatibility antigen, DR beta 5 chain	3127	Up	Up
	Q95IE3	HLA class II histocompatibility antigen, DRB1-12 beta chain	3123	Up	/
	Q30134	HLA class II histocompatibility antigen, DRB1-8 beta chain	3123	Up	/
Complement	P13987	CD59 glycoprotein	966	/	/
	P00736	Complement C1r subcomponent	715	/	Up
	P06681-1	Complement C2	717	/	Up
	P01024	Complement C3	718	Up	Up
	P0C0L4-1	Complement C4-A	720	Up	Up
	P01031	Complement C5	727	Up	Up
	P13671	Complement component c6	729	/	Up
	P10643	Complement component C7	730	Up	Up
	P07357	Complement component C8 alpha chain	731	/	/
	P07358	Complement component C8 beta chain	732	/	/
	P02748	complement component C9	735	/	Up
	P08174-1	Complement decay-accelerating factor	1604	/	/
	P00751-1	Complement factor B	629	/	/
	P17927	Complement receptor type 1	1378	/	/
Coagulation	P01009-1	Alpha-1-antitrypsin	5265	/	/
	P08697-1	Alpha-2-antiplasmin	5345	/	Up
	P01008	Antithrombin-III	462	Up	Up
	P00740	Coagulation factor IX	2158	Up	Up
	P12259	Coagulation factor V	2153	Up	Up
	P00451	Coagulation factor VIII	2157	/	/
	P00742	Coagulation factor X	2159	Up	Up
	P00488	Coagulation factor XIII A chain	2162	/	/
	P05160	Coagulation factor XIII B chain	2165	Up	Up
	P02671-1	Fibrinogen alpha chain	2243	/	/
	P02675	Fibrinogen beta chain	2244	/	/
	P02679	Fibrinogen gamma chain	2266	/	/
	P05546	Heparin cofactor 2	3053	Up	Up
	P36955	Pigment epithelium-derived factor	5176	Up	Up
	P05155	Plasma protease C1 inhibitor	710	Up	Up
	P00734	Prothrombin	2147	Up	Up
	Q8IW75	Serpin A12	145264	/	/
	Q96P63	Serpin B12	89777	/	/

(Continued)

TABLE 1 | Continued

Category	Accession	Description	Entrez Gene ID	Up in MV[a]	Up in Exo[b]
	P29508	Serpin B3	6317	/	/
	P50454	Serpin H1	871	/	/
Nanovesicle marker/Tetraspanin	P21926	CD9 antigen	928	/	Up
	P08962	CD63 antigen	967	/	/
	P60033	CD81 antigen	975	/	Up
	P27701	CD82 antigen	3732	Up	Up
Vesicle trafficking	Q99816	Tumor susceptibility gene 101 protein	7251	/	/
	Q15836	Vesicle-associated membrane protein 3	9341	/	/
	O95183	Vesicle-associated membrane protein 5	10791	/	/
	Q99698-1	Lysosomal-trafficking regulator	1130	/	/
	Q13571	Lysosomal-associated transmembrane protein 5	7805	/	/
	P11279	Lysosome-associated membrane glycoprotein 1	3916	/	/
	P13473-1	Lysosome-associated membrane glycoprotein 2	3920	/	/
	Q86Y82	Syntaxin-12	23673	/	/
	P32856	Syntaxin-2	2054	/	/
	O43752	Syntaxin-6	10228	/	/
	O15400	Syntaxin-7	8417	/	/

[a] *Up indicates proteins found overexpressed in NK-cell-derived microvesicle (NKMV) (abundance ratio NKMV/NKTotExtr > 1.5 and adjusted P < 0.05).*
[b] *Up indicates proteins found overexpressed in NKExo (abundance ratio NKExo/NKTotExtr > 1.5 and adjusted P < 0.05).*

reduced under the same conditions when IL-10/TGFβ cytokines were added, although even in this suppressive milieu, NKEVs induced the release of TNFα and RANTES by PBMCs (**Figure 7B**). In contrast, if we depleted $CD14^+$ cells from PBMCs, the direct stimulatory effect of NKMV and NKExo in T cells was less evident, although we could measure a significant increase in the percentage of $CD25^+$ CD4 T cells in the presence of NKMV (**Figure S7A**). These data suggest a role of monocytes in NKEV-mediated T-cell activation on the one hand, but that NKEVs may also influence T cells in their absence on the other hand. In the presence of CD3/CD28 stimulation and/or soluble IL-10 and TGFβ for 72 h, we could detect no differences in CD25 upregulation of $CD3^+$-gated T cells in PBMCs, suggesting that NKMV or NKExo did not influence this process (**Figure 7C**). Similar results were obtained in the absence of $CD14^+$ cells, although as for unstimulated CD4 T cells, a nonsignificant upregulation of CD25 expression by CD4 T cells was detectable in the presence of NKMV (**Figure S7B**). In PBMC experiments, we also concomitantly measured PD-1 expression on CD3/CD28 stimulated T cells and observed that the presence of NKMV and NKExo led to a decrease in fluorescence intensity of this immune checkpoint, an effect that was accentuated by adding TGFβ and IL-10 (**Figure 7D**). Cytokine detection in conditioned media of PBMCs collected at the end of stimulation showed remarkable amounts of granzyme B majorly deriving from NKEVs. Interestingly, statistical analysis revealed a significant inverse correlation between granzyme B and PD-1 geometric mean fluorescent intensity (gMFI) of T cells (**Figure 7E**). In summary, our results suggest that NKEVs may contribute to T-cell activation.

Effects of NKEVs on Monocytes

We studied the effects of NKEVs on monocytes in PBMCs in the presence or absence of 24 h LPS to recapitulate tolerogenic conditions (36). Additional experiments were performed on purified $CD14^+$ cells to evaluate the direct effects of NKEVs. NKEV-conditioned monocytes were also tested for their stimulatory activity on autologous activated T cells. In PBMCs, we could assess a statistically significant increase in the costimulatory molecules CD80 and CD86 on gated $CD14^+$ cells in the presence of NKMV. If LPS was present in the cultures, we observed a downregulation of CD80 and CD86, which was countered by NKMV (**Figure 8A**). These data suggest that NKMV possess stimulatory properties that lead to an upregulation of costimulatory molecules even if monocytes were exposed to tolerogenic LPS stimulation. Similar results were obtained for HLA-DR expression, where we could observe an induction mediated also by NKExo. Again, the activating potential of NKEVs was evident in the presence of LPS (**Figure 8B**). Compared to monocytes in PBMCs, purified $CD14^+$ cells displayed less upregulation of the costimulatory molecules CD80-86 and HLA-DR if cultured with NKEVs. We could measure an increase in fluorescence intensity in monocytes only in one out of three healthy donors (**Figure 8C**). NKEV-conditioned monocytes were also tested for their stimulatory potential on autologous T cells in the presence of CD3/CD28 stimulation. Excess NKEVs were removed from monocytes before incubation with the CD14-depleted PBMCs. Results showed that, in one case out of three, NKEV-conditioned monocytes displayed an increased stimulatory activity on T-cell proliferation with respect to unconditioned ones, indicating

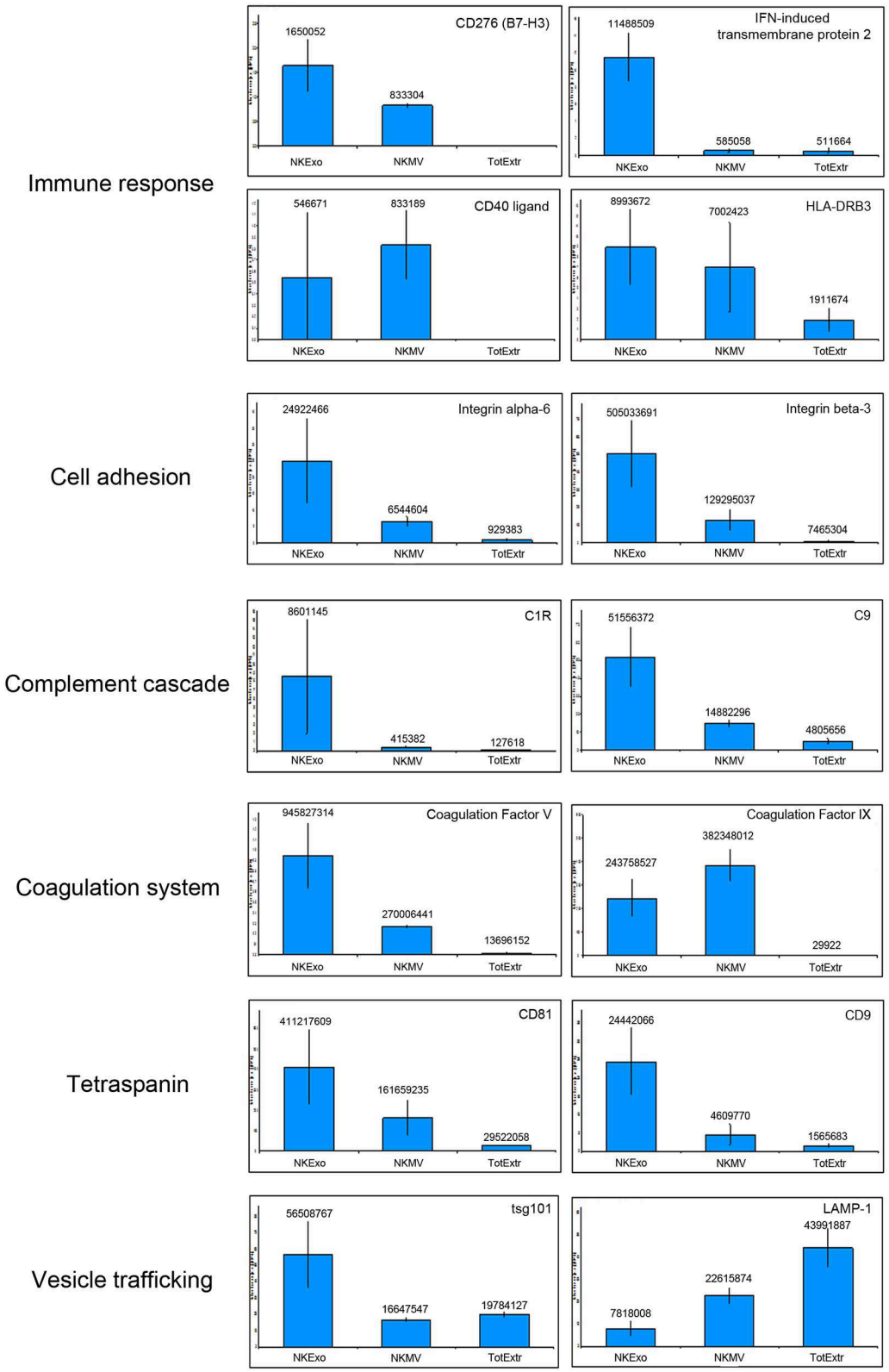

FIGURE 5 | Protein abundances of specific proteins quantified by liquid chromatography–MS/MS (LC-MS/MS). Representative proteins grouped in the indicated categories are shown. Each panel shows the protein median abundance of the three replicates of NKExo, NK-cell-derived microvesicle (NKMV), NK total cell extract (TotExtr). The bars indicate the standard error for each sample group.

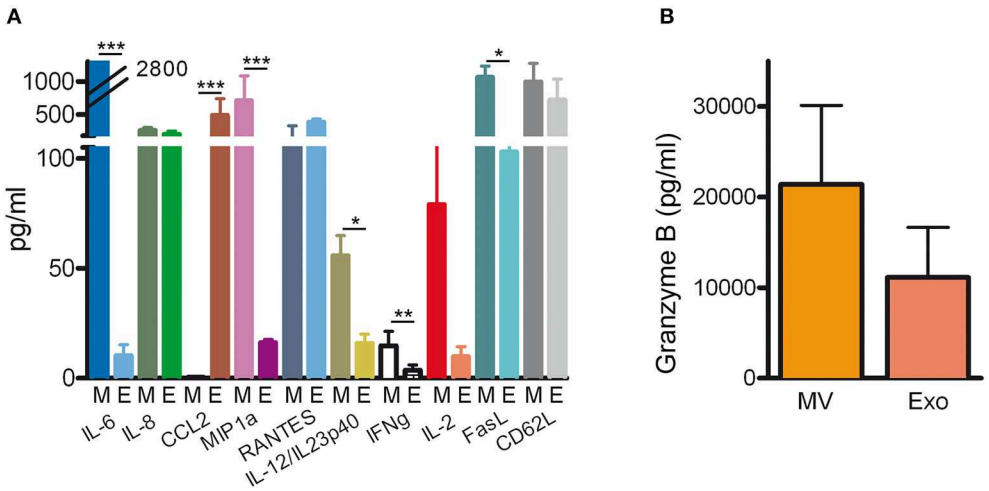

FIGURE 6 | Cyto- and chemokine profiling natural killer cell-derived extracellular vesicles (NKEVs). **(A)** Cytokine bead array for the indicated soluble factors in NK-cell-derived microvesicle (NKMV) (M) and NKExo (E). **(B)** Granzyme B expression by cytometric bead array (CBA) assay in NKMV (MV) and NKExo (Exo). Results are shown for NKEVs produced by different healthy donors ($n = 3$). Statistical significance was achieved by paired t-test. Means ± SD are plotted, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

that NKEVs can indeed positively influence activated T cells (**Figure 8D**).

Effects of NKEVs on NK Cells

The effects of NKEVs on NK cells were investigated in PBMCs from healthy donors cultured for 72 h in the presence or absence of TGFβ and IL-10. NK cells, gated as $CD56^{+}$ in the $CD3^{neg}$ gate (37), displayed an increase in percentage if NKEVs were present. This effect was particularly evident for the NKMV fraction, which maintained its stimulatory activity also in the TGFβ/IL-10 condition (**Figure 9A**, upper panels). Upon evaluation of the NK cell subpopulations, we could determine higher levels of $CD56^{bright}$ cells, the IFNγ-producing population, also in the presence of TGFβ/IL-10. The cytotoxic $CD56^{dim}CD16^{+}$ cells increased only in the absence of TGFβ/IL-10, while $CD56^{dim}CD16^{neg}$ resting NK cells decreased (**Figure 9A**, lower panels). Differences were statistically significant in the case of total NK cells (**Figure 9B**, left upper panel) and $CD56^{bright}$ NK cells (**Figure 9B**, right upper panel), where effects were mediated by both NKMV and NKExo. In the presence of TGFβ/IL-10, only effects mediated by NKMV maintained their statistical significance. No statistically significant differences could be assessed for $CD56^{dim}CD16^{+}$ and $CD56^{dim}CD16^{neg}$ NK cells (**Figure 9B**, lower panels). We also evaluated the activity of NKEVs on NK cells in PBMCs depleted from monocytes, in the presence or absence of CD3/CD28 T-cell stimuli. Similarly to what we observed in PBMCs, also in the absence of monocytes, NKMV led to a statistically significant increase in NK cells (**Figure 9C**, upper left panel). Interestingly, the presence of T-cell stimuli led to a significant increase in percentage of $CD56^{dim}$ NK cytotoxic cell population concomitantly with a trend to decrease in $CD56^{dim}CD16^{neg}$ and $CD56^{bright}$ NK cells (**Figure 9C**), an effect we could not detect if monocytes were present (data not shown).

Circulating NK-Derived Exosomes and PBL/NK Cells in Healthy Donors and Melanoma Patients

Several studies correlate cancer with NK-cell absence or dysfunction, highlighting the value of NK cells in cancer immunosurveillance. It has been demonstrated that lessened NK-cell-mediated cytotoxicity is often associated with increased risk of cancer development (5). With this premise, we first characterized freshly isolated peripheral blood lymphocyte (PBL) from melanoma patients and compared them with those from healthy donors (**Figure 10A**). The percentage of CD3, CD4, CD8, CD16, and CD56 cells in cancer patients' PBL was significantly decreased with respect to healthy donors, as shown in **Figure 10A**. Next, we evaluated the ability of NK cells to expand using the method previously reported (22). After 15 days, melanoma-patient-derived PBL cultures contained only few alive cells, and these expressed a different pattern of differentiation cluster compared to cells expanded from healthy donors' PBL (**Figures 10B,C**). In particular, healthy donor NK cells proliferated 30–40 times more and showed 80–95% of differentiation toward the NK phenotype, displaying higher expression of CD56 and CD16 molecules. On the opposite, melanoma patients' PBL neither proliferated nor differentiated into NK cells, expressing essentially CD3 and CD4 molecules (**Figure 10C**).

To test if this NK cell function deficiency could be detected in the liquid biopsy, we performed a homemade ELISA test (NKExoELISA) for the capture and relative quantification of NKEVs in human plasma. NKExoELISA detects NKExo in at least 1 ml of plasma and displays sensitive results, thus excluding

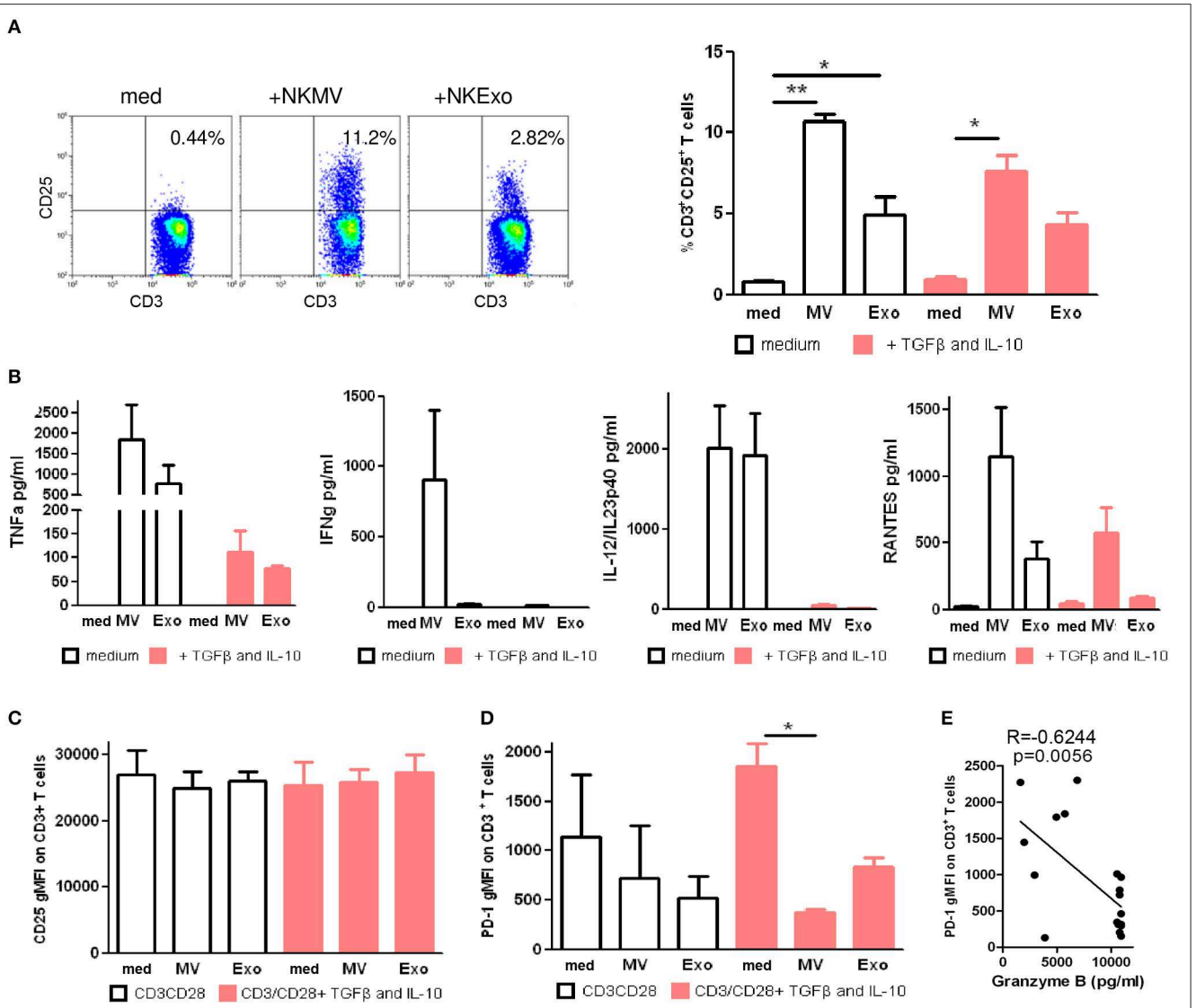

FIGURE 7 | Effects of natural killer cell-derived extracellular vesicles (NKEVs) on T cells. **(A)** Flow cytometry analysis of CD25 expression by $CD3^+$ gated T cells in peripheral blood mononuclear cells (PBMCs) evaluated after 72 h of culture with NK-cell-derived microvesicle (NKMV) and NKExo. Left panels: representative dot plots of a healthy donor. Right panel: the graph shows the results obtained with PBMCs of different healthy donors ($n = 3$), in the presence or absence of transforming growth factor beta (TGFβ)/interleukin (IL)-10 (10 ng/ml each). **(B)** Cytometric bead array (CBA)-measured cytokine production of 72 h PBMCs cultured as described in **(A)**. **(C)** Geo mean fluorescence intensity (gMFI) of CD25 expression of $CD3^+$ gated T cells in CD3/CD28 activated PBMCs in the presence or absence of NKMV and NKExo and/or TGFβ/IL-10 (10 ng/ml each). **(D)** PD-1 gMFI on gated $CD3^+$ T cells as in **(C)**. **(E)** Pearson correlation analysis of PD-1 gMFI and GRZB production. Results are shown for different healthy donors ($n = 3$). Statistical significance was achieved by paired *t*-test. Means ± SD are plotted, $^*p < 0.05$; $^{**}p < 0.01$.

MV. Probably, the major size of MV, compared to exosomes, prevented the antibody from recognizing and binding the antigen exposed on microvesicles. With the NKExoELISA, we capture intact circulating exosomes by a polyclonal antibody anti-tsg101, a typical exosome marker, and then detect exosomes released specifically by NK cells with a monoclonal anti-CD56 antibody (**Figure 10D**, graphical scheme). The analysis performed on the plasma of healthy donors compared to melanoma patients clearly showed that the amount of all circulating exosomes in melanoma patients was higher than in healthy donors ($tsg\text{-}101^+/CD9^+$ exosomes), while the fraction of $tsg\text{-}101^+/CD56^+$ exosomes (NKExo) was significantly lower (**Figure 10E**).

DISCUSSION

In view of a potential future application of NKEVs in support of cancer therapies, we studied EVs released into the extracellular milieu by NK cells from healthy donors to provide a photograph of the physiological content of these nanovesicles. We characterized NKEVs, both NKMV and NKExo, produced by human *ex vivo*-expanded healthy donor NK cells by NTA and electron microscopy and by LC-MS/MS mass spectrometry to get deeper insight into their morphology and protein composition. We also provided first evidence of their effects on immune cells in a healthy donor setting. Furthermore, with the intent

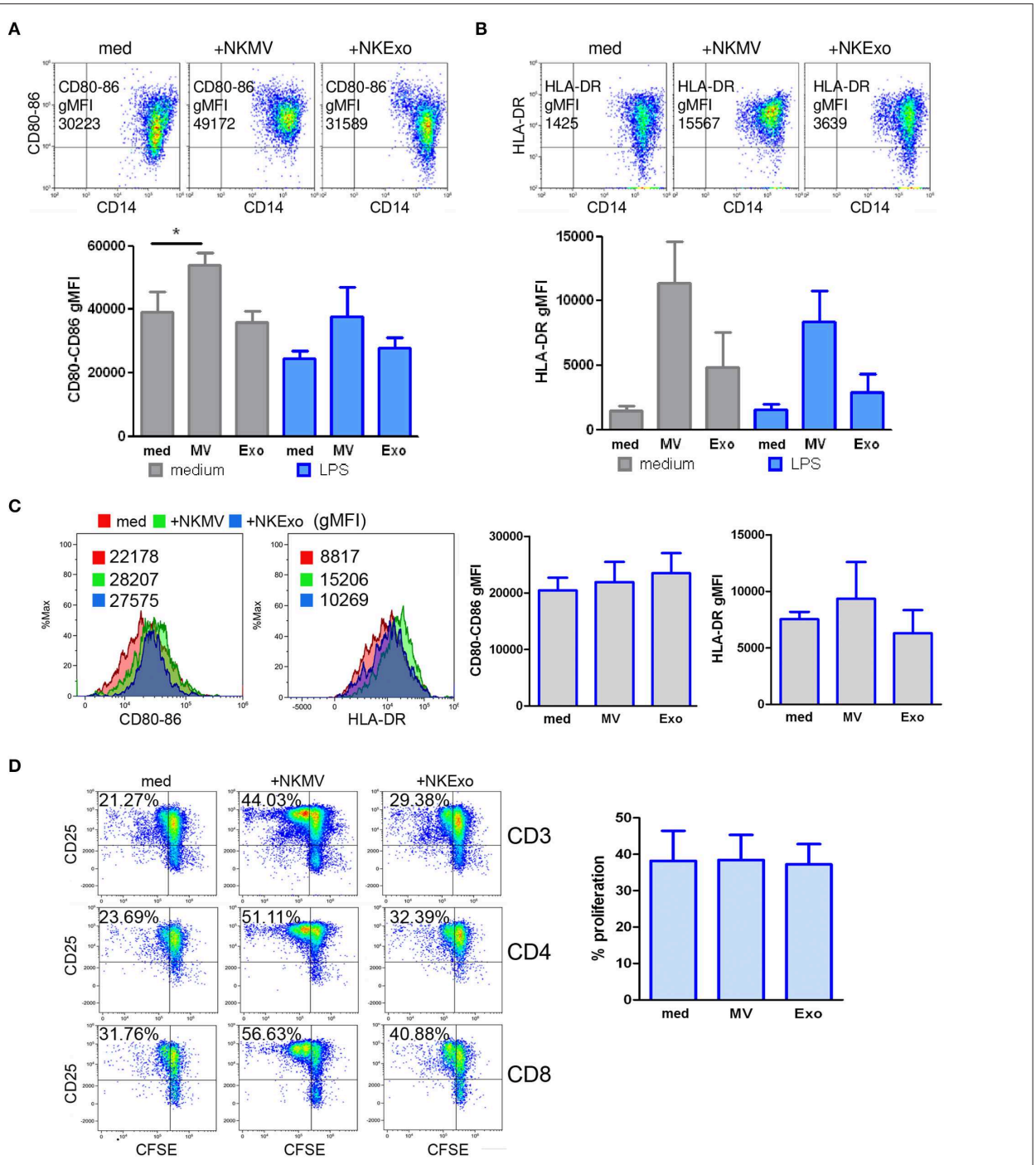

FIGURE 8 | Effects of natural killer cell-derived extracellular vesicles (NKEVs) on monocytes. **(A)** Flow cytometry analysis of CD80–CD86 geo mean fluorescence intensity (gMFI) of gated $CD14^+$ cells in peripheral blood mononuclear cells (PBMCs) cultured in the presence or absence of NK-cell-derived microvesicles (NKMV), NKExo, and/or lipopolysacharride (LPS) for 24 h. Upper panels: representative dot plots showing CD80–CD86 expression in the presence of NKMV and NKExo, lower panel: graphical summary of different healthy donors. **(B)** Flow cytometry of human leukocyte antigen DR isotype (HLA-DR) gMFI of $CD14^+$ gated monocytes as in **(A).** Results for different donors ($n = 3$) are shown. **(C)** Effects of NKEVs on isolated monocytes, measured by flow cytometry after 24 h culture of $CD14^+$ cells with NKMV or NKExo. Left panels: gMFI of CD80-86 and HLA-DR expression by monocytes of one healthy donor. Right panel: results from different healthy donors ($n = 3$). **(D)** Stimulatory potential of monocytes preconditioned with NKMV and NKExo. Left panels: flow cytometry analysis of 72 h proliferation and CD25 expression by CD3, CD4, and CD8 T cells cultured in the presence of monocytes (medium), monocytes preconditioned with NKMV or NKExo. Right panels: graphical summary showing results from different healthy donors ($n = 3$). Percentage of proliferation is indicated. Statistical significance was achieved by paired *t* test. Means ± SD are plotted, $^*p < 0.05$.

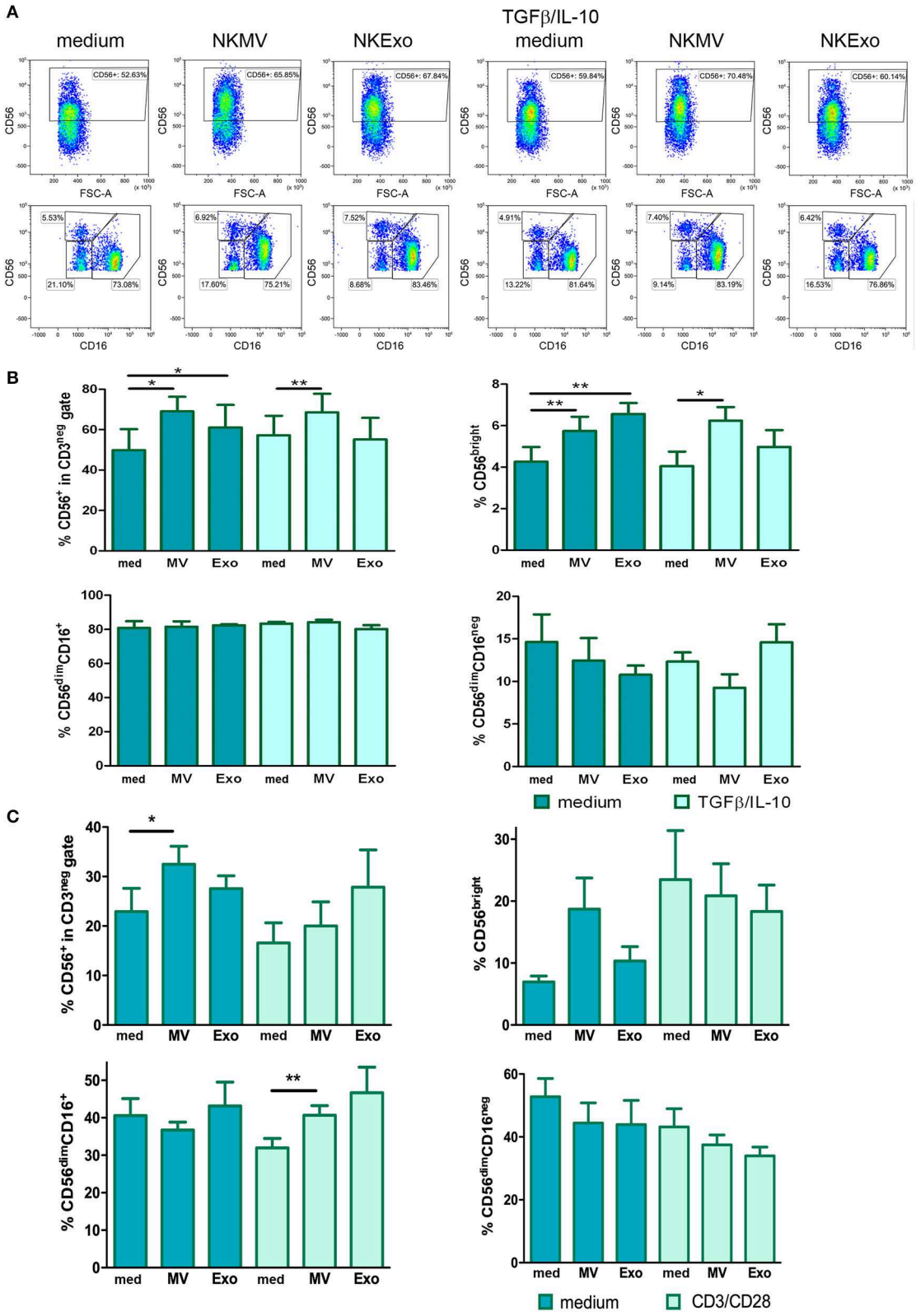

FIGURE 9 | Effects of natural killer cell-derived extracellular vesicles (NKEVs) on NK cells. **(A)** Upper panels: representative dot plots showing CD56^{+} total NK cells in the CD3^{negative} lymphocyte gate after culturing PBMCs for 72 h in the presence or absence of NK-cell-derived microvesicle (NKMV) and NKExo and/or transforming

(Continued)

FIGURE 9 | growth factor beta (TGFβ)/interleukin (IL)-10 (10 ng/ml each). Lower panels: representative dot plots showing the percentage of NK cell subpopulations in the $CD56^{+}$ gate for each culture condition as in upper panels: $CD56^{bright}$, $CD56^{dim}CD16^{+}$, and $CD56^{dim}CD16^{neg}$ NK cells. **(B)** Graphs show the results obtained for total NK cells and each subpopulation shown in **(A)** for different healthy donors ($n = 3$). **(C)** Graphs show total NK cells and NK subpopulations after 72 h culture of the $CD14^{negative}$ fraction in the presence or absence of NKMV or NKExo and/or CD3/CD28 stimulation. Results were obtained with cells from different donors ($n = 3$). Statistical significance was achieved by paired *t* test. Means ± SD are plotted, $^{*}p < 0.05$; $^{**}p < 0.01$.

of exploiting NKExo as surrogate of circulating NK cells, we conducted a homemade immunoenzymatic test to capture and quantify NKExo from human plasma. Our NKExoELISA is the first attempt to measure the human NK cell status via EVs and allowed us to quantify lower amounts of NKExo in the plasma of melanoma patients with respect to healthy donors, likely reflecting the biological defects of circulating NK cells in cancer patients.

We found that NK cells, in addition to exosomes, also release microvesicles. The size that we measured for NKExo, 124.8 ± 3.8 mean and 91.5 ± 6.7 mode, corresponds to the size defined for exosomes (30–150 nm), while NKMVs ranged from 315.2 ± 4.8 mean and 210.2 ± 7.1 mode, consistent with the one described for membrane-derived EVs (150–1,000 nm). Mass spectrometry showed that both NKExo and NKMV express specific biomarkers, high levels of cytoskeletal (actin, myosin, ERM proteins), cytosolic [14-3-3 protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)], heat shock (e.g., HSP60, HSP70), antigen presentation (i.e., MHC-I, MHC-II), and plasma membrane proteins, i.e., the tetraspanins CD9, CD63, CD81, CD82, as well as proteins involved in vesicle trafficking, such as Tsg101 and several syntaxins. The expression of all these proteins on NKEVs is in accordance with the definition of EVs given by the International Society for Extracellular Vesicles (ISEV) (35, 38). Their protein composition postulates an involvement of NKEVs in cell adhesion, immune response, homing, cytotoxicity, coagulation, and complement cascade. NKMV and NKExo share several proteins, but with different degrees of significance, as in the case of proteins involved in complement activation, immune response, and proteolysis, resulting in more enrichment in NKExo than in NKMV. On the other side, the retinoid and lipoprotein metabolic processes resulted in significant enrichment in NKMV. NKEVs express several cell adhesion molecules (CAMs). The integrins facilitate cell–extracellular matrix adhesion and activate internal signaling transduction pathways (39). Integrin-mediated sensing, stiffening, and remodeling of the tumor stroma are key steps in cancer progression supporting invasion, acquisition of cancer stem cell characteristics, and drug resistance (40). The expression of several integrins by NKEVs could disturb the functionality of the integrins expressed by cancer cells, entering for example in competition with integrin ligands in the tumor microenvironment (41). Other important CAMs expressed by NKEVs are selectins, which are molecules mediating physiological responses such as inflammation, immunity, and hemostasis and involving in constitutive lymphocyte homing (42). Elevated levels of selectin ligands in carcinomas are associated with poor prognosis (43), and selectins can activate signaling cascades that regulate immune responses within a tumor microenvironment (44). Probably, the expression of these CAMs implies an involvement of NKEVs in the complex cellular crosstalk, both in physiological and pathological conditions. As expected, NKEVs carry a large variety of proteins involved in immune responses: CD276 (B7-H3), an immune checkpoint (45) expressed by NK cells that is involved in self-tolerance and costimulation of innate immunity by augmenting proinflammatory cytokine release of TNFα, IFNγ, IL-6, IL-1beta, and IL-12p70 from LPS-stimulated monocytes/macrophages; CD55 and CD59 complement regulators that could enable EVs to escape from complement attack, thus potentially prolonging their circulatory availability and stability (46); CD97, an activation-induced antigen on leukocytes and a critical mediator of host defense that binds CD55 and that is upregulated to promote adhesion and migration to sites of inflammation (47). It has been acknowledged that exosomes released by immune cells may act as antigen-presenting vesicles, stimulating antitumoral immune responses, or as inducers of tolerogenic effects suppressing inflammation (48). In fact, it has been demonstrated that although unstimulated NK cells do not express MHC class II, activated NK cells express HLA-DR and can initiate MHC class-II-dependent $CD4^{+}$ T-cell proliferation (49, 50). Early studies showed that immune-cell-derived EVs carry MHC classes I and II (51). Surprisingly, our experiments show that also NKEVs express MHC classes I and II. These data suggest that NKEVs could have a role not only in MHC class I self-recognition but also in antigen presentation. NKEVs express multiple cytotoxic proteins, such as perforin, FasL, granzyme A and B, granulysin (22, 25), as well as molecules that activate NK-mediated cytolysis, i.e., CD40L. Indeed, reports indicate that NKEVs can trigger multiple killing mechanisms (52). NK cells and probably also NKEVs may possess regulatory functions (6). By means of IFNγ and TNF, NK cells can promote the maturation of DCs, which in turn activate NK cells via IL-12. We report in this study that NKEVs also express several TNF receptors and ligands and carry different types of interferon-induced transmembrane proteins (IFITM). Therefore, in a specular way to the NK cells, the NKEVs could influence adaptive immune responses by directly acting on T and B cells. In the inflamed lymph node, NK cells promote the priming of $CD4^{+}$ T helper type 1 cells by secreting IFNγ (53), a phenomenon that could me mediated also by NKEVs. In addition, NKEVs carry different interferon regulatory factors (IRFs). Regarding coagulation-related proteins, this is the first report showing their expression by NKEVs and with regulatory functions, i.e., factors V, VIII, IX, X, and XIII, fibrinogen, prothrombin, anticoagulant antithrombin, protein S, and protein C. In cancer, coagulation facilitates tumor progression through the release of platelet granule contents, inhibition of NK cells, and recruitment of macrophages (54–56). Plasma exosomes in epithelial ovarian cancer seem to cover a potential role in

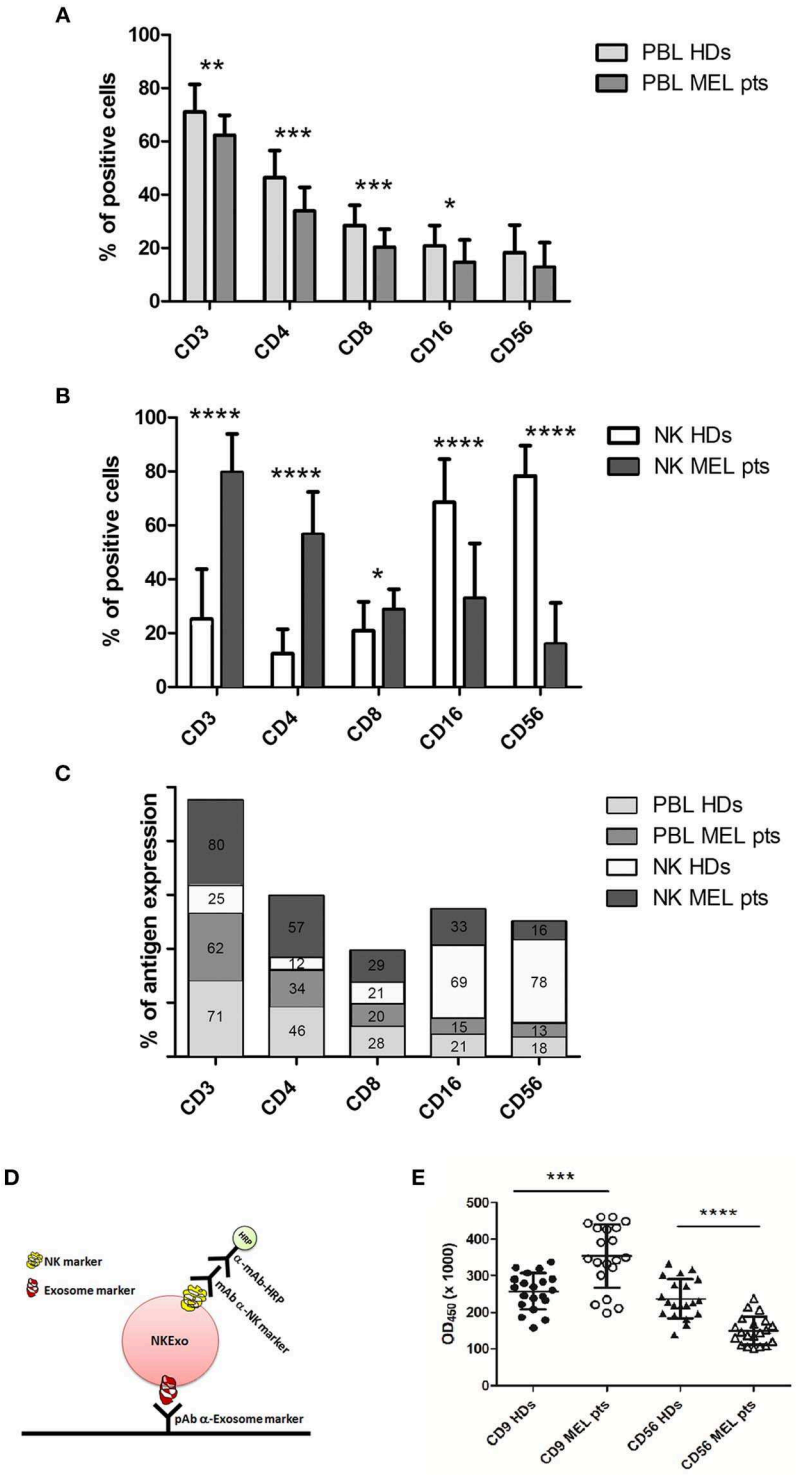

FIGURE 10 | Characterization of circulating peripheral blood lymphocyte (PBL) and natural killer (NK) cells and quantification of plasma NKExo. **(A)** Immune phenotype analysis of freshly circulating PBL from healthy donor (HDs) and melanoma patients (MEL pts). **(B)** Immune phenotype analysis of 15 days-expanded NK cells from HDs and melanoma patients PBL. **(C)** Percent of indicated CD antigen expression in freshly PBL and 15 days expanded NK cells of HDs and melanoma patients. **(D)** Graphic scheme of "homemade" ELISA to detect and quantify NKExo in human plasma. (**(E)** Quantification of all circulating exosomes, by tsg-101 capture and CD9 detection and also of NKExo by tsg-101 capture and CD56 detection in plasma of HDs and melanoma patients, using the NKExoELISA test. Statistical significance was achieved by unpaired *t* test. Means ± SD are plotted, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

the coagulation cascade (57), and treatment of cancer patients with anticoagulant drugs led to diminished metastasis (56). Thus, NKEVs could contribute to coagulation homeostasis and cancer surveillance.

Given that NKEVs contained numerous proteins involved in immune regulation, we were not surprised to detect modulatory effects on different immune cells. Our experiments were performed with healthy donors' PBMCs cultured with NKEVs derived from healthy donors' NK cells to assess the physiological properties of these vesicles. In this setting, we also included conditions aimed at recapitulating an immune suppressive state. Overall, our results show that NKEVs exhibit stimulatory activities on monocytes, T cells, and NK cells in a PBMC context and to a lesser extent also on isolated monocytes and on T and NK cells in the CD14-depleted fraction, potentially suggesting that a direct and indirect stimulation by NKEVs, which could occur simultaneously in a PBMC setting, may represent an optimal condition for stimulation by NK-cell-derived EVs. Results of PBMC studies showed that NKEVs induced the expression of HLA-DR and costimulatory molecules on monocytes albeit the presence of LPS, a condition aimed at mimicking a tolerogenic environment (36). Again, in a PBMC setting, NKEVs stimulated the upregulation of CD25 by T cells, while in the presence of CD3/CD28 triggering they induced a downregulation of PD-1 in gated T cells that correlated with the presence of granzyme B, this latter deriving mostly from NKEVs but potentially also from CD3/CD28-stimulated cells. Although attenuated, these effects were still detectable in PBMC-NKEV cocultures containing TGFβ and IL-10, a condition we included to recapitulate an immune compromised microenvironment, such as in cancer (58). The NKEV-mediated stimulatory activity on the NK cell population was detectable in PBMCs as well as in CD14-depleted PBMCs, showing an increase in percentage of $CD56^{+}$ total NK cells and the $CD56^{bright}$ and $CD56^{dim}$ subpopulations. NKEVs could engage with NK cells and amplify their inflammatory response, as reported for monocyte–NK cell interaction (59). Most of the observed effects were primarily mediated by the NKMV fraction, although also NKExo displayed stimulatory properties. This could depend on the higher content of stimulatory immune factors such as IL-2 and IFNγ, which we measured in NKMV with respect to NKExo. These soluble factors were detected by cytokine bead array in 15 μg NKMV and 30 μg NKExo protein after isolating them from the same volume of supernatant. This kind of measurement did not take into account the differences in vesicle size or concentration, thus suggesting that in half the protein amount, the MV carry more of some of the measured cytokines with respect to Exo. The increased stimulatory potential of MV might also depend on the bigger dimension of these EVs, which could facilitate a stronger EV–cell contact via enhanced available interaction surface. Thanks to their rich repertoire of adhesion molecules, NKEVs may bind to target cells to deliver their activating signals via surface–surface interaction directly. In fact, we hypothesize this to represent the main type of interaction with T and NK cells. In contrast, the interaction of monocytes with NKEVs may also be conditioned by internalization of the vesicles, potentially leading to a different response by monocytes as compared to surface–surface interaction. In addition, the differences in donor cells' response that we observed upon conditioning isolated monocytes with NKEVs and their consequent stimulatory potential on T-cell proliferation may suggest interdonor variability. In the absence of CD3/CD28 stimuli, NKMV induced the activation of CD4 T cells, a phenomenon that could rely on HLA-DR expressed by NKEVs, as previously shown for NK cells (50). The observed immunomodulatory effects still need further investigation; nonetheless, our results may represent a starting point for future evaluation of NKEVs stimulatory potential also on cancer-patient-derived immune cells, including myeloid-derived suppressor cells and regulatory T cells, major contributors to the detrimental phenomenon of immune suppression in cancer (60). Here, the activity of NKEVs could potentially contribute reversing immune suppression.

We performed a homemade ELISA test (NKExoELISA) for the capture and quantification of NK exosomes derived from human plasma of healthy donors and cancer patients. We previously developed a similar assay to detect and quantify plasma exosomes expressing Rab5B/CD63 or Rab5B/caveolin plasma exosomes and demonstrated that $CD63^{+}$ exosomes or caveolin-1^{+} exosomes were significantly increased in melanoma and prostate cancer patients as compared to healthy donors (61, 62). Here, we performed a homemade ELISA that is able to capture specifically exosomes derived from NK cells. By NKExoELISA, we demonstrated that in melanoma patients, the amount of circulating NKExo is lower than in healthy donors. This last result is correlated with a low proliferation and no differentiation toward the NK phenotype of circulating PBLs of melanoma patients if subjected to the same *in vitro* NK expansion protocol as for healthy donor PBL (22).

Taken together our data suggest that NKExo and NKMV could cover a promising role in the support of NK-mediated immunosurveillance, potentially sustaining therefore a wide range of disease therapies. In particular, the use of NKEVs in combination with NK cells or immune checkpoint-based therapies may contribute to improving cancer treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Azienda Policlinico Umberto I, University Sapienza, Rome, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LL and VH were responsible for the study design, wrote and critically reviewed the manuscript, and supervised the study. CF and ES wrote the manuscript, provided technical support for exosome isolation and characterization, and performed many experiments. SCe contributed figures and tables and with EI expanded and characterized NK cells and PBL and also contributed to the discussion of mass spectrometry results. SCam and MC provided results of mass spectrometry and related figures. CC, SF, AC, and PS performed immunomodulation experiments. GP and SCal provided the plasma of melanoma patients and patient informed consent. LB and FI provided results of transmission and scanning electron microscopy and related images.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00262/full#supplementary-material

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