MOLECULAR STRATEGIES AIMED TO BOOST NK CELL-BASED IMMUNOTHERAPY OF CANCER

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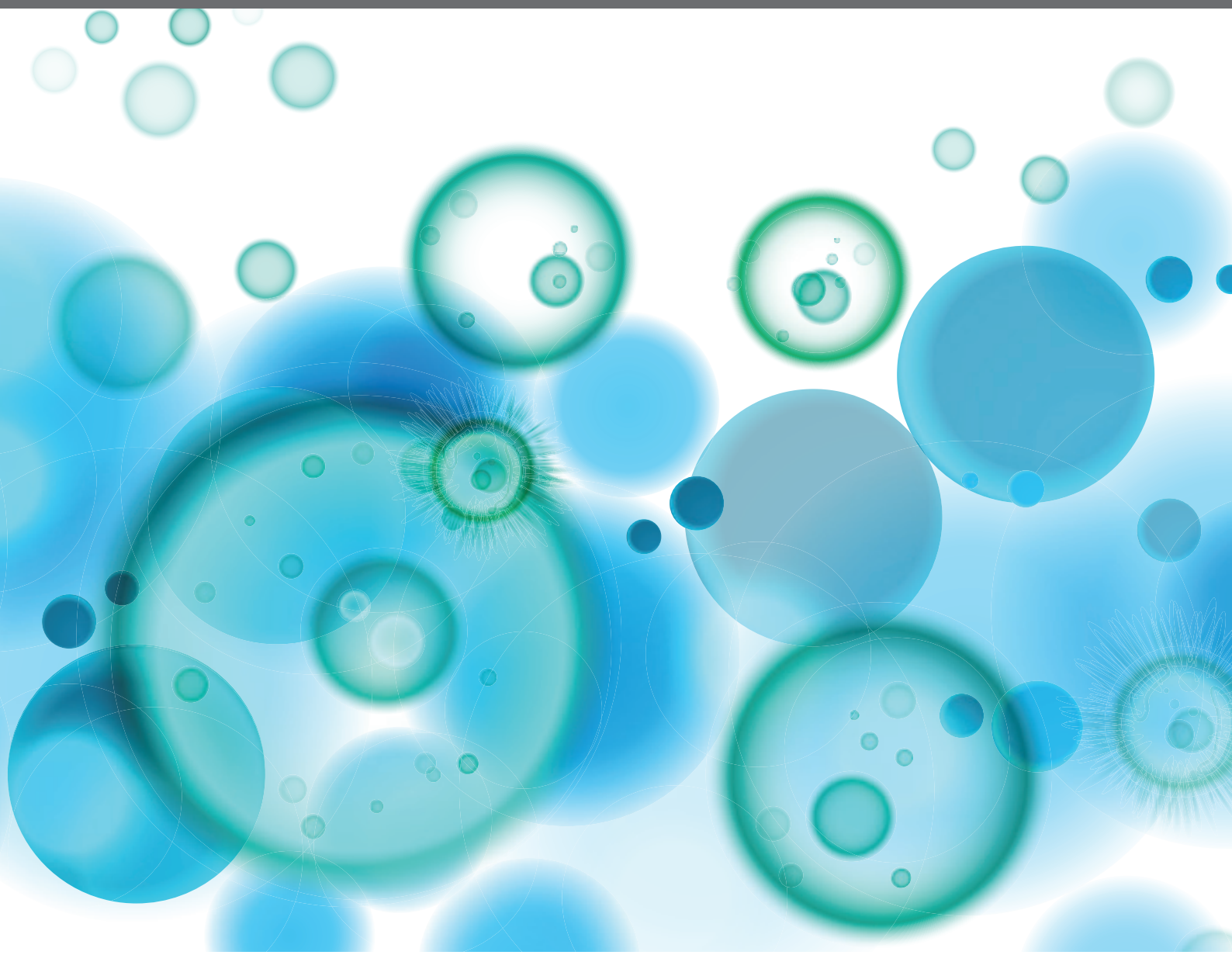

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MOLECULAR STRATEGIES AIMED TO BOOST NK CELL-BASED IMMUNOTHERAPY OF CANCER

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In this Research Topic, we would like to honor the memory of Prof. Vito Pistoia and pay tribute to his scientific contributions to the field of Cancer Immunity and Immunotherapy.

Topic Editor Daniel Olive is the co-founder and shareholder of company Imcheck Therapeutics. All other topic editors declare no competing interests with regards to the Research Topic subject.

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EDITORIAL
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Editorial: Molecular Strategies Aimed to Boost NK Cell-Based Immunotherapy of Cancer

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Keywords: NK cells, NK cell-based immunotherapy, cancer immunotherapy, immune checkpoint inhibitors, adoptive transfer of NK and CAR-NK cells, ligands for NK cell-activating receptors

Editorial on the Research Topic

Molecular Strategies Aimed to Boost NK Cell-Based Immunotherapy of Cancer

Natural Killer (NK) cells represent the first line of defense against aberrant cells, playing a crucial role in counteracting tumor development. Although NK cells are able to circulate in many tissues and rapidly recognize and eliminate tumor cells, a combination of deregulated molecular networks, occurring in tumor microenvironment (TME), allows the immune escape of tumor cells from NK cell-mediated surveillance, thus contributing to tumor progression. The expression of immune checkpoint molecules contributes to the functional exhaustion of NK cells. To overcome the NK cell limitations in the fight against tumor cells, several molecular strategies have been recently adopted at a preclinical and clinical level. Therefore, the use of monoclonal antibodies (mAbs) that neutralize the immune checkpoint molecules and the adoptive transfer of *ex vivo* expanded and activated NK cells or chimeric antigen receptor (CAR)-modified NK cells represent the main NK cell-based immunotherapeutic approaches currently adopted to treat tumors. Of note, NK cells, unlike T cells, do not cause graft-versus-host disease (GvHD) allowing a safe and successful NK cell adoptive transfer in an allogenic setting. Furthermore, although the use of CAR-T cells has shown potent antitumor efficacy, their application is restricted to an autologous setting. Therefore, CAR-NK cells constitute an off-the-shelf product with enormous immunotherapeutic potential.

All of these issues have been extensively described in the reviews collected in this Research Topic, also raising several questions about approaches not yet fully investigated.

Sun and Sun claimed that blocking with checkpoint inhibitors can restore functional exhaustion of NK cells and may complement the limitation of T cell-based immunotherapy. The authors described preclinical studies and clinical application of neutralizing mAbs targeting NKG2A, KIRs TIGIT, CD96, LAG-3, and TIM-3 NK cell-inhibitory receptors.

Minetto et al. focused on the expression of HLA-I on tumor cells and how it can influence the anti-tumor NK cell-mediated functions. These authors described recent NK cell-mediated anti-tumor clinical approaches based on the use of mAbs recognizing NK cell-inhibitory receptors and immune checkpoint inhibitors, alone or in combination with other compounds.

Bi and Tian described the benefits mediated by immune checkpoint inhibitors on NK cells. The authors argued that PD-1^+ NK cells, displaying a stronger potential than PD-1^- NK cells, can be functionally compromised by PD-L1 which is highly expressed in the TME. Therefore, PD-1/PD-L1 blockade can restore PD-1^+ NK cell functions. In addition, the authors proposed the therapeutic targeting of other molecules involved in functional suppression of NK cells, such as E3 ubiquitin ligase Cbl-b, IL-1R8, the negative regulator of IL-15 CIS, and A2A adenosine receptor.

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In addition to these approaches, Zhang et al. proposed other strategies to overcome the NK cell suppression in TME, such as the clinical use of TGF-β neutralizing mAb Fresolimumab and TGFβR1 inhibitor Galunisertib in solid tumors. The authors contemplated the use of NK cells knocked down for *TGFBR2* or *SMAD3*, or the use of CAR-modified NK cells containing TGF-β type II receptor. In addition, they envisaged the use of CMV-induced adaptive memory NK cells and CAR NKG2C$^+$ CD57$^+$ adaptive NK cells for clinical implication.

Vacca et al. focused on different strategies adopted for NK cell-based immunotherapy. In addition to the use of cytokines, mAbs and checkpoint inhibitors, all factors that increase the efficacy of the adoptive transfer of NK cells and CAR-NK cells, the authors underlined the efficacy of αβT cell- and B cell-depleted haploidentical hematopoietic stem cells (HSC) transplantation in which the infusion of donor NK cells and γδT cells, together with HSC, resulted in reduced leukemia relapses and infections.

Melaiu et al. focused mainly on solid tumors and described the immune evasion mechanisms occurring in TME leading to the NK cell exhaustion, together with the preclinical and clinical NK cell-based immunotherapeutic studies. They described the susceptibility of cancer stem cells (CSCs) to NK cell-mediated killing as evaluated in preclinical models. Moreover, they reported studies on NK cell distribution, phenotype, and function in several solid tumor tissues.

Molfetta et al. described other immune evasion strategies, such as the downregulation and cleavage of ligands for NK cell-activating receptors through transcriptional, post-transcriptional and post-translational mechanisms. The authors argued that chemotherapy drugs known to inhibit the ubiquitination and SUMOylation, which are mechanisms involved in the negative regulation of ligands for NK cell-activating receptors, may be exploited for their immunomodulatory effects, thus suggesting their clinical use in combination with conventional therapies.

Sayitoglu et al. in a research article reported new data of genetically modified (GM)-NK-92 cells activity in sarcoma. The authors explored the profile of ligands for NK cell-activating receptors in sarcoma primary cells and underlined the importance of PCNA, CD112, and CD115 expression for NK cell-mediated recognition. They developed a screening platform to assess the efficiency of 14 different activating receptors by generating GM-NK-92 cells over-expressing each individual receptor. The authors show that DNAM-1 and NKG2D GM-NK-92 cells elicited an increase NK cell-degranulation and cytotoxicity against primary sarcoma cells. Moreover, DNAM-1 GM-NK-92 degranulated efficiently also in response to other solid tumor cell lines.

Heinze et al. in a research article described the NK cell anti-tumor efficacy against neuroblastoma (NB) cells by comparing cytokine-induced killer (CIK) with *ex vivo* expanded NK cells, activated with IL-2, IL-15, and IL-21. The authors compared also two different protocols of NK cell isolation and showed that CD3/CD19-depleted PBMCs NK cells expanded higher that CD56-enriched cells. NK cells were assayed in cytotoxic activity against NB spheroidal culture. In addition, IL-21, in combination with IL-15, enhanced NK cell proliferation thus suggesting that the use of IL-15+IL-21 expanded NK cells from CD3/CD19-depleted PBMCs may be promising for a new NK cell-based immunotherapy.

Müller et al. in a research article optimized the protocol leading to the generation of PBMC-derived CD19-CAR-NK cells. These authors compared lentiviral and alpharetroviral, in combination with two different transduction enhancers such as Retronectin and Vectofusin-1. They demonstrated that the transduction of primary NK cells with RD114-TR pseudotyped retroviral vectors, in combination with Vectofusin-1, generated CD19-CAR-NK cells with a highly efficient cytotoxicity against CD19-expressing lymphoblastic cells.

Last but by no means least, we would like to dedicate this series of NK cell-based immunotherapy papers to the memory of Professor Vito Pistoia (Pediatric Hospital Bambino Gesù, Rome) who tragically passed away in 2018. This collection is a fitting tribute to his creative scientific contributions and his unwavering dedication to the field of Cancer Immunity and Immunotherapy.

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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REVIEW
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NK Cell Dysfunction and Checkpoint Immunotherapy

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NK cells play important roles in the innate immune responses against tumors. The effector function of NK cells relies on the integration of activating and inhibitory signals. Emerging checkpoint receptors and molecules are being revealed to mediate NK cell dysfunction in the tumor microenvironment. Inhibition of some NK cell surface checkpoint receptors has displayed the potential to reverse NK cell dysfunction in tumors, and to boost anti-tumor immunity, both in clinical trials (anti-KIR and anti-NKG2A), and in preclinical studies (e.g., anti-TIGIT, and anti-CD96). To fully exploit the potential of NK–based checkpoint immunotherapy, more understanding of the regional features of NK cells in the tumor microenvironment is required. This will provide valuable information regarding the dynamic nature of NK cell immune response against tumors, as well as novel checkpoints or pathways to be targeted. In this Review, we discuss recent advances in the understanding of NK cell dysfunction in tumors, as well as emerging strategies of NK-based checkpoint immunotherapy for tumors.

Keywords: inhibitory receptors, checkpoint blockade, immune evasion, immune tolerance, regional immunity

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INTRODUCTION

Limited response rates of T cell–based checkpoint immunotherapies against CTLA-4 (CytoToxic Lymphocyte Antigen 4) and/or PD-1 (Programmed-cell Death protein 1)/PD-L1 indicate that additional checkpoints/pathways exist to suppress efficient tumor immunity (1–3). Moreover, tumors usually escape T cell immune surveillance by downregulating the expression of major histocompatibility complex (MHC) class I to compromise the tumor antigen presentation pathway (4–6), making these tumors difficult to be recognized by T cells. However, these tumors are highly susceptible to NK cell elimination via the "missing-self" recognition (7, 8). On the other hand, tumors with low mutational loads usually trigger less effective T cell responses than tumors with high mutational loads (9–11). Nevertheless, even tumors with low mutational loads should be recognized and killed by NK cells. These alternative features suggest that NK cells could serve as the major anti-tumor effector cells where tumors should develop mechanisms of escaping T cell surveillance, thus providing additional benefits to T cell–based immunotherapies. Therefore, NK cells represent an emerging target for tumor immunotherapies (12, 13).

On the other hand, multiple intrinsic, and extrinsic immune suppressive checkpoints/pathways exist to prevent NK cells from fully displaying the anti-tumor potentials in the tumor microenvironment (14, 15). Among these checkpoints/mechanisms that inhibit tumor-associated NK cell functions, targeting some of the checkpoint receptors of NK cells by monoclonal antibodies

has been shown to unleash the anti-tumor effector function of tumor-associated NK cells, highlighting NK cells as a potential target for checkpoint immunotherapy. However, a lot of further work lies ahead to unveil the dynamics of anti-tumor NK cell responses, as well as the regional features of tumor-infiltrating NK cells, not only in the tumor immune suppression landscape, but also in the settings of tumor immunotherapies. Unless we have a better understanding of the basic biology of tumor-associated NK cells, we cannot rationally design strategies that efficiently harness the anti-tumor potential of NK cells.

ROLE OF NK CELLS IN TUMOR IMMUNITY

The roles of NK cells in tumor surveillance are well-established. Since cytolytic activity is one of the features of NK cell effector functions, decreased cytolytic activity of NK cells has been associated with higher tumor incidence (16, 17), indicating that NK cell cytolytic function is normally required for tumor control. Among various types of tumors, the role of NK cells in the control of blood cancers and tumor metastasis is especially well-recognized. For example, the expression levels of NK cell activating receptors, NKp30 and NKG2D, in lymph nodes with metastasis from tumor patients was negatively correlated with the levels of metastasis (18). In patients with metastatic prostate cancers, NK cells from patients with longer overall survival and castration resistance display high expression levels of activating receptors and high cytotoxicity (19). In mouse models of metastasis, the depletion of NK cells, as well as genetic deficiency of IFN-γ or perforin, resulted in higher levels of metastasis in mice (20, 21). Besides the role in the surveillance against blood cancers and tumor metastasis, the infiltration of NK cells into solid tumors also affects the tumorigenesis (22), at least in some tumors from the colon (23), the stomach (24), lung (25), and the renal (26).

In addition to the role of NK cells in direct tumor surveillance, NK cells also contribute to T cell anti-tumor immunity. In mouse models, NK cells facilitated the accumulation of T-bet$^+$CD4$^+$ T cells in the tumor region (27), promoted the production of effector molecules, TNF-α and IFN-γ, by tumor-infiltrating CD8$^+$ T cells, suppressed the expression of exhaustion marker PD-1 on these CD8$^+$ T cells (28), and promoted the induction of tumor-specific T cell memory (29). *In vitro* data suggest that NK cells might facilitate the differentiation of anti-tumor Th1 cells via production of IFN-γ in an NKG2D-dependent manner (27). Also, NK cells are required for the accumulation of conventional type I dendritic cells (cDC1) in tumors in mouse models, as NK cells produce CCL5 and XCL1 chemoattractants (30). Such recruitment of cDC1 is critical for T cell anti-tumor immunity. In human cancers, intratumoral CCL5, XCL1, and XCL2 transcripts correlated with gene signatures of both NK cells and cDC1, and were associated with increased overall patient survival (30). This evidence highlights the role of NK cells as a "helper" in formation of an efficient anti-tumor T cell response.

The "helper" effects of NK cells are important in the context of T cell–based checkpoint immunotherapy. Although anti-PD-1 immunotherapy largely targets T cells, the frequency of intratumoral NK cells was found to correlate with patient responsiveness to PD-1 blockade immunotherapy, and with increased overall survival (31). These intratumoral NK cells formed clusters with intratumoral stimulatory dendritic cells, and thus played a role in stimulating anti-tumor T cell activity (31). In line with this, data from mouse models showed that depletion of NK cells abrogated the efficacy of PD-L1 blockade immunotherapy (28). The presence of NK cells prevented formation of a more exhausted status of tumor-infiltrating CD8$^+$ T cells even under conditions of PD-L1 blockade, as evidenced by decreased expression of degranulation marker CD107a, and effector cytokines, TNF-α and IFN-γ, and increased expression of exhaustion marker PD-1 by CD8$^+$ T cells, after NK cell depletion (28). Therefore, by facilitating an efficient anti-tumor T cell response, NK cells contribute to the PD-1/PD-L1 checkpoint immunotherapy. Also, higher levels of intratumoral NK cells might serve as a biomarker to predict better clinical response to PD-1/PD-L1 checkpoint immunotherapy.

NK CELL ACTIVATION

Unlike T cells that majorly use antigen-specific T cell receptors (TCR) to recognize target cells for activation, the activation of NK cells relies on the integration of signals from an array of cell surface activating and inhibitory receptors (7, 32, 33). NK cell activation receptors (33–36) include CD16, natural killer gene 2D (NKG2D), natural cytotoxicity receptors (NCRs), activating KIRs in humans (Ly49D and Ly49H in mice), CD226, as well as the signaling lymphocytic activation molecule (SLAM) family of receptors (SFRs).

On the other hand, NK cell inhibitory receptors (37–39), potentially druggable targets in tumor immunotherapy, are referred to as "checkpoint" receptors, which involve killer inhibitory receptors (KIRs), CD94/NKG2A, T cell immunoreceptor with Ig, and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT), CD96, T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3), PD-1, CTLA-4, lymphocyte activation gene 3 (LAG-3), and V domain immunoglobulin suppressor of T cell activation (VISTA).

The triggering of NK cell activation usually involves two modes: "missing-self" recognition and "induced-self" recognition (8, 40–42). "Missing-self" recognition happens when the target cells display lower or even absent surface expression of MHC I molecules, which is usually linked with viral infection or cellular transformation. This would result in dampened inhibitory signaling from the MHC-I-binding KIRs or CD94/NKG2A (and Ly49 family members in mice), leading to activation of NK cells. Alternatively, "induced-self" recognition requires the engagement of stress-induced or virus-encoded ligands on target cells by germline-encoded activating receptors.

Besides the balance of surface receptors-mediated signaling, priming also affects strength of NK cell effector activity. Stimulation by infections, cytokines [e.g., type I interferon (IFN), interleukin-15 (IL-15), IL-12, IL-18, IL-21 and

IL-1β; either alone or in combinations], and pathogen-associated molecular patterns (PAMPs) can prime NK cells by lowering the threshold for further activation (43), and by inducing expression of effector molecules (44, 45).

Downstream of the surface receptors are common signaling molecules that regulate the triggering and strength of NK cell activation and responses upon ligand engagement or cytokine stimulation (13, 46). For NK cell activating surface receptors, downstream signals converge on SH2 domain-containing leukocyte phosphorylation of 76 kDa (SLP-76)-mediated phosphorylation of Vav1, which is negatively regulated by the E3 ubiquitin ligase, Casitas B-lineage lymphoma-b (Cbl-b) (47). On the other hand, IL-15, the NK cell key cytokine signals through the JAK-STAT pathway, which is inhibited by cytokine-inducible Src homology-2 (SH2)-containing protein (CIS) (48). These molecules are also emerging "checkpoints" in tumor immunotherapy.

NK CELL DYSFUNCTION IN TUMORS

Upon activation, NK cells normally form conjugations with target cells, and release cytotoxic granules containing perforin and granzymes for target cell lysis (49–51), or induce target cell apoptosis via TNF-α, FasL, and TRAIL (32, 52, 53). Besides, NK cells are responsible for early and rapid production of anti-tumor effector cytokine IFN-γ (54, 55). However, the tumor microenvironment possesses unique regional immune features compared with the peripheral and other immune organs, resulting in the impaired effector functions of tumor-associated NK cells (14, 15). Firstly, NK cells usually display decreased percentages along tumor progression (56). Secondly, the "quality" of single NK cells is also compromised, as shown by lower effector molecules expression of IFN-γ, CD107a, granzyme B, FasL, TRAIL, and perforin in tumor-infiltrating NK cells as assessed by intracellular staining for flow cytometry (27, 57–61). Notably, the decreases in both "quantity" and "quality" of NK cells in tumors were reported to positively connect with each other (62), indicating that the dysfunction of tumor-associated NK cells is multi-aspect.

The suppressed expression of effector molecules by NK cells suggests that the NK-specific signaling/transcriptional program should be altered in the tumor microenvironment. IL-15 is expressed in the tumor microenvironment, required for establishing normal levels of NK cell anti-tumor immune response (63). However, IL-15 signaling is compromised for NK cells in tumors (64). Therapeutic application of exogeneous IL-15 directed to the tumor sites activated and recruited NK cells in mouse models (65), indicating that IL-15 signaling is essential for NK cell anti-tumor immunity. Furthermore, expression of key transcriptional factors, Eomes and T-bet, are also decreased in NK cells in tumor-bearing mice (66, 67). In line with these alterations, tumor-associated NK cells displayed defective maturation status both in mice (64, 68, 69) and in humans (67, 70, 71). Such hypomaturation status of NK cells has been associated with reduced overall survival and relapse-free survival of patients with acute myeloid leukemia (AML) (72). Together, these defects contribute to compromising the effector program of NK cells in tumors.

The effector function of NK cells is sustained by cellular metabolism (73–76). However, in the tumor microenvironment, not only tumor cells (77–79), but also NK cells display dysregulated metabolism (80). The dysregulated metabolic status would lead to the dysfunctional status of NK cells (81–83), as well as other immune effector cells (84). In a KRas - driven tumor model in the lung, fructose-1,6-bisphosphatase (FBP1) was highly up-regulated in lung NK cells from mice bearing advanced lung tumors, compared with lung NK cells from normal mice (80). FBP1 functions as a rate-limiting enzyme in gluconeogenesis, facilitating gluconeogenesis, and inhibiting glycolysis. Up-regulated FBP1 in NK cells in the tumor settings suppressed the glycolysis of NK cells, compromised their viability, and effector functions (80).

NK cell's dysfunctional status in tumors is accompanied by a series of phenotypic alterations. Multiple NK cell activating receptors have been reported to be down-regulated in tumors. For example, NK cells express decreased levels of NKG2D in various types of cancers (57, 59, 85). In addition, DAP10, the adaptor for transducing NKG2D receptor signaling, was also found to decrease in the chronic viral infection setting, and probably also in tumors, which might further add to the compromised NKG2D signaling (86). Other NK cell activating receptors reported to show decreased expression in tumors include CD16 (57), NCRs (57, 59, 85), and CD226 (56, 57, 85, 87). The downregulation of activating receptors could be restored in remission (57), suggesting that this detrimental regulation of NK cell activating receptors expression is an active suppression mechanism by the tumor microenvironment.

The detrimental regulation of NK cell receptors also involves the upregulation of inhibitory receptors. For example, TIGIT expression on mouse NK cells was up-regulated during tumor progression. In humans, the constitutive expression of TIGIT on NK cells was further up-regulated in tumor regions compared with peritumoral regions in colorectal tumors (28). Other inhibitory receptors reported to be upregulated on NK cells in tumors involve CD96 (88), NKG2A (60), and PD-1 (89–92). The immune suppressive cytokines, such as IL-10 and TGF-β, might contribute to the upregulation of inhibitory receptors (60, 93).

One recently discovered aspect regarding the dysfunction of tumor-associated NK cells is the intratumoral differentiation of NK cells. The dissection of the lineage differences between NK cells and ILC1s has been revealed in multiple tissues (94, 95). This complexity has recently been extended to the tumor microenvironment. TGF-β receptor signaling in NK cells was found to mediate the intratumoral differentiation of conventional anti-tumor effector NK cells into ILC1-like cells, which were unable to control tumor growth (96, 97).

NK–BASED CHECKPOINT IMMUNOTHERAPY

The success of T cell–based checkpoint immunotherapy has revolutionized the treatment for cancer, which has established a

concept that unleashing the potential of anti-tumor immunity is capable of controlling tumors. At the same time, the limited responsiveness of current checkpoint immunotherapies is driving the area toward discovering new "checkpoints" on not only T cells, but also on other immune cells, such as on NK cells (**Figure 1**). On the other hand, recent studies have shed light on the potential of targeting some common signaling regulators to stimulate the anti-tumor activity of immune cells. These molecules have broadened the conventional concept of "checkpoints," representing new areas in NK–based tumor immunotherapy.

KIR

The history of NK–based checkpoint immunotherapy started with blocking KIRs on NK cells. Early studies on bone marrow transplantation with acute myeloid leukemia patients showed that 5-year recurrence rate was 75% for donor's NK-KIR matched with recipient's MHC I molecule. However, strikingly, for those mismatched cases, this number dropped to 0% (98). This and other related evidence together indicate that absence of the matching/recognition between NK cell inhibitory receptor KIRs and target cell MHC I molecules would trigger the activity of NK cells, leading to the rejection of these target cells. This lays the rationale of KIR blockade for stimulating NK cell activity against tumors. In clinical trials, KIR blockade showed limited side effects (99). Neither KIR single blockade (NCT01687387), nor combined with anit-CTLA-4 (NCT01750580), displayed better efficacy than anti-CTLA-4 alone. However, combination blockade of KIR and PD-1 showed the trend of increased objective response rate for advanced head and neck tumor patients previously treated with chemotherapy (NCT01714739). Moreover, preliminary data from KIR blockade in combination with 5-azacytidine, a DNA methyltransferase inhibitor, as a therapy for refractory or relapsed AML showed that 20 percent of the first 25 patients responded to therapy, with two patients achieving complete remission (CR) (NCT02399917). These preliminary data have given us confidence on more ongoing clinical trials on KIR blockade alone or combined therapies (e.g., combined with anti-CD20 in NCT02481297, with anti-PD-1, and 5-azacytidine in NCT02599649, or with anti-SLAMF7 in NCT02252263). In addition, more investigations on new indications and the data mining on previous clinical trials might further reveal the potential of KIR blockade in tumor immunotherapy.

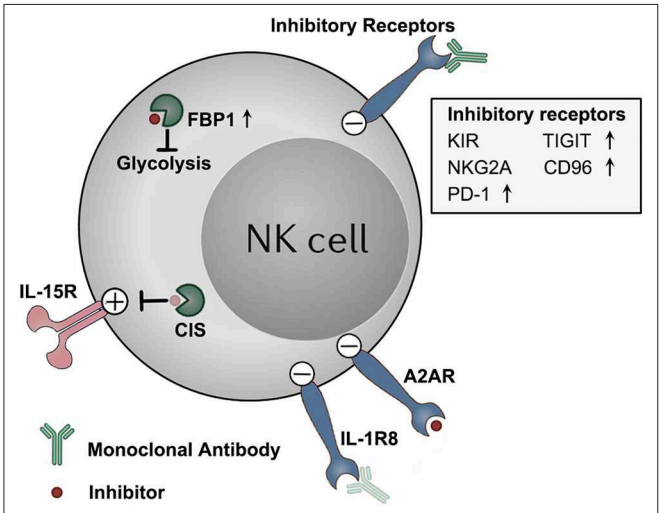

FIGURE 1 | Targeting NK cell dysfunction via checkpoint inhibition tumor immunotherapies. NK cells display a dysfunctional status in tumors, along with detrimental upregulation of some checkpoint molecules (e.g., TIGIT, CD96, PD-1, and FBP1). These molecules, as well as other constitutively expressed checkpoint molecules (e.g., KIRs, NKG2A, A2AR, and IL-1R8), functions to impair the anti-tumor effector potentials of NK cells in tumors. These checkpoint molecules represent potential targets for NK–based immunotherapy. Therapeutically targeting these molecules by blocking monoclonal antibodies or small molecule inhibitors might unleash NK cells from those immune suppressive mechanisms, and boost NK cell anti-tumor activity. Antibody and inhibitor symbols in light color (those for CIS and IL-1R8) indicate that the agents have not been developed yet.

TIGIT

TIGIT is an inhibitory receptor expressed majorly on T cells and NK cells (100, 101). TIGIT binds ligands CD155 and CD112. The inhibitory functions of TIGIT depend on the intracellular ITIM and ITT-like domain (101, 102), on the competition with activating receptors CD226 for ligand interactions (103), as well as on the ligand signaling from CD155 upon interaction (100). The interaction of TIGIT with its ligands suppressed the cytolytic activity and IFN-γ production of NK cells *in vitro* (101, 104). Such effects are critical for its role in maintaining NK cell self-tolerance in acute inflammations (105) and tissue regenerations (106), which, however, in turn lead to the exhausted status of NK cells in tumor settings, promoting the progression of tumors (28).

The expression of checkpoint inhibitory receptor TIGIT on NK cells was up-regulated along tumor progression in mice (28). The tumor-associated expression pattern of TIGIT is not dependent on the adaptive immune system, since TIGIT was also highly expressed on tumor-infiltrating NK cells in SCID mice (28). In humans, TIGIT is constitutively expressed by NK cells, which was up-regulated on NK cells in tumor regions compared with NK cells from peri-tumoral regions (28). Consistently, $CD103^+$ tumor resident NK cells displayed higher expression of TIGIT than $CD103^-$ circulating NK cells. In addition, tumor-infiltrating NK cells from tumor patients with lymph node invasion expressed higher levels of TIGIT, compared with those from patients without lymph node invasion. These studies demonstrate that the expression of TIGIT on NK cells is associated with tumor progression both in humans and in mice. Importantly, while the expression pattern of two well-established T cell checkpoint receptors, PD-1 and CTLA-4, are more restricted on tumor-infiltrating T cells, TIGIT was highly expressed not only on T cells, but also on NK cells in tumors (28), highlighting TIGIT as a checkpoint receptor more specific to NK cells.

Monoclonal antibody blocking TIGIT reversed the exhaustion status of tumor infiltrating NK cells (28). Such effect adds to the T cell -stimulating effects on both regulatory T cells (107) and effector T cells (103) by TIGIT blockade. With these supportive

results from preclinical studies, therapeutic blockade of TIGIT in advanced cancers is currently being tested in various clinical trials alone in NCT03119428 and NCT03628677, or in combination with anti-PD1 in NCT03119428 and NCT03628677, or with anti-PD-L1 in NCT03563716. Therefore, blocking the checkpoint receptor TIGIT represents a potential strategy to explore in immunotherapy.

NKG2A

NKG2A is a cell surface inhibitory receptor expressed both on T cells and on NK cells, which forms a heterodimer with CD94 (108). In mice, about 40–60% of NK cells both in the peripheral and inside the tumors express NKG2A. In humans, more than half of NK cells are NKG2A$^+$ not only in the blood, but also in tumors [e.g., squamous cell carcinoma of the head and neck (SCCHN)] (109). The expression of NKG2A on NK cells could be further up-regulated upon IL-15 stimulation (109). Importantly, among NKG2A$^+$ NK cells, there is a subset co-expressing NKG2A and PD-1. On the other hand, non-classical MHC-I, HLA-E, is the ligand of NKG2A in humans, which is widely expressed by various types of tumors (e.g., lung, pancreas, stomach, colon, head and neck, and liver tumor tissues) (109). In mice, the ligand of NKG2A is Qa-1^b. Binding of NKG2A/CD94 to its ligand suppresses the effector functions of T and NK cells by recruitment of the SHP-1 tyrosine phosphatase to the ITIM in the intracellular domain of NKG2A.

Blockade or abrogated expression of NKG2A rescued HLA-E-mediated suppression of cytotoxicity and IFN-γ production by NK cells *in vitro* (109, 110), and rendered NK cells with enhanced efficacy against HLA-E$^+$ tumors *in vivo* upon infusion (110). Combined blockade of both NKG2A and PD-1 synergistically stimulated the degranulation of NKG2A$^+$PD-1$^+$ NK cells in HLA-E$^+$PD-L1$^+$ target cell co-culture (109). Disruption of NKG2A-Qa-1^b interaction by knocking out Qa-1^b on tumor cells promoted NK cell–dependent anti-tumor efficacy (109). Consistent with these functional studies, the expression of NKG2A and HLA-E in hepatocellular carcinoma (HCC) tissues correlated with poor prognosis of HCC patients (60).

Monalizumab, a humanized anti-NKG2A antibody, enhanced NK cell activity against various tumor cells and rescued CD8$^+$ T cell function in combination with PD-1/PD-L1 axis blockade (109). Importantly, combined blockade of both NKG2A and PD-1/PD-L1 exhibited synergistic anti-tumor efficacy with improved survival compared with PD-1/PD-L1 blockade alone (109). This efficacy was shown to be NK-dependent, since NK cell depletion greatly shortened the prolonged survival of mice (109). In addition, since NKG2A is also up-regulated on CD8$^+$ T cells in tumors, blockade of NKG2A would also stimulate CD8$^+$ T cell–dependent responses (109).

Monalizumab also stimulated NK cell ADCC (antibody-dependent cell-mediated cytotoxicity) activity against antibody-coated target cells *in vitro* (109). In a phase II clinical trial NCT02643550 for the treatment of SCCHN, monalizumab combined with cetuximab, an anti-EGFR monoclonal antibody, resulted in a partial response rate of 31%, and stable disease at 54%, compared with the historical data of 13% objective response rate for single agent cetuximab, showing that NKG2A blockade has the potential of treating tumor patients in combination with tumor-targeting antibodies. Besides, NKG2A single blockade for the therapy of gynecologic malignancies (NCT02459301), or in combination with ibrutinib (a BTK inhibitor) for the therapy of chronic lymphocytic leukemia (CLL) (NCT02557516), are also under clinical trials.

CD96

CD96 is a transmembrane glycoprotein Ig superfamily receptor expressed on T cells and NK cells. CD96 was earlier found to mediate the adhesion between NK cells and tumor cells to facilitate NK cell cytolysis (111). Later, the use of CD96$^{-/-}$ mice revealed the role of CD96 as an important checkpoint for NK cell effector functions. Loss of CD96 rendered NK cells with hyper-production of IFN-γ in mice challenged with LPS (112). In chemical-induced tumor models, mice deficient in CD96 displayed more resistance to tumor growth in both an NK and IFN-γ –dependent manner (112). In HCC patients, the percentage and intensity of CD96 on NK cells, as well as the numbers of CD96$^+$ NK cells, were higher for tumor-infiltrating NK cells compared with NK cells from peri-tumoral tissues (88). CD96$^+$ NK cells are more severely dysfunctional compared with CD96$^-$ NK cells, as evidenced by lowered expression of IFN-γ and TNF-α, as well as lower gene expression levels of *Tbx21*, *Prf1* and *Gzmb*, and increased gene expression levels of *Il-10* and *Tgf-β* (88). Importantly, high expression levels of CD96, or its ligand CD155 in tumors of HCC patients, was associated with poor disease prognosis (88).

Therapeutic blockade of CD96 in tumor metastasis models confirmed its role as a checkpoint receptor on NK cells. CD96 blockade was shown to inhibit experimental metastases in three different models (113). The efficacy was dependent on NK cells, CD226, and IFN-γ, but not dependent on activating Fc receptors (113). Furthermore, when combined with CD96 blockade, anti-CTLA-4, anti-PD-1, or chemotherapy was more effective. Co-blockade of CD96 and PD-1 resulted in increased local NK cell IFN-γ production and infiltration (113). These studies demonstrate that CD96 checkpoint blockade represents a potential immunotherapy strategy targeting NK cells.

PD-1

Compared with the checkpoint receptors discussed above, the expression of PD-1 on NK cells is relatively minor. The levels of PD-1 on NK cells could be substantially up-regulated upon viral infections or in specific tissue/organs (114). In tumors in both humans and mice, NK cells displayed higher expression of PD-1 above baseline, although not at a high percentage (115, 116). PD-1$^+$ NK cells, unlike TIGIT$^+$, or CD96$^+$ NK cells, displayed stronger effector potentials than PD-1$^-$ NK cells, as shown by higher levels of IFN-γ and granzyme B upon IL-2 stimulation *in vitro* (116). However, in the tumor microenvironment where PD-L1 is expressed at high levels, this subset of PD-1$^+$ NK cells might be dysfunctional under effects of the inhibitory signaling from interaction between PD-1 and PD-L1. Based on these studies, PD-1/PD-L1 blockade might therefore reverse the dysfunctional status of PD-1$^+$ NK cells in this context, adding to the benefits from enhanced T cell responses upon PD-1/PD-L1 blockade.

Cbl-b

Genetic deletion of the E3 ubiquitin ligase Cbl-b, or treatment with a small molecule targeting the substrate TAM tyrosine kinase receptors Tyro3, Axl and Mer, was shown to efficiently enhance anti-metastatic activity of NK cells in mouse models (47). In addition, the anticoagulant warfarin stimulated the anti-metastatic activity of NK cells via Cbl-b/TAM receptors (47). These data indicate that the Cbl-b/TAM pathway is a "checkpoint" that normally suppresses NK cell anti-tumor activity, and that therapeutically targeting this pathway might unleash the anti-metastatic potential of NK cells.

IL-1R8

Interleukin-1 receptor 8 (IL-1R8), a negative regulator of Toll-Like and Interleukin-1 Receptor family signaling, is highly expressed on NK cells, and is increased during NK cells maturation (117). Also, IL-1R8 is expressed by tumor cells (e.g., in breast cancers) (118). Not only the expression of IL-1R8 in NK cells, but also that in the tumor cells, inhibited NK cell activation and NK -mediated control of tumor growth and metastasis, highlighting its role as a checkpoint for NK cell tumor immunity (117, 118). Therefore, therapeutically targeting IL-1R8 might represent a potential strategy to boost NK cell anti-tumor immunity.

CIS

CIS is a negative regulator of IL-15 signaling by inhibiting the downstream JAK-STAT pathway. CIS expression was increased in NK cells upon cellular activation, such as in response to IL-15 (48). NK cells deficient in *Cish* showed increased JAK-STAT signaling, and enhanced proliferation, survival, IFN-γ production, and cytotoxicity against tumors (48). Mice deficient in *Cish* were resistant to melanoma, prostate, and breast cancer metastasis *in vivo* (48). The combination of *Cish* deficiency with targeted therapies or immune checkpoint blockade therapies displayed further improved control of metastasis (119). These data demonstrate that CIS acts as a potent intracellular checkpoint to target in NK cell-mediated tumor immunity.

A2A Adenosine Receptor

Adenosine is an endogenous purine nucleoside that binds adenosine receptors. High levels of adenosine is present in the tumor microenvironment (120), impairing both the anti-tumor effector functions and maturation of NK cells (69, 121) via the A2A adenosine receptor on NK cells. Either inhibition of the adenosine -generating enzymes, CD73 or CD39, or blockade of A2A adenosine receptor, displayed immune stimulatory and anti-tumor effects in mouse models (122–124). Furthermore, a combination of A2A receptor inhibitors and PD-1 blockade significantly reduced metastasis of $CD73^+$ tumors and prolonged the survival of mice compared with monotherapy alone (125, 126). Notably, the combination therapy depended on NK cells and IFN-γ, and to a less extent, $CD8^+$ T cells (125). Recently, monoclonal antibodies targeting human membrane-associated and soluble forms of CD39 and CD73, respectively, were reported to efficiently block the hydrolysis of immunogenic ATP into immunosuppressive adenosine, and could restore the activation of cancer patient-derived T cells (127). Importantly, the CD39-inhibiting antibody increased the anti-tumor activity of the ATP-inducing chemotherapeutic drug oxaliplatin in a human CD39 knockin mouse preclinical model (127). Therefore, targeting the A2A adenosine receptor pathway can enhance NK cell anti-tumor activity, and might synergize with T cell–based checkpoint immunotherapy or immunogenic chemotherapy.

PERSPECTIVE

In conclusion, the well-documented role of NK cells in tumor surveillance has been further substantiated by recent progresses in NK-based checkpoint blockade immunotherapy, which targets NK cells to stimulate anti-tumor responses. More importantly, some strategies displayed the potentials to further improve current T cell–based immunotherapies. These studies indicate that NK-based immunotherapy represents a promising direction worthy of further investigations, especially in the current age of tumor immunotherapy.

Among these studies, while most have confirmed the roles of NK cells in controlling blood cancers and tumor metastasis, some have also proposed a role for NK cells in surveillance against solid tumors with evidence from either mouse models or clinical relevance, at least in some contexts (22). Based on this limited yet ever-growing evidence, it is not unreasonable to assume that NK cells might fully exhibit their anti-tumor effector potentials even in solid tumors, provided that we could be able to remove some of the either current or unknown checkpoints 1 day. In order to do so, apparently, a long way still lies ahead. Most of the studies on NK cell biology have been performed in normal mice, instead of in the immune suppressive landscape of tumors. As discussed in the above sections, intratumoral NK cells are mostly dysfunctional, and display alterations in many aspects compared with peripheral NK cells in normal mice. Not only NK-intrinsic biology, but also various NK-extrinsic factors from the tumor microenvironment governs the actual responses of intratumoral NK cells, making it difficult to interpret our basic knowledge for NK cell biology locally in the tumors. Therefore, more studies are required on the tumor regional features of NK cells.

In addition, the current age of immunotherapy urges the focus of investigations in tumor immunology to be on why so many patients are unresponsive to therapies, and how to increase the response rates. This requires us to look into the mechanisms regulating NK cell functions not only in the tumor immune suppressive landscape, but also in the settings of tumor immunotherapies, hopefully leading to novel strategies to improve current therapies.

Currently, the physiological roles in tumor surveillance by NK cells, as well as the therapeutic potential, of many surface receptors on NK cells have been demonstrated (e.g., KIR, TIGIT, NKG2A, CD96, and PD-1), while those of many others still remain to be shown (e.g., LAG-3 and TIM-3). For the ongoing interest in searching for novel "checkpoints" to target, it is important to describe the expression pattern of specific

checkpoint receptors on NK cells (as well as their ligands on other cells), as well as on T cells, in specific tumors, in specific tumor stage, and in specific therapeutic settings. The underlying rationale is that only when the checkpoint (as well as its ligand) is expressed at functional levels, should the targeting be antagonizing its function. As we discussed in the above sections, checkpoint molecules display different expression patterns: (1) some are constitutively and stably expressed by NK cells; (2) some are normally absent or lowly expressed, and are up-regulated upon stimulation; (3) some are constitutively expressed normally, and are further up-regulated in special contexts. However, more detailed information is required. For example, in future studies on checkpoints, we also need to pay attention to whether the expression pattern of a specific checkpoint molecule is different in "hot" tumors and in "cold" tumors, and whether it is altered in hosts receiving anti-tumor therapies. The intensity of anti-tumor immune responses, as well as the immune suppression by the microenvironment, might be different under these different contexts, which might affect the expression levels of these checkpoint molecules. Therefore, detailed information is required for the complete description of the spatiotemporal expression profile of these checkpoint molecules. Only when we have sufficient knowledge about the immune checkpoint landscapes of specific tumor microenvironments could we rationally design therapeutic strategies precisely targeting the functional checkpoints for the proper indications at the right time.

Collectively, accumulating evidence has indicated a role of NK cells in surveillance against not only blood cancers and metastasis, but also solid tumors, at least in some contexts. In recent years, a lot of progress has been made regarding the role of NK cells, as well as the role of NK cell checkpoints in anti-tumor immunity. Targeting those checkpoints displayed the potential of boosting NK cell activity against tumors. Importantly, some might improve current T cell–based checkpoint immunotherapies. Although a lot remains to be understood, recent studies demonstrate the promise that further investigations into the regional features of NK cells in tumors might give rise to novel checkpoint immunotherapies in future.

AUTHOR CONTRIBUTIONS

JB and ZT conceived and wrote the manuscript.

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REVIEW
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The Rise of NK Cell Checkpoints as Promising Therapeutic Targets in Cancer Immunotherapy

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Checkpoint immunotherapy that targets inhibitory receptors of T cells, thereby reversing the functional exhaustion of T cells, marks a breakthrough in anticancer therapy. The success of T cell-directed checkpoint inhibitors of CTLA-4 and PD-1/PD-L1 has opened a new approach for cancer immunotherapy and resulted in extensive research on immune checkpoints. However, it is only in recent years that research on NK cell exhaustion and potential checkpoints impacting NK cells has become popular. NK cells, as the major player in innate immunity, are critical for immune surveillance, particularly the control of metastasis and hematological cancers. The balance between activating and inhibitory signals fine tunes the activation and effector functions of NK cells, and transformed cells modulate NK cells by upregulating negative signaling that "exhausts" NK cells. Exhausted NK cells with excessive expression of inhibitory receptors (checkpoint molecules) are impaired in the recognition of tumor cells as well as antitumor cytotoxicity and cytokine secretion. Therefore, an understanding of the potential checkpoint molecules involved in NK cell exhaustion is particularly important in terms of NK cell-targeted cancer immunotherapy. In this review, we summarize recent advances in NK cell checkpoint inhibitors and their progress in clinical trials. Moreover, we highlight some of the latest findings in fundamental NK cell receptor biology and propose potential NK cell checkpoint molecules for future immunotherapeutic applications.

Keywords: NK cell, NK cell exhaustion, inhibitory receptor, checkpoint inhibitor, cancer immunotherapy

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INTRODUCTION

Excessive negative regulation of immune cells by inhibitory receptors (checkpoint molecules) results in functional exhaustion of these cells, which is one of the major reasons for tumor escape. The activation and function of immune cells are regulated through activating and inhibitory receptors on these cells, and establishing an equilibrium between activating and inhibitory signaling is critical because it assures effective control against pathogenic factors (such as tumors, viruses, and bacteria) meanwhile helping to avoid self-directed attacks (such as autoimmune disease) (1, 2). The negative feedback provided by inhibitory receptors is the key to immune regulation; however, unfortunately, tumor cells can take advantage of this negative feedback system, as they upregulate the surface expression of corresponding ligands to ingratiate excessive expression of inhibitory receptors

on immune cells that automatically leads to reduced activation and functional exhaustion of these cells (3, 4). Antibodies that specifically target these inhibitory checkpoints can effectively block the interaction between the checkpoint molecule and its ligands, thereby reversing the functional exhaustion of immune cells and restoring antitumor immunity.

Checkpoint immunotherapy targeting checkpoint molecules that reverses functional exhaustion in immune cells marks a major breakthrough in anticancer therapy. Blocking inhibitory receptors on T cells to reverse functional exhaustion in these cells has made great progress. T cells from cancer patients highly express inhibitory receptors including cytotoxicity T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), etc., which contribute to T cell functional exhaustion (5, 6). Blocking these checkpoint molecules can effectively reverse T cell exhaustion and restore the antitumor capacity of T cells. During the past 10 years, the efficacy and feasibility of checkpoint immunotherapy have been verified in clinical settings, and antibodies targeting CTLA-4, PD-1/programmed death-ligand 1 (PD-L1), TIM-3, LAG-3, or TIGIT have entered clinical trials (7, 8). Monoclonal antibodies (mAbs) targeting CTLA-4 and PD-1/PD-L1 were approved by the U.S. Food and Drug Administration (FDA) in 2011 and 2014, respectively. The combined use of an anti-CTLA-4 mAb with an anti-PD-1 mAb showed better efficacy than either antibody used as a monotherapy (9–11).

However, although remarkable clinical benefits derived from anti-CTLA-4 and anti-PD-1/PD-L1 antibody therapies have been noted in some patients, there are still many patients who do not respond to these treatments. Currently, researchers are still trying to understand these "non-responders;" additional unknown inhibitory pathways that suppress immune responses could be an explanation for nonresponse. On the other hand, tumors escape T cell-mediated immunity by downregulating the expression of major histocompatibility complex class I (MHC-I) molecules; these MHC-I-null tumor cells are not attacked by T cells, but they are still targets of natural killer (NK) cells (12). Moreover, Zhang et al. demonstrated the significance of NK cells in cancer immunotherapy and noted that mAb targeting checkpoint molecule TIGIT, which is expressed by both T cells and NK cells, could improve the antitumor immunity of both T and NK cells. Furthermore, they found that TIGIT blockade was effective even in the absence of T cells and B cells, highlighting the importance of NK cells in checkpoint-targeted immunotherapy (13, 14).

NK cells are innate lymphocytes that play a critical role in the early defense against transformed cells, and they are particularly important in the control of cancer metastasis and hematological cancers (1, 15–17). NK cells can directly kill tumor cells, secrete various cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α to initiate antitumor responses, and recruit other immune cells into the antitumor response (1, 18, 19). Alterations in NK cells, for example, excessive expression of inhibitory receptors or reduced expression of activating receptors, can result in impaired cytotoxicity against tumor cells and a decreased ability to recruit other immune cells (20–22). Some checkpoint molecules in cytotoxic T lymphocytes (CTLs), such as PD-1, LAG-3, TIGIT, and TIM-3, are shared with NK cells. Blockade with checkpoint inhibitors that reverses the functional exhaustion of NK cells opens a new strategy for cancer immunotherapy that may complement the limitations of T cell-based immunotherapy. This review summarizes recent advances in NK cell checkpoint molecules in humans (**Figure 1**) and the corresponding antibodies being studied in clinical trials (**Tables 1**, **2**). In addition, we highlight some of the latest findings in fundamental NK cell receptor biology that may provide a fundamental basis for future NK cell-based immunotherapeutic applications.

KILLER CELL LECTIN-LIKE RECEPTOR FAMILY

NKG2A

NK group 2 member A (NKG2A) is a type II membrane receptor that forms a heterodimer with CD94 (23). NKG2A binds a human leukocyte antigen (HLA) class I molecule (HLA-E) (24) and transduces inhibitory signaling that suppresses the cytokine secretion and cytotoxicity of NK cells (25–27). $NKG2A^+$ NK cells infiltrate the tumor microenvironment, and increased expression of NKG2A in NK cells has been observed in patients with non-small cell lung cancer (28, 29), breast cancer (30), colorectal cancer (31, 32), acute myeloid leukemia (33, 34), hepatocellular carcinoma (35), breast cancer (36), cervical cancer (32), etc. A large proportion of NK cells with high NKG2A expression has also been found in tumor-draining lymph nodes (36). NK cells with elevated NKG2A expression are functionally exhausted and associated with a poor prognosis in human hepatocellular carcinoma (35). NKG2A expression predominantly increases on $CD56^{dim}$ NK cells compared to $CD56^{bright}$ NK cells, and these $NKG2A^+$ $CD56^{dim}$ NK cells are functionally exhausted and highly correlated with massive tumor size in human hepatocellular carcinoma (35).

Due to the strong capability of NKG2A to suppress NK cells, blockade of NKG2A is effective in restoring functions of NK cells. A mAb targeting NKG2A, namely, monalizumab (formerly IPH2201), has been tested in both phase I and phase II clinical trials (**Table 2**). NKG2A is overexpressed in

Abbreviations: NK, Natural killer; CTL, Cytotoxic T lymphocyte; PBMC, Peripheral blood mononuclear cell; MDSC, Myeloid-derived suppressor cell; mAb, Monoclonal antibody; IFN, Interferon; TNF, Tumor necrosis factor; IL, Interleukin; KO, Knockout; HLA, Human leukocyte antigen; ADCC, Antibody-dependent cell-mediated cytotoxicity; MHC, Major histocompatibility complex; FDA, Food and Drug Administration; MCA, Methylcholanthrene; PD-1, Programmed death-1; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; TIM-3, T cell immunoglobulin and mucin domain 3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; KIR, Killer cell immunoglobulin-like receptor; LAG-3, Lymphocyte activating gene 3; FGL1, Fibrinogen-like protein 1; NKG2A, NK group 2 member A; Gal-9, Galectin-9; CAF, Carcinoma-associated fibroblast; PEBL, Protein expression blocker; HMGB1, High mobility group box 1 protein; CEACAM-1, Carcinoembryonic antigen-related cell adhesion molecule 1.

TABLE 1 | Potential NK cell checkpoint molecules in cancer.

Targets	Monoclonal antibody	Expression distribution	Ligand	Signaling motif
KILLER CELL LECTIN-LIKE RECEPTOR FAMILY				
NKG2A	Monalizumab, IPH2201	$CD8^+$ T cells and NK cells	HLA-E	ITIM
KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR FAMILY				
KIR	IPH2101, 1-7F9, Lirilumab, and IPH4102	$CD4^+$, $CD8^+$ T cells, NK cells	MHC class I molecules	ITIM/ITAM
IMMUNOGLOBULIN SUPERFAMILY				
TIGIT	MTIG7192A, OMP-313M32, and AB154	$CD4^+$, $CD8^+$ T cells, NK cells	CD155, CD112, CD113	ITIM/ITT
CD96	–	$CD4^+$, $CD8^+$ T cells, NK cells	CD155	ITIM/YXXM
LAG-3	Sym022, BMS-986016, Relatlimab, IMP321, and Eftilagimod Alpha	$CD4^+$, $CD8^+$ T cells, NK cells, B cells, and dendritic cells	MHC class II molecules, Fibrinogen-like Protein 1	KIEELE
TIM-3	sym 023, TSR-022, LY3321367, BGB-A425, and MBG453	$CD4^+$, $CD8^+$ T cells, dendritic cells, NK cells, and monocytes	Gal-9, phosphatidylserine, HMGB1, Ceacam-1	Tyrosine

NK cells from chronic lymphocytic leukemia patients, and blocking NKG2A with monalizumab is sufficient to restore the direct cytotoxicity of NK cells against HLA-E-expressing tumor cells (37). Treatment with IPH2201 has been shown to trigger $NKG2A^+$ NK cell-mediated lysis of HLA-E^+ target cells *in vitro* and abolish HLA-E^+ leukemia and lymphoma tumors in xenograft mouse models of human neoplastic disease (NOD-SCID mice injected with HLA-E^+ Epstein-Barr virus-positive cells or acute myeloid leukemia cells) (38). Interestingly, although NKG2A is predominantly expressed by NK cells, a study by the Vivier group showed that blockade of NKG2A enhanced the effector functions of both NK cells and $CD8^+$ T cells in mice and humans (32). The use of monalizumab not only promoted human NK cell antibody-dependent cell-mediated cytotoxicity (ADCC) against various tumor cells but also rescued the function of $CD8^+$ T cells when combined with PD-1 blockade (32). This group also reported impressive clinical outcomes: the use of monalizumab combined with cetuximab (an anti-EGFR antibody) in previously treated patients with squamous cell carcinoma of the head and neck showed a 30% response rate with limited side effects [fatigue (17%), pyrexia (13%), and headache (10%)] (32). Interestingly, a study by Kamiya et al. showed that $NKG2A^{null}$ NK cells, which were generated through transduction of anti-NKG2A protein expression blockers (PEBLs), exhibited relatively high cytotoxicity against HLA-E^+ tumor cells; moreover, this method generated more potent cytotoxicity than blockade with an anti-NKG2A mAb (39), suggesting a new method for developing NKG2A-targeted cancer immunotherapy.

KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR FAMILY

KIRs

The killer-cell immunoglobulin-like receptors (KIRs) on human NK cells include both activating and inhibitory receptors, among which the inhibitory KIRs exhibit an inhibitory signaling motif and are named with the convention KIRxDL (40). KIR2DL and KIR3DL specifically bind to HLA-C and HLA-A/B allotypes, respectively (41, 42). KIR2DL includes KIR2DL1 and KIR2DL2/3, which bind distinct HLA-C allotypes to suppress the activation and effector functions of NK cells (41). Tumor cells induce the upregulated expression of KIRs on NK cells; for example, the expression of KIR2DL2 and HLA-C1 is significantly elevated in breast cancer patients (43); KIR2D (L1, L3, L4, and S4) and KIR3DL1 are expressed on tumor cells and TILs from non-small cell lung cancer patients, and patients without expression of KIR2D (L1, L3, L4, and S4) or KIR3DL1 on their tumor cells or TILs exhibit extended overall survival (44). KIR centromeric B haplotype is associated with significant risks of multiple basal cell carcinoma and squamous cell carcinoma, suggesting that interactions between KIRs and HLA molecules may modify the risks of basal cell carcinoma and squamous cell carcinoma (45). Interestingly, patients with bile duct cancer show multiple alterations at KIR gene loci (46), and genetic variations in KIRs are also present in non-small cell lung cancer patients who are resistant to anti-PD-1 monotherapy (47).

Due to their impressive suppressive effect on NK cells, human mAbs targeting KIRs have shown some clinical benefits. Lirilumab (1-7F9, IPH2101) targeting KIR2DL1, KIR2DL2, and KIR2DL3 increases NK cell cytotoxicity against autologous acute myeloid leukemia blasts and mediates the lysis of HLA-C-expressing tumor cells both *in vitro* and *in vivo* (48). Lirilumab also enhances NK cell activity against autologous multiple myeloma cells by preventing inhibitory KIR-ligand interactions (49). Phase I studies of lirilumab in patients with acute myeloid leukemia, hematological malignancies or solid tumors have shown that lirilumab can effectively block KIRs with mild adverse events (50, 51). However, a study by Carlsten et al. demonstrated that lirilumab not only reduced KIR2D expression on NK cells but also rapidly reduced NK cell functions, resulting in significantly diminished overall responses (52). On the other hand, IPH4102 targeting KIR3DL2 shows encouraging clinical activity in patients with relapsed or refractory cutaneous T-cell lymphoma, particularly those with Sézary syndrome (53).

An *in vitro* study found that stimulation with IL-12/IL-15/IL-18 also downregulated the expression of

TABLE 2 | Clinical trials based on potential NK cell checkpoint inhibitors in cancer.

	Registry	Disease	Intervention	Phase	Status	Enrollment	Sponsors and collaborators
TIGIT	NCT03119428	Locally advanced cancer, metastatic cancer	OMP-313M32/Nivolumab	I	Active	30	OncoMed Pharmaceuticals, Inc.
	NCT03563716	Non-small cell lung cancer	MTIG7192A/Atezolizumab	II	Active	120	Genentech, Inc.
	NCT03628677	Non-small Cell Lung Cancer, squamous cell carcinoma of the head and neck, renal cell carcinoma, breast cancer, colorectal cancer, melanoma, bladder cancer, ovarian cancer, endometrial cancer, merkel cell carcinoma, gastroesophageal cancer	AB154/AB122	I	Recruiting	242	Arcus Biosciences, Inc.
	NCT02794571	Advanced/metastatic tumors	Atezolizumab/MTIG7192A	I	Recruiting	300	Genentech, Inc.
KIR	NCT01750580	CANCER, NOS	Lirilumab/Ipilimumab	I	Completed	22	Bristol-Myers Squibb
	NCT01714739	CANCER, NOS	Lirilumab/Nivolumab/ Ipilimumab	I/II	Active	337	Bristol-Myers Squibb
	NCT03203876	Advanced cancer	Lirilumab/Nivolumab/ Ipilimumab	I	Active	21	Bristol-Myers Squibb Ono Pharmaceutical Co.Ltd
	NCT01222286	Smoldering multiple myeloma	IPH2101	II	Completed	30	Innate Pharma
	NCT00999830	Multiple myeloma	IPH2101	II	Completed	27	Innate Pharma
	NCT00552396	Multiple myeloma	Anti-KIR (1-7F9)	I	Completed	32	Innate Pharma
	NCT01256073	Acute myeloid leukemia	IPH2101	I	Completed	21	Innate Pharma
	NCT01687387	Acute myeloid leukemia	IPH2102	II	Completed	152	Innate Pharma
	NCT02481297	Leukemia, chronic lymphocytic leukemia, lymphocytic leukemia	Lirilumab/Rituximab	II	Active	8	M.D. Anderson Cancer Center Bristol-MyersSquibb
	NCT02593045	Cutaneous T-Cell lymphoma	IPH4102	I	Active	60	Innate Pharma
	NCT03902184	Lymphoma, mycosis fungoides/sezary syndrome	IPH4102 + Gemcitabine + Oxaliplatin	II	Recruiting	250	Innate Pharma
TIM-3	NCT03489343	Metastatic cancer, solid tumor, lymphoma	Sym023	I	Recruiting	48	Symphogen A/S
	NCT02817633	Advanced or metastatic solid tumors	TSR-022/TSR-042/TSR-033	I	Recruiting	819	Tesaro, Inc.
	NCT03680508	Adult primary liver cancer, advanced adult primary liver cancer, localized unresectable adult primary liver cancer	TSR-022 + TSR-042	II	Not yet recruiting	42	University of Hawaii
	NCT03311412	Metastatic cancer, solid tumor, lymphoma	Sym021/Sym022/Sym023	I	Recruiting	102	Symphogen A/S
	NCT03099109	Solid tumor	LY3321367/LY3300054	I	Recruiting	196	Eli Lilly and Company
	NCT03744468	Locally advanced or metastatic solid tumors	BGB-A425/tislelizumab	I/II	Recruiting	162	BeiGene
	NCT03961971	Glioblastoma multiforme	MBG453	I	Not yet recruiting	15	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins NovartisPharmaceuticals

(Continued)

TABLE 2 | Continued

	Registry	Disease	Intervention	Phase	Status	Enrollment	Sponsors and collaborators
	NCT03066648	Leukemia, myelodysplastic syndromes, preleukemia, bone marrow diseases, hematologic diseases	Decitabine/PDR001/ MBG453	I	Recruiting	175	Novartis Pharmaceuticals
	NCT02608268	Advanced malignancies	MBG453/PDR001	I/II	Recruiting	250	Novartis Pharmaceuticals
LAG-3	NCT03489369	Metastatic cancer, solid tumor, lymphoma	Sym022	I	Recruiting	30	Symphogen A/S
	NCT03311412	Metastatic cancer, solid tumor, lymphoma	Sym021/Sym022/Sym023	I	Recruiting	102	Symphogen A/S
	NCT02061761	Hematologic neoplasms	BMS-986016/BMS-936558	I/II	Recruiting	132	Bristol-Myers Squibb
	NCT02966548	Cancer	Relatlimab/Nivolumab	I	Recruiting	45	Bristol-Myers Squibb Ono Pharmaceutical Co.Ltd
	NCT01968109	Neoplasms by site	Relatlimab/Nivolumab/ BMS-986213	I/II	Recruiting	2000	Bristol-Myers Squibb
	NCT03459222	Advanced cancer	Relatlimab/Nivolumab/ BMS-986205/Ipilimumab	I/II	Recruiting	230	Bristol-Myers Squibb
	NCT02658981	Glioblastoma, gliosarcoma, recurrent brain neoplasm	BMS 986016/Anti-PD-1/Anti-CD137	I	Recruiting	100	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins National Cancer Institute (NCI) Bristol-MyersSquibb
	NCT03044613	Gastric cancer, esophageal cancer, gastroesophageal cancer	Nivolumab/Relatlimab/ Carboplatin/Paclitaxel/ Radiation	I	Recruiting	32	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Bristol-MyersSquibb
	NCT03623854	Chordoma, locally advanced chordoma, metastatic chordoma, unresectable chordoma	Nivolumab/Relatlimab	II	Recruiting	20	Jonsson Comprehensive Cancer Center National Cancer Institute(NCI)
	NCT03493932	Glioblastoma	Nivolumab/BMS-986016	I	Recruiting	20	National Institute of Neurological Disorders and Stroke (NINDS)
	NCT03743766	Melanoma	Relatlimab/Nivolumab	II	Recruiting	42	John Kirkwood Bristol-MyersSquibb
	NCT00351949	Stage IV renal cell carcinoma	IMP321	I	Completed	24	Immutep S.A, Umanis
	NCT03252938	Solid tumors, peritoneal carcinomatosis	IMP321/Avelumab	I	Recruiting	50	IKF Klinische Krebsforschung GmbH at Krankenhaus Nordwest
	NCT00349934	Metastatic breast cancer	IMP321	I	Completed	33	Immutep S.A, Umanis
	NCT03625323	Non-small cell lung cancer, squamous cell carcinoma of head and neck	Eftilagimod alpha/Pembrolizumab	II	Recruiting	109	Immutep S.A, Merck Sharp & Dohme Corp.
	NCT02614833	Stage IV breast adenocarcinoma	IMP321/Paclitaxel	II	Active	241	Immutep S.A.
	NCT02676869	Stage IV and stage III melanoma	IMP321/Pembrolizumab	I	Active	24	Immutep Australia Pty. Ltd.
NKG2A	NCT02921685	Hematological malignancy	Monalizumab (IPH2201)	I	Recruiting	18	Institut Paoli-Calmettes InnatePharma

(Continued)

TABLE 2 | Continued

	Registry	Disease	Intervention	Phase	Status	Enrollment	Sponsors and collaborators
	NCT02557516	Chronic lymphocytic leukemia	Monalizumab	I/II	Active	22	Innate Pharma
	NCT02459301	Gynecologic cancer	IPH2201	I	Active	59	Canadian Cancer Trials Group
	NCT02643550	Head and neck neoplasms	Monalizumab/Cetuximab/ Anti-PD-L1	I/II	Recruiting	140	Innate Pharma AstraZeneca
	NCT02671435	Advanced solid tumors	Durvalumab (MEDI4736)/Monalizumab (IPH2201)	I/II	Recruiting	501	MedImmune LLC
	NCT03822351	Unresectable stage III non-small cell lung cancer	Durvalumab/Monalizumab/ Oleclumab	II	Recruiting	300	MedImmune LLC
	NCT03833440	Non-small cell lung cancer	Durvalumab (MEDI4736)/Monalizumab/ Oleclumab (MEDI9447)/AZD6738	II	Not yet recruiting	120	Assistance Publique Hopitaux De Marseille
	NCT02331875	Squamous cell carcinoma of the oral cavity	IPH2201	I/II	Terminated	3	Innate Pharma
	NCT03088059	Squamous cell carcinoma of head and neck	Afatinib/Palbociclib/ IPH2201/Durvalumab/ Niraparib/BAY1163877	II	Recruiting	340	European Organization for Research and Treatment of Cancer

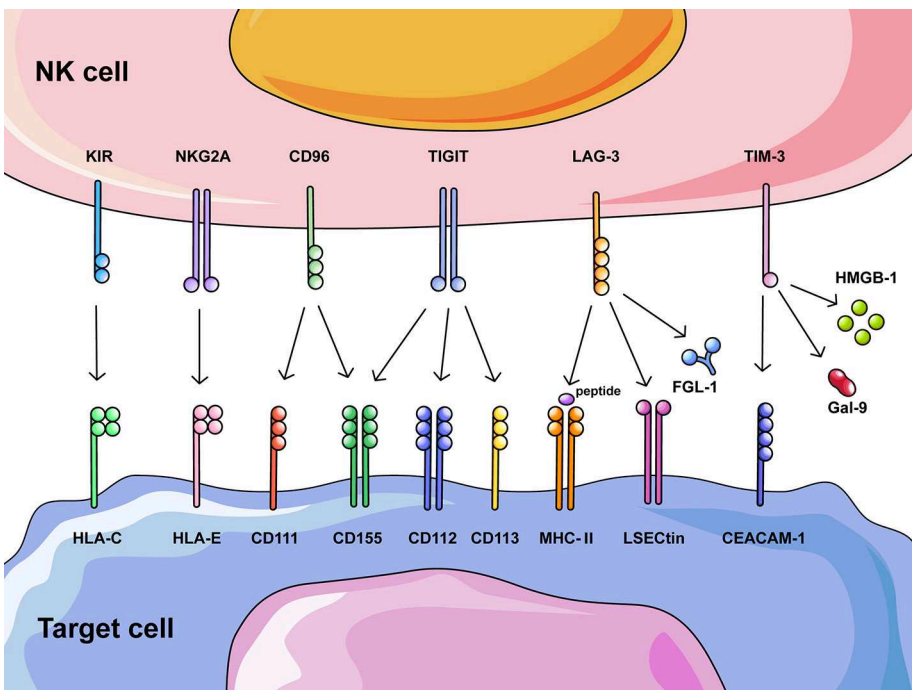

FIGURE 1 | Overview of potential NK cell checkpoint molecules and their corresponding ligands. Recognition and clearance of tumor cells by NK cells are regulated through activating and inhibitory receptors on NK cells that bind their corresponding ligands on tumor cells. Increased expression of ligands on tumor cells induces altered expression of inhibitory receptors on NK cells, excessive negative regulation results in functional exhaustion of NK cells. This figure summarizes inhibitory receptors on NK cells that could also act as checkpoints in cancer immunotherapy, including HLA class I-specific receptors (KIR and NKG2A) and those recognizing ligands other than HLA class I molecules (CD96, TIGIT, LAG-3, and TIM-3).

KIR2DL2/3, KIR2DL1, and KIR3DL1 on peripheral blood NK cells, resulting in reduced inhibitory KIR signaling and elevated CD16-dependent cytotoxicity (54). Furthermore, these IL-12/IL-15/IL-18-stimulated NK cells showed increased cytotoxicity against tumor cells (54).

IMMUNOGLOBULIN SUPERFAMILY

TIGIT

TIGIT is an immunoglobulin protein that belongs to the CD28 family (55, 56). It was discovered as a surface receptor on T cells that recognizes CD155 in 2009 (57); however, TIGIT is also expressed on NK cells and interacts with other ligands, such as CD112 and CD113 (56).Together with CD226 and CD96, TIGIT participates in a balanced system to control the activation and function of T cells and NK cells. Unlike CD96, which inhibits only IFN-γ production in NK cells and has no effects on cytotoxicity, TIGIT can inhibit NK cell cytotoxicity directly through its ITIM domain in both humans and mice (58, 59). A study showed that the cytotoxicity of YTS NK cells (human NK cell line) transfected with TIGIT was strongly inhibited by CD155-transfected 721.221 cells and this inhibition could be blocked with an anti-TIGIT mAb *in vitro* (58). Furthermore, blockade of TIGIT has also been shown to significantly increase mouse NK cell-mediated killing of CD155-expressing B12 cells and enhance the secretion of IFN-γ (59). In an *in vivo* study, NK cells isolated from TIGIT-transgenic mice produced reduced amounts of IFN-γ after incubation with Yac-1 cells (murine T cell lymphoma cell line), while NK cells isolated from TIGIT$^{-/-}$ mice produced increased amounts of IFN-γ. The suppression of IFN-γ production was mediated by TIGIT-CD155 ligation through the NF-κB pathway (60). In humans, NK cells with lower levels of TIGIT isolated from healthy individuals were shown to have a higher ability to secrete cytokines, degranulate, and kill target cells than those with higher TIGIT expression (61), suggesting the ability of TIGIT to regulate immune responses.

The expression of TIGIT is highly variable among different cancer types. The highest expressions of TIGIT in lymphocytes are found in Hodgkin's lymphoma, Warthin's tumors, medullary breast cancer, intestinal stomach cancer, and seminoma, while the lowest expressions of TIGIT in lymphocytes are found in renal oncocytoma, papillary renal cell cancer, desmoid tumors, pancreatic neuroendocrine cancer, chromophobic renal cell cancer, and adrenocortical cancer (62). Indeed, TIGIT$^{-/-}$ mice show no resistance to lung metastasis in three different experimental lung metastasis models (B16F10, murine melanoma cell line; RM-1, murine prostate cancer cell line; E0771, murine breast cancer cell line) (63); moreover, TIGIT expression on NK cells is not significantly different between pancreatic cancer patients and healthy controls (64). Furthermore, a reduced proportion of TIGIT^{+} NK cells has been observed in the intratumoral region of hepatocellular carcinoma compared to the peritumoral region (65).

In contrast, TIGIT is overexpressed on CD8^{+} tumor-infiltrating lymphocytes (TILs) and tumor antigen-specific CD8^{+} T cells from melanoma patients and often coexpressed with the inhibitory receptor PD-1 (66). Coblockade of TIGIT and PD-1 can reverse dysfunctions in CD8^{+} TILs and antigen-specific CD8^{+} T cells by increasing their proliferation and effector functions (66). TIGIT expression was also found to be significantly increased in CD4^{+} T cells from chronic lymphocytic leukemia patients, and an increased number of TIGIT^{+} CD4^{+} T cells was found in patients with advanced disease stage (67). Moreover, TIGIT$^{-/-}$ was shown to significantly inhibit tumorigenicity in both CT26 tumor-bearing BALB/c mice and MC38 tumor-bearing C57BL/6 mice, whereas an anti-TIGIT mAb significantly inhibited tumor growth in both of these colorectal tumor models (68). Furthermore, a study showed that TIGIT$^{-/-}$ mice intravenously injected with B16 melanoma cells had relatively few lung metastases and improved overall survival (13). It is also important to note that TIGIT inhibits IFN-γ secretion of both CD8^{+} T cells and NK cells in the above mentioned colorectal tumor models; interestingly, CT26 tumor-bearing TIGIT knockout (KO) mice develop tumors early after NK cells are depleted, suggesting that NK cells and T cells collaborate to eliminate tumors (68).

The Tian group has demonstrated that TIGIT is highly expressed on tumor-infiltrating NK cells and associated with NK cell exhaustion in different tumor models [CT26 colon cancer, 4T1 breast cancer, B16 melanoma, and fibrosarcoma induced by methylcholanthrene (MCA)] and patients with colon cancer (13). NK cell-specific TIGIT KO in mice results in significantly prolonged survival, while TIGIT blockade inhibits NK cell exhaustion in colon tumors, breast tumors, and MCA-induced fibrosarcomas (13). Surprisingly, they showed that anti-TIGIT mAb could reduce tumor mass and slow tumor growth in T cell-deficient mice; in addition, NK cell deficiency resulted in an increased metastasis and number of exhausted CD8^{+} T cells, and abolished the effect of TIGIT blockade even in the presence of TIGIT-expressing CD8^{+} T cells (13). Notably, the therapeutic effects of anti-TIGIT mAbs, anti-PD-L1 mAbs or anti-TIGIT mAbs combined with anti-PD-L1 mAbs all depended on the presence of NK cells (13), indicating the importance of NK cells in checkpoint-targeted immunotherapy. Other studies have also indicated the importance of TIGIT^{+} NK cells in the tumor microenvironment. For example, blocking TIGIT could increase cytokine production by NK cells after an incubation with trastuzumab-coated breast cancer cells (69); the proportion of TIGIT^{+} NK cells was significantly increased in the peripheral blood mononuclear cell (PBMC) population of non-muscle invasive bladder cancer patients compared to that of healthy controls (70); endometrial tumor-resident CD103^{+} NK cells expressed higher levels of TIGIT than circulating CD103^{-} NK cells, and tumor-resident NK cells from patients with lymph node invasion showed significantly higher expressions of TIGIT than those from patients with no lymph node invasion (71); and TIGIT^{+} NK cells showed increased susceptibility to functional suppression by CD155-expressing myeloid-derived suppressor cells (MDSCs) (72). Currently, several clinical trials (phase I and phase II) focused on testing the feasibility of targeting this new pathway and improving therapeutic effects through combination with existing immunotherapies are either active or recruiting (**Table 2**).

CD96

CD96 is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily (73, 74). It was identified as a key receptor on NK cells that recognizes the ligand CD155 in 2004 (75) and was initially identified as a possible costimulatory receptor. However, 10 years later, its inhibitory characteristics

were revealed by the Smyth group (76). Their study showed that CD96 competes with CD226 for CD155 binding and negatively regulates IFN-γ secretion in NK cells (77); however, it does not affect the direct killing of tumor cells by NK cells. Furthermore, $CD96^{-/-}$ mice are resistant to MCA-induced fibrosarcoma and experimental lung metastasis modeled by injecting B16F10 melanoma cells (77), and blocking CD96 with a mAb inhibits experimental metastases in three different models (B16F10 melanoma, 3LL lung carcinoma, and RM-1 prostate cancer) (63). Blockade of the CD96-CD155 interaction was also shown to be effective in controlling lung metastases in NCR2-transgenic mice injected with B16-PDGFD cells (78). An anti-CD96 mAb was shown to be superior to other well-characterized checkpoint inhibitors, such as anti-CTLA-4 and anti-PD-1 antibodies, and the combination of an anti-CD96 mAb with an anti-CTLA-4 or anti-PD-1/PD-L1 mAb could further inhibit experimental lung metastases (63). Notably, although CD96 was also expressed by T cells, the control of metastases by an anti-CD96 mAb appeared to be dependent on NK cells, CD226 and IFN-γ production (63), suggesting a non-negligible role for NK cells in cancer immunotherapy. Further, blocking CD96 reduced the number of B16F10 metastases in $Tigit^{-/-}$ mice compared to wildtype mice, indicating the synergistic potential of blocking CD96 and TIGIT in treating cancer (63).

The structural basis for the CD96-CD155 interaction involves the "ancillary key" motif that is critical for CD155 recognition; moreover, CD96 and CD155 interact via the "lock-and-key" docking mode (79). However, surprisingly, a comparison between three anti-CD96 mAbs, including two that block the CD96-CD155 interaction (3.3 and 6A6) and one that does not block this interaction (8B10), revealed that although the two blocking mAbs showed higher potency than the non-blocking mAb in the control of metastases, it was not necessary to block the CD96-CD155 interaction to promote NK cell antimetastatic functions (80, 81). In contrast, another study using a transgenic mouse model of resectable pancreatic ductal adenocarcinoma showed that a mAb targeting the CD96-CD155 interaction (6A6) significantly reduced distant metastases, while a mAb that did not target the CD96-CD155 interaction (8B10) showed no effect on the frequency of metastases (82). One possible explanation for the contradictions occurred involves various microenvironmental cues in *in vitro* vs. *in vivo* settings, given that the microenvironment in an *in vivo* experiment is much more complicated and involves various cell-cell interactions and consequences (for example, cytokine secretions, etc.) following these interactions, which may contribute to the differences raised between the studies.

In humans, a significantly decreased percentage of $CD96^+$ NK cells in pancreatic cancer patients and associations of this decreased percentage with lymph node metastasis and tumor histological grade were observed (64), suggesting a possible protective role for $CD96^+$ NK cells in pancreatic cancer. Contradictorily, another study showed increased serum levels of soluble CD96 in NK cells from late-stage melanoma patients (83). In addition, a study noted an elevated proportion and number of $CD96^+CD56^{dim}$ NK cells in hepatocellular carcinoma tissues, and these NK cells were functionally exhausted with impaired IFN-γ and TNF-α productions (65). Furthermore, patients with higher $CD96^+$ NK cell infiltration within tumors have been shown to exhibit relatively short disease-free survival times (65). These studies suggest a protumor role for $CD96^+$ NK cells in melanoma and hepatocellular carcinoma.

LAG-3

LAG-3 is a negative coinhibitory receptor expressed on T cells and NK cells that binds MHC class II (MHC-II) molecules on target cells (84, 85). LAG-3 also interacts with LSECtin, a cell surface lectin that belongs to the C-type lectin receptor superfamily, to inhibit IFN-γ production by effector T cells (86). Recently, Chen and colleagues identified fibrinogen-like protein 1 (FGL1), a liver-secreted protein, as an MHC-II-independent ligand for LAG-3 in both humans and mice (87). Previous studies have shown that LAG-3 negatively regulates the proliferation and activation of T cells (88, 89) and that it also interacts with FGL1 to inhibit antigen-mediated T cell responses both *in vitro* and *in vivo* (87). LAG-3 and PD-L1 coregulate the exhaustion of $CD8^+$ T cells, and compared to anti-PD-L1 mAb or anti-LAG-3 mAb monotherapy, dual blockade of LAG-3 and PD-L1 increases the number and effector functions of functional virus-specific $CD8^+$ T cells (90), suggesting that the combination of anti-LAG-3 and anti-PD-L1 antibodies results in an improved reversal of exhaustion.

LAG-3 has been shown to suppress immune responses in several tumors, including Hodgkin's lymphoma, gastric cancer, breast cancer, and other solid tumors (91). In studies of squamous cell carcinoma mouse models, both $CD8^+$ and $CD4^+$ TILs coexpressed LAG-3 and PD-1, and dual blockade of LAG-3 and PD-1 significantly suppressed tumor growth (92). LAG-3 has been detected in TILs from 41.5% of non-small cell lung cancer patients and associated with the checkpoint molecules PD-1 and TIM-3 (93). Moreover, elevated LAG-3 expression has been associated with reduced progression-free survival in patients with advanced non-small cell lung cancer treated with PD-1 blockade (93). Elevated expression of LAG-3 has also been observed in patients with peripheral T cell lymphoma or NK/T cell lymphoma (94). LAG-3^+ TIL numbers are increased in MHC-II^+ tumors (lung cancer, melanoma, and breast cancer), and MHC-II^+ tumors acquire immunosuppressive signals through LAG-3; thus, combined PD-1/PD-L1 and LAG-3 blockade can provide a particular advantage against MHC-II^+ tumors (95). Interestingly, expression of the newly identified ligand FGL1 has also been shown to be upregulated in cancer, and blockade of the FGL1-LAG-3 interaction stimulates immune responses and exhibits therapeutic effects on mouse tumor models (MC38 colon cancer and Hepa1-6 liver cancer) (87). Furthermore, compared to monotherapy, an anti-FGL1 mAb or anti-LAG-3 mAb in combination with an anti-B7-H1 mAb significantly reduces tumor burden and prolongs survival (87).

An early study in 1996 indicated that mice lacking the *lag3* gene exhibited reduced lysis of Yac-1 cells; in addition, polyclonal antibodies against LAG-3 could reduce NK-mediated lysis of Yac-1 cells but leave MHC-II-deficient target cells intact, suggesting the existence of an independent mode of natural

killing through LAG-3 (96). However, this independent mode of natural killing has not been observed with human NK cells, and blockade of LAG-3 has no effect on the natural killing of various target cells (97). Notably, although studies on LAG-3$^+$ NK cells are limited, the role of these cells in antitumor immunity should not be neglected. A study showed that using IL-12 to boost the cytotoxicity of NK cells in a lung cancer model (BALB/c mice injected with 4T1 cells) increased the NK cell population expressing high levels of coinhibitory molecules, including LAG-3, which limited NK cell-mediated antimetastatic activity (98). The combination of an anti-LAG-3 mAb with IL-12 significantly reduces lung metastasis, whereas monotherapy fails to achieve this effect (98). Furthermore, synergy between an anti-LAG-3 mAb and IL-12 contributes to the increased efficacy of IL-12 immunotherapy in breast cancer, which is solely dependent on NK cells, suggesting that LAG-3 is applicable in not only T cell-mediated immunotherapies but also NK cell-mediated antimetastatic immunotherapies (98).

TIM-3

TIM-3 is a type I glycoprotein that binds galectin-9 (Gal-9), high mobility group box 1 protein (HMGB1), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) on target cells to act as an NK cell coreceptor (91, 99, 100). An early *in vitro* study showed that an NK92 cell line overexpressing TIM-3 secreted an increased amount of IFN-γ, while TIM-3 blockade resulted in reduced IFN-γ production (101). However, although human TIM-3$^+$ NK cells are functional in terms of cytokine production and cytotoxicity, they become suppressed when TIM-3 is cross-linked with antibodies (102), suggesting that an interaction between TIM-3 and its ligand can result in NK cell dysfunction.

TIM-3 can mediate cell exhaustion and suppress immune responses under both chronic viral and cancerous conditions. For example, TIM-3 mediates suppression of NK cells in chronic hepatitis B patients, while TIM-3 blockade results in increased NK cell cytotoxicity both *in vitro* and *ex vivo* (103). TIM-3 is highly expressed in various tumor types, including gastrointestinal stromal tumor (104), lung adenocarcinoma (105), perineural squamous cell carcinoma (106), melanoma (107), gastric cancer (108), acute myeloid leukemia (109), colon cancer (110), bladder cancer (70), renal cell carcinoma (111), pancreatic cancer (112), glioma (113), anaplastic thyroid cancer (114), peripheral T cell lymphoma, NK/T cell lymphoma (94), etc. Significant overexpression of TIM-3 has been observed in peripheral NK cells from non-muscle invasive bladder cancer patients (70); moreover, TIM-3 is expressed in TILs from 25.3% of non-small cell lung cancer patients and associated with the expression of PD-1 and LAG-3 (93). TIM-3 expression was found to be higher on peripheral NK cells from glioma patients than on those from healthy controls, and these TIM-3$^+$ NK cells showed a reduced capability for IFN-γ production and correlated with the proportion of Ki-67$^+$ tumor cells (113). Furthermore, TIM-3 expression is upregulated on NK cells in late-stage melanoma patients, and blockade of TIM-3 reverses NK cell exhaustion in these patients (107).

TIM-3 functions as a potential prognostic marker in several tumor types. Upregulated expression of TIM-3 in peripheral NK cells from lung adenocarcinoma patients correlates with decreased overall survival, while blockade of TIM-3 enhances cytotoxicity and IFN-γ production in peripheral NK cells (105). Overexpression of TIM-3 in NK cells from gastric cancer patients has been associated with an advanced tumor stage (108). In addition, a study found that endometrial tumor-resident CD103$^+$ NK cells expressed higher levels of TIM-3 than circulating CD103$^-$ NK cells, and tumor NK cells from patients with lymph node invasion showed significantly higher expression of TIM-3 than those from patients with no lymph node invasion (71). Bladder cancer patients have high levels of TIM-3$^+$ NK cells and Gal-9$^+$ tumor cells, and patients with relatively low levels of TIM-3$^+$ NK cells and Gal-9$^+$ tumor cells have an improved prognosis (115). Furthermore, TIM-3$^+$ NK cells are defective in esophageal cancer, and relatively high TIM-3 expression on NK cells correlates with a poor prognosis in esophageal carcinoma (116).

However, contradictorily, studies have also reported stimulatory functions of TIM-3 (117). For example, after short-term stimulation with anti-CD3/CD28 antibodies, TIM-3 can enhance the secretion of IL-2 and signaling pathways that lead to T-cell activation (118, 119). TIM-3 engagement during antigen stimulation directly promoted CD8 T cell differentiation through mTORC1 (120). Furthermore, activation of human T cells was not affected by the presence of Gal-9 or antibodies to TIM-3 (121), which also reported a contradictory role of Gal-9 as a ligand for TIM-3. These studies suggest that the use of anti-TIM-3 should be particularly careful because TIM-3 also plays an activating role under certain circumstances, whether its antibodies act as agonist or antagonist remains to be questioned. Both anti-murine and anti-human TIM-3 antibodies bind to TIM-3 in a manner that interfere with the binding of TIM-3 to both phosphatidylserine and CEACAM1, the understanding of the interaction between TIM-3 and its ligands plays an important role in the screening of anti-TIM-3 antibody candidates (122).

Some studies have explored the reasons underlying the upregulation of TIM-3 expression on NK cells in the tumor microenvironment. One study reported that the LPHN1/PKC/mTOR-TIM-3-Gal-9 pathway in human acute myeloid leukemia induced high levels of Gal-9 secretion and the release of soluble TIM-3 (109). Gal-9 impaired the killing of tumor cells by NK cells, whereas soluble TIM-3 impaired the ability of T cells to produce IL-2, contributing to the breakdown of immune surveillance and thus to the progression of tumors (109). Another proposed mechanism is that MHC-I-deficient tumors induce coexpression of TIM-3 and PD-1 on NK cells, resulting in functional NK cell exhaustion in both tumor-bearing mice and cancer patients; functional recovery in these exhausted NK cells induced by vaccination requires IL-21 produced by NKT cells (110). Furthermore, carcinoma-associated fibroblasts (CAFs) have been shown

to promote the expression of TIM-3 in pancreatic cancer patients (112). Sustained IL-15 stimulation upregulates TIM-3 expression on both T and NK cells (123). TIM-3 expression can also be induced by TNF-α through the NF-κB signaling pathway (116). In addition, IL-27/NFIL3 signaling axis has been identified crucial for the induction of Tim-3, IL-10 and T-cell dysfunction (124).

PERSPECTIVE

The success of mAbs targeting CTLA-4 and PD-1 has shed light on cancer immunotherapy, and restoring exhausted T cells has shown promising clinical outcomes in some patients. However, there were still many patients who are nonresponsive to these treatments. However, recent findings indicate that improved survival highly correlates with the frequency of DNAM-1^{+}CD56^{dim} NK and NKp46^{+}CD56^{dim} NK cells after treatment with anti-CTLA-4 in patients with malignant mesothelioma (125). Hsu and Hodgins et al. demonstrated in multiple tumor models that PD-1 is upregulated on the most activated and functionally responsive intratumoral NK cells, suggesting that the efficacy of PD-1 blockade depends in part on inducing an NK cell-based antitumor response (126). Thereof, we propose that NK cell-targeted immunotherapy may provide an alternative or complementary approach to overcome the limitations of T cell immunotherapy and combination with NK cell immunotherapy could increase the response rate of T cell-targeted treatments. NK cells are critical for immunosurveillance, particularly in the control of metastasis and hematological cancers. A study by the Tian group indicated the importance of NK cells in checkpoint immunotherapy; TIGIT blockade prevented NK cell exhaustion in the absence of T cells and B cells, and an anti-TIGIT mAb improved T cell responses in an NK cell-dependent manner (13). These findings suggest that certain checkpoint molecules expressed by both T cells and NK cells may exert a greater effect on NK cells than on T cells and that NK cells could be essential for the T cell-mediated antitumor response in such a scenario.

The recent success of anti-NKG2A mAb on clinical trials unleashes its role as a promising checkpoint inhibitor in treating cancers with minimum side effects, and the success of NKG2A blockade also points out the importance of NK cells in anti-tumor immunity and advances the idea that combined reversal of both T and NK cell exhaustion is truly important in anti-tumor immunotherapy. Anti-NKG2A could be the third potential checkpoint inhibitor approved by the FDA following anti-CTLA-4 and anti-PD-1/PD-L1. Another promising checkpoint molecule targeting NK cells is TIGIT based on the results observed by the Tian group (13). However, due to its constitutive expression on peripheral human NK cells, more studies are required to fulfill its role as a checkpoint in treating cancer. The balance between CD96, TIGIT, and CD226 is critical for proper immune responses by NK cells. The accumulation of CD96 in NK cells in hepatocellular carcinoma patients disrupts the balance between these three receptors, which subsequently results in NK cell dysfunction and exhaustion (65); therefore, careful examination of the CD96-TIGIT-CD226 system should be involved in developing immunotherapies targeting these receptors. In addition to CD155, CD112, and CD113, human TIGIT can also bind to the Fap2 protein of *Fusobacterium nucleatum*, and the interaction between TIGIT and Fap2 inhibits NK cell cytotoxicity (127). This finding unleashes a new mechanism of tumor immune evasion that depends on the bacterium and identifies new possibilities for NK cell immunotherapy. Alterations in KIR and HLA gene loci affect NK cell functions, which should be considered when developing immunotherapies against KIRs (46). The interaction between LAG-3 and the newly defined ligand FGL1 suppresses the functions of T cells, and whether FGL1 also interacts with LAG-3 on NK cells merits further research (87).

Other checkpoint molecules on NK cells have been proven to be potential targets, and further experiments are needed to prove these novel targets for NK-based immunotherapy (22, 128). There are ways to improve the efficacy of NK cell immunotherapy. For example, NKG2A^{null} NK cells are more effective than NKG2A^{+} NK cells treated with an anti-NKG2A mAb, suggesting a new immunotherapeutic approach using NKG2A^{null} NK cells (39, 129). In addition, cytokines can enhance the efficacy of mAbs (130), and a combination of cytokine treatment with checkpoint immunotherapy may boost the effects of mAbs. Furthermore, we believe that combined blockade of checkpoint molecules expressed by T cells and NK cells could unleash antitumor immunity mediated by innate and adaptive populations, which not only improve overall antitumor immune responses but also allow the two approaches to complement each other; this strategy might be the solution for the "non-responders." Accumulating evidence suggests that NK cell-targeted immunotherapy is highly feasible; however, our knowledge of the inhibitory mechanisms in NK cells is still inadequate, and more fundamental research is required to identify the best inhibitory pathways to be targeted for future clinical applications.

AUTHOR CONTRIBUTIONS

HS and CS were involved in the search and analysis of the literature, design and writing of the manuscript, and revision of the manuscript.

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MINI REVIEW
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Post-translational Mechanisms Regulating NK Cell Activating Receptors and Their Ligands in Cancer: Potential Targets for Therapeutic Intervention

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Efficient clearance of transformed cells by Natural Killer (NK) cells is regulated by several activating receptors, including NKG2D, NCRs, and DNAM-1. Expression of these receptors as well as their specific "induced self" ligands is finely regulated during malignant transformation through the integration of different mechanisms acting on transcriptional, post-transcriptional, and post-translational levels. Among post-translational mechanisms, the release of activating ligands in the extracellular milieu through protease-mediated cleavage or by extracellular vesicle secretion represents some relevant cancer immune escape processes. Moreover, covalent modifications including ubiquitination and SUMOylation also contribute to negative regulation of NKG2D and DNAM-1 ligand surface expression resulting either in ligand intracellular retention and/or ligand degradation. All these mechanisms greatly impact on NK cell mediated recognition and killing of cancer cells and may be targeted to potentiate NK cell surveillance against tumors. Our mini review summarizes the main post-translational mechanisms regulating the expression of activating receptors and their ligands with particular emphasis on the contribution of ligand shedding and of ubiquitin and ubiquitin-like modifications in reducing target cell susceptibility to NK cell-mediated killing. Strategies aimed at inhibiting shedding of activating ligands and their modifications in order to preserve ligand expression on cancer cells will be also discussed.

Keywords: activating NK cell receptors, ligands for NK cell activating receptors, post-translational modifications, shedding, ubiquitin modification

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INTRODUCTION

Natural Killer (NK) cell activation is tuned by the integration of signals derived from inhibitory receptors for Major Histocompatibility Complex (MHC) class I molecules and from activating receptors that bind either non self-molecules associated to pathogens or self-molecules up-regulated in stress conditions including malignant transformation (1, 2).

Among activating receptors, Natural-Killer receptor group 2, member D (NKG2D), DNAX-associated molecule1 (DNAM-1), and the Natural Cytotoxicity Receptors (NCRs) play a pivotal role in NK cell-mediated tumor surveillance as revealed by an increased incidence of spontaneous malignancies or impaired tumor clearance

in mice deficient for these receptors (3–7). In human, NCR expression may represent a prognostic biomarker in acute myeloid leukemia (AML) and solid tumors (8–10). Moreover, the engagement of the low affinity receptor for IgG (CD16) by means of natural or therapeutic monoclonal antibodies can also contribute to tumor clearance through antibody-dependent cellular cytotoxicity (ADCC) (11, 12).

NK Cell Activating Receptors and Their Ligands on Tumor Cells

NKG2D is a C-type lectin receptor not exclusively expressed on NK cells but also found on NKT, $CD8^+\alpha\beta$ T cells, γδ T cells, and activated $CD4^+\alpha\beta$ T cells (13–15). In humans, NKG2D binds to the adaptor DNAX activating protein 10 (DAP10), responsible for signal propagation. In murine activated NK cells, a shorter NKG2D isoform can either associate with DAP10 or DAP12, an alternative signal transducing adaptor (16, 17).

Human NKG2D ligands (NKG2DLs) belong to two families of polymorphic molecules structurally related to MHC class I: the MHC class I related proteins (MIC)A/B which possess α1, α2, and α3 domains similar to MHC molecules and six UL16 binding proteins (ULBP1-6) characterized by α1 and α2 domains (15, 18, 19). MICA and MICB are generally transmembrane proteins, while ULBP proteins can be transmembrane (ULBP4 and 6) or GPI-linked (ULPB1-3 and 5) molecules. Murine NKG2DLs include Rae-1α-ε, MULT1, and H60a-c and are expressed either as transmembrane or GPI-linked molecules (18).

DNAM-1 belongs to the immunoglobulin receptor family and is expressed not only on NK cells but also on monocytes, T cells, and subsets of B cells (14, 20, 21). It binds to Nectin2/CD112 and PVR/CD155 both members of the Nectin/Nectin-like family of adhesion molecules (22–24), and it associates with the integrin LFA1 to transduce intracellular signals.

Natural cytotoxicity receptors comprise NKp46, NKp44, and NKp30 immunoglobulin-like receptors that are not exclusively expressed on NK cells but also on innate lymphoid cells (ILCs) of group 1 (ILC1) and a subset of ILC3, γδ T cells, and a population of cytotoxic T lymphocytes (25, 26). Only ortholog of NKp46 is expressed in mice (26).

NKp30 and NKp46 associate with the signal transducing adaptors CD3ζ and FcεRIγ while NKp44 mainly signals through the DAP12 adapter. Splicing variants of NKp44 and NKp30 endowed with inhibitory signal capability have been described and are associated with worst prognosis in cancer patients (9, 27).

NCRs interact with several ligands that are either pathogen-encoded or self-molecules and include cell surface and intracellular proteins that reach the surface in infected or transformed cells (28). However, the ligands expressed on tumor cells have not been fully identified yet.

Each NCR has the ability to recognize a specific configuration of heparan sulfate proteoglycans expressed in the context of tumor microenvironment, and this binding can modulate receptor function (28).

Ligands for NKp30 include B7-H6 belonging to the B7 family and only expressed on tumor cells, the intracellular protein HLA-B associated transcript 3 (BAT3), also known as BAG6, and galectin-3 (29–31). The first two ligands bind to and activate NKp30 while the released form of galectin-3 inhibits anti-tumor NKp30 function.

NKp44 interacts with the Proliferating Cell Nuclear Antigen (PCNA), which is aberrantly expressed on the surface of tumor cells. This binding preferentially engages an inhibitory isoform of NKp44 and negatively regulates NK cell functions (32). Interaction between NKp44 and a subset of HLA-DP molecules has been recently reported (33) demonstrating that HLA class II molecules may impact on NK cell activity. Of note, NKp44 can be triggered by specific tumor-derived soluble growth factors (34) and by Nidogen-1, an extracellular matrix protein (35). NKp46 recognize viral ligands including hemagglutinins as well as tumor ligands of still unknown identity.

Most of the above mentioned NK cell activating ligands, including NKG2DLs and B7-H6, are absent in normal cells but their expression is induced upon neoplastic transformation, thus rendering tumor cells more susceptible to NK cell-mediated killing (29, 36–41).

On the other hand, PVR and Nectin2 are expressed on healthy cells (21) but their amount is up-regulated on epithelial and hematological tumor cells promoting NK cell cytotoxicity (7, 42–44).

POST-TRANSLATIONAL MECHANISMS MODULATING MEMBRANE EXPRESSION OF NK CELL ACTIVATING LIGANDS ON TUMOR CELLS

During malignant transformation different stressful stimuli are responsible for the induction of NK cell activating ligands at transcriptional and post-transcriptional levels and the molecular mechanisms implicated have been partially identified (18, 45). Moreover, increasing evidence demonstrate that post-translational mechanisms including the release of ligands for NK cell activating receptors as soluble forms as well as ligand modification by the Ubiquitin (Ub) or Ub-like pathways are used by tumor cells to dampen activating ligand surface expression in order to evade NK cell recognition (**Figure 1A**).

Mechanisms Implicated in the Release of Ligands for NK Cell Activating Receptors

Most of the information regarding soluble ligands in cancer patients comes from studies performed on NKG2DLs. These molecules are present in the sera of patients affected by hematological or solid malignancies, and their level correlate with tumor stage and poor prognosis (46–53). More recently, B7-H6, BAG6, and PVR soluble forms have been found in the sera of patients affected by different type of tumors suggesting a relationship between soluble ligand expression and cancer progression (54–58).

Generation of soluble ligands relies on different mechanisms including alternative splicing, exosome secretion and proteolytic

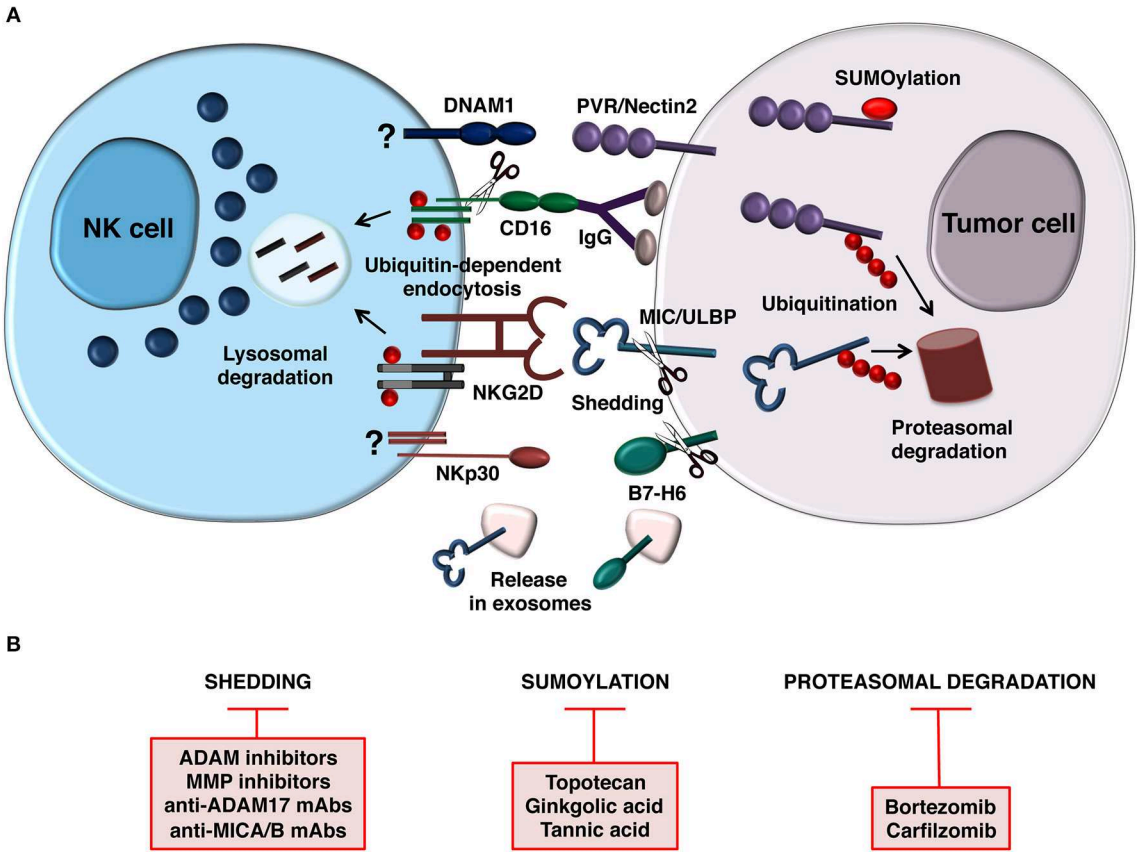

FIGURE 1 | Post-translational mechanisms controlling NK cell-mediated recognition of tumor cells. **(A)** Model depicting how NCR (B7-H6), NKG2D, and DNAM1 ligand expression is prevented by post-translational mechanisms on target cell surface (right). Release of B7-H6 and NKG2D ligands on exosomes is also depicted. In addition to regulate ligand expression on tumor cell, ubiquitin modification also provides a signal for internalization and trafficking of NKG2D and CD16 on NK cells (left). CD16 is also downmodulated by metalloproteinase-mediated shedding. Mechanisms regulating DNAM-1 and NCR (NKp30) downmodulation are currently unknown. **(B)** Therapeutic strategies aimed to prevent post-translational mechanisms affecting activating ligand and receptor expression.

cleavage. Soluble PVR isoforms are generated by alternative splicing (59) and have an inhibitory effect on DNAM-1 mediated tumor immunity (54). In addition, alternative splicing gives rise to ULBP-4/5 secreted ligands that can impair NK cell target recognition *in vitro* (60, 61).

Exosomes represents nanovesicles derived from the endosomal compartment (62) and have been involved in the secretion of NKG2D and NKp30 ligands but not of DNAM-1 ligands (63). Differently from the proteolytic-mediated release, expression of activating ligands on the exosome surface should retain their biological activity by keeping the integral-molecule. A number of studies have shown that NKG2DLs from both MIC and ULBP families are expressed on the surface of exosome-like vesicles released from ovarian cancer (63), melanoma (64), and prostate cancer cells (65). Remarkably, NKG2DLs such as ULBP3 and ULBP1 (66) or the allelic variant MICA*008 (67, 68) that are glycosylphosphatidylinositol (GPI)-anchored proteins, are preferentially released via exosomes.

In regard to NKp30Ls, the nuclear protein BAG6 is secreted on exosomes and stimulates NK cell activity (69), whereas the cell surface ligand B7-H6 can be released in its soluble form associated to exosomes or through protease-mediated cleavage (57, 70, 71). Although several stress conditions can increase exosome secretion from cancer cells (72–75), it is still uncertain whether the release of NKG2DLs or B7-H6 through exosome-like vesicles could result in the diminution of their expression on the cell surface.

Concerning the shedding process, MICA, MICB, and ULBP2 are cut by metalloproteinases belonging to two distinct families, the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) (76–81), whereas the B7-H6 proteolytic cleavage occurs through a mechanism mainly dependent on ADAM enzymes (57). A recent study has shown that some ULBP4 isoforms are sensitive to the protease cleavage (82). Both MMPs and ADAMs proteases undergo modulation of their activity and expression in the course of neoplastic transformation (83, 84) and in response to cancer therapy (85–88). Disparate sensitivity to the proteases has been described for distinct NKG2DLs and/or allelic variants and isoforms. For instance, the generation of soluble MICA can be affected by

polymorphisms as shown for the MICA*008 allele that is resistant to the protease-mediated cleavage. Moreover, the MICA-129 dimorphism, producing a valine to methionine swap at position 129, influenced the MICA cleavage process but the mechanism behind has to be defined (89, 90). In addition, proteolytic cleavage can be affected by fatty acylation and palmytolation that mediate MICA/B recruitment to membrane microdomains (78, 91).

Differently from the exosome-mediated release, the proteolytic cleavage of NKG2DLs and B7H6 has been associated to a reduction of cell surface ligands, thus its inhibition could be accomplished as a promising approach to keep the ligands on cancer cell surface and to promote anti-cancer immune response.

Activating Ligand Modification by Ub and Ub-Like Pathways

Recent evidences reveal a role for ubiquitination and SUMOylation in the regulation of NK cell ligand expression on tumor cells.

Ubiquitination and SUMOylation are reversible modifications whereby Ub and small Ub-like modifier (SUMO), respectively, are covalently bound to a target protein through the action of enzymes frequently up-regulated during malignant transformation (92–95).

Once modified, proteins undergo different fate depending on the type of modification.

Proteins modified by poli-Ub chains are generally targeted to proteasomal degradation (95) whereas the addition of single Ub molecules to one or more lysine residues promote non-degradative fates including regulation of membrane protein endocytosis (96). SUMOylated substrates undergo conformational changes that in turn modify their interaction with other proteins or their enzymatic activity without inducing a degradative fate (94).

Little is currently known about the role of these modifications in the regulation of NK cell ligand expression during malignant transformation.

Ubiquitination of MICA/B has been demonstrated in Kaposi's sarcoma-associated herpesvirus infected cells: the viral E3 Ub ligase K5 induces modification of both NKG2DLs and their intracellular retention (97). Moreover, in healthy cells the murine ULBP-1 ortholog MULT-1 undergoes constitutive ubiquitination and lysosomal degradation (98, 99). Interestingly, stress conditions including UV radiation and heat shock prevent MULT-1 ubiquitination and increase its surface expression (98). Thus, these results support a negative role for the Ub pathway in the regulation of NKG2DL expression.

In tumor cells a direct implication of the Ub pathway has not been formally reported but several data demonstrate that surface expression of human NKG2DLs is regulated by a rapid protein turnover. In melanoma cells, an immature form of MICA accumulates in the endoplasmic reticulum and is targeted to degradation (100). MICB is internalized and retained intracellularly in several tumor cell lines (101), while in Multiple Myeloma (MM) cells the constitutive internalization of MICB is followed by its lysosomal degradation (102). Similarly, the GPI-linked ligand ULBP1 is continuously removed from plasma membrane and targeted to proteasomal degradation (103). Regarding DNAM-1 ligands, in hepatocellular carcinoma the activation of Unfolded Protein Response (UPR) inhibits PVR surface expression and promotes protein degradation (104). In line with this result, ubiquitination and SUMOylation negatively regulates surface expression of Nectin2 and PVR on tumor cells (105, 106). Ubiquitinated Nectin2 is retained in intracellular compartments but also targeted to proteasomal degradation (106) whereas SUMOylation of PVR promotes its intracellular retention without inducing protein degradation (105). Inhibition of Ub and SUMO pathways increases Nectin2 and PVR surface expression and renders tumor cells more sensitive to NK cell-mediated killing.

Although these findings are currently limited to NKG2D and DNAM-1 ligands, they provide novel insights into the mechanisms underlying activating ligand expression in diseased cells and reveal novel potential targets for therapeutic intervention.

LIGAND INDUCED DOWN-MODULATION OF NK CELL ACTIVATING RECEPTOR EXPRESSION

Tumor progression also implies the inability of NK cells to kill tumor cells as consequence of ligand mediated down-regulation of activating receptors (**Figure 1A**).

However, receptor down-regulation may be affected by the presence of soluble or membrane-bound ligands as well as by their affinity and/or avidity (19).

A decreased in NCR expression levels was observed in NK cells derived from patients affected by myeloid leukemia and other tumors upon the interaction with their respective ligands (8, 107).

Reduced NKp30 surface expression has been also detected on NK cells derived from ovarian carcinoma and neuroblastoma patients as a result of chronic stimulation either with B7-H6-expressing tumor cells or soluble B7-H6 (55, 56, 108). Moreover, the presence of soluble BAG6 has been associated with a low transcriptional levels of different NKp30 isoforms (58, 109).

DNAM-1 engagement, by membrane-bound ligands but not their soluble counterpart, is also followed by receptor down-modulation and impairment of NK cell functions in patients affected by different tumors including MM, ovarian carcinoma and AML (44, 110, 111). However, the mechanisms underlying these effects are still undefined.

For other activating receptors including NKG2D and CD16, mechanisms of ligand-induced down-modulation have been elucidated.

NKG2D stimulation by ligands expressed on tumor cells as well as by soluble ligands promotes receptor endocytosis and the decrease of NKG2D-dependent functions (46, 76, 112–115). In regard to released ligands, those associated to exosomes show a higher avidity and a more efficient ability to induce receptor down-regulation compared to shed ligands (66, 67).

In humans, internalization of ligand-engaged NKG2D receptors requires DAP10 ubiquitination and is followed by

lysosomal degradation (116). However, MICA is more efficient than ULBP2 in promoting receptor ubiquitination (114).

Ub modification has been also implicated in the down-modulation of CD16 in response to antibody-coated tumor cells (117–119). Indeed, CD16 clearance from NK cell surface is mainly induced by Ub-dependent endocytosis of aggregated receptors followed by degradation of CD16ζ subunit and the associated kinases (114, 117, 120). However, CD16 down-regulation can also occur as a consequence of metalloproteinase-induced receptor shedding (121–124). Regardless, NK cell-mediated ADCC, natural cytotoxicity, and the efficacy of antibody-based therapies resulted impaired (118, 119, 125).

Altogether these results demonstrate that activating receptor expression is modulated in tumor microenvironment by the interaction with ligand-expressing cells, thus impairing NK cell ability to counteract tumor development.

TARGETING POST-TRANSLATIONAL MECHANISMS REGULATING NK CELL-MEDIATED RECOGNITION AND KILLING OF CANCER CELLS

All these post-translational mechanisms represent potential targets for therapeutic intervention (**Figure 1B**). Ligand shedding blocking can be achieved by the usage of inhibitors of MMPs and ADAMs enzymes (57, 77, 126). Since ADAM10 and ADAM17 sheddases play a prominent role in B7-H6 (57) and NKG2DL cleavage (77, 78, 80, 127), the selective targeting of such enzymes might be promising for anticancer therapy. Recently, by performing an *in vitro* drug screen using an FDA-approved drug library, lomofungin was found to strongly decrease ADAM17 activity in hepatocellular carcinoma leading to the impairment of MICA shedding and has been proposed as new drug candidate for immunotherapy in liver cancer (128). Most of the compounds able to inhibit ADAM catalytic activity are hydroxamate-based and are either selective for ADAM17 or inhibitors of both ADAM10 and ADAM17 (129). Of interest, the synthesis of new selective ADAM10 inhibitors able to impair NKG2DL shedding in Hodgkin's lymphoma cell models has been reported (130). ADAM10 and ADAM17 are expressed at high levels on the surface of glioblastoma-initiating cells thus contributing to an immunosuppressive phenotype through the ULBP2 cleavage. Specific inhibition of these enzymes preserved cell surface ULBP2 leading to increased glioblastoma cell recognition and killing by NK cells (131). Remarkably, *in vivo* experiments using athymic nu-/nu- mice implanted with subcutaneous HeLa tumors demonstrated that systemic MMPi treatment resulted in the reduction of MICA serum levels and a concomitant augmentation of MICA expression on cancer cells reinforcing the immune cell therapy mediated by cytokine-induced killer cells (132). Of interest, adoptively transferred NK cells displaying high levels of surface NKG2D determined the clearance of soluble MICA in neuroblastoma patients by preserving NK cell cytotoxicity via non-occupied NKG2D (133).

Another appealing strategy to specifically inhibit MICA/B proteolytic cleavage concerns the generation of antibodies targeting the MIC protein domain involved in the proteolytic cleavage (134). Interestingly the usage of these antibodies limited MICA/B shedding in human cancer cells and repressed cancer cell growth in *in vivo* models (134). More recently, the glycosylation-engineered epitope mapping (GEM) method allowed to the identification of a number of epitopes relevant for MICA/B shedding inhibition (135).

In general, such antibodies as well as metalloproteinase inhibitors could be used in combination with other therapies aimed at the enhancement of ligand expression on the surface of cancer cells including DNA damaging agents (127), radiations (87), and chemotherapeutic drugs (132). Our group has shown that the combined use of metalloproteinase inhibitors and genotoxic drugs enhanced NK cell-mediated killing of multiple myeloma cells by preserving MIC molecules on the cell surface (127). To date, ADAMs inhibitors have been largely unsuccessful in clinical trials, but they remain a viable and desirable therapeutic target based on preclinical studies.

Strategies aimed at inhibiting ADAM17-mediated CD16 cleavage from the surface of NK cells could be also promising. Beyond the usage of inhibitors, recent advances in generating function-blocking antibodies of ADAM17 are emerging. The monoclonal antibody MEDI3622 has been shown to block CD16A cleavage from activated human NK cells allowing to an increased IFNγ production in the course of ADCC (136).

Proteasome inhibitors can change the fate of ubiquitinated ligands. Bortezomib (Velcade) and Carfilzomib (Kyprolis) have been already used as chemotherapeutic drugs for relapsed MM patients (137–139) and for the treatment of mantle cell lymphoma (140).

In line with our findings (106), previous reports demonstrated that low doses of bortezomib increase NK cell activating ligand, including Nectin2 (141–143). Whether those drugs can directly affect ligand expression stabilizing ubiquitinated Nectin2 and/or SUMOylated PVR is currently unknown.

Regarding the SUMO pathway, the FDA-approved drug Topotecan has been shown to affect SUMOylation in glioblastoma multiforme (144). Moreover, natural compounds including ginkgolic acid and tannic acid (145, 146) have been found to possess anti-cancer activities by targeting the SUMO pathway (147).

All of these compounds hold great promise to be developed into novel and efficient anti-cancer drugs.

CONCLUSION AND THERAPEUTIC PERSPECTIVES

On tumor cells, several activating ligands are subjected to protease-mediated cleavage with a consequent dramatic reduction of their surface expression. A similar effect is also achieved upon ubiquitination or SUMOylation of NKG2D and DNAM-1 ligands, which are retained intracellularly and/or degraded.

On NK cells, the Ub pathway may also contribute to down-regulate the surface expression of activating receptors.

In conclusion, all these post-translational mechanisms act to reduce NK cell-mediated surveillance against tumors and represent potential targets for therapeutic intervention.

Several inhibitors have been developed and their use in combination with conventional therapies represent a useful tool to potentiate NK-cell mediated recognition and killing of tumor cells.

AUTHOR CONTRIBUTIONS

RM, AZ, AS, and RP participated in the conception, writing, and elaboration of the final version of the manuscript.

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The Synergistic Use of IL-15 and IL-21 for the Generation of NK Cells From CD3/CD19-Depleted Grafts Improves Their *ex vivo* Expansion and Cytotoxic Potential Against Neuroblastoma: Perspective for Optimized Immunotherapy Post Haploidentical Stem Cell Transplantation

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Neuroblastoma (NB) is the most common solid extracranial tumor in childhood. Despite therapeutic progress, prognosis in high-risk NB is poor and innovative therapies are urgently needed. Therefore, we addressed the potential cytotoxic capacity of interleukin (IL)-activated natural killer (NK) cells compared to cytokine-induced killer (CIK) cells for the treatment of NB. NK cells were isolated from peripheral blood mononuclear cells (PBMCs) by indirect CD56-enrichment or CD3/CD19-depletion and expanded with different cytokine combinations, such as IL-2, IL-15, and/or IL-21 under feeder-cell free conditions. CIK cells were generated from PBMCs by *ex vivo* stimulation with interferon-γ, IL-2, OKT-3, and IL-15. Comparative analysis of expansion rate, purity, phenotype and cytotoxicity was performed. CD56-enriched NK cells showed a median expansion rate of 4.3-fold with up to 99% NK cell content. The cell product after CD3/CD19-depletion consisted of a median 43.5% NK cells that expanded significantly faster reaching also 99% of NK cell purity. After 10–12 days of expansion, both NK cell preparations showed a significantly higher median cytotoxic capacity against NB cells relative to CIK cells. Remarkably, these NK cells were also capable of efficiently killing NB spheroidal 3D culture in long-term cytotoxicity assays. Further optimization using a novel NK cell culture medium and a prolonged culturing procedure after CD3/CD19-depletion for up to 15 days enhanced the expansion rate up to 24.4-fold by maintaining the cytotoxic potential.

Addition of an IL-21 boost prior to harvesting significantly increased the cytotoxicity. The final cell product consisted for the major part of $CD16^-$, NCR-expressing, polyfunctional NK cells with regard to cytokine production, CD107a degranulation and antitumor capacity. In summary, our study revealed that NK cells have a significantly higher cytotoxic potential to combat NB than CIK cell products, especially following the synergistic use of IL-15 and IL-21 for NK cell activation. Therefore, the use of IL-15+IL-21 expanded NK cells generated from CD3/CD19-depleted apheresis products seems to be highly promising as an immunotherapy in combination with haploidentical stem cell transplantation (SCT) for high-risk NB patients.

Keywords: immunotherapy, NK cells, CD3/CD19 depletion, CIK cells, IL-21, IL-15, *ex vivo* expansion, neuroblastoma

INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and causes 15% of cancer-related mortality in children. The majority of cases are diagnosed before the age of 5 years, and 30% of cases are diagnosed within the first year of life. Around half of the patients are currently classified as high-risk for disease relapse, with a 5-year event-free survival (EFS) of <40% despite intensive multimodal therapy (1–3). Current therapeutic approaches for high-risk NB include surgery, radiotherapy [iodine (I-131) Metaiodobenzylguanidine (MIBG) therapy or external beam radiation] and myeloablative chemotherapy, followed by autologous stem cell rescue. Furthermore, immunotherapies using monoclonal antibodies against NB cell membrane antigens such as anti-GD2 (e.g., Dinutuximab ch14.18/SP2/0; Dinutuximab-beta ch14.18/CHO) have gained increasing clinical significance (4, 5).

In addition, for children with relapsed or refractory high-risk NB, hematopoietic stem cell transplantation (SCT) has been shown to be a feasible and promising treatment that can induce long-term remission in some patients with tolerable side effects (6–8). In this context, haploidentical SCT from mismatched family donors is an important therapeutic option for patients lacking a human leukocyte antigen (HLA)-matched donor. The removal of potentially alloreactive T cells from the graft by CD3/CD19-depletion allows HLA barriers to be overcome and reduces the induction of harmful graft-versus-host-disease (GvHD). While the risk of EBV post-transplant lymphoproliferative disease (PTLD) is reduced by depletion of $CD19^+$ B cells, CD3/CD19-depleted grafts also seem to facilitate engraftment and to support the graft-versus-leukemia/tumor (GvL/T) effect (9).

The beneficial GvL/T effect is predominantly mediated by NK cells that are key players of the innate immune system and potent effector cells participating in the defense of viral-infected and malignant cells (10). NK cells compromise ∼6–12% of lymphocytes in the peripheral blood (PB) and are characterized by surface expression of CD56 and the lack of T cell antigens such as CD3 or T cell receptors ($CD56^+CD3^-$). Human NK cell subsets in the PB can further be subdivided into a major $CD56^{dim}CD16^+$ highly cytotoxic population, expressing the Fcγ receptor III CD16 and therefore exerting antibody-dependent cell-mediated cytotoxicity (ADCC), and a minor $CD56^{bright}CD16^-$ immune regulatory population with a potent cytokine producing capacity (11).

NK cell cytotoxicity is mediated by a balanced system processing signals from activating and inhibitory receptors (12, 13). Activating receptors, such as natural cytotoxicity receptors (NCRs) and the NK group 2D (NKG2D) receptor can be triggered by an enhanced surface expression of stress-induced ligands on abnormal cells (14, 15). Inhibitory signals are mainly mediated by killer immunoglobulin-like receptors (KIR) that recognize major histocompatibility complex (MHC) class I molecules, which are highly present on endogenous healthy cells. Most transformed and virally infected cells down-regulate their MHC class I surface expression ("missing-self") to evade cytotoxic T cell recognition, which renders them sensitive to NK cell killing (16, 17).

Based on the donor cell mediated GvL/T effect, adoptive post-transplantation immunotherapeutic strategies using donor-derived immune cells have been established. To further increase the anti-tumor efficacy, donor cells can be *ex vivo* expanded and stimulated by different cytokines, generating highly activated NK or cytokine-induced killer (CIK) cell products. A synergistic effect of haploidentical SCT and cell-based immunotherapy seems probable, but few clinical results from the treatment of high-risk NB have yet been published (18–20).

Manufacturing sufficient effector cell doses for repetitive cell applications to overcome suppressive tumor escape mechanisms still remains a challenge. Therefore, we analyzed the expansion capacity as well as the cytotoxic potential upon different cytokines of either CD56-enriched or CD3/CD19-depleted NK cells compared to CIK cells against human NB cell lines *in vitro*.

MATERIALS AND METHODS

Generation of NK Cells and CIK Cells

NK cells and CIK cells were generated from PBMCs from buffy coats of healthy donors by density gradient centrifugation. In experiments directly comparing NK and CIK cell proliferation and cytotoxicity (**Figures 1**, **2**) the NK cells and CIK cells were

generated from PBMCs isolated from buffy coats of the same donor. Donors gave their written informed consent and the use of the buffy coats was approved by the medical ethics committee of the University Hospital Frankfurt (approval no. 329/10). CIK cells were generated and cultured as described previously (21). In short, PBMCs were adjusted to a concentration of 3 × 10^6 cells/ml in X-VIVO^{TM}10 medium (Lonza, Verviers, Belgium) supplemented with 5% heat-inactivated human fresh frozen plasma (FFP) (DRK-Blutspendedienst Baden-Württemberg-Hessen, Frankfurt/Main, Deutschland) and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher, Waltham, MA, USA) in tissue culture-treated plates (Thermo Fisher, Waltham, MA, USA; Sarstedet, Nümbrecht, Germany; Corning NY, USA). On day 0 they received 1,000 U/ml IFN-γ (Imukin®, Boeringer Ingelheim Pharma, Germany). On day 1, 100 ng/ml OKT- (anti-CD3 antibody, MACS GMP CD3 pure, Miltenyi Biotec, Bergisch Gladbach, Germany) and 500 U/ml IL-2 (ProleukinS, Novartis Pharmaceuticals, Horsham, UK) were added. On days 3–4 and 7–8 the cell density was set to a concentration of 1 × 10^6 cells/ml and cells were treated with 50 ng/ml IL-15 (Peprotech, Rocky Hill, CT, USA). CIK cells were harvested on days 10–12 (**Figure 1A**).

NK cells were isolated from PBMCs using either the EasySep® Human NK cell enrichment kit or EasySep® Human CD3 and CD19 positive selection kits (STEMCELL Technology, Vancouver, Canada). Cells were adjusted to a concentration of 3 × 10^6 cells/ml in either X-VIVO^{TM}10 medium or NK MACS® medium supplemented with 1% NK MACS supplements (Miltenyi Biotec, Bergisch Gladbach, Germany) and 5% human plasma (HP) type AB and 1% penicillin/streptomycin in 48-well cell culture plates (Thermo Fisher Scientific, Roskilde, Denmark). Analogous to CIK cells, NK cell density was adjusted to 1 × 10^6 cells/ml on subsequent culture days. In detail, we suspended, harvested and counted cells, took out needed cells, diluted to 1 × 10^6/ml with fresh medium and added cytokine in the corresponding concentration. To optimize *ex vivo* cultivation of NK cells, different stimulation protocols were tested. In the IL-15^{low} protocol, NK cells were stimulated with 10 ng/ml IL-15 every 3–4 days for a period of either 10–12 or 15 days (**Figures 1A**, **3A**, **5A**). In the IL-2+IL-15^{low} protocol NK cells received 500 U/ml IL-2 on day 0 and 10 ng/ml IL-15 every 3–4 days until days 10–12 (**Figure 1A**). For the IL-15^{low}+IL-21 protocol NK cells were cultivated with 10 ng/ml IL-15 every 3–4 days including day 11. On day 13, 25 ng/ml IL-21 (Peprotech Rocky Hill, CT, USA) was added, and cells were harvested on day 15 (**Figures 3A**, **5A**). In the IL-15^{gap}+IL-21 protocol, cells were cultured for 11 days with addition of 10 ng/ml IL-15 every 3–4 days and then collected and centrifuged. The supernatant was removed in order to abolish traces of cytokines. On day 13, 10 ng/ml IL-15 and 25 ng/ml IL-21 were added (**Figure 3A**) (22, 23).

NB Cell Lines

The human NB cell lines SK-N-SH (ATCC® HTB-11™) and SK-N-AS (ATCC® CRL-2137™) were cultured in IMDM medium (Gibco, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, Thermo Fisher, Waltham, MA, USA) at 37°C and 5% CO_2. The cells were split once or twice a week using Accutase solution (Sigma-Aldrich, St. Louis, MO, USA) to detach adherent cells from the cell culture flask. MHC class I is expressed to a low extent on SK-N-SH and at higher levels on SK-N-AS cells (24, 25).

Flow-Cytometric Analysis

Flow cytometric analysis was performed on a FACS Canto 10c and a FACS Celesta instrument (both BD Biosciences, San Jose, CA, USA). NK cells and CIK cells were analyzed for the expression of various surface molecules on day 0 and the day of harvest. Flow cytometric phenotyping was performed for the most promising protocols on the day of harvest. The following monoclonal antibodies were used:

AF488: NKp30 (#210845), NKG2C (#134591) (both R&D Systems Wiesbaden, Germany), APC: CD57 (NK-1, Biolegend, San Diego, CA, USA), CD178/FASL (NOK-1), NKG2D (ON72, Beckman Coulter, Krefeld, Germany), CD107a (H4A3, BioLegend), APC-A700: CD16 (3G8, Biolegend), CD56 (N901, Beckman Coulter), BB515: CD19 (HIB19), BUV395: CD3 (SK7), BUV737: CD20 (2H7), BV421: CD56 (NCAM16.2), Zombie Violet (BioLegend), BV510: CD45 (HI30), BV605: CD25 (2A3), CD69 (FN50), BV711: CD14 (M5E2, Biolegend), FITC: CD3 (UCHT1, Beckman Coulter), CD226 (DX11), KIR2D (NKVFS1, Miltenyi Biotec, Bergisch Gladbach, Germany), IFNy (B27), PC7: CD19 (HIB19, Biolegend), CD184/CXCR4 (12G5), PE: CD16 (3G8, Biolegend), NKG2A (Z199, Beckman Coulter), NKp44 (P44-8.1), NKp46 (9E2, Biolegend), PD1 (PD1.3.1.3, Miltenyi), TRAIL (RIK-2, Biolegend), CD56 (NCAM16.2), PE Vio770: 2B4 (REA112, Miltenyi), PerCP: CD3 (UCHT1, Biolegend), CD14 (TÜK4, Miltenyi), CD19 (HIB19, Biolegend), CD16 (3G8, BioLegend), V450: CD3 (UCHT1), CD14 (MøP9), CD19 (HIB19) All antibodies were purchased from BD Biosciences if not otherwise specified.

Depending on the panel either strongly diluted DAPI (Biolegend, San Diego, CA, USA) (experiments in **Figures 1**, **2**) or 7-Actinomycin D (7-AAD) (BD Biosciences San Jose, CA, USA) (experiments in **Figures 3–6**) was used for the assessment of cell viability. Absolute lymphocyte subset counts were calculated via a dual platform using the flow cytometric data and cell counts acquired via a COULTER Ac.T diff Analyzer (Beckman Coulter, Krefeld, Germany). Flow-cytometric data was analyzed using FlowJo-software (Tree Star Inc., Ashland, OR, USA).

Europium Release Assay

To test the cytotoxicity of CIK and NK cells against the human NB cell lines SK-N-SH and SK-N-AS, the non-radioactive europium release assay was used as described previously (26) and modified for adherent cell lines (27). In short, human NB cell lines (target cells) were labeled with BATDA (Perkin Elmer, Boston, MA, USA) under constant shaking (400 rpm) at 37°C, 5% CO_2 to prevent cell adhesion. Cells were washed in medium supplemented with Probenecid (Santa Cruz, Dallas, Texas, USA), an inhibitor of multidrug-resistance associated proteins, to avoid spontaneous release of TDA into the supernatant. Target cells

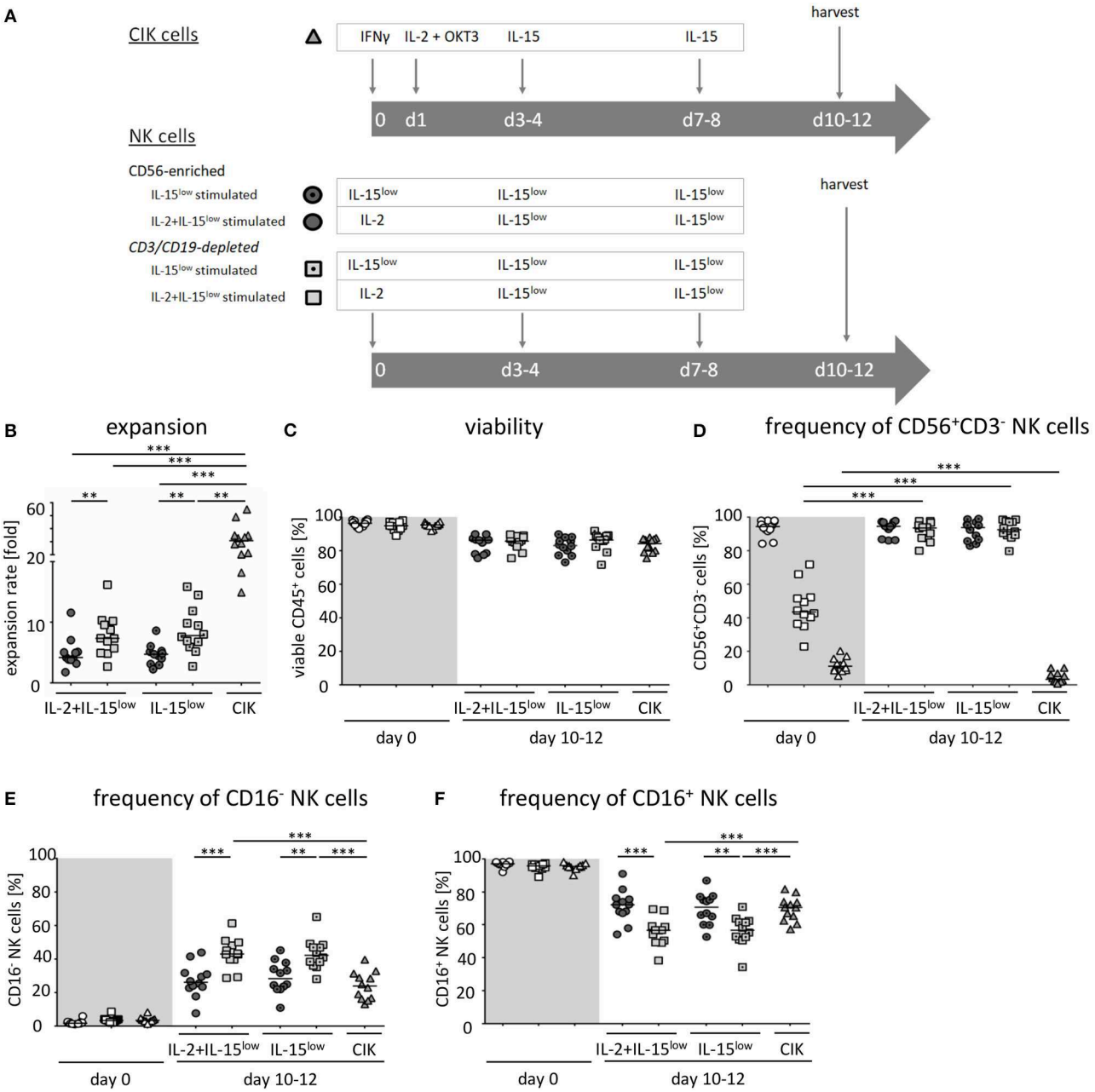

FIGURE 1 | *Ex vivo* expansion and characterization of CIK and NK cells. **(A)** CIK cells were expanded by *ex vivo* stimulation with IFNγ, IL-2, OKT3, and IL-15 over a period of 10–12 days (△). NK cells were purified by either CD56-enrichment (●●) or CD3/CD19-depletion (⊡□) and *ex vivo* stimulated with IL-2+IL-15 (●□) or solely IL-15 (●⊡) for 10–12 days. **(B)** CD56-enriched NK cells expanded 4.3-fold independent of the use of IL-15 alone or in combination with IL-2 (●●), NK cells in CD3/CD19-depleted cell products expanded significantly higher under both cytokine stimulation conditions (median 7.5-fold) (⊡□). CIK cells showed highest expansion rate of 30.8-fold, referring to the total cell composite of NK, T and NK-like T cells (△) **(C)** All three cell products showed a high viability of >95% following purification procedure on day 0 (○□△) and remained at >85% during expansion procedure independent of the cytokine additive. **(D)** CD56-enriched cells on day 0 contained a median 94.5% NK cells, CD3/CD19-depleted cells products contained a median 43.5% NK cells, and CIK cells a median 11.5%. Upon cytokine stimulation for 10–12 days, NK cell purity remained high (94.6 and 93.8%) in CD56-enriched, and significantly increased in CD3/CD19-depleted cell products (93.6 and 92.5%) under IL-2+IL-15 or IL-15 stimulation, respectively. In contrast, the frequency of NK cells decreased to only 3.3% during CIK cell expansion. **(E)** The frequency of the $CD16^-$ NK cell subpopulation significantly increased during IL-2+IL-15 or IL-15 cytokine stimulation, especially within CD3/CD19-depleted NK cell products (CD56-enriched 26.1 and 28.3%; CD3/CD19-depleted 43.0 and 42.3%), while the $CD16^+$ NK cell subpopulation correspondingly decreased **(F)** ($n = 12$ independent experiments, median fold expansion rate day 10–12 compared to day 0, gated on viable cells (DAPI negative): $CD56^+CD3^-$NK cells **(B)**, $CD45^+$ cells **(C)**, $CD56^+CD3^-$NK cells **(D)**, $CD16^-$ NK cells **(E)**, $CD16^+$ NK cells **(F)**. Differences were considered significant for $p < 0.01$ (**) and $p < 0.001$ (***).

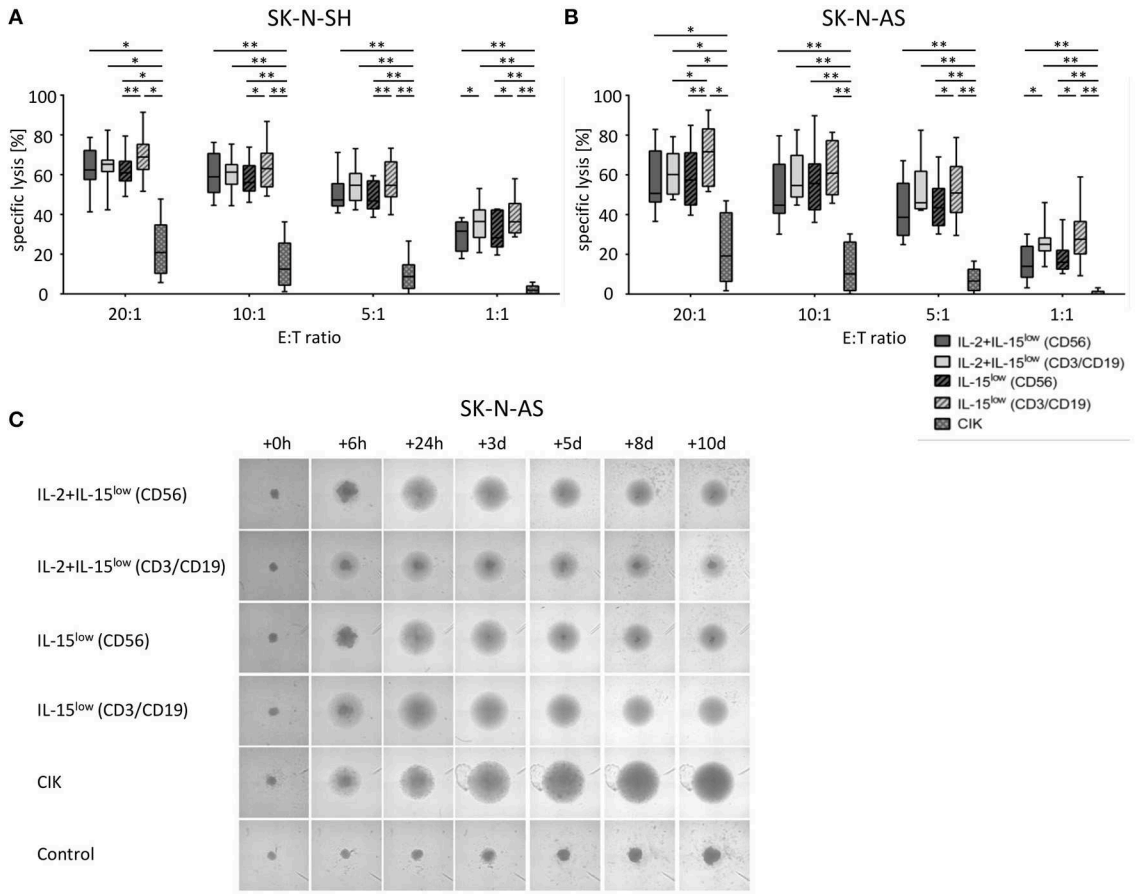

FIGURE 2 | Cytotoxic potential and long-term cytotoxicity of CIK and NK cells against NB target cells. Specific lysis of the NB cell lines SK-N-SH **(A)** and SK-N-AS **(B)** was evaluated by Europium release assay. Both NB cell lines were efficiently lysed by either CD56-enriched (▬▬) or CD3/CD19-depleted (▭▭) NK cells; while CD3/CD19-depleted NK cells (▭) killed significantly better relative to CD56-selected (▬) NK cells at all E:T ratios. In addition, there was an upward tendency for IL-15 to be superior to IL-2+IL-15 treatment, which was statistically relevant for CD3/CD19-depleted NK cells vs. SK-N-AS cells at the 20:1 E:T ratio, only. CIK cell-mediated cytotoxicity was significantly lower with a median cell lysis of 12.5% for SK-N-SH and 10.1% for SK-N-AS cells (all E:T ratio 10:1). Effector to target (E:T) ratios 20:1, 10:1, 5:1 and 1:1, $n = 8$–9 independent results, experiments performed in triplicate, incubation time: 3 h, box-and-whisker plots show median, 25th−75th percentiles, Min-Max. **(C)** Tumor spheroids were produced from 10,000 SK-N-AS cells and co-incubated with 200,000 CIK or NK cells. As a control the dynamics of tumor spheroids without effector cells were observed. The cultures were imaged via a Celigo cell cytometer after 6 h, 24 h, 3, 5, 8 and up to 10 days. All NK cells sustained tumor growth but only IL-15 stimulated NK cells generated from CD3/CD19 depleted PBMCs were able to completely eradicate tumor cells. Also CIK cells infiltrated and lysed NB tumor spheroids ($n = 1$ representative of 3 independent experiments). Differences were considered significant for $p < 0.05$ (*), $p < 0.01$ (**).

were co-cultured with NK cells or CIK cells (effector cells) for 3 h in triplicates at effector-to-target (E:T) ratios of 20:1, 10:1, 5:1, 1:1, and 0.5:1 at 37°C. Target cells were also cultured in medium without effector cells to determine spontaneous cell lysis. The maximum lysis was obtained by adding 16% Triton-X solution (AppliChem, Darmstadt, Germany) to target cells. After co-incubation, 20 µl of supernatant was taken from each well and incubated for 15 min in the dark and under shaking (250 rpm) with 200 µl of europium solution (Perkin Elmer, Boston, MA, USA). The fluorescence signal, which directly correlates to tumor cell lysis, was measured by a multilabel plate reader (Victor 1420 multilabel counter, Perkin Elmer, Boston, MA, USA). Percentage of lysis was calculated as follows: specific lysis = [(lysis – spontaneous lysis)/(maximal lysis – spontaneous lysis)]*100.

Co-culture in the Presence of Tumor Spheroids

Three-dimensional (3D) tumor spheroids were obtained by placing 10,000 SK-N-AS cells into one well each of an ultra-low attachment (ULA) 96-well round-bottom plate without additional coating (Corning Incorporated, Corning NY, USA). Culture plates were centrifuged at 1,000 g for 10 min. Every 3 to 4 days, half of the medium was replaced and on day 4 200,000 CIK or NK cells were added per well. The dynamics of the tumor spheroid were automatically imaged using a Celigo cell cytometer (Nexcelom Bioscience, Lawrence, MA, USA) with the colony counting embryoid body application at time points of 0, 6, 24 h and subsequently every 24 h for a total of 10 days (28, 29).

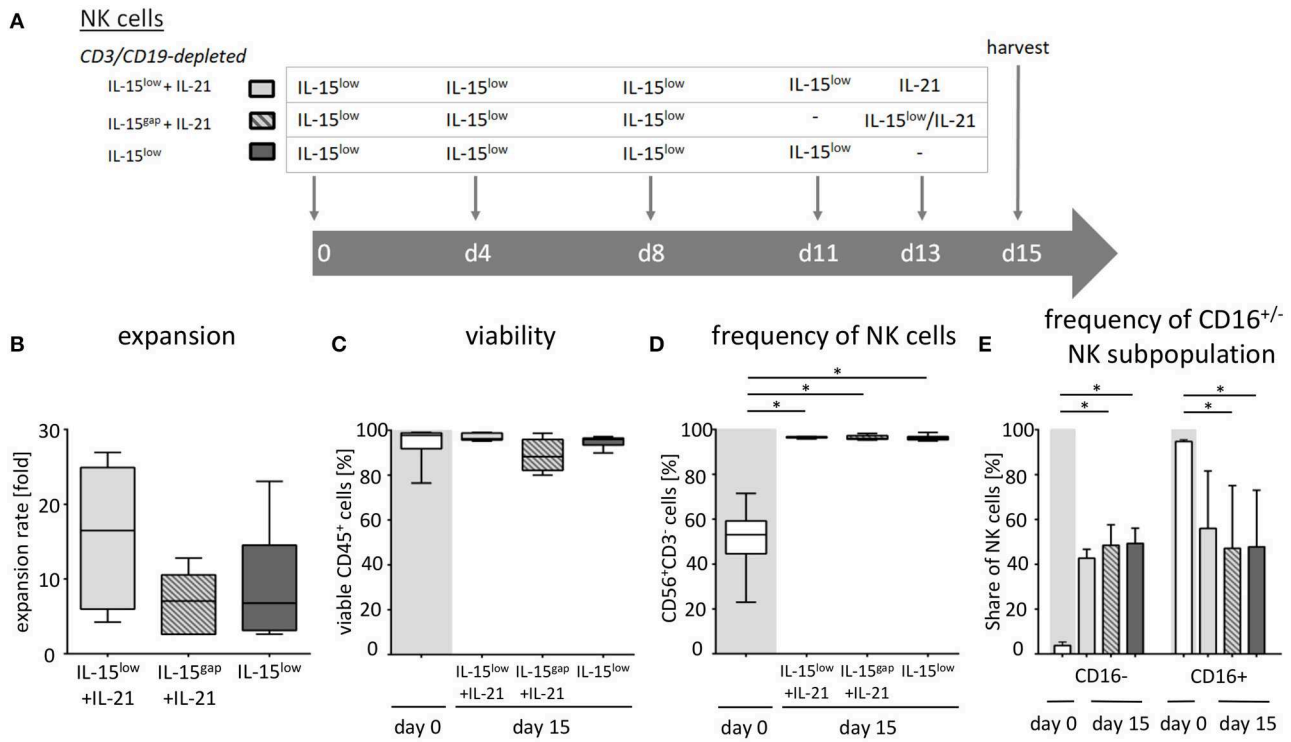

FIGURE 3 | *Ex vivo* expansion and characterization of IL-15 and IL-15+IL-21 stimulated NK cells following CD3/CD19-depletion. **(A)** NK cells were purified by CD3/CD19-depletion and *ex vivo* stimulated with IL-15 (■) or with a combination of IL-15 and IL-21 (■■) for 15 days. On day 11, cells were either treated with IL-15 (IL-15^{low}■■) or the supernatant was removed and no cytokines were added (IL-15^{gap}■). IL-21 was added as a cytokine boost 48 h prior to harvest. **(B)** NK cells in the IL-15^{low} protocol (■) expanded 6.8-fold. An IL-21 boost was able to further enhance proliferation, irrespective of gap or continuous treatment. Expansion rates reached 7.1-fold in the IL-15^{gap}+ IL-21 protocol (■) and 16.5-fold in the IL-15^{low}+ IL-21 protocol (■) (statistically not significant differences). **(C)** All cell products showed a high viability of median 97.5% following the purification procedure on day 0 (white symbols gray background) and remained >80% during the expansion procedure independent of the cytokine additive. However, the gap treatment led to the lowest viability (■). **(D)** Purified CD3/CD19-depleted cells on day 0 contained a median 53.1% NK cells. Upon cytokine stimulation for 15 days, NK cell purity significantly increased in CD3/CD19-depleted cell products regardless of the cytokine combination. **(E)** The frequency of the CD16$^-$ NK cell subpopulation significantly increased during *ex vivo* stimulation within all protocols ($n = 5$–6 independent experiments, **(B)** median fold expansion rate on day 15 compared to day 0, **(C)** gated on viable 7-AAD$^-$ CD45$^+$ cells, **(D)** CD56$^+$CD3$^-$ NK cells, **(E)** CD16$^-$ NK cells. Box-and-whisker plots show median, 25th–75th percentiles, Min-Max. Bar graphs show median and interquartile range. Differences were considered significant for $p < 0.05$ (*).

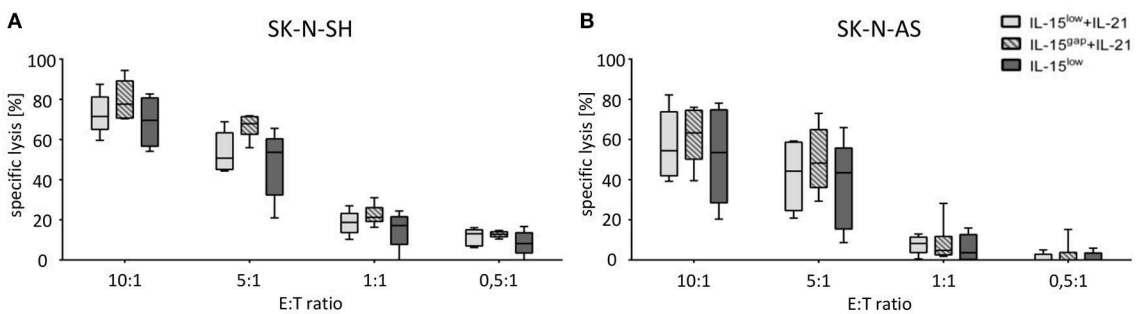

FIGURE 4 | Cytotoxic potential of IL-15+IL-21 stimulated CD3/CD19-depleted NK cells against NB target cells. Specific lysis of the NB cell lines SK-N-SH **(A)** and SK-N-AS **(B)** was evaluated by Europium release assay. Both NB cell lines were efficiently lysed by CD3/CD19-depleted NK cells. Median target cell lysis of NK cells treated in the IL-15^{low} protocol (■) was 69. Five percent for SK-N-SH and 53.5% for SK-N-AS cells (E:T ratio 10:1). An additional IL-21 boost elevated cytotoxic activity of IL15-stimulated NK cells to a median cell lysis of 71.5% (IL-15^{low} + IL-21■) and 77.6% (IL-15^{gap} +IL-21■) for SK-N-SH and 54.4% (IL-15^{low}+IL-21) and 63.3% (IL-15^{gap} +IL-21) for SK-N-AS cells (all E:T ratio 10:1) (statistically not significant differences). Effector to target (E:T) ratios 10:1, 5:1, 1:1, and 0.5:1, $n = 5$–6 independent results, experiments performed in triplicate, incubation time: 3 h, box-and-whisker plots show median, 25th–75th percentiles, Min-Max.

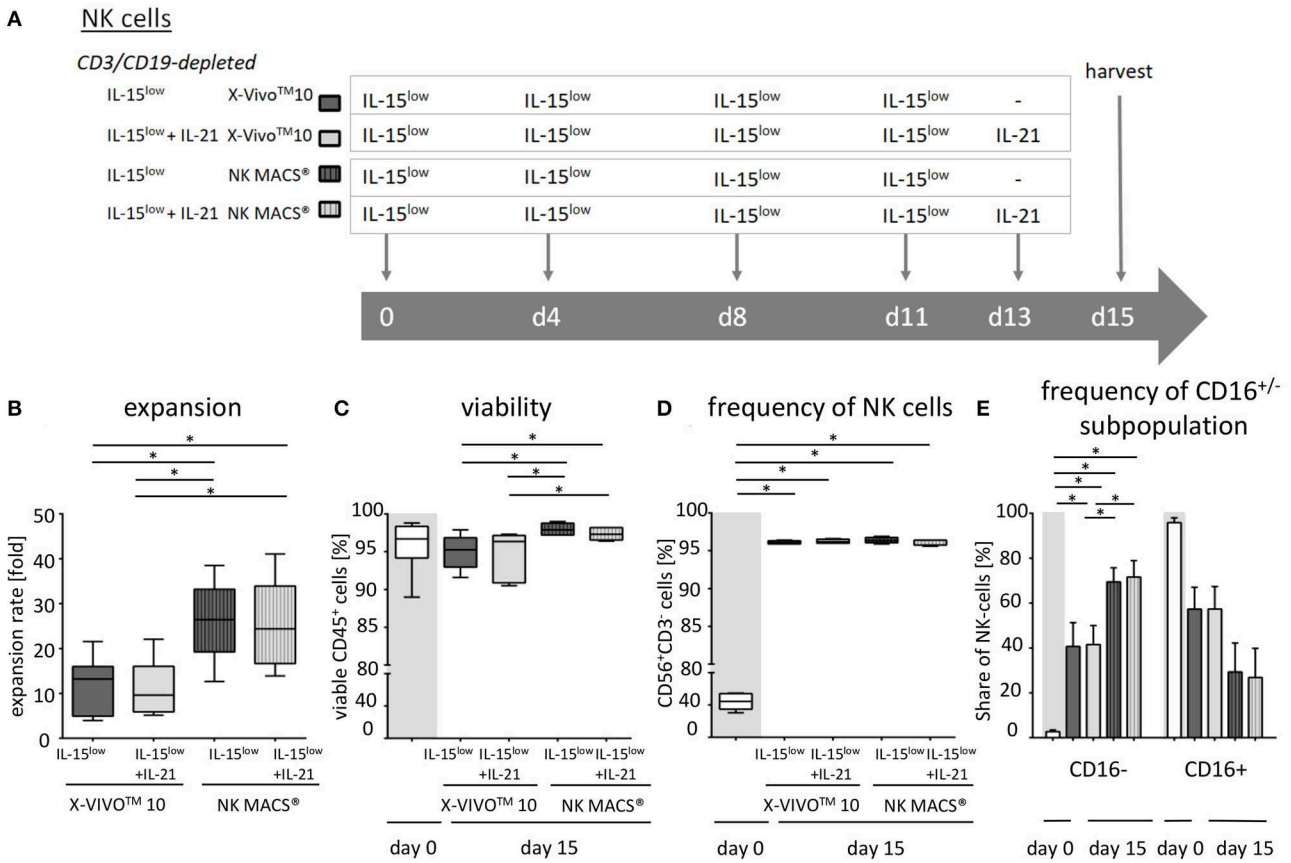

FIGURE 5 | *Ex vivo* expansion and characterization of IL-15+IL-21 stimulated CD3/CD19-depleted NK cells cultured in optimized NK cell medium. **(A)** NK cells were purified by CD3/CD19-depletion and *ex vivo* stimulated with solely IL-15 (▬▬) or with a combination of IL-15 and IL-21 (▭▭) for 15 days. IL-21 was added as a cytokine boost 2 days prior to harvest. NK cells were cultured in X-VIVO™10 medium (▬▭) or NK MACS® medium (▮▭). **(B)** NK cells in X-VIVO™10 medium expanded 13.2-fold (IL-15^{low}) ▬ and 9.6-fold (IL-15^{low}+ IL-21▭). Expansion rates were significantly higher in NK MACS® medium at 26.4-fold (IL-15^{low} ▮) and 24.4-fold (IL-15^{low}+IL-21▭). **(C)** All cell products showed a high viability with a median 96.5% following the purification procedure on day 0 (white symbols gray background) and remained >90% during the expansion procedure, independent of the cytokine additive. *Ex vivo* cultivation in NK MACS® medium even led to viability >96%. **(D)** Purified CD3/CD19-depleted cells on day 0 contained a median 54.4% NK cells. Upon cytokine stimulation for 15 days NK cell purity significantly increased in CD3/CD19-depleted cell products to >95% in all protocols. **(E)** The frequency of the CD16$^-$ NK cell subpopulation significantly increased during *ex vivo* stimulation. In total, 42.7% (IL-15^{low} ▬) and 42.6% (IL-15^{low}+ IL-21▭) of NK cells were CD16$^-$ after cultivation in X-VIVO™10 medium. Percentages of CD16$^-$ cells were significantly higher after cultivation in NK MACS® medium: 69.5% (IL-15^{low} ▮) and 71.6% (IL-15^{low}+ IL-21▭) ($n = 6$ independent experiments, **(B)** median fold expansion rate day 15 compared to day 0, gated on: **(C)** viable 7-AAD$^-$ CD45$^+$ cells, **(D)** CD56$^+$CD3$^-$ NK cells, **(E)** CD16$^-$ NK cells. Box-and-whisker plots show median, 25th–75th percentiles, Min-Max. Bar graphs show median and interquartile range. Differences were considered significant for $p < 0.05$ (*).

CD107a Degranulation Assay and IFN-γ Analysis

Intracellular IFN-γ expression and degranulation potential of cytokine stimulated NK cells cultured in X-VIVO™10 or NK MACS® media was assessed with cells harvested on day 15 of cultivation. Cells were incubated in 96-well-U-bottom-plates for a total time of 4 h, either co-incubated with SK-N-AS target cells (E:T ratio 1:1) or stimulated with cytokines at 37°C and 5% CO_2. SK-N-AS target cell stimulation started from the beginning of co-incubation. After 1 h, cells were incubated with anti-human CD107a, followed by additional 2 h incubation with Monensin/ GolgiStop™ (BD Biosciences). Simultaneously, IL-12 (Peprotech, 96 ng/ml) and IL-18 (MBC, 98.7 ng/ml) were added to the wells not stimulated so far. After the total incubation time of 4 h, cells were washed, blocked with 1 μg of human IgG and stained with Zombie Violet™ Fixable Viability Kit (Biolegend, 5 μl, pre-diluted 1:10 in DPBS) for live/death discrimination. Post washing, cells were stained for 20 min with CD45, CD56, CD16, CD3, CD14, and CD19 in brilliant stain buffer. After a further washing step, they were fixed with formaldehyde solution (3.7% final concentration) by a 15 min incubation. In the end, permeabilization and intracellular staining took place by incubating cells with saponin buffer (0.2% saponin, 1% bovine serum albumin, Sigma Aldrich) and anti-human IFN-γ for further 30 min. For final analysis, cells were washed with perm/wash buffer solution (BD Biosciences, prediluted 1:10 with distilled water), and measured by flow cytometry (Canto 10C).

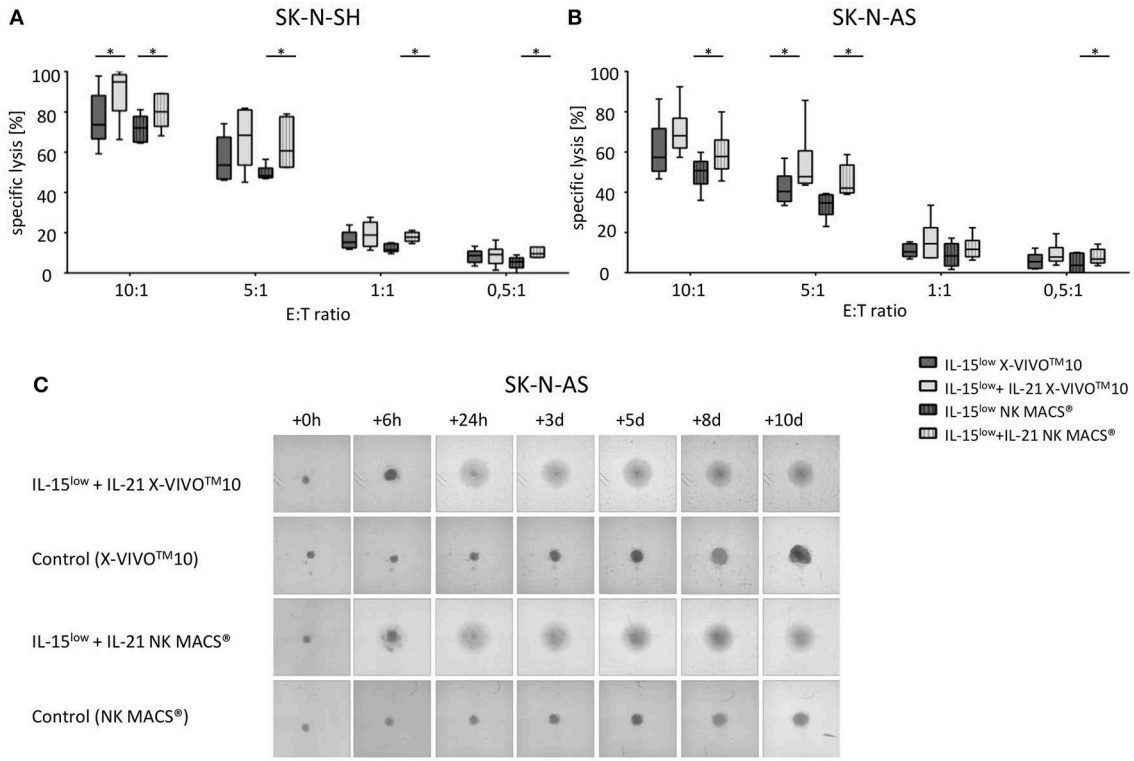

FIGURE 6 | Cytotoxic potential and long-term cytotoxicity after optimization of NK cell cultivation. Specific lysis of the NB cell lines SK-N-SH **(A)** and SK-N-AS **(B)** was evaluated by Europium release assay. Both NB cell lines were efficiently lysed by CD3/CD19-depleted NK cells. Median target cell lysis of NK cells treated in the IL-15^{low} protocol in X-VIVO^{TM}10 (▭) was 73.6% for SK-N-SH and 57.4% for SK-N-AS. An IL-21 boost (▭) significantly elevated the cytotoxic activity of IL15-stimulated NK cells in X-VIVO^{TM}10 medium to a median cell lysis of 94.92% for SK-N-SH and 68.09% for SK-N-AS cells. IL-21 also significantly increased target cell lysis in NK MACS® medium. Cultivation in NK MACS® medium resulted in slightly lower cytotoxic activity with median cell lysis of 70.3% (IL-15^{low} ▭) and 80.1% (IL-15^{low}+ IL-21▭) against SK-N-SH and 50.7 and 57.8% against SK-N-AS (all E:T ratio 10:1). E:T ratios 10:1, 5:1, 1:1, and 0.5:1, $n = 6$ independent results, experiments performed in triplicate, incubation time: 3 hours, box-and-whisker plots show median, 25th–75th percentiles, Min-Max. **(C)** Tumor spheroids were produced from 10,000 SK-N-AS cells and co-incubated with 200,000 NK cells. As a control the dynamics of tumor spheroids without effector cells were observed in both cell culture media. The cultures were imaged via a Celigo cell cytometer after 6 h, 24 h, 3, 5, 8 and up to 10 days. IL-15^{low}+IL-21 stimulated NK cells grown in both cell culture media were able to completely eradicate tumor spheroids in this 10 day long-term cytotoxicity assay ($n = 1$ representative of 3 independent experiments). Differences were considered significant for $p < 0.05$ (*).

Statistical Analysis

Results were statistically analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The data were compared by a Wilcoxon matched paired signed rank test. Differences were considered significant for $p < 0.05$ (*), 0.01 (**), and 0.001 (***).

RESULTS

Ex vivo Expansion and Characterization of CIK and NK Cells

Aiming to manufacture sufficient effector cell doses and to evaluate the most promising cell population to target NB, we compared different cell purification (CD56-enriched vs. CD3/CD19-depleted NK cell) and cytokine stimulation procedures (IL-15 vs. IL-2+IL-15) (**Figure 1A**). For better comparison, NK cell stimulation was adjusted to the well-established stimulation protocol for GMP-grade CIK cell production (30).

Highly purified CD56-enriched NK cells showed a median expansion rate of 4.3-fold (range 1.8–11.6) after 10–12 days, independent of the use of IL-15 alone or in combination with an initial IL-2 boost at day 0 (**Figure 1B**, **Table 1**). In contrast, NK cells within CD3/CD19-depleted cell products expanded significantly higher with both cytokine stimulation settings (median 7.5-fold, range 2.7–16.2) (**Figure 1B**). CIK cells showed the most prominent expansion rate of 30.8-fold, referring to the total cell composite of NK, T and NK-like T cells (**Figure 1B**).

All three cell products showed a high viability of >95% following the purification procedure on day 0 (**Figure 1C**, **Table 1**). Independent of the cytokine additive, median viability of CD56-selected as well as CD3/CD19-depleted NK and CIK cells remained >85% during expansion (**Figure 1C**).

Composition of the different cell products was evaluated regarding the frequency of $CD3^-CD56^+$ NK cells (**Figure 1D**, **Table 1**) (incl. immune regulatory $CD56^{bright}CD16^-$ (**Figure 1E**, **Table 1**) and cytotoxic $CD56^{dim}CD16^+$ subpopulations (**Figure 1F**, **Table 1**), $CD3^+$ T cells (**Supplementary Figure 1A**), $CD3^+CD56^+$ NK-like T cells (**Supplementary Figure 1B**),

TABLE 1 | Summary statistics to experiments shown in **Figure 1**.

Purification method		**CD56-enriched**	**CD3/CD19-depl.**	**CD56-enriched**	**CD3/CD19-depl.**
NK cell stimulation		**IL-2+IL-15**		**IL-15**	
NK cell expansion rate	[x-fold]	4.2 (1.8–11.6)	7.4 (2.7–16.2)	4.7 (2.3–8.6)	7.8 (2.7–15.9)
Viability	[%] of WBC	86.3 (75.5–89.9)	85.7 (75.5–89.7)	83.1 (73.1–89.8)	86.4 (71.6–91.8)
Purity NK cells	[%] of WBC	94.6 (86.1–97.8)	93.6 (80–97.7)	93.8 (82.9–99)	92.5 (79.8–98.6)
$CD16^-$ NK cells	[%] of NK	26.1 (7.6–43.9)	43.0 (28.7–61.3)	28.3 (10.9–45.2)	42.3 (28.0–65.1)
$CD16^+$ NK cells	[%] of NK	72.2 (54.2–91)	56.7 (38.3–69.5)	70.7 (52.7–86.9)	56.8 (34.3–70.9)
Ratio $CD16^-/CD16^+$		0.4	0.7	0.4	0.8
T cell contamination	[%] of WBC	1.0 (0.07–5)	0.03 (0–8.4)	0.34 (0.05–2.5)	0.05 (0–1.5)
NKT cell contamination	[%] of WBC	0.52 (0.05–3.5)	0.07 (0–1.1)	0.16 (0.02–1.2)	0.03 (0–1.2)
B cell contamination	[%] of WBC	0.04 (0–4)	0.12 (0.01–3)	0.08 (0–4)	0.09 (0.04–2.4)

$CD56^+CD3^-$ NK cells were analyzed on day of harvest (day 10–12). Median (range) of expansion rate, purity, viability, NK cell subpopulations and ratios, as well as T, NK-like T and B cell contamination was assessed by flow cytometric analyses. NK, natural killer; NKT, NK-like T; IL, interleukin; WBC, white blood cell; CD3/CD19-depl., CD3/CD19-depleted.

and $CD19^+$ B cells (**Figure 1C**). Composition of the cell products used for expansion differed distinctly depending on the purification procedure. CD56-enriched cell products mainly consisted of highly purified NK cells of in median 94.5%, with minimal overall T, NK-like T and B cell contamination of 0.02%. CD3/CD19-depleted cells products consisted of in median 43.5% NK cells as well as minimal overall T and NK-like T contamination of 0.02%, but with a slightly higher B cell amount. Notably, CD3/CD19-depleted cell products additionally compromised a median of 33.9% (range 16.4–51.8%) monocytes. Thereby, monocytes analyzed in CD3/CD19-depleted cell products highly expressed the IL-15 receptor alpha (IL-15Rα, CD215) on the surface, and expression even further increased early in co-culture ($n = 3$, data not shown). Isolated PBMCs serving as starting material for CIK cell generation contained 11.5% NK cells, 54.7% T cells, 3.7% NK-like T cells and 4.4% B cells.

Upon IL-2+IL-15 and IL-15 cytokine stimulation, CD56-enriched NK cell products contained >94% NK cells, <1.0% T cells, <0.52% NK-like T cell and <0.08% B cells. Thereby, T and NK-like T cell contamination was significantly higher in CD56-enriched NK cell products and if IL-2 was added. CD3/CD19-depleted NK cell products reached >92.5% NK cell purity also without relevant T, NK-like T and B cell contamination. Again, T cell contamination was higher if IL-2 was added, although not statistically relevant (**Figure 1**, **Supplementary Figure 1**, and **Table 1**).

Following expansion, CIK cells contained 3.3% NK cells (range 0.6–10.1%), 84.2% T cells (range 69.6–97.4), 6.2% NK-like T cells (range 1.2–20.7), and 0.6% B cells (range 0–0.7%). Thereby, the content of T and NK-like T cell increased while the NK cell count decreased.

Overall, IL-15 stimulated CD3/CD19-depleted NK cell products revealed the lowest T and NK-like T cell contamination of all NK cell products. In addition, the $CD16^-$ immune regulatory NK cell subpopulation significantly increased during cytokine stimulation, especially within the CD3/CD19-depleted NK cell products. As shown in **Table 1**, the ratio of $CD16^-/CD16^+$ NK cells on the day of harvest was the lowest, when NK cells were purified by CD56 enrichment leading to a NK cell product consisting of predominantly $CD16^+$ NK cells (ratio 0.4). NK cells expanded from CD3/CD19 depleted cell products led to a higher proportion of $CD16^-$ NK cells in the final cell product (ratio 0.7 for IL-2+IL-15 and 0.8 for IL-15 stimulation) independent of the cytokine combinations.

Cytotoxic Potential of CIK and NK Cells Against NB Target Cells

Using the Europium release assay, the NK or CIK cell-mediated cytotoxicity against two different NB cell lines, SK-N-SH (**Figure 2A**) and SK-N- AS (**Figure 2B**), was evaluated. Both NB cells lines were efficiently lysed by either CD56-enriched or CD3/CD19-depleted NK cells with escalating E:T ratios. Thereby, NK cells isolated by CD3/CD19-depletion killed significantly better relative to CD56-selected NK cells in all E:T ratios. The median specific lysis of SK-N-SH cells by IL-15^{low} treated NK cells was 62.4%, 58.9%, 47.3% and 31.6% for CD56-enriched NK cells and 68.9, 63.0, 54.6, and 36.3% for CD3/CD19-depleted NK cells at E:T ratios of 20:1, 10:1, 5:1 and 1:1, respectively. In addition, there was a clear tendency for IL-15 to be superior to IL-2+IL-15 treatment. Notably, NK cells expanded after CD3/CD19-depletion with IL-15^{low} revealed the highest cytotoxic potential against both target cell lines. In contrast, CIK cell-mediated cytotoxicity was significantly lower in all E:T ratios; e.g., 12.5% (range 1.2–36.3%) and 10.1% (range 0.1–30.3%) CIK cells vs. SK-N-SH and SK-N-AS at the 10:1 ratio. Of note, CIK cell killing correlated with the amount of NK cells present in the individual heterogeneous CIK cell composite (data not shown).

To further address long-term cytotoxicity and mimic tumor lysis in a 3D setting, we used a tumor spheroid model. NB tumor spheroids built of SK-N-AS cells were co-incubated with CIK or NK cells following different stimulation procedures (**Figure 2C**). All NK cells were able to quickly infiltrate and lyse the tumor spheroids. However, only co-incubation with IL-15-stimulated NK cells generated from CD3/CD19-depleted PBMCs led to a complete lysis with no reoccurrence of target cells. CIK cells also infiltrated tumor spheroids and lysed target cells

resulting in complete lysis of the tumor spheroids. In the total observation period of 10 days no tumor regrowth could be seen as well.

Optimizing *ex vivo* NK Cell Activation Protocol for CD3/CD19-Depleted Cells by IL-21

As NK cells expanded with IL-15 after CD3/CD19-depletion showed the highest expansion rate and cytotoxic capacity against NB cell lines, we further aimed to optimize this protocol based on previous data indicating a further gain in NK cell cytotoxicity by a boost with IL-21 (23). Therefore, the cytokine stimulation has been prolonged by the addition of IL-21 prior to harvesting. In addition, a break of cytokine exposure prior to the IL-21 boost has been tested as this short "gap" in cytokine addition has been reported to improve NK cell expansion (22) (**Figure 3A**, **Table 2**). *Ex vivo* expansion with IL-15^{low} for 15 days led to a median expansion rate of 6.8-fold (range 2.6–23.1) (**Figure 3B**, **Table 2**). An additional boost with IL-21 on day 13 of culture was able to further enhance proliferation up to a median 16.5-fold (range 4.2–26.9), but not to a statistically relevant extent. If cells received only medium and no cytokines at day 11 prior to the IL-21 boost on day 13 (IL-15^{gap}+IL-21), NK cells expanded 7.1-fold (range 2.6–12.8), only.

Following CD3/CD19-depletion 97.8% of all CD45$^+$ cells were viable (**Figure 3C**, **Table 2**). Median NK cell viability on the day of harvest was 96% following IL-15 stimulation, while viability in both protocols containing an IL-21 boost was slightly lower, but not to a significantly relevant extent (96.2% in the IL-15^{low}+IL-21 and 88.3% in the IL-15^{gap}+IL-21 protocol). CD3/CD19-depleted cell products consisted in median of 53% NK cells (range 23.0–71.5%) (**Figure 3D**). By *ex vivo* cultivation for 15 days the NK cell purity could be significantly elevated up to 96.2, 96.5, and 96.7% for IL-15^{low}, IL-15^{gap}+IL-21, and IL-15^{low}+IL-21, respectively. Importantly, there was no significant T or B cell contamination. All NK cell products contained a median amount of 0.01% T cells (range 0–0.04%) and 0.02% B cells (range 0–0.18%) (**Table 2**). Following CD3/CD19-depletion, the majority of NK cells (94.7%) can be assigned to the CD16$^+$ cytotoxic NK cell subpopulation (**Figure 3E**, **Table 2**). Upon *ex vivo* cultivation the percentage of CD16$^-$ immune regulatory NK cells significantly increased in all three cytokine stimulation protocols up to 49.4% IL-15^{low}, 42.7% IL-15^{low}+IL-21, and 48.5% IL-15^{gap}+IL-21, respectively.

Next, the percentage of lysed target NB cells within the optimized NK cell expansion protocols was evaluated by Europium release assay. Both NB cell lines, SK-N-SH (**Figure 4A**) and SK-N-AS (**Figure 4B**), were efficiently lysed by CD3/CD19-depleted NK cells with escalating E:T ratios. The median specific lysis of SK-N-SH cells by IL-15^{low} treated NK cells was 69.5% (range 54.1–82.7%) at an E:T-ratio of 10:1. NK cells further stimulated with IL-21 in the IL-15^{low}+IL-21 protocol were able to lyse 71.5% (range 60.0–87.4%) and those cultured in the IL-15^{gap}+IL-21 protocol lysed 77.6% (range 70.3–94.3%) of the SK-N-SH cells at an E:T-ratio of 10:1. In summary, the additional IL-21-boost was able to slightly further enhance cytotoxicity. Remarkably, NK cells were even able to lyse SK-N-SH cells at an E:T ratio of 0.5:1 with a median lysis of 8.2% (range 0–16.7%) in the IL-15^{low} protocol, 13.06% (range 6.2–16.1%) following the IL-15^{low}+IL-21 treatment and 12.24% (range 10.5–14.7%) in the IL-15^{gap}+IL-21 condition (**Figure 4A**). NK cell mediated killing of SK-N-AS cells was still very effective but slightly lower compared to SK-N-SH cells. NK cells cultured in the IL-15^{low}+IL-21 protocol were able to lyse 54.4% (range 39.2–82.2%) and those cultured in the IL-15^{gap}+IL-21 protocol 63.3% (range 39.5–76.0%) of SK-N-AS cells at an E:T-ratio of 10:1 (**Figure 4B**). Overall, NK cells stimulated according to the IL-15^{gap}+IL-21 protocol showed the highest cytotoxic potential against both NB cell lines, although differences were not statistically relevant.

Further Optimization of the *ex vivo* NK Cell Activation Protocol by Specialized NK Cell Medium

To investigate if NK cell expansion can be further enhanced, we included an optimized cell culture medium for the cultivation and expansion of human NK cells, the NK MACS® medium (Miltenyi Biotec) in our stimulation protocols with either continuous IL-15 treatment or with an additional IL-21 boost 2 days prior to cell harvest on day 15 (**Figure 5A**, **Table 3**). Comparing the effect of an IL-21 boost to the treatment with IL-15, NK cell expansion was slightly, but not significantly, lower after the boost. Culturing in NK MACS® medium led to a significantly higher expansion rate compared to culturing in X-VIVO™10 medium, which was also used in all previously shown experiments. While NK cells expanded 13.2-fold (range 4–21.6) under IL15 stimulation and 9.6-fold (range 5.1–22.1) after an additional IL-21 boost in X-VIVO™10 medium, expansion rates increased up to 26.4-fold (range 12.7–38.5) and 24.4-fold (range 13.9–41.1), respectively when NK MACS® medium was used (**Figure 5B**, **Table 3**).

However, NK cell expansion was not better in NK MACS® compared to that in X-VIVO™10 medium over the entire *ex vivo* cultivation process. Within the first 4 days in culture, NK cell counts decreased and first expansion could be seen in the time period between days 4 and 8. Interestingly, expansion rates during this time point were higher in X-VIVO™10 medium, but after 8 days of culture, NK cell proliferation was significantly better in NK MACS® medium. Further, a continuous increase in expansion could be seen until the day of harvest in NK MACS® medium, while NK cells in X-VIVO™10 medium proliferated until day 15, but with much lower rates than those during the beginning of cultivation (data not shown).

Viability on the day of isolation by CD3/CD19-depletion amounted to 96.7%, which decreased slightly after 15 days of culture in X-VIVO™10 medium. Median viability on the day of harvest of IL-15^{low} treated NK cells was 95.3 and 96.4% after the IL-21 cytokine boost. Culturing NK cells in NK MACS® medium for 15 days increased the median viability up to 98% following IL-15^{low} treatment and up to 97.3% within the IL-15^{low}+IL-21 stimulation protocol (**Figure 5C**, **Table 3**).

CD3/CD19-depleted cell products consisted prior to expansion (day 0) in median of 44.4% NK cells with T and B cell content of 0.04 and 0.06% (**Figure 5D**). Fifteen days

TABLE 2 | Summary statistics to experiments shown in **Figure 3**.

Purification method		**CD3/CD19-depl.**		
NK cell stimulation		**IL-15+IL-21**	**IL-15+IL-21^{gap}**	**IL-15**
NK cell expansion rate	[x-fold]	16.5 (4.2–26.9)	7.1 (2.6–12.8)	6.8 (2.6–23.1)
Viability	[%] of WBC	96.2 (95.2–99.1)	88.3 (80–98.7	96.0 (89.9–97.2)
Purity NK cells	[%] of WBC	96.7 (95.8–96.9)	96.0 (95.3–98.3)	96.2 (94.9–98.7)
CD16$^-$ NK cells	[%] of NK	42.7 (16.4–46.7)	48.5 (19.2–57.6)	49.4 (18.1–56.1)
CD16$^+$ NK cells	[%] of NK	56.0 (51.6–81.6)	47.1 (39.7–75.1)	47.8 (40.2–73)
Ratio CD16$^-$/CD16$^+$		0.7	1.0	1.0
T cell contamination	[%] of WBC	0.0 (0–0.01)	0.02 (0–0.04)	0.005 (0–0.37)
NKT cell contamination	[%] of WBC	0.19 (0.06–0.3)	0.24 (0.09–0.44)	0.16 (0.46–0.38)
B cell contamination	[%] of WBC	0.02 (0–0.03)	0.03 (0.01–0.11)	0.01 (0–0.18)

CD56$^+$CD3$^-$ NK cells were analyzed on day of harvest (day 10–12). Median (range) expansion rate, purity, viability, NK cell subpopulations and ratios, as well as T, NK-like T and B cell contamination was assessed by flow cytometric analyses. NK, natural killer; NKT, NK-like T; IL, interleukin; WBC, white blood cell; CD3/CD19-depl., CD3/CD19-depleted.

TABLE 3 | Summary statistics to experiments shown in **Figure 5**.

Purification method		**CD3/CD19-depl.**			
NK cell stimulation		**IL-15**	**IL-15+IL-21**	**IL-15**	**IL-15+IL-21**
		X-VIVO10		**NK MACS**	
NK cell expansion rate	[x-fold]	13.2 (4–21.6)	9.6 (5.1–22.1)	26.4 (12.7–38.5)	24.4 (13.9–41.1)
Viability	[%] of WBC	95.3 (91.6–97.9)	96.4 (90.5–97.3)	98 (97.2–99)	97.3 (96.4–98.2)
Purity NK cells	[%] of WBC	96.1 (95.9–96.4)	96.2 (96–96.6)	96.4 (95.9–96.9)	96.4 (95.6–96.4)
CD16$^-$ NK cells	[%] of NK	40.7 (32–51.3)	41.5 (31.9–50)	69.5 (55.7–75.7)	71.6 (58.0–78.9)
CD16$^+$ NK cells	[%] of NK	57.3 (43.7–67.1)	57.4 (47–67.4)	29.3 (20.7–42.2)	26.9 (17.5–39.8)
Ratio CD16$^-$/CD16$^+$		0.7	0.7	2.4	2.7
T cell contamination	[%] of WBC	0.0 (0–0)	0.0 (0–0.04)	0.0 (0–0.01)	0.0 (0–0.01)
NKT cell contamination	[%] of WBC	0.14 (0.11–0.22)	0.21 (0.1–0.25)	0.14 (0.06–0.22)	0.15 (0.13–0.23)
B cell contamination	[%] of WBC	0.06 (0–0.18)	0.14 (0.02–0.18)	0.07 (0.01–0.18)	0.07 (0.02–0.16)

CD56$^+$CD3$^-$ NK cells were analyzed on day of harvest (day 10–12). Median (range) expansion rate, purity, viability, NK cell subpopulations and ratios, as well as T, NK-like T and B cell contamination was assessed by flow cytometric analyses. NK, natural killer; NKT, NK-like T; IL, interleukin; WBC, white blood cell; CD3/CD19-depl., CD3/CD19-depleted.

of *ex vivo* culture led to a high NK cell purity of around 96%, which was independent of cell culture medium and cytokine stimuli (96.1% IL-15^{low} and 96.2% IL-15^{low}+ IL-21 after culture in X-VIVO^{TM}10 medium and 96.4% IL-15^{low} and 96.4% IL-15^{low}+ IL-21 in NK MACS® medium). As observed in the previous results, there was hardly any T or B cell contamination. All NK cell products were almost T and B cell free (range of 0–0.04% T cells and 0–0.18% B cells) (**Table 3**). Directly after CD3/CD19-depeletion, the predominant NK cell population at 95.9% consisted of CD16$^+$ cytotoxic cells (day 0) and decreased to 57.3 and 57.4% under cultivation with IL-15^{low} or IL-15^{low}+IL-21 in X-VIVO^{TM}10, respectively (day 15, **Figure 5E**, **Table 3**). Interestingly, the cultivation in NK MACS® medium led to an even more pronounced effect with significantly higher proportions of CD16$^-$ cells, which made up 69.5% of NK cells following culturing with IL-15^{low} and 71.6% after an additional IL-21-boost displayed by a switch in the CD16$^-$/CD16$^+$ ratio to 2.4 and 2.7, respectively (**Figure 5E**, **Table 3**).

Cytotoxic Potential and Long-Term Cytotoxicity After Optimization of NK Cell Cultivation

The additional IL-21 boost 2 days prior to harvest was able to significantly enhance cytotoxicity compared to cultivation with only IL-15^{low} (**Figure 6A**). This observation was seen in both NK cell media but was even more pronounced for NK cells cultivated in NK MACS® medium. Here, even at an E:T ratio of 0.5:1, the addition of IL-21 led to an significant increase in target cell lysis. However, specific cell lysis was slightly higher after *ex vivo* culture in X-VIVO^{TM}10 medium compared to that in NK MACS® medium. At an E:T ratio of 10:1, the specific lysis of SK-N-SH cells by IL-15^{low}-treated NK cells in X-VIVO^{TM}10 medium was 73.6% (range 59.2–97.8%). An IL-21 boost elevated lysis up to 94.9% (range 66.3–100%). In NK MACS® medium, lysis was 72.1% (range 64.6–81.1%) for IL-15^{low} and 80.1% (range 68.1–89.2%) for IL-15^{low}+IL-21 (**Figure 6A**). At an E:T ratio of 10:1, the specific lysis of SK-N-AS cells by IL-15^{low} stimulated NK

cells in X-VIVO^{TM}10 medium was 57.4% (range 46.9–86.3%), and an IL-21 boost elevated the lysis up to 68.1% (range 57.4–92.4%). In NK MACS® medium, lysis was 50.7% (range 36.0–59.8%) (IL-15^{low}) and 57.8% (range 45.6–78.0% (IL-15^{low}+ IL-21) (**Figure 6B**). Remarkably, NK cells did not only lyse NB cells at high E:T ratios but also at very low ones. Median specific lysis of SK-N-SH cells at an E:T of only 0.5:1 was 8.7% (range 3.5–13.3%) for IL-15^{low} X-VIVO^{TM}10, 9.2% (range 1.4–16.4%) for IL-15^{low}+IL-21 X-VIVO^{TM}10, 5.6% (range 0–9.0%) for IL-15^{low} NK MACS® and 9.6% (range 7.7–13.0%) for IL-15^{low}+IL-21 in NK MACS® medium.

To further address long-term cytotoxicity, NB tumor spheroids built of SK-N-AS cells were co-incubated with IL-15^{low}+IL-21 stimulated NK cells cultured in either X-VIVO^{TM}10 NK or MACS® medium. As already demonstrated in the short-term cytotoxicity assays, no differences between both media were observed in long-term cytotoxicity assays using tumor spheroids. Regardless what culture medium was used, IL-15^{low}+IL-21 stimulated NK cells were able to quickly infiltrate and completely lyse the tumor spheroids (**Figure 6C**).

IFN-γ Production and Expression of the Degranulation Marker CD107a

Degranulation assays detecting CD107a as a marker for stimulation-induced granule exocytosis and intracellular IFN-γ staining were performed comparing NK cells previously cultured with IL-15^{low} or IL-15^{low}+IL-21 in X-VIVO^{TM}10 or NK MACS® media. As degranulation/cytokine production stimulus either SK-N-AS NB target cells (E:T ratio 1:1) or IL-12+IL-18, mimicking stimulation by dendritic cells, was used (**Figure 7**). Both NK cell subpopulations were capable of IFN-γ production upon cytokine stimulation and target cell co-incubation and higher levels of IFN-γ^{+} cells were detected following cytokine stimulus compared to tumor cell co-incubation, which was statistically significant for the CD16^{-} subset. Between both culture media similar effects were observed, except CD16^{-} NK cells grown in X-VIVO^{TM}10 produced significantly more IFN-γ upon IL-12+IL-18 cytokine stimulation. Target cell co-incubation and cytokine stimulation led to a high CD107a expression in the different NK cell subpopulations, thereby more pronounced within the CD16^{-} NK cell population. Only small differences were seen between both cell culture media, but throughout all experiments, the additional IL-21 boost during NK cell cultivation enhanced IFN-γ and CD107a expression, which was noticeable in all measurements and was even statistically significant in $n = 2$ settings.

Phenotype Analysis of NK Cells and CD16^{-} and CD16^{+} Subpopulations

Extensive flow cytometric analysis revealed that the IL-21 boost had no significant influence on the expression of a broad repertoire of NK cell characteristic cell surface markers when compared to stimulation with IL-15^{low} only (**Figure 8A**). Notably, the activating cytotoxicity receptors NKp44 and NKG2D and the activation marker CD69 showed higher expression on NK cells following cultivation in X-VIVO^{TM}10 medium compared to NK MACS® medium. However, all other surface markers tested were equally expressed on NK cells cultured in either X-VIVO^{TM}10 or NK MACS® medium.

Irrespective of culture medium-dependent differences, also variations in the receptor expression of CD16^{-} and CD16^{+} NK cell subpopulations were observed (**Figures 8B,C**). Receptors associated with cytotoxicity, particularly NKG2D which plays a crucial role in NK cell killing, were equally expressed on both NK cells subsets following expansion by IL-15^{low} and IL-15^{low}+IL-21; NKp44 and NKp46 even to a higher extend in CD16^{-} NK cells. In the same line, expression of CD25, the α-chain of the IL-2/IL-15 receptor, seemed to be correlated to activation. By contrast, the death receptor FASL and the maturation marker CD57 were considerably higher expressed on CD16^{+} NK cells, which was expected as the CD56^{dim}CD16^{+} subset is described as the mature, while CD16^{-} NK cells are the more immature NK cell subpopulation.

DISCUSSION

Current therapy for high-risk NB includes surgery, myeloablative conditioning and autologous SCT, but the occurrence of relapse remains still a major limit (31, 32). Despite therapeutic improvements, the overall survival is not satisfying, and alternative treatment options are urgently needed. To benefit from the GvL/T effect, allogeneic SCT was considered for NB patients. However, there was no difference in the outcome of patients treated with autologous purged and matched allogeneic SCT (33). Haploidentical SCT is an option to enhance the GvL/T effect but carries an increased risk of severe GvHD, mainly mediated by alloreactive donor T cells. An effective strategy to prevent the occurrence of lethal GvHD is the *in vitro* depletion of T cells. Specific depletion techniques are available to remove T and B cells from the grafts (CD3/CD19- or TCRαβ/CD19-depletion) while preserving cells that are beneficial regarding engraftment and GvL/T effect such as NK cells (9, 34, 35). Notably, HLA class I expression is reduced on NB cells which makes them valid targets for NK cell lysis (10, 36). The feasibility of haploidentical SCT for solid tumors including NB was shown, unfortunately with limited effect (6, 8, 37–39). Most studies included small cohorts of patients who suffered from diverse primary tumors (6, 38) that relapsed following autologous SCT (6) or were in non- or partial remission at the time-point of haploidentical SCT (6, 8, 38, 39). Illhardt et al. treated 26 patients suffering from refractory or relapsed metastatic NB showing that patients reaching complete remission before haploidentical SCT had a significantly better prognosis compared to patients with proven residual tumor load. Unfortunately, event-free and overall-survival in the whole cohort at 5 years post SCT were 19% and 23%, respectively, enforcing the urgent need for improved treatment options (8).

Adoptive immunotherapy following SCT is a promising approach to further strengthen GvL/T to combat relapse in high-risk solid tumors, such as NB. Within the last decade, different cell-based immunotherapeutic strategies were adopted and refined, including the infusion of donor lymphocytes (DLI),

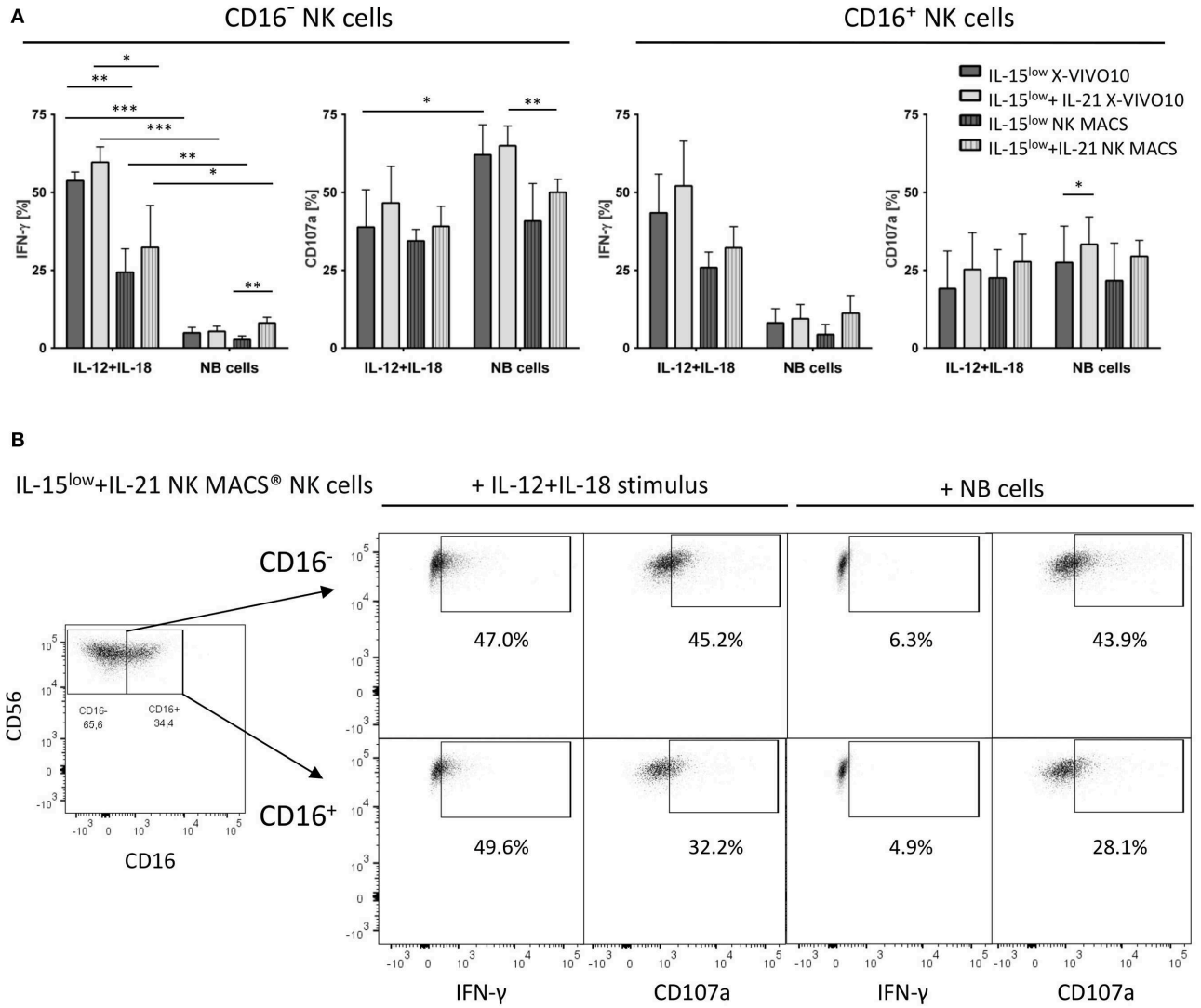

FIGURE 7 | CD107a degranulation and IFN-γ production. **(A)** Intracellular IFN-γ expression and degranulation potential indicated by CD107a expression of cytokine stimulated NK cells cultured in X-VIVO™10 or NK MACS® media was assessed after 15 days of cultivation with the IL-15^{low} or IL-15^{low}+IL-21 stimulation protocol. Cells were either co-incubated with SK-N-AS NB target cells (E:T ratio 1:1) or stimulated with IL-12+IL-18 mimicking stimulation by dendritic cells. After the total incubation time of 4 h, cells were stained and measured by flow cytometry. IFN-γ and CD107a expression was compared to unstimulated cells in each cultivation setting used as negative control. Both NK cell subsets produced IFN-γ upon cytokine stimulation and target cell co-incubation, with higher levels after the cytokine stimulus, which was statistically significant for the CD16$^-$ subset. Similar effects were seen in both media, except CD16$^-$ NK cells grown in X-VIVO™10 produced significantly more IFN-γ upon IL-12+IL-18 cytokine stimulation. Target cell co-incubation and cytokine stimulation led to a high CD107a expression in both NK cell subpopulations, especially within the CD16$^-$ NK cell population. Only small differences were seen between both cell culture media. Throughout all experiments, the additional IL-21 boost during NK cell cultivation enhanced IFN-γ and CD107a expression, which was even statistically significant in $n = 2$ settings (light gray vs. dark gray bars). Summary data show mean and SEM percentage of CD107a$^+$ and IFN-γ$^+$ NK cells ($n = 4$ independent results). **(B)** FACS plots show IFN-γ and CD107a expression in both CD16$^-$ and CD16$^+$ NK cell subpopulations of IL-15^{low}+IL-21 NK cells grown 15 days in NK MACS® media. This stimulation protocol led to an outgrowth of the CD16$^-$ NK cell subpopulation resulting in an inverse CD16$^-$/CD16$^+$ distribution. The short-term stimulus of IL-12+IL-15 and target cell co-incubation, demonstrated that both NK cell subpopulations are capable of IFN-γ production and CD107a expression. Thereby CD107a expression was higher present on CD16$^-$ NK cells, while IFN-γ was produced equally by both subsets. FACS plots gated on viable Zombie Violet$^-$ CD3$^-$CD56$^+$ NK cells (density plots show one representative result from $n = 4$ independent experiments). Differences were considered significant for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

NK cells with and without prior cytokine stimulation, and CIK cells (18, 20, 40–42). CIK cells have shown high anti-leukemic potential, while demonstrating only low alloreactive potential. Through their heterogeneous composition, including a majority of CD3$^+$ T cells, CD3$^+$CD56$^+$ NK-like T cells and a small amount of CD3$^-$CD56$^+$ NK cells, CIK cells can mediate different killing mechanisms involving T cell receptor-dependent and non-HLA-restricted cytotoxicity using various receptors and signaling pathways (27, 40, 43, 44). In a previous work, we showed that CIK cells were able to attack NB by lysing between

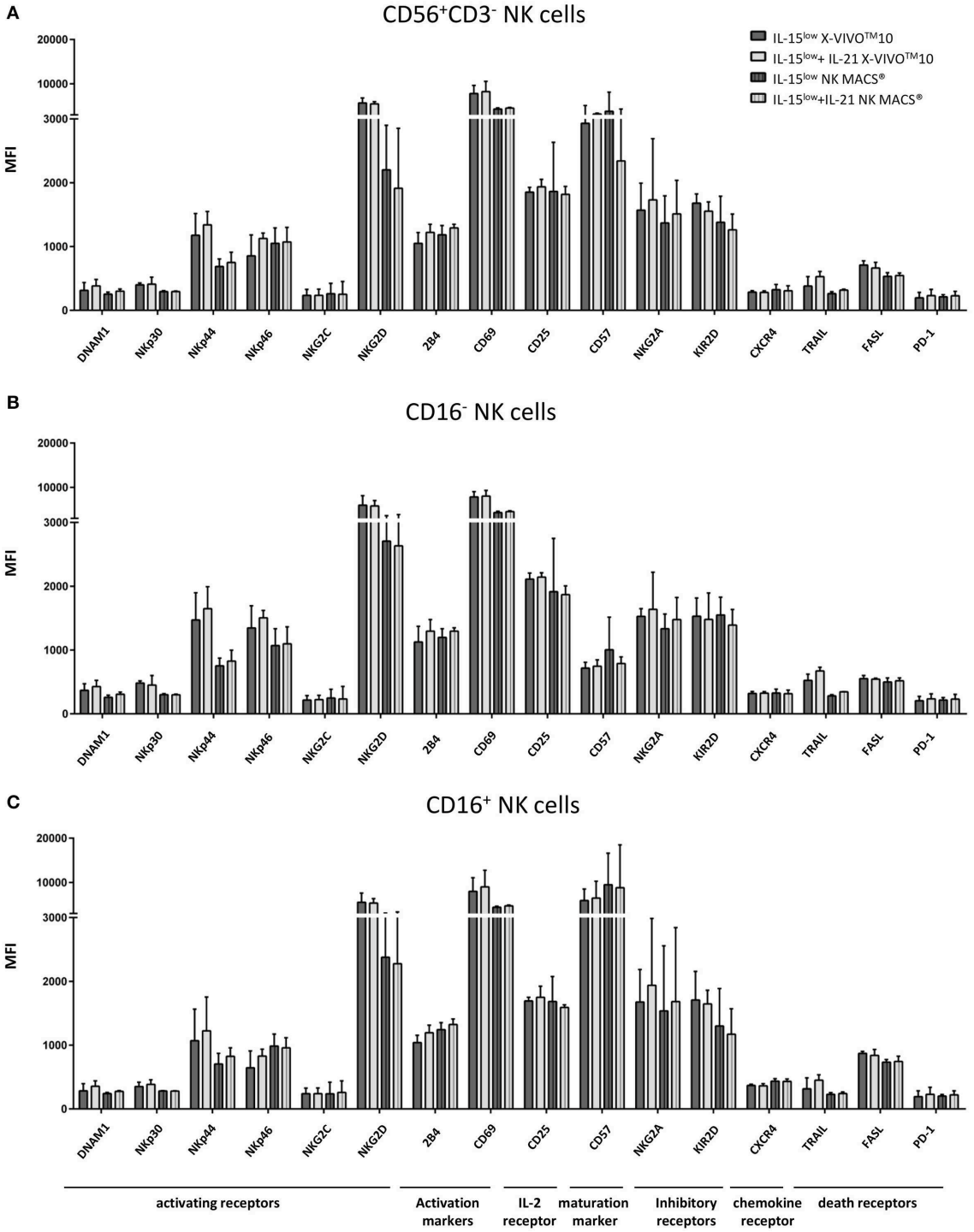

FIGURE 8 | Phenotype analyses of NK cells and $CD16^-$ and $CD16^+$ subpopulations. **(A)** Expression of various surface markers on NK cells including $CD16^-$ **(B)** and $CD16^+$ **(C)** NK cell subpopulations on the day of harvest. No significant differences between stimulation with IL-15 solely (▬▥) or in combination with IL-21 (□▤) could be seen. The activating receptors NKp44 and NKG2D and the activation marker CD69 showed higher expression on NK cells cultured in X-VIVO™10 medium (▬□) compared to ones cultured in NK MACS® medium (▥▤). While the $CD16^+$ NK cell population expressed higher levels of maturation marker CD57, the inhibitory receptor NKG2A and the death receptor FASL, $CD16^-$ NK cells expressed the activating receptors NKp44 and NKp46 as well as the α-chain of the IL-2/IL-15 receptor CD25 to a higher extend (statistically not relevant differences). $n = 4$, independent results, median fluorescence intensity (MFI), bar graphs show median and interquartile range, gated on viable 7-AAD$^-$ NK cells using FMO (fluorescence minus one) controls for each antigen.

14.7 and 19.8% of different NB cell lines, such as UKF-NB-3, UKF-NB4 and SK-N-SH, at an E:T ratio of 20:1 (27). Hence, repeated CIK cell immunotherapy might support the patient's immune system to combat NB relapse, but increased killing rates are desirable. An encouraging approach might be the engineering of CAR-CIK cells as already shown for refractory or advanced soft tissue sarcoma (28).

Another immunotherapeutic strategy that was successfully applied in NB patients is the infusion of allogeneic NK cells (18, 19, 45). The expression of stress-induced activating receptor ligands MHC class I chain-related protein A and B (MICA/B) combined with reduced expression of HLA class I surface molecules to escape T cell mediated killing, renders NB an optimal target for NK cell mediated cytotoxicity (46). Within clinical trials, we and others applied highly purified NK cells with and without prior *ex vivo* IL-2 stimulation (19, 20, 47). Thereby, we have demonstrated feasible manufacturing under GMP conditions and the safe application of haploidentical NK cells on day +40 and +100 post SCT in 16 pediatric patients suffering from high-risk malignancies (4 of them with high-risk NB stadium IV). Two of the four patients with NB treated with IL-2-stimulated NK cells showed a notable response. One patient is still alive 9.3 years after SCT and NK cell immunotherapy whereas the second patient remained in complete remission (CR) for 5.8 years. However, despite a high lytic activity of the activated donor NK cells *in vitro* and these promising clinical responses, the continuation of this clinical study was limited by an NK cell expansion rate of a median 4-fold, which did not ensure repetitive high-dose NK cell infusions. In addition, the manufacturing procedure was associated with enormously high expenses.

In substantial research studies, various cytokine combinations with and without feeder cell addition were tested to investigate the improvement of NK cell proliferation and cytotoxicity against a broad range of tumor entities (23, 48, 49). The establishment of a protocol without feeder cells seems advantageous as cell products produced with feeder cells cannot simply be infused, but must be thoroughly tested for clinical safety first (50–52). In this work, we generated NK cells with IL-2, IL-15 and/or IL-21 using either CD3/CD19-depleted or CD56-enriched PBMCs as starting material. Thereby, our analyses revealed that NK cells generated from CD3/CD19-depleted starting material expanded significantly faster compared to conventionally generated CD56-enriched NK cells. This might be due to autologous accessory cells of the CD3/CD19-depleted cell product, meaning co-cultured non-NK cells, such as monocytes and DCs, that can facilitate NK cell expansion and stimulation by cytokine secretion and/or direct cell-to-cell-interaction (53–55). Monocytes analyzed early in co-culture of CD3/CD19-depleted NK cell products expressed the high affinity IL-15 receptor alpha (IL-15Rα, CD215) on the surface, indicating a possible mechanism of trans-presentation of IL-15 to NK cells by the IL-2Rβ and γ chain receptor complex. Similar to the recently described design of a IL-15 superagonist by complexing IL-15 and IL-15Rα in solution, this may as well enhance the biological activity of IL-15 in the expansion procedure of NK cells when co-cultured with accessory cells such as monocytes following CD3/CD19-depletion. This effect might explain the enhanced NK cell proliferation of CD3/CD19-depleted PBMCs compared to highly purified CD56-enriched NK cell cultures (56–58). Unlike to classical feeder cells, these accessory cells are not of third-party origin and do not need to be inactivated or irradiated. With regard to an intended clinical cell application, this is of crucial importance, because these cells are not going to be removed prior to clinical cell application. Moreover, in the clinical haploidentical setting that carries an elevated risk of developing GvHD, the amount and possible expansion of residual T cells needs to be carefully monitored. Importantly, the NK cell product generated from CD3/CD19-depleted primary material consisted of only 39% NK cells at culture start, but reached a purity of almost 99%, without relevant T or B cell contamination (<0.2%).

We further showed that NK cells isolated by CD3/CD19-depletion exerted significantly higher cytotoxic activity than those isolated by CD56-enrichment. These findings are coherent to findings published by Williams et al. (59). In addition to the common 2D cytotoxicity tests, we performed lysis experiments with tumor spheroids. Hereby, using the Celigo cell cytometer, we were able to investigate killing over an extended time period up to 10 days and in a 3D structure closer to *in vivo* tumor conditions (60–62). All NK cells were able to suppress tumor growth and the superior lysis of CD3/CD19-depleted cells could be affirmed. However, especially NK cells generated from CD3/CD19-depleted PBMCs cultured with IL-15 mediated remarkably high cytotoxicity in short-term and long-term killing of the 3D spheroid structure without reoccurrence of target cells. Interestingly, while CIK cells showed significantly lower short-term killing capacity, in the long-term cytotoxicity assay against 3D NB structures, CIK cells eradicated tumor cells as well. This was probably due to their persistence and proliferative capacity under these specific *in vitro* conditions. Furthermore, CIK-cell-mediated killing is a process that is known to need more time than NK cell-induced killing (63).

Although IL-2-stimulated NK cells were successfully administered in several clinical studies, we and others showed advantageous expansion rates and cytotoxicity with IL-15 stimulation (50, 64). IL-2 is described as a relevant cytokine for the maintenance of induced regulatory T cells and also for the activation and proliferation of T cells which might be of importance regarding tumor-escape mechanisms, but also for GvHD development (65–67).

Therefore, encouraging results were published by Choi et al. treating 41 adult patients suffering mainly from leukemia with haploidentical NK cell immunotherapy generated from CD3-depleted primary material stimulated with IL-15, IL-21 and hydrocortisone over a period of 13 to 20 days. The median cell dose given in this study was with 2×10^8 NK cells kg/BW ∼10–20 times higher compared to previous studies leading to a significant reduction in leukemia progression (68). Experience with NK cell expansion from CD3/CD19-depleted primary material was also published by Williams et al. and van Ostaijen-ten Dam et al., however their cultivation with IL-2 and/or IL-15 was limited to overnight and 5 days, resulting in lower cell expansion compared to our protocol (59, 69).

Here, we could see that NK cells isolated by CD56-enrichment and cultured with IL-2 in addition to IL-15 showed higher T cell contamination (median 1%) than NK cells cultured with IL-15 only. Stimulation with IL-21 is known to enhance NK cell cytotoxicity, while hampering IL-2- and IL-15-driven expansion and viability (50, 70–72). As already shown by Wagner et al. (23), an additional short-term IL-21 boost to IL-15^{low}-cultured NK cells prior to harvest further significantly increased cytotoxicity and partly also NK cell expansion. They described, that with the IL-15+IL-21 boost protocol, expansion rates ranged between 2- and 10-fold depending on the donor, exhibiting an average 4.5-fold increase on day 10 of culture. In our experiments, the effect of the IL-21 boost on NK cell expansion was also to some extend variable and donor-dependent, while IL-21 consistently increased NK cell cytotoxicity, in median 8–10% when NK cells were cultured in the NK cell optimized medium NK MACS®. Remarkably, this gain in cytotoxicity was even more significant than described by Wagner et al. (23).

Importantly, our data highlight that cultivation of NK cells from CD3/CD19-depleted primary material in NK MACS® medium compared to X-VIVO^{TM}10 medium resulted in significantly higher expansion rates up to 26.4-fold compared to 13.2-fold following IL-15^{low} stimulation, which was combined with higher purity and viability, but slightly lower cytotoxicity against NB cell lines. Oberschmidt et al. reported a median expansion of 5.9-fold after 14 days of *ex vivo* cultivation of NK cells in NK MACS® medium with the addition of IL-2/IL-15 and initial IL-21 after CD3-depletion and CD56-selection from leukapheresis products using the CliniMACS® Prodigy device (73). Significantly higher expansion rates using NK MACS® compared to X-VIVO^{TM}10 media were also described by Klöß et al. when cultivating NK cells (74).

In our study NK cell subsets were analyzed for the distribution of the CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} subpopulation. As all stimulation protocols induced an upregulation of the NK cell marker CD56, leading to a complete CD56^{bright} phenotype, only a discrimination of CD16^{+} and CD16^{-} NK cells was used to define distinct subpopulations in the resulting cell products. Upon cytokine stimulation we further observed an increase of the CD16^{-} NK cell population. This was in correlation with a previous report describing a reduction of CD16^{+} NK cells and an increase of CD16^{-} NK cells upon IL-15 and IL-15+IL-21 simulation (23). It has been shown earlier that CD16^{-} NK cells have a high proliferating capacity. Klingemann et al. reported that *ex vivo* culture of CD56 cells leads primarily to expansion of the CD16^{-} cell population, when stimulated with IL-2 or IL-2+IL-15 in X-VIVO^{TM}10 media for 14 days (75). Interestingly, our data showed that the increase of the CD16^{-} proportion was more pronounced when cells where generated by CD3/CD19-depletion and even led to an inverse distribution in the proportion of CD16^{-} and CD16^{+} NK cell subsets when cultured in NK MACS® compared to X-VIVO^{TM}10 medium (see **Tables 1–3**). This outgrowth of the CD16^{-} subpopulation correlated with a significantly increased expansion capacity when NK cells were cultured in NK MACS® medium, indicating that CD16^{-} NK cells are the major subpopulation stimulated under these given cell culture conditions.

When addressing the impact of different culture conditions on the NK cell proliferation capacity, the culture in the presence of NK MACS® led to the highest expansion rates, while expansion was significantly lower in the other tested media X-VIVO^{TM}10. This observation is in line with a report by Klöß et al. on a detailed comparison of different GMP-compliant expansion media. In addition, they also describe an altered proportion of CD16^{-} and CD16^{+} NK cell subsets in all culture batches containing NK MACS® medium, with in median 49.6% (range 42.2–51.5) CD16$^{dim/-}$ and 50.4% (range 48.6–57.2) CD16^{+} containing NK cells (74). These findings are also consistent with a study describing a wide range of CD16^{-}/CD16^{+} ratio between 0.2 and 2.3 (median 0.9) when CD3-depleted CD56-enriched NK cells were cultured in NK MACS® medium + IL-2/IL-15 and initial IL-21 for 14 days using the CliniMACS Prodigy device (73).

Regarding a future clinical application of NK cells stimulated by IL-15^{low}+IL-21 in NK MACS® for 15 days, the characterization of the final NK cell phenotype and function is of crucial interest. Importantly, cytotoxic activity did not differ between both cell culture medium conditions, as we have demonstrated in short- and long-term cytotoxicity experiments, thereby indicating that the CD16^{-} population also exerts potent killing mechanisms. Moreover, both NK cell subsets were capable of IFN-γ production as well as CD107a expression. Contact with NB tumor cells or cytokine stimulation with IL-12+IL-18, mimicking activation by dendritic cells, led both to a CD107a expression in both NK cell subpopulations, and was even more pronounced within the CD16^{-} NK cell population. Interestingly, in correlation to the natural NK cell function *in vivo,* the cytokine stimulus induced a high cytokine (IFN-γ) production in both NK cell subsets, while NK cells responded to the NB cell co-incubation with enhanced CD107a expression. Notably, especially the CD16^{-} NK cell population showed poly-functional characteristics being capable of both cytokine secretion and degranulation.

These findings are in line with recent publications investigating the systemic IL-15 administration in cancer patients. Dubois et al. describe a dramatic expansion with a 358-fold increase of CD56^{bright} NK cells exceeding the CD56^{dim} NK cell populations upon continuous i. v. IL-15 infusions. As expected, CD56^{bright} NK cells responded with increased cytokine release to various stimuli. Importantly, CD56^{bright} NK cells also gained the ability to kill various target cells. In addition, IL-15 treatment resulted in increased amounts of the cytolytic molecules granzymes A and B and perforin in CD56^{bright} NK cells, what conclusively led the authors to state that IL-15 infusions appear to equip CD56^{bright} NK cells to become cytotoxic and therefore enabled the whole NK cell population to mediate antitumor response (76, 77). Our results are also consistent with recent findings describing CD56^{bright} NK cells exhibiting potent antitumor responses with multiple cell functions such as degranulation, cytotoxicity and cytokine production following IL-15 priming (78). Wagner et al. postulate that IL-15 unleashes potent antitumor effector function of CD56^{bright} NK cells. Traditionally, CD56^{bright}CD16^{-} NK cells are described as cells with low antitumor responsiveness that are primarily involved in immune regulatory functions like cytokine

secretion. However, these studies in correlation to our report indicate that this definition may not be transferable to *in vitro* cytokine stimulated NK cells.

In addition, also receptors associated with cytotoxicity, i.e., DNAM1, NKp30 and particularly NKG2D which plays a crucial role in NK cell killing, were equally expressed in both NK cell subsets after IL-15^{low} and IL-15^{low}+IL-21 treatment. NKp44 and NKp46 even to a higher extend in CD16^{-} NK cells, underlining the gain in cytotoxic potential of this subset. In our study, we observed that an additional IL-21 boost to continuous IL-15^{low} treatment significantly enhanced NK cell cytotoxicity, which was even more pronounced when NK cells were cultured in NK MACS® medium. However, also in accordance with our previous reports, surface expression of the corresponding receptors did not correlate with these findings (23). Klöß et al. found no differences in the expression of the markers NKp30, NKp44, NKp46, and NKG2D between NK cells cultured in X-VIVO™10 and NK MACS® medium (74), while Oberschmidt et al. reported the upregulation of NKG2D, NKp30, and NKp44 on NK cells cultured for 14 days in NK MACS® medium with IL-2/IL-15 and initial IL-21 relative to unstimulated cells (73).

Overall, in our study we address whether donor NK cells may provide a potent cell-based immunotherapeutic approach to treat high-risk NB patients in addition to haploidentical SCT. We report on our efforts to optimize the cytokine stimulation protocol with regard to expansion rate and cytotoxic activity of NK cells generated from CD3/CD19-depleted products in comparison to CD56-enriched NK cells or CIK cells activated by our well-established manufacturing procedure. In sum, our data revealed that NK cells have a significantly higher cytotoxic potential to combat NB than CIK cell products. Although CIK cells have shown compelling results in the treatment of hematological malignancies, NK cells seem superior regarding the targeting of NB.

As an important step toward clinical application, these results could be verified in a first *proof of principle experiment* in which we analyzed NK cells from a clinical CD3/CD19-depleted G-CSF stimulated stem cell leukapheresis product using the IL-15^{low}+IL-21 stimulation protocol. Expansion rate, viability, and cytotoxicity against NB target cells were similar to NK cells isolated from PBMCs. On the base of our finding, for subsequent studies we favor the use of NK cells generated from CD3/CD19-depleted cell products stimulated in a 15 day expansion procedure using IL-15^{low}+IL-21 and NK MACS medium. Up-scaling experiments toward the translation into a GMP-compliant expansion protocol are ongoing, as well as the establishment of a Xenograft mouse model to verify our data *in vivo*.

As one major limitation of repeated NK cell immunotherapy is the generation of sufficient amounts of NK cells to apply clinically relevant doses, this optimized expansion and stimulation protocol to *ex vivo* cultivate NK cells from grafts of a haploidentical donors might be used in future clinical studies. In this context, the use of IL-15- and IL-21-expanded NK cells generated from CD3/CD19-depleted apheresis products seem to be highly promising as additional immunotherapy in combination with haploidentical SCT for high-risk NB patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Goethe University Frankfurt, Germany (approval no. 329/10). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH, CC, and EU conceived and designed the experiments. AH, BG, JF, TM, and LG performed the experiments. AH, BG, CC, and SH analyzed the data. EU, PB, and TK coordinated and supervised the research. CC, EU, ER, JF, SH, MM, and JS discussed the results. AH, BG, CC, EU, and MB wrote the paper with support from all co-authors. All authors approved the final version of 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.2019.02816/full#supplementary-material

Supplementary Figure 1 | Characterization of CIK and NK cells supplemental to **Figure 1**. **(A)** CD56-enriched NK cell products contained a median of 1.0 and 0.3% T cells and CD3/CD19-depleted NK cell products a median of 0.03% and 0.05% T cells following IL-2+IL-15 and IL-15 stimulation, respectively. T cell

contamination was significantly higher in CD56-enriched NK cell products and if IL-2 was added. Frequency of T cells within the CIK cell bulk increased following cytokine stimulation from 54.7% up to 84.2%. **(B)** CD56-enriched NK cell products contained a median of 0.5% and 0.16% NK-like T cells and CD3/CD19-depleted NK cell products in median 0.08% and 0.03% NK-like T cells following IL-2+IL-15 and IL-15 stimulation, respectively. Frequency of NK-like T cells within the CIK cell bulk increased following cytokine stimulation from 3.7% up to 6.2%. **(C)** CD56-enriched NK cell products contained a median 0.04% and 0.08% B cells and CD3/CD19-depleted NK cell products contained a median 0.1% and 0.1% B cells following IL-2+IL-15 and IL-15 stimulation, respectively. Frequency of B cells within the CIK cell bulk decreased following cytokine stimulation from 6.5% on day 0 to 0.06% ($n = 12$ independent experiments, median fold expansion rate day 10–12 compared to day 0), gated on viable cells (DAPI negative): $CD3^+$ T cells **(A)**, $CD3^+CD56^+$ NK-like T cells **(B)**, and $CD19^+$ B cells **(C)**. Differences were considered significant for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

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Harnessing NK Cells for Cancer Treatment

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In the last years, natural killer (NK) cell-based immunotherapy has emerged as a promising therapeutic approach for solid tumors and hematological malignancies. NK cells are innate lymphocytes with an array of functional competences, including anti-cancer, anti-viral, and anti-graft-vs.-host disease potential. The intriguing idea of harnessing such potent innate immune system effectors for cancer treatment led to the development of clinical trials based on the adoptive therapy of NK cells or on the use of monoclonal antibodies targeting the main NK cell immune checkpoints. Indeed, checkpoint immunotherapy that targets inhibitory receptors of T cells, reversing their functional blocking, marked a breakthrough in anticancer therapy, opening new approaches for cancer immunotherapy and resulted in extensive research on immune checkpoints. However, the clinical efficacy of T cell-based immunotherapy presents a series of limitations, including the inability of T cells to recognize and kill HLA-I^{neg} tumor cells. For these reasons, new strategies for cancer immunotherapy are now focusing on NK cells. Blockade with NK cell checkpoint inhibitors that reverse their functional block may overcome the limitations of T cell-based immunotherapy, mainly against HLA-I^{neg} tumor targets. Here, we discuss recent anti-tumor approaches based on mAb-mediated blocking of immune checkpoints (either restricted to NK cells or shared with T cells), used either as a single agent or in combination with other compounds, that have demonstrated promising clinical responses in both solid tumors and hematological malignancies.

Keywords: NK cells, NK cell receptors, immune checkpoint blockade, immunotherapy, solid tumors, hematological malignancies, adoptive NK cell therapy

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NK CELL: AN "EFFICIENT" TOOL FOR IMMUNOTHERAPY

Immunotherapy is an innovative approach for the treatment of cancer and is based on the idea of harnessing the immune system to target tumors. Recently, immunotherapy, and in particular immune checkpoint (IC) blockade therapy, has represented a significant step forward for cancer treatment (1–5). Two inhibitory ICs, CTLA-4 (6) and PD-1 (7), received great attention, since the inhibition of CTLA-4 or PD-1 signaling significantly improved the survival of patients with metastatic solid cancers. Given the clinical efficacy of PD-1 and/or CTLA-4 blockade in patients with untreatable solid and hematological cancers (1–5, 8), much attention has been given to IC receptors and their cognate ligands.

Currently, one of the major challenges in immune-oncology is the understanding of the mechanisms of IC inhibitor resistance (indeed, only a fraction of patients respond to immunotherapy), to increase the proportion of patients benefitting from such treatment, and to control treatment toxicity.

A critical point is that the clinical effect of the PD-1/PD-Ls blockade has been conventionally attributed to the restoration of cytotoxic lymphocyte activity. However, a partial or complete loss of HLA-I expression is one of the most frequent mechanisms of tumor escape from T-cell control in different human tumor types (9). In this scenario, the role of the "innate counterpart" of cytotoxic T cells, the natural killer (NK) cells, which show the ability to recognize and kill tumor cells regardless of HLA-I expression, appears to be crucial (10–12).

NK cells were first identified in the mid-1970s as a unique lymphocyte subset able to detect and rapidly kill abnormal cells without prior sensitization or recognition of specific tumor antigens, thus preventing the development of many cancers (13, 14). In the late 1980s, the observation that NK cells could kill a lymphoma cell line that had lost MHC class I surface molecules, while the original MHC class I+ cells were resistant to lysis, led to the formulation of the "missing self-hypothesis" that stated that NK cells are able to sense the absence of "self" MHC class-I molecules on target cells (15, 16). In the 1990s, this hypothesis was confirmed by the discovery of inhibitory (17, 18) and activating NK receptors (19). In humans, the main inhibitory receptors are represented by the inhibitory killer Ig-like receptors (KIRs), recognizing allotypic determinants shared by groups of HLA class-I alleles (20, 21) and by the CD94/NKG2A heterodimer (22), specific for the non-classical HLA-E molecule.

Inhibitory KIRs are type I molecules with two (KIR2D) or three (KIR3D) highly polymorphic extracellular Ig-like domains followed by long (L) cytoplasmic tails harboring two ITIMs, able to transduce an inhibitory signal through the recruitment of tyrosine phosphatases. The four main inhibitory KIRs are specific for epitopes shared by distinct groups of HLA class I allotypes. In particular, KIR2DL1 recognizes HLA-C2 epitope, while KIR2DL2/L3 recognizes HLA-C1 epitope. KIR3DL1 is specific for HLA-B or HLA-A molecules sharing the Bw4 public epitope (Bw4I80 or Bw4T80), and KIR3DL2 binds HLA-A*03 and -A*11 allotypes (18, 21).

The activating NK cell receptors include a series of non-HLA-specific receptors and co-receptors able to induce NK cell triggering by directly interacting with ligands overexpressed or expressed *de novo* on tumor-transformed or virus-infected cells (23–25).

These findings indicate that autologous cells are not killed by NK cells thanks to an appropriate expression of all self-HLA alleles, while a wide spectrum of tumor types can be killed due to the loss of HLA molecules and to the expression/overexpression of ligands for NK cell activating receptors (**Figure 1**). During NK cell differentiation, CD94/NKG2A is the first HLA-I-specific receptor expressed by appearing on the most immature CD56bright NK cell subset. After several maturation steps, CD56bright cells become CD56dim, lose NKG2A, and acquire KIR receptors (26–28). The most mature NK cells are KIR+ and NKG2A– and express the marker of terminal differentiation CD57 (29).

Under normal conditions, the HLA-I-specific inhibitory receptors recognize autologous cells and prevent auto-reactive responses. However, under pathological conditions, these receptors function as ICs, by blocking the cytotoxic activity of NK cells against those tumors that maintain the expression of HLA-I molecules (11, 30).

In order to restore NK cell activity against HLA-I+ tumor cells, novel immunotherapies have been developed, based on the use of therapeutic monoclonal antibodies anti-pan-KIR2D (lirilumab) (https://www.innate-pharma.com/en/pipeline/lirilumab-first-class-anti-kir-mab-licensed-bristol-myers-squibb) and anti-NKG2A (monalizumab) (https://www.innate-pharma.com/en/pipeline/monalizumab-anti-nkg2a-mab-partnered-astrazeneca) mimicking "missing-self" response by disrupting the interaction between these ICs and their ligands. Therefore, NK cells can efficiently kill tumor cells that have lost HLA-I expression, thus becoming resistant to T lymphocytes, but also HLA-I+ cancers when blockers of ICs are used (**Figure 1**). These agents are currently used in phase I/II clinical trials on a range of hematologic and solid tumors as monotherapy or in combination with other agents, including other forms of IC blockade (31–37).

Notably, NK cells may also express non-HLA class I-specific inhibitory receptors such as PD-1 (38). This receptor was originally discovered on T cells and was found to exert a sharp inhibitory effect on their anti-tumor activity. In healthy donors, PD-1 is expressed on a subset of fully mature (KIR+NKG2A–CD57+) NK cells from HCMV+ individuals (38). Higher proportions of PD-1+ NK cells can be detected in patients affected by different types of tumors (36, 38, 39).

The finding that NK cells from cancer patients express PD-1 IC coupled with the observation that the use of anti-PD-1 or anti-PD-L1 monoclonal antibodies improve the anti-tumor activity of NK cells (36, 38, 39) (**Figure 1**) is clinically relevant for patients with tumors displaying a T-cell-resistant (HLA class Ineg) phenotype.

Recent data strongly suggest a possible role for NK cells in immunotherapeutic strategies targeting the PD-1/PD-L1 axis particularly against HLA-I-deficient tumor cells (40, 41).

NK cells also express additional constitutive or inducible IC shared with T cells, recognizing additional ligands other than HLA class I molecules. These include CTLA-4, T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif domains (TIGIT), and CD96 (12, 42–44).

Here, we review recent developments to improve NK cell responses against solid and hematological tumors mainly focusing on NK cell ICs.

NK CELL-BASED THERAPY IN SOLID TUMORS

Although the ability of NK cells to destroy solid tumors has been questioned, their capacity to prevent metastatic

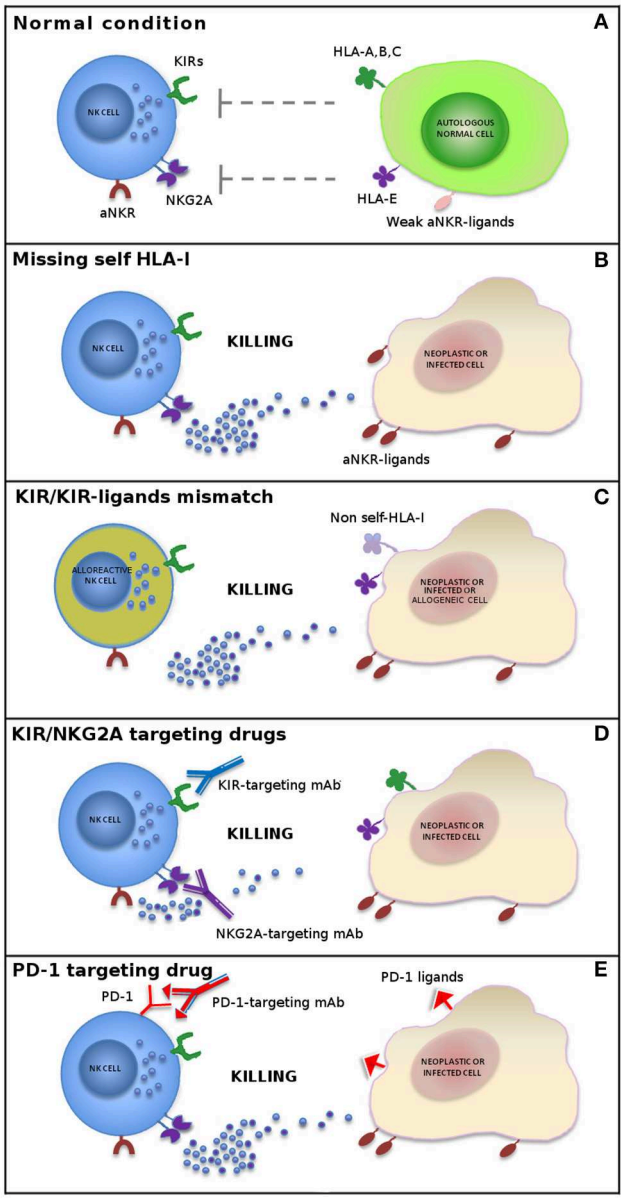

FIGURE 1 | Mechanisms of NK cell-mediated killing. In physiological conditions, NK cell activity is tightly regulated by a complex interplay between inhibitory and activating receptors that prevents killing of normal autologous cells expressing an appropriate level of all self-HLA alleles and low/negative levels of ligands for non-HLA-specific activating receptors (aNKR) **(A)**. Downregulation of HLA-I molecules on neoplastic or infected cells induces NK-mediated killing by a "missing-self" recognition mechanism. NK cell activating receptors are co-responsible in inducing NK cell triggering by interacting with ligands (aNKR-ligands) overexpressed or expressed *de novo* on tumor-transformed or virus-infected cells **(B)**. Allogeneic (alloreactive) donor NK cells are able to kill neoplastic cells of the recipient expressing non-self allotypic determinants on HLA-I molecules ("KIR/KIR-ligand mismatch") and to control infections with a limited risk of toxicity (e.g., GvHD and HvG) **(C)**. The use of inhibitors of classical NK cell immune checkpoints (i.e., KIR and NKG2A) **(D)** or immune checkpoints shared with T cells (e.g., PD-1) **(E)** or, finally, a combination of these approaches represents new promising strategies in NK cell-based immunotherapy.

dissemination by killing circulating cancer cells is well-known. However, tumor cells frequently develop strategies to evade NK cell immunosurveillance including changes at the tumor cell level (e.g., abnormal expression of ligands for activating and inhibitory receptors) and changes in tumor microenvironment (e.g., immunosuppressive cytokines), resulting in tumor escape and cancer progression (12, 45–48). Exact mechanisms and manipulation strategies to durably and reproducibly enhance NK cell function *in vivo* are not known. However, the use of IC blockade, including lirilumab and monalizumab, to create a condition of "missing-self" recognition (consequent to the antibody-mediated disruption of pan-KIR2D or NKG2A/HLA-I interactions) may represent a promising novel therapeutic approach to cure tumor patients (49).

HLA-E is one of the emerging suppressive ligands in human tumors, and its expression negatively correlates with the overall survival (OS) of cancer patients (48). NKG2A is expressed on NK cells but also on T cells infiltrating different types of solid tumors (36, 50, 51). These findings suggested that NKG2A/HLA-E interaction could suppress the cytotoxic lymphocyte functions directly in the tumor microenvironment.

The IgG4 anti-NKG2A antibody monalizumab is currently in clinical development for the treatment of various solid tumors, either as single-agent or in combination with other compounds. In the initial clinical experiences, single-agent intravenous monalizumab was administered to patients affected by advanced gynecologic malignancies (including ovarian, endometrial, and cervical carcinomas), divided into a dose-ranging cohort and an expansion cohort, for a total of 58 patients. The drug was generally well-tolerated but achieved only short-term disease stabilization as best response (NCT02459301) (37).

In another phase II study, monalizumab was administered in combination with the anti-epithelial growth factor receptor (EGFR) antibody cetuximab in patients affected by squamous cell carcinoma of the head and neck (SCCHN) (NCT02643550). Cetuximab represents an established therapeutic approach to SCCHN acting through induction of antibody-dependent cell cytotoxicity through CD16 (FcγRIII) receptor expressed on NK cells (52). The rationale of this combination lies on the evidence that SCCHN tumors were strongly positive for HLA-E and were infiltrated with CD8+ T and NK cells, suggesting a potential sensitivity to NKG2A inhibitors. The regimen was well tolerated, being characterized mostly by grade 1–2 adverse events, and the interim analysis reported an overall response rate (ORR) of 31% and a disease stabilization rate of 54%. Although preliminary, these data appear encouraging (37).

With regard to the combination of anti-NKG2A with PD-1/PD-L1 disrupting agents, a combination of monalizumab and durvalumab has been evaluated in a first-in-human dose-escalation/dose-expansion phase I trial in patients with metastatic microsatellite-stable colorectal cancer (MSS-CRC). The rationale of this study was supported by preclinical models (https://www.innate-pharma.com/sites/default/files/180205asco_15poster_09.pdf) and was based on the hypothesis that the inhibition of NKG2A might improve the efficacy of an

anti-PD-L1 antibody in a patient population characterized by poor response to PD-1/PD-L1 antibodies. The study included 40 patients in the MSS-CRC expansion cohort. The treatment was well-tolerated; in the expansion cohort, three responses and 11 disease stabilizations were observed, with a disease control rate of 24% at 16 weeks (https://ascopubs.org/doi/abs/10.1200/JCO.2018.36.15_suppl.3540). Currently, other clinical trials involving the combination of monalizumab with durvalumab in different solid tumors are ongoing (NCT03794544; NCT02671435).

The efficacy and safety of the first-in-class anti-pan-KIR2D agent lirilumab was explored in several clinical trials. In a first-in-human phase I trial, escalating doses of lirilumab were administered to patients with solid tumors (breast, kidney, or ovarian carcinoma) or hematologic malignancies. No dose-limiting toxicities were reported and maximum tolerated dose was not reached for doses up to 10 mg/kg (53). With regard to combinations including lirilumab, a phase I/II trial explored the safety of increasing doses of lirilumab in combination with the anti-PD-1 antibody nivolumab or with the anti-CTLA-4 antibody ipilimumab in a population of patients with solid tumors (136 with nivolumab; 22 with ipilimumab). Both combinations were well-tolerated, encouraging further developments. Although definitive efficacy results are not available yet, data from 29 patients with SCCHN in the lirilumab-nivolumab cohort showed an ORR equal to 24%, with durable responses. Notably, increased PD-L1 expression was strongly associated with improved probability of objective response (https://news.bms.com/press-release/bristolmyers/interim-phase-12-data-show-encouraging-clinical-benefit-lirilumab-combina). Currently, other trials designed to explore the potential role of lirilumab plus nivolumab are being conducted in populations of patients with SCCHN and invasive bladder cancer in the neoadjuvant setting (NCT03532451 and NCT03341936).

Interestingly, in those tumors resistant to anti-PD-1 immune-therapies, an up-regulation of alternative immune checkpoints, including TIM-3, has been observed. In this context, therapeutic approaches combining the administration of anti-TIM-3 and anti-PD-1 antibodies showed that the adaptive resistance to PD-1 blockade can be overcome (54).

TIM-3 is a checkpoint receptor that binds several ligands including galectin-9 (Gal-9) (55), phosphatidylserine on apoptotic cells (56), high mobility group box 1 (56), and CEA-related cell adhesion molecule-1 (57). High frequencies of circulating and/or tumor infiltrating TIM-3+ NK cells have been found in different types of malignant tumors (58–60). The increased surface levels of TIM-3 on NK cells in cancers induce NK cell impairments (61), while TIM-3 blockade results in increased NK cell cytotoxicity both *in vitro* and *ex vivo* (59, 62, 63).

TIM-3 functions as a potential prognostic marker in several tumor types, such as lung adenocarcinoma, gastric cancer, bladder cancer, and esophageal cancer (58, 59, 62, 63).

On the other hand, contradictorily, studies have also reported stimulatory functions of TIM-3 (64). These divergent functions are likely associated with the existence of multiple and different TIM-3 ligands.

An anti-TIM-3 (Sym023) antibody has been developed and is currently being tested in phase I clinical trials in patients with advanced, unresectable, and metastatic solid tumor malignancies or lymphomas that are refractory to currently available therapies, in monotherapy or in combination with anti-PD-1 or anti-LAG-3 antibodies (NCT03489343 and NCT03311412). Additional phase I studies of anti-TIM-3 antibodies have been activated in patients with advanced solid tumors, as a monotherapy or in combination with an anti-PD-1 antibody (NCT02817633, NCT03680508, NCT04139902, and NCT03744468).

LAG-3 is a negative co-inhibitory receptor expressed on T cells and NK cells that binds MHC class II (MHC-II) molecules, the C-type lectin receptor LSECtin, and a fibrinogen-like protein 1 (FGL1) on target cells (65–68). LAG-3 has been shown to suppress immune responses in several tumors, including Hodgkin's lymphoma, gastric cancer, breast cancer, and other solid tumors (69). Thus, the use of anti-LAG-3 antibodies in combination with anti-PD-1 immunotherapy has been proposed to restore T cell function (70). Although the specific role of LAG-3 on NK cells remains to be fully clarified, this inhibitory immune checkpoint is currently considered a good target for immunotherapy because of its potential to activate both T and NK cells. In this context, different anti-LAG-3 antibodies are currently being used in phase I and phase II clinical trials as single drugs in metastatic cancer, solid tumor, and lymphoma (NCT03489369 and NCT03250832) or in association with other immune checkpoints inhibitors, including anti-TIM-3 antibody, in multiple myeloma patients (NCT04150965), with anti-PD-1 antibodies in treating patients with glioblastoma (NCT02658981), solid tumors (NCT01968109), advanced malignancies including lymphoma (NCT03005782), and SCCHN (NCT04080804) or other anti-PD-1 agents in solid tumor patients (NCT02676869). A number of additional LAG-3 antibodies are currently in preclinical development.

TIGIT and CD96 are co-inhibitory receptors expressed on both T and NK cells and compete with the activating NK cell receptor DNAM-1 for binding to PVR (CD155) and Nectin-2 (CD112) (71). These receptors participate in a balanced system to control NK cell effector functions. Indeed, following interaction with their ligands, TIGIT inhibits NK cell cytotoxicity directly through its ITIM domain, whereas CD96 hampers NK cell IFN-γ production (72), thus counterbalancing DNAM-1-mediated activation. The expression of TIGIT is highly variable among different cancer types. It has been recently demonstrated that TIGIT is highly expressed on tumor-infiltrating NK cells and associated with NK cell exhaustion in different tumor models and patients with colon cancer (73). Notably, the therapeutic effects of anti-TIGIT, anti-PD-L1, or anti-TIGIT antibodies combined with anti-PD-L1 antibodies depended on the presence of NK cells (73), indicating the importance of NK cells in checkpoint-targeted immunotherapy. Currently, several ongoing clinical trials (phase I and phase II) focus on testing the feasibility of targeting TIGIT pathway and improving therapeutic effects through combination with existing immunotherapies, including anti-PD-1 agents (NCT04150965, NCT03119428, NCT04047862, and NCT03563716), mainly in solid tumor patients.

Recently, results from clinical studies have demonstrated the safety of the infusion of allogeneic NK cells for immunotherapy of both hematological malignancies and solid tumors (74) despite the microenvironment rich of NK-inhibiting factors and the limited ability of infiltration of immune cells. While generally safe, allogenic NK cell infusions generally showed poor anti-tumor activity.

Therefore, strategies designed to improve the efficacy of adoptive transfer of NK cells are currently being explored and include associations with IC inhibitors or chemotherapy, induction of chemokine expression that can improve NK cell migration and trafficking into tumors, as well as concurrent administration of cytokines with activating effect on NK cells. However, while some of such approaches achieved encouraging results, none of these has evolved into an established protocol; hence, additional efforts in this setting are warranted (75–79). Relevant trials are reported in **Table 1**.

NK CELLS TO TREAT HEMATOLOGICAL MALIGNANCIES

Current approaches of NK cell immunotherapy for hematological malignancies involve methods for *in vivo* potentiation of NK cell proliferation and activity; adoptive transfer of NK cells from autologous and allogeneic sources, and NK cell lines; genetic modification of NK cells; and, similar to solid tumor, the use of IC blockade (12, 83).

Following the introduction of haploidentical hematopoietic stem cell transplantation (haplo-HSCT), the potential anti-leukemic activity of donor-derived alloreactive NK cells has been observed (84–87). After engraftment, NK cells are the first lymphocyte subset that appears in peripheral blood, suggesting their role as graft-vs.-leukemia effector cells, in the absence of GvHD (84, 88–90). The generation of alloreactive NK cells (i.e., NK cells expressing inhibitory KIRs that are not engaged by any of the HLA-I alleles present on allogeneic target cells) results in improved clinical outcome in haplo-HSCT thanks to their ability to kill not only leukemic blasts (GvL) but also patient dendritic cells (DCs) (91) and T cells, thus preventing GvHD and HvG reaction, respectively (92–95).

Given the crucial role of alloreactive NK cells in mediating safe and durable anti-tumor immunity in patients receiving KIR/HLA-C-mismatched transplantation, different strategies, including miRNA targeting the expression of HLA-C-specific KIRs (i.e., KIR2DL1, KIR2DL2/L3) (96) or therapeutic antibody blocking these inhibitory KIRs (lirilumab) for HLA-C, were generated to simulate the mechanism of "missing self" condition.

The IgG4 antibody lirilumab prevents inhibitory signals from pan-KIR2D, increasing NK cell-mediated tumor killing of AML *in vitro* and *in vivo* (31). This anti-pan-KIR2D antibody had acceptable safety without significant toxicity or autoimmunity in AML and multiple myeloma (MM) patients (33, 97). Although lirilumab failed to show significant efficacy as a monotherapy in MM, dual immune therapy with lenalidomide showed a good response on relapsed/refractory (R/R) MM in clinical trials (81, 97). Lirilumab is currently being widely tested in combination with other therapeutics, including rituximab (an anti-CD20 antibody), and other forms of IC blockade, such as nivolumab in R/R non-Hodgkin's lymphoma, Hodgkin's lymphoma (HL), and MM patients (NCT01592370) (34, 97).

In R/R HL patients (98–102), IC inhibitors have shown good activity; indeed, nivolumab is currently approved for this indication by the Food and Drug Administration and the European Medicines Agency (103). Interestingly, the Hodgkin neoplastic cells (Reed Sternberg cells) hyper-express PD-L1 but have low/negative expression of HLA-I molecules. This means that NK cells that exert their killing activity mainly against HLA-Ineg targets, but not cytotoxic T CD8+ cells, may be the primary effectors in the immune response induced by nivolumab in HL patients. In this view, an increase in cytotoxic NK cell population during IC blockade treatment in HL patients has been recently observed (104). In order to better elucidate the exact mechanism of action of nivolumab in HL and to improve the efficacy in terms of complete response (CR) in R/R HL, an innovative clinical protocol based on the combined application of high-dose chemotherapy with autologous stem cell transplant (ASCT) and early post-transplant administration of nivolumab, supported by autologous lymphocytes re-infusions (ALI), has been recently proposed (https://doi.org/10.1182/blood-2018-99-118901). Preliminary observations support the hypothesis that NK cells play a primary role in response to nivolumab in HL patients (82, 104). Furthermore, from a clinical point of view, preliminary data are encouraging, as all six R/R patients treated so far achieved CR (82).

Leukemic cells have been shown to overexpress HLA-E, a ligand for NKG2A. This provides the rationale for the use of monalizumab for the treatment of leukemia. The use of monalizumab in HSCT has recently also been proposed to induce NK cell alloreactivity in the first weeks after transplant when almost all reconstituting NK cells are NKG2Apos, in order to limit opportunistic infection and leukemia relapse (105). Moreover, monalizumab is currently under evaluation in several phase I/II clinical trials in monotherapy (35) or in combination with other therapeutic antibodies, such as the Bruton's tyrosine kinase inhibitor ibrutinib, in patients with R/R or previously untreated chronic lymphocytic leukemia (NCT02557516).

A phase I clinical trial evaluating the hypomethylating agent decitabine together with either PDR001 (anti-PD-1 antibody), MBG453 (anti-TIM-3 antibody), or their combination is currently recruiting patients with R/R AML patients not eligible for intensive therapy, as well as high-risk myelodysplastic patients (NCT03066648).

Another anti-TIM-3 antibody (Sym023) is currently being tested in phase I clinical trials in patients with lymphoma refractory to currently available therapies, in monotherapy or in combination with anti-PD-1 (Sym021) or anti-LAG-3 (Sym022) antibodies (NCT03489343 and NCT03311412).

The anti-LAG-3 antibody BMS-986016 is currently being used in phase I and phase II clinical trials in combination with nivolumab in subjects with relapsed or refractory HL, and relapsed or refractory diffuse large B cell lymphoma

TABLE 1 | Relevant clinical trials involving immune checkpoint blockade in NK cells.

Trial	Targets	Setting	Agent	Phase	Pts	Results
SOLID TUMORS						
Tinker et al. (37)	NKG2A	Advanced gynecological solid tumors	Monalizumab	I	58	Manageable safety profile; short term response
NCT02643550	NKG2A; EGFR	Advanced squamous cell carcinoma of the head and neck	Monalizumab plus cetuximab	I/II	31	Manageable safety profile; ORR: 31%; DCR: 54%
Segal et al. (80)	NKG2A; PD-L1	Metastatic microsatellite-stable colorectal cancer	Durvalumab plus monalizumab	I	40	Ongoing; preliminary data show manageable safety profile and DCR: 24% at 16 weeks
NCT03794544	NKG2A; PD-L1	Resectable non-small cell lung cancer	Durvalumab plus monalizumab	II	160 (estimated)	Ongoing
Vey et al. (53)	KIR2D	Solid and hematologic malignancies	Lirilumab	I	37	No reported dose-limiting toxicity
NCT03203876	KIR2D; PD-1; CTLA-4	Solid tumors	Lirilumab plus nivolumab with or without ipilimumab	I/II	21 (estimated)	Ongoing
NCT03532451	KIR2D; PD-1	Resectable bladder cancer	Nivolumab with or without lirilumab	I	43 (estimated)	Ongoing
NCT03341936	KIR2D; PD-1	Resectable squamous cell carcinoma of the head and neck	Nivolumab plus lirilumab	II	58 (estimated)	Ongoing
NCT03489343	TIM-3	Advanced solid tumors or lymphomas	Sym023	I	48 (estimated)	Ongoing
NCT03311412	TIM-3; LAG-3	Advanced solid tumors or lymphomas	Sym021 with or without Sym022 or Sym023	I	102 (estimated)	Ongoing
NCT02817633	TIM-3	Advanced solid tumors	TSR-022	I	873 (estimated)	Ongoing
NCT03680508	TIM-3; PD-1	Liver cancer	TSR-022 plus TSR-042	II	42 (estimated)	Ongoing
NCT04139902	TIM-3; PD-1	Resectable melanoma	TSR-042 with or without TSR-022	II	56 (estimated)	Ongoing
NCT03744468	TIM-3; PD-1	Solid tumors	BGB-A425 plus tislelizumab	I/II	162 (estimated)	Ongoing
NCT03489369	LAG-3	Advanced solid tumors or lymphomas	Sym022	I	30 (estimated)	Ongoing
NCT03250832	LAG-3	Advanced solid tumors	TSR-033 alone or in combination with PD-1 blocking agents	I	200 (estimated)	Ongoing
NCT04150965	LAG-3; TIGIT	Multiple myeloma	Elotuzumab	I/II	104 (estimated)	Ongoing
NCT02658981	LAG-3; PD-1	Recurrent glioblastoma	BMS-986016 with or without nivolumab	I	100 (estimated)	Ongoing
NCT01968109	LAG-3; PD-1	Advanced solid tumors	BMS-986016 with or without nivolumab	I/II	2,000 (estimated)	Ongoing
NCT03005782	LAG-3; PD-1	Advanced solid tumors	REGN3767 with or without REGN2810	I	589 (estimated)	Ongoing
NCT04080804	PD-1; LAG-3; CTLA4	Advanced head and neck squamous cell carcinoma	Nivolumab with or without BMS-986016 or ipilimumab	II	60 (estimated)	Ongoing
NCT02676869	LAG-3; PD-1	Advanced melanoma	IMP321 plus pembrolizumab	I	24 (estimated)	Ongoing
NCT03119428	TIGIT; PD-1	Advanced solid tumors	OMP-313M32 with or without nivolumab	I	33 (estimated)	Ongoing
NCT04047862	TIGIT; PD-1	Advanced solid tumors	BGB-A1217 plus tislelizumab	I	39 (estimated)	Ongoing
NCT03563716	TIGIT; PD-L1	Advanced non-small cell lung cancer	MTIG7192A plus atezolizumab	II	135 (estimated)	Ongoing
HEMATOLOGICAL MALIGNANCIES						
Vey et al. (33)	KIR2D	Acute myeloid leukemia	IPH2101	I	23	Manageable safety profile
Korde et al. (81)	KIR2D	Smoldering multiple myeloma	IPH2101	II	9	Failure to meet the primary endpoint (50% decline in M-protein)
NCT01592370	KIR2D; PD-1	Multiple myeloma	Lirilumab plus nivolumab (among multiple arms including nivolumab)	I/II	375 (estimated; multiple arms)	Ongoing

(Continued)

TABLE 1 | Continued

Trial	Targets	Setting	Agent	Phase	Pts	Results
Guolo et al. (82)	PD-1	Relapsed or refractory Hodgkin lymphoma	Nivolumab supported by the reinfusion of unselected autologous lymphocytes	I/II	7	Manageable safety profile; fast immune recovery
NCT02557516	KIR2D	Chronic lymphocytic leukemia	Monalizumab plus ibrutinib	I/II	22 (estimated)	Ongoing
NCT03066648	PD-1; TIM-3	Acute myeloid leukemia; high-risk myelodysplastic syndrome	PDR001 and/or MBG453 in combination with Decitabine	I	235 (estimated)	Ongoing

References and additional details can be found in the text. For published trials, we included the name of the first author, while for ongoing trials, we included NCT identifier. The trials are reported in the same order of the main text. Pts, patients.

(DLBCL) (NCT02061761) or as a single drug in lymphoma (NCT03489369). Relevant trials are reported in **Table 1**.

CONCLUDING REMARKS

Several molecular mechanisms regulating the anti-tumor activity of NK cells have been discovered over the last decades. However, further characterization of the main immunosuppressive pathways developed by tumor cells to evade NK cell recognition is still needed. Analysis of NK cells and tumor cells and their relationship is necessary to define personalized immunotherapy procedures in cancer patients.

The combined blockade of checkpoint molecules expressed by T cells and NK cells could trigger antitumor immunity mediated by innate and adaptive populations, allowing the two approaches to complement each other. NK cell-targeted immunotherapy may provide an alternative, or a complementary approach, to overcome the limitations of T-cell immunotherapy. In addition, combination with NK cell immunotherapy could increase the response rate of treatments targeting T cells (**Figure 1** and **Table 1**).

In conclusion, considering the excellent outcome of some patients, future efforts should be addressed to identify the best inhibitory pathways to target for future clinical applications. Moreover, further studies should aim at improving NK cell-based immunotherapy by targeting the tumor-induced NK cell inhibition, thus promoting the maximal anti-tumor effect of these innate effectors.

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

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Exploiting Human NK Cells in Tumor Therapy

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NK cells play an important role in the innate defenses against tumor growth and metastases. Human NK cell activation and function are regulated by an array of HLA class I-specific inhibitory receptors and activating receptors recognizing ligands expressed *de novo* on tumor or virus-infected cells. NK cells have been exploited in immunotherapy of cancer, including: (1) the *in vivo* infusion of IL-2 or IL-15, cytokines inducing activation and proliferation of NK cells that are frequently impaired in cancer patients. Nonetheless, the significant toxicity experienced, primarily with IL-2, limited their use except for combination therapies, e.g., IL-15 with checkpoint inhibitors; (2) the adoptive immunotherapy with cytokine-induced NK cells had effect on some melanoma metastases (lung), while other localizations were not affected; (3) a remarkable evolution of adoptive cell therapy is represented by NK cells engineered with CAR-targeting tumor antigens (CAR-NK). CAR-NK cells complement CAR-T cells as they do not cause GvHD and may be obtained from unrelated donors. Accordingly, CAR-NK cells may represent an "off-the-shelf" tool, readily available for effective tumor therapy; (4) the efficacy of adoptive cell therapy in cancer is also witnessed by the αβT cell- and B cell-depleted haploidentical HSC transplantation in which the infusion of donor NK cells and γδT cells, together with HSC, sharply reduces leukemia relapses and infections; (5) a true revolution in tumor therapy is the use of mAbs targeting checkpoint inhibitors including PD-1, CTLA-4, the HLA class I-specific KIR, and NKG2A. Since PD-1 is expressed not only by tumor-associated T cells but also by NK cells, its blocking might unleash NK cells playing a crucial effector role against HLA class I-deficient tumors that are undetectable by T cells.

Keywords: NK cells, inhibitory checkpoints, innate immunity, immunotherapy, anti-tumor therapy

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INTRODUCTION

Natural killer (NK) cells play a central role in innate defenses against viruses and tumors. They belong to a family of innate lymphoid cells (ILC) that do not express receptors encoded by rearranged genes. NK cell function is regulated by an array of inhibitory and activating receptors. Inhibitory receptors that play a major role in the control of NK cell function are those specific for HLA-class (Cl)-I molecules. Killer Ig-like receptors (KIRs) recognize allotypic determinants shared by different HLA-Cl-I alleles, while CD94/NKG2A is specific for the non-classical HLA-E (1). The

fact that NK cell inactivation is required to spare healthy cells implied the existence of activating receptors recognizing ligands on target cells. The prototypes and most important ones in tumor cell detection and killing are NKp46, NKp44, and NKp30, collectively called "natural cytotoxicity receptors" (NCRs) (2). While in an autologous setting all NK cells express one or more inhibitory receptors for self HLA-Cl-I, in an allogeneic setting, it may occur that KIRs expressed by a subset of NK cells do not recognize HLA-Cl-I alleles on allogeneic cells, and the lack of inhibition may result in killing of target cells (3). NK cells with these characteristics were named "alloreactive" NK cells. Although NK cells display a potent anti-tumor activity and are thought to participate to the control of tumor growth and metastatic spread, the tumor microenvironment may sharply inhibit their effector functions (4, 5). This inhibitory effect is due to the tumor cells themselves as well as to other cells present in the tumor microenvironment and frequently involves both the downregulation of activating surface receptors and the *de novo* expression of inhibitory checkpoints (primarily PD-1) (6, 7). In this contribution, we will briefly discuss different therapeutic strategies (**Table 1**), which allow to successfully exploit NK cell-mediated anti-tumor activity as well as novel promising approaches that may offer important new tools in cancer treatment.

BOOSTING *IN VIVO* NK CELLS WITH IMMUNE STIMULATORY CYTOKINES

In cancer patients, NK cells frequently display an impaired function (6, 27). Thus, primary strategies in immunotherapy are aimed to boost *in vivo* NK cell-mediated antitumor activity. One approach is based on the *in vivo* administration of cytokines, such as IL-2 and IL-15, that determine NK cell activation, differentiation, and expansion (8, 28–32). IL-2 administration was approved in the 1990s for the treatment of metastatic RCC and melanoma patients (33–35). Two major obstacles in IL-2-based therapy are the dose-associated toxicity (primarily vascular leakage) and the induction of T regulatory (Treg) cell activation and expansion, thus resulting in inhibition of NK cell function (9, 10). Recently, IL-2 variants, with lower affinity for IL-2Rα subunit (highly expressed by Treg cells), have been designed (11, 36, 37). In addition, PEGylated IL-2 (also known as NKTR-214) that binds CD122 (IL-2Rβ), expressed by both T and NK cells, is able to boost preferentially these cells and their anti-tumor responses. This therapeutic treatment is currently under investigation in clinical trials for solid tumors (13). The use of IL-15 may represent a better therapeutic option as it can selectively sustain NK cells without inducing Treg expansion. However, the clinical use of IL-15 is limited because of its short *in vivo* half-life (38). Notably, IL-15 induces a rapid expansion of memory $CD8^+$ T cells, thus favoring anti-tumor activity. The effect of IL-15 administration combined with checkpoint inhibitors (anti-CTLA-4 and/or anti-PD-1 mAbs) is currently under investigation in patients with cancers refractory to other therapies. To improve the anti-tumor effect of NK cells, ALT-803, an IL-15 superagonist complex, is also being assessed in phase I studies either alone (14) or in combination with checkpoint inhibitors (39). An emerging approach is based on bi- or tri-specific killer cell engagers (BiKEs and TriKEs) binding CD16 or NKG2D on NK cells and tumor antigens, thus favoring the interaction between NK cells and tumor cells. Notably, "TriKEs" also contain a modified IL-15 linker to improve NK cell survival and proliferation (15, 40, 41). An additional prospect is the use of IL-12, a molecule that enhances cytokine production and cytotoxicity by NK cells (16).

TABLE 1 | Human NK cell-based immunotherapeutic approaches in tumors.

1. **Adoptive NK cell therapies**
- Infusion of IL-2- or IL-15-activated NK cells or lymphokine-activated lymphocytes (LAK and CIK) (8–11);
- Infusion of allogeneic "off-the-shelf" CAR-NK cells directed to tumor antigens (12).

2. **NK cells in haplo-HSCT to cure high-risk leukemia**
- Transplant of "pure" donor $CD34^+$ cells. $NKG2A^+$ NK cells are detectable after 2 weeks, while KIR^+, cytolytic NK cells only after 6–8 weeks. Central role of NK cells in GvL, especially of "alloreactive" NK cells (13, 14);
- Transplant of αβT- and B cell-depleted mononuclear cells. Donor NK cells and γδT cells, being present in the graft, are immediately available for the control of infections and leukemia relapses. Better clinical outcome, particularly in AML (15–19).

3. **mAbs blocking inhibitory checkpoints in NK cells**
- The disruption of PD1/PD-L1 interactions unleashes both $PD1^+$ T and NK cells. Major effect of NK cells in case of HLA-Cl-I^- tumors (20–24);
- Blocking of NKG2A expressed by both NK and tumor-infiltrating T cells results in killing of HLA-E^+ tumors (i.e., most tumors) (25, 26);
- Combined blocking of NKG2A and PD1 in case of PD-$L1^+$ tumors (25, 26);
- Combined use of NKG2A-blocking mAb and mAb specific for tumor antigens (e.g., EGFR): "unlocked" NK cells mediate ADCC (25, 26).

ADOPTIVE IMMUNOTHERAPY WITH CYTOKINE-INDUCED NK CELLS

Clinical trials have been attempted since 1980s in which NK cell-containing cell suspensions isolated from patients with metastatic melanomas were expanded *in vitro* in the presence of IL-2 and infused back into the patients. While a relevant effect was detected in some cases, primarily in metastatic lesions such as lung metastases, other tumor localizations were not affected. These studies were important because they provided the first evidence that such "lymphokine-activated killers" (LAK) could exert their anti-tumor effect also *in vivo*. Relevant toxicity was mostly related to the concomitant administration of high dosages of IL-2 (17, 18). Evolutions of such pioneering studies, based on adoptive cell therapy, were the use of IL-15 and, recently, the use of NK cells engineered with chimeric antigen receptor (CAR, see below). Although NK cells to be used in adoptive tumor therapy are usually derived from peripheral blood (PB), other sources have also been proposed. For example, the pleural fluid of primary or metastatic tumors contains high numbers of functional NK cells (19), which acquire strong cytotoxicity upon short culture intervals with IL-15 or IL-2 *in vitro*. Since large volumes of such fluids are routinely discarded, NK cells could be recovered and reinfused systemically or in the pleural cavity after *in vitro*

culture with IL-15 (8). Such loco-regional treatment might contribute to the control of pleural localizations of the tumor. In general, the infusion of potent effector cells with anti-tumor activity is an important approach in tumor immunotherapy because it may greatly amplify the effect of endogenous cells. The relevance of infusing mature effector cells in cancer patients is also underscored in the αβT- and B-cell-depleted haploidentical hematopoietic stem cell transplantation (HSCT), in which leukemia relapses and infections are sharply reduced as compared to the HSCT setting with "purified" $CD34^+$ cells, thanks to the co-infusion of mature γδT cells and NK cells (see below).

ROLE OF NK CELLS IN THE THERAPY OF HIGH-RISK LEUKEMIA IN HAPLOIDENTICAL HSCT

HSCT represents the life-saving therapy for acute leukemia poorly responsive to chemotherapy, relapsing, or with adverse cytogenetic characteristics. Unfortunately, it is possible to find a HLA compatible donor only for ∼60% of patients (42, 43). Thus, T-depleted haplo-identical HSCT has been developed in an attempt to rescue those patients for whom no alternative therapeutic option is available. Haplo-HSCT is based on the infusion of "megadoses" of purified $CD34^+$ cells extensively depleted of T cells in order to avoid life-threatening GvHD. In this transplantation setting, donor NK cells may express KIR that do not recognize any of the HLA-Cl-I alleles of the patient (44, 45). Notably, NK cells are the first donor lymphoid cells detectable in patients' PB after transplantation. In pediatric patients, this occurs after ∼2 weeks. However, such NK cells are represented by relatively immature $CD56^{bright}$ cells, expressing NKG2A but not KIR (KIR expression is required for NK cell alloreactivity). Appearance of mature KIR^+ NK cells requires an additional 4–6 weeks. In this T-depleted HSCT setting, NK cells play a major role in graft-vs.-leukemia (GvL) (46). The anti-leukemia effect has been related to NK cell alloreactivity in different studies, pioneered by Ruggeri et al., in adult AML (20, 21, 45). Indeed a clear correlation was found between the frequency of alloreactive NK cells and the clinical outcome (22, 44). Of note, a subset of NK cells derived from CMV-seropositive donors could undergo expansion in transplanted patients upon CMV reactivation after HSCT. These NK cells expressed NKG2C, CD57, and displayed epigenetic modifications identical to those present in memory T cells. These characteristics confer to NK cells a strong cytolytic activity, including a "memory-like" behavior in response to NKG2C triggering, and are associated to a better clinical outcome (23–25, 47, 48). In pediatric patients receiving "megadoses" of purified $CD34^+$, the survival probability at 5 years was very good for patients with high-risk ALL, reaching over 70% in the presence of NK alloreactivity and ∼40% in its absence, the overall survival being ∼60%. In patients with AML, survival reached ∼40% in case of NK alloreactivity, but only ∼20% in its absence, the overall survival being ∼30%. Notably, all deaths occurred early, during the first few weeks/months after transplant, primarily due to leukemia relapses or infections (20, 49). In an attempt to fill the temporal gap between transplant and the generation of mature KIR^+ alloreactive NK cells, a novel graft manipulation has been developed. This is based on the selective depletion of TCR αβ T cells (responsible of GvHD) and B cells (to prevent B cell malignancies in immunocompromised individuals). With this graft manipulation, the infused mononuclear cells also contain, in addition to HSC (including not only $CD34^+$ but also $CD34^-$ precursors), effector cells such as mature ($CD56^{dim}$) NK cells and TCRγδ T cells, both capable of anti-leukemia activity (12, 26, 50). In addition, the graft contained different myeloid cell types, including monocytes and low-density monocytic or polymorphonuclear (PMN) myeloid cells (51, 52). The immediate availability of cells capable of killing leukemia blasts and controlling virus reactivation or infections had a major positive impact. Indeed the overall survival probability was ∼70% not only for ALL but also for AML patients. An unexpected finding was that NK cell-mediated alloreactivity did not appear to play a significant role (49). While it is conceivable that the NK cell function may be offset by a predominant GvL effect of γδT cells (greatly expanded *in vivo* thanks to the use of zoledronic acid) (53), we could not exclude that also other mechanisms may impair NK alloreactivity. Indeed we recently found that myeloid-derived suppressor cells (MDSC), particularly abundant in the graft, exert a potent inhibitory effect on NK cell function (54). These data suggest a possible effect also *in vivo* and offer a clue for applying an additional step in the graft manipulation to further remove MDSC. The rescue of NK cell function may contribute to increase the clinical outcome, particularly by preventing leukemia relapses, still representing ∼25% of total deaths.

Taken together, these data support the notion that NK and other cells of the innate immunity may play a relevant role in the therapy of high-risk leukemia. Notably, HSC from different sources give rise to other innate lymphoid cells (ILC), particularly ILC3. ILC3 cells contribute to tissue repair and regeneration of lymphoid tissues and are likely to play a major role in the integrity of such tissues severely compromised by the chemo/radiotherapy given to patients prior to HSCT (55).

BLOCKING OF INHIBITORY CHECKPOINTS/RECEPTORS TO UNLEASH NK CELLS

NK cells express inhibitory receptors such as the HLA-Cl-I-specific KIRs and CD94/NKG2A that may function as true inhibitory checkpoints (56). The lack of interactions with their cognate HLA class I ligands on target cells leads to cytolytic activity and cytokine production. This may occur in an autologous environment in the case of tumors or viral infections, as well as in an allogeneic setting such as the haplo-HSCT (see above).

While KIRs and NKG2A are constitutively expressed by mature NK cells, the expression of other inhibitory checkpoints involved in the homeostasis of immune responses, including PD-1, TIGIT, TIM-3, and CD96, is inducible (57). Such

de novo-expressed checkpoint regulators have been shown to inhibit the NK cell function upon interaction with their ligands on tumor cells (19, 58). We will focus on PD-1 since it is a major checkpoint receptor involved in the control of immune responses, and the therapeutic use of blocking antibodies disrupting the PD-1/PD-L1 axis represents a major breakthrough in the cure of highly aggressive tumors.

While PD-1 expression has been first reported in T lymphocytes, recent studies revealed that, in pathological conditions, such as CMV infections and tumors, it may be expressed also by NK cells. The expression of PD-1 by NK cells is controversial; indeed PB-NK cells derived from both healthy donors (HD) and neoplastic patients were originally reported to express low levels, if any, of PD-1. On the other hand, PD-1^{bright} NK cells were found in the PB and, more abundantly, in ascitic fluid of ovarian carcinoma patients (58), as well as in pleural effusions of patients with primary and metastatic tumors (19) and in Hodgkin lymphoma. Notably, both PD-1 mRNA and PD-1 protein are present in the cytoplasm of NK cells isolated from HD (59), although the molecular mechanisms leading to its surface expression are still poorly defined.

Under physiological conditions, PD-1 acts as a brake in the regulation of immune responses, playing a relevant role in the induction and maintenance of T cell tolerance. However, in cancer patients, it may impair T cell- and NK cell-mediated responses against tumor cells. In these cases, immunotherapy with mAbs disrupting the PD-1/PD-L1 interaction has shown great effectiveness, particularly in melanoma and lung carcinomas with responses to therapy reaching 20–40% in different clinical trials. Importantly, therapeutic blockade of inhibitory checkpoints in NK cells may be effective also in HLA-Cl-I^{neg} tumors, a condition that frequently occurs in metastatic carcinomas (as a result of tumor escape from cytolytic T cell-mediated control) (60, 61). Nevertheless, the majority of patients do not benefit from the anti-PD-1/PD-L1 treatment. Thus, prediction of clinical responses to PD-1/PD-L1 blockade represents a major issue also in view of important side effects and of the high treatment cost. In this context, an important approach is the evaluation of PD-L1 expression on tumor cells. However, its predictive value is still unsatisfactory due to several technical limitations, such as the use of different mAbs, different diagnostic materials (biopsies vs. surgical specimen, cytology), and different operators (62–64). For this reason, current researches are aimed to identify additional checkpoints to be targeted, either alone or in combination. In this context, the actual potential of blocking TIGIT, TIM-3, CD96, or LAG-3 is currently under investigation. Importantly, a recent study by Vivier's group has highlighted the use of anti-CD94/NKG2A blocking mAb in tumor therapy (65). NKG2A^{+} cells represent >50% of PB-NK cells and may express either the CD56^{bright} or the CD56^{dim} phenotype. While CD56^{bright} NKG2A^{+} NK cells are primarily cytokine producers, CD56^{dim} NKG2A^{+} cells also display potent cytolytic activity and DC editing capability (66). NKG2A is also expressed by T lymphocytes, either upon prolonged stimulation via TCR (67) or upon exposure to TGF- β (68), an immunosuppressive cytokine often present in the tumor microenvironment. This *de novo* NKG2A expression may lead to the impairment of T cell function, including anti-tumor activity (67). Accordingly, blocking of NKG2A can unleash not only NK cells but also tumor-infiltrating T cells with potential anti-tumor activity. In addition, HLA-E, the NKG2A ligand, is expressed in many highly aggressive tumors (e.g., lung, head and neck, colon, pancreas, and liver), and most cells in the tumor are HLA-E^{+}. Accordingly, blocking of NKG2A may result in potent anti-tumor effect in different cancers. In tumors expressing both HLA-E and PD-L1, the combined blocking of NKG2A and PD-1/PD-L1 axis can enhance NK cell cytotoxicity and rescue T cell function. Notably, in a murine model, this combined treatment also resulted in T cell proliferation and T cell memory induction. Finally, in HLA-E^{+} tumors, expressing tumor-associated antigens, NKG2A blockade could increase the therapeutic efficacy of other mAbs (for example, anti-EGFR mAb), favoring the NK cell triggering via the CD16-mediated antibody-dependent cytotoxicity (ADCC) (65, 69). These different scenarios involving NKG2A blockade are promising because they may occur in many tumors and involve important synergies with other checkpoint inhibitors or therapeutic antibodies directed to tumor antigens. In addition, these studies emphasize the importance of harnessing NK cell-mediated anti-tumor activity while, so far, the immunotherapeutic strategies have been mostly focused on potentiating T cell anti-tumor responses.

CONCLUDING REMARKS

It is now clear that cells of the innate immunity, in particular NK cells, play a relevant defensive role in the control of tumor growth and metastases. As shown by many experimental evidences, both *in vitro* and *in vivo*, such anti-tumor effect is related both to direct cytolytic activity and to the production of cytokines that activate other effector cells and promote useful TH1 adaptive responses. Therefore, therapeutic approaches that trigger and/or reconstitute NK cell function and proliferation are crucial in tumor immunotherapy. In addition, NK cells engineered with CAR, targeting tumor antigens, are highly promising. Indeed CAR-NK cells could complement or even replace CAR-T cells in view of their particularly potent cytolytic activity and their peculiar homing capability (70–72). Importantly, in case of loss of the targeted tumor antigen, CAR-NK cells could still exert their anti-tumor activity, particularly in the absence of KIR/HLA ligand matching. In addition, CAR-NK cells, genetically modified to over-express either molecules mediating tumor killing or cytokines able to sustain NK cell proliferation/function (e.g., IL-15), may represent a further valuable tool for adoptive cell therapy of cancer (73, 74). Notably, since NK cells do not cause GvHD, they may be obtained from unrelated donors, thus overcoming major limitations of autologous T cell therapy (time needed for preparation and high costs) and providing a rapid access to an "off-the-shelf" life-saving therapy. Indeed given the possibility to better plan treatments with standardized approaches and appropriate cell numbers, donor-derived allogeneic CAR-NK cells may represent the next generation of cell-based therapies of cancer. **Table 2** summarizes recent or ongoing clinical trials based on the use of adoptively infused NK cells.

TABLE 2 | Selected recent/ongoing trials of NK-based adoptive therapy of cancer.

Identifier	Trial	Status	Phase	NK cells/drug	Tumor
NCT00900809	QUILT-3.018: Neukoplast^{TM} (NK-92) for the treatment of refractory or relapsed acute myeloid leukemia	Recruitment completed	I	NK-92	Acute myeloid leukemia
NCT03027128	QUILT-3.028: study of haNK^{TM} for infusion in subjects with metastatic or locally advanced solid tumors	Recruitment completed	I	NK-92	Solid tumor
NCT03383978	Intracranial injection of NK-92/5.28.z cells in patients with recurrent HER2-positive glioblastoma (CAR2BRAIN)	Recruiting	I	NK-92/5.28.z	Glioblastoma
NCT02573896	Immunotherapy of relapsed refractory neuroblastoma with expanded NK cells	Recruiting	I	CAR-NK-Ch14.18 lenalidomide	Neuroblastoma
NCT02280525	Cord blood Natural Killer (NK) cells in leukemia/lymphoma	Active, not recruiting	I	NK Cells, lenalidomide, rituximab (anti-CD20), fludarabine, cyclophosphamide, cytarabine	Leukemia
NCT02481934	Clinical trial of expanded and activated autologous NK cells to treat multiple myeloma (NK-VS-MM)	Completed	I	NK Cells, lenalidomide, bortezomib	Multiple myeloma
NCT03415100	Pilot study of NKG2D-ligand targeted CAR-NK cells in patients with metastatic solid tumors	Recruiting	I	CAR-NK cells targeting NKG2D ligands	Solid tumors
NCT01974479	Pilot study of redirected haploidentical natural killer cell infusions for B-lineage acute lymphoblastic leukemia	Suspended	I	Anti-CD19 redirected NK cells	B-cell acute lymphoblastic leukemia
NCT03579927	CAR.CD19-CD28-zeta-2A-iCasp9-IL15-transduced cord blood NK cells, high-dose chemotherapy, and stem cell transplant in treating participants with B-cell lymphoma	Not yet recruiting	I/II	Cord blood-NK Cells Autologous HSCT carmustine Cytarabine, etoposide, filgrastim, melphalan, rituximab (anti-CD20)	CD19 positive, mantle cell lymphoma, recurrent diffuse large B-cell lymphoma, recurrent follicular lymphoma, refractory B-cell non-Hodgkin lymphoma, refractory diffuse large B-Cell lymphoma, refractory follicular lymphoma
NCT03056339	Umbilical and Cord Blood (CB) derived CAR-engineered NK cells for B lymphoid malignancies	Recruiting	I/II	iC9/CAR.19/IL15 transduced CB-NK Cells, fludarabine, cyclophosphamide, mesna, AP1903	B-lymphoid malignancies, acute lymphocytic leukemia, chronic lymphocytic leukemia, non-Hodgkin lymphoma
NCT02839954	CAR-pNK cell immunotherapy in MUC1 positive relapsed or refractory solid tumor	Unknown	I/II	Anti-MUC1 CAR-NK cells	Hepatocellular carcinoma, NSCLc, pancreatic carcinoma, triple-negative invasive breast carcinoma, malignant glioma of brain, colorectal carcinoma, gastric carcinoma
NCT02892695	PCAR-119 bridge immunotherapy prior to stem cell transplant in treating patients with CD19 positive leukemia and lymphoma	Unknown	I/II	Anti-CD19 CAR-NK cells	Acute/chronic lymphocytic leukemia, follicular lymphoma, mantle cell lymphoma, B-cell prolymphocytic leukemia, diffuse large cell lymphoma
NCT02742727	CAR-pNK cell immunotherapy in CD7 positive leukemia and lymphoma	Unknown	I/II	Anti-CD7 CAR-NK cells	Acute myeloid leukemia, precursor T-cell lymphoblastic leukemia-lymphoma, T-cell prolymphocytic leukemia, T-cell large granular lymphocytic leukemia, peripheral T-cell lymphoma, angioimmunoblastic T-cell lymphoma, extranodal NK/T-cell lymphoma, nasal type enteropathy-type intestinal T-cell lymphoma

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Influence of the Tumor Microenvironment on NK Cell Function in Solid Tumors

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Natural killer (NK) cells are a population of innate lymphoid cells playing a pivotal role in host immune responses against infection and tumor growth. These cells have a powerful cytotoxic activity orchestrated by an intricate network of inhibitory and activating signals. The importance of NK cells in controlling tumor growth and in mediating a robust anti-metastatic effect has been demonstrated in different experimental mouse cancer models. Consistently, high density of tumor-infiltrating NK cells has been linked with a good prognosis in multiple human solid tumors. However, there are also tumors that appear to be refractory to NK cell-mediated killing for the presence of an immunosuppressive microenvironment affecting NK cell function. Immunotherapeutic strategies aimed at restoring and increasing the cytotoxic activity of NK cells in solid tumors, including the adoptive transfer of NK and CAR-NK cells, are currently employed in preclinical and clinical studies. In this review, we outline recent advances supporting the direct role of NK cells in controlling expansion of solid tumors and their prognostic value in human cancers. We summarize the mechanisms adopted by cancer cells and the tumor microenvironment to affect NK cell function, and finally we evaluate current strategies to augment the antitumor function of NK cells for the treatment of solid tumors.

Keywords: natural killer cells, tumor microenvironment, solid tumors, immune checkpoint inhibitors, cellular metabolism, cancer stem cells, hypoxia, adoptive transfer of NK and CAR-NK cells

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INTRODUCTION

Natural killer (NK) cells are a specialized population of innate lymphoid cells (ILCs) that mediates cytotoxic functions against damaged, infected, and pre-malignant cells through an intricate network of signals that allow for rapid activation (1). NK cell cytotoxicity is mainly regulated by the secretion of effector molecules (IFN-γ, TNF-α, NO, IL-2, IL-12, IL-15, IL-18, and IL-21), and the interplay between inhibitory and activating signals originating at the cell surface from NK cell-inhibitory receptors (NK-IRs) and NK cell-activating receptors (NK-ARs), respectively. NK-IRs promote the effector function upon interaction with ligands expressed on normal and healthy cells. Conversely, NK-ARs recognize ligands encoded by pathogens (2), or induced by cellular stress during viral infections (3), or cellular growth factors (4). The downregulation of inhibitory ligands and the expression of ligands for NK-ARs on cancer cells can trigger NK cells to kill them and secrete cytokines, such as IFN-γ and TNF-α. Thus, blocking interaction between NK-IRs and their ligands or enhancing NK-ARs–ligand binding may represent a promising strategy to generate an antitumor activity.

NK cells in the peripheral blood, spleen, and bone marrow can infiltrate other tissues. In human, two distinct subsets of NK cells have been identified based on CD56 expression levels. The $CD56^{bright}$ NK cell subset, representing the majority of NK cells in the peripheral blood, is specialized in the secretion of cytokines and chemokines in response to IL-12, IL-15, and IL-18, and in the regulation of adaptive immunity. The $CD56^{dim}$ NK cell subset is both cytotoxic and cytokine-producing and expresses high levels of CD16 (also known as FcγRIII), which is responsible for antibody-dependent cellular cytotoxicity (ADCC). The recruitment of NK cells in the inflamed tissues is regulated by the expression of several chemokine receptors, including CXCR3 that binds to the tumor-derived chemokine ligands CXCL9, CXCL10, and CXCL11 (5). Although the presence of tumor-infiltrating NK cells confers a favorable outcome in many tumors, in others, their function is impaired by soluble modulators secreted in the tumor microenvironment (TME). In this Review, we discuss recent evidence for a direct role of NK cells in controlling tumor expansions and summarize the mechanisms adopted by tumor cells and the TME to affect NK cell functions in solid tumors. Finally, we evaluate novel therapeutic strategies to enhance the antitumor function of NK cells for the treatment of solid tumors.

ROLE OF NK CELLS IN THE IMMUNOSURVEILLANCE OF SOLID TUMORS

NK cells control tumor growth by interacting directly with tumor cells or affecting the function of other populations of innate and adaptive immunity in the TME. The importance of NK cells in antitumor immunity has been established in different experimental mouse tumor models.

Depletion of NK cell populations prior to tumor transplantation has been shown to cause a more aggressive phenotype with metastatic tumors (6–8). Specifically, the use of mutant mice with developmental and functional alterations of NK cells allowed a better understanding of the role of these cells in antitumor immunity. NCR1/NKp46 is directly involved in the killing of melanoma and Lewis lung carcinoma cells and in the formation of metastases (9). Indeed, $NCR1^{-/-}$ mice underwent a more aggressive tumor development compared to wild type (WT) mice. Mice knockout for Mcl1, which is required to sustain the *in vivo* survival of NK cells, were characterized by the total absence of NK cells and a rapid development of metastatic melanomas (10). A similar observation was reported in IL-$2rg^{-/-}$ and $TLR3^{-/-}$ mice (11, 12). TLR3 is known to limit B16F10 lung metastasis through the production of IFN-γ by NK cells. The lack of TLR3 signaling downregulates NK cell function following cytokine stimulation, leading to defective immune responses unable to constrain metastatic diseases (12). DNAM-$1^{-/-}$ mice developed fibrosarcoma and papilloma in response to chemical carcinogens significantly more frequently than WT mice (13). Tbx21, also known as T-bet, is a transcription factor involved in the differentiation of NK cells. $Tbx21^{-/-}$ mice injected intravenously with melanoma or colorectal carcinoma cells were more susceptible to metastasis formation compared to WT mice (14). The ability of NK cells to invade the primary tumors and migrate in the metastatic site is dependent on the heparanase. Mice lacking heparanase specifically in NK cells ($Hpse^{fl/fl}$ $NKp46^{-iCre}$ mice) were more susceptible to develop lymphoma, metastatic melanoma, prostate carcinoma, or mammary carcinoma when challenged with the carcinogen methylcholanthrene (15). These observations suggest that NK cells play a prominent role in controlling tumor growth and in mediating a robust anti-metastatic effect.

Further evidence for the role of NK cells in controlling tumor development and dissemination derived from the ability of these cells to target and eliminate cancer stem cells (CSCs), a subset of cells with self-renewal ability involved in the generation and evolution of tumors (16). CSCs exhibit a typical surface expression profile consisting of low levels of MHC class I, CD54 and PD-L1, and high expression of CD44 (17). The susceptibility of CSCs to NK cell-mediated killing has been reported in different tumor models (18, 19). An *in vivo* study reveals that activated NK cells transferred in NSG mice harboring orthotopic pancreatic cancer xenografts were able to preferentially kill CSCs, leading to a significant reduction of both intratumoral CSCs and tumor burden (20). Additionally, in colorectal cancer, CSCs upregulated the NK-ARs NKp30 and NKp44 and were susceptible to NK cell-mediated killing (19). Similarly, glioblastoma-derived CSCs showed an increased susceptibility to NK cell killing by both allogeneic and autologous IL-2 and IL-15 activated NK cells (21). Melanoma cell lines with CSC features exposed to IL-2-activated allogeneic NK cells showed an increased susceptibility to NK cell-mediated killing through upregulation of the DNAM-1 ligands, such as PVR and Nectin-2 (22). Breast cancer CSCs showed sensibility to IL-2- and IL-15-treated NK cells and increased expression of NKG2D ligands, such as ULBP1, ULBP2, and MICA (23).

CSCs are also considered an important source of resistance to standard anti-cancer therapies. Following chemotherapy and radiation therapy treatments, CSCs upregulate ligands for NKG2D such as MICA and MICB, resulting in an increase of NK cell cytotoxicity (24, 25).

NK cells are able to target and shape CSC-undifferentiated tumors, thereby leading to a selection of a differentiated tumor subset (26). After selection, NK cells down-modulate their surface receptors, lose their cytotoxicity, and become anergized, but continue to produce IFN-γ and TNF-α, which drive differentiation of the remaining stem cells. This results in an increased expression of MHC class I, CD54, and PD-L1 and reduction of CD44 on CSC surface. These cells exhibit a decreased proliferation rate, inability to invade or metastatize and increased susceptibility to chemotherapeutic and radiotherapeutic agents (26, 27).

Despite the role of NK cells in targeting CSC/undifferentiated tumors, some authors have highlighted an association between the stage of differentiation and sensitivity to NK cell-mediated cytotoxicity. Studies conducted on patients with pancreatic tumors or oral squamous carcinoma stem cells revealed that although CSCs/undifferentiated tumors were susceptible to NK cell-mediated cytotoxicity, they remained significantly resistant to chemotherapeutic and radiotherapeutic agents. Conversely,

differentiated tumors grew slower and were resistant to primary NK cell-mediated cytotoxicity with high susceptibility to chemotherapeutic and radiotherapeutic agents (27, 28).

The role of NK cells in controlling tumor growth is also supported by several evidence in human specimens. The first studies date back to the late 1980s (29–31). Several authors have studied the phenotype of circulating NK cells and their cytotoxic activity in cancer patients compared to healthy donors (32). Other authors have investigated the prognostic role of tumor-infiltrating NK cells (33), but due to the lack of immunohistochemical markers that unambiguously identify NK cells, current information is limited. Different methods are used to attribute a prognostic value to specific immune cell subsets. The most reliable is the one that evaluates survival analysis stratifying the subjects according to the median cutoff value for density of immune cell subsets.

Denkert et al. showed that a higher density of NK cells is an indicator of good prognosis in breast cancer (33). A pooled analysis of 3,771 patients treated with neoadjuvant therapy revealed the presence of intratumoral immune cell types, including NK cells, significantly associated with better prognosis of HER2-positive and triple negative breast cancer patients (33). Trastuzumab treatment was associated with a significant increase of tumor-infiltrating NK cells and expression of granzyme B and TiA1 in breast cancer compared with controls (34). Moreover, the expression of NCR1 and other NK cell-associated genes, i.e., CD1d, DNAM-1, CRTAM, CD96, and NCR3/NKp30, was associated with prolonged disease-free survival (DFS) of breast cancer patients (34). High number of intratumoral NK cells was considered an optimal biomarker of response to the anti-HER2 antibody-based treatment. Indeed, the presence of these cells was significantly associated with a complete response and extended DFS (35). The same authors showed that the number of circulating $CD57^+$ NK cells was inversely correlated to tumor-infiltrating NK cells and predicted resistance to HER2-specific antibody treatment in HER2-positive primary breast cancers (36). From a clinical perspective, baseline screening for high circulating $CD57^+$ NK cells could be implemented for the identification of patients with primary resistance to neoadjuvant treatment with HER2 therapeutic antibodies, complementing the positive predictive value of tumor-infiltrating NK cells in diagnostic biopsies (27).

Melanoma patients were characterized by expansion of $CD56^{dim}CD57^{dim}CD69^+CCR7^+KIR^+$ tumor-infiltrated NK cells (37). These cells showed robust cytotoxicity against autologous tumor cells compared to those derived from tumor-free ipsilateral lymph nodes of the same patients (37). Melanoma cells isolated from metastatic lymph nodes were efficiently lysed by circulating NK cells expressing high levels of NKG2D, NKp30, DNAM-1, and CD62L (38).

Recently, the prognostic role of NK cells has been investigated through the expression of genes specific for NK cell function. NK cell gene signatures were associated with a strong survival advantage for metastatic melanomas (39). More recently, Barry et al. defined a clear link between NK cells and dendritic cells (DC), demonstrating that NK cells produce FLT3LG cytokine that controls the intratumoral level of $BDCA\text{-}3^+$ DCs. The authors also showed that the high expression of NK and DC gene signatures was associated with a better OS in two independent datasets of melanoma patients (40).

High levels of tumor-infiltrating NK cells have been associated with a good prognosis also in other human cancers, including gastrointestinal stromal tumor (41–43), neuroblastoma (44), head and neck cancer (45), and prostate cancer (46). A more complex and heterogeneous landscape has been outlined for other types of neoplasms. A gene signature able to predict tissue NK cell content and prognosis of renal cell carcinoma patients was proposed (47). Two distinct groups of renal cancer patients have been identified based on the level of tumor-infiltrating NK cells (48). In both groups, NK cells were not cytolytic but differed for CD16 expression levels. NK cells from tumors with high NK cell content (>20% of the lymphocyte population) were $CD16^{bright}$, whereas those from tumors with low NK cell content (<20%) were $CD56^{dim}$. *In vitro* experiments showed that unlike the low-NK group, the high-NK group was able to acquire cytotoxic function against K562 cells (48). An early study reported that low NK cell cytotoxicity was predictive of colon cancer recurrence, independently of other prognostic factors (30). These data were confirmed in patients with metastatic colorectal cancer treated with the mAb 17-1A (49). Recently, density and tissue distribution of NK cells were investigated in 112 primary colorectal cancer samples (50). Despite high concentration of chemokines known to promote NK cell infiltration, colorectal cancers were only scarcely infiltrated by these cells as compared to normal mucosa, thus suggesting the presence of soluble factors in the TME preventing NK cell infiltration.

The tissue distribution of NK cells was studied in different subtypes of lung cancer. A high number of intratumoral $CD57^+$ NK cells, as evaluated by IHC analysis, was significantly associated with a better clinical outcome in squamous cell lung cancer patients (51). A gene expression study conducted on 148 blood samples discovered valid prognostic innate immune markers (52). Among the screened genes, the enhanced expression of *NCR3* was associated with better overall survival (OS) in non-small cell lung cancer patients. More recently, a low number of circulating $NKp46^+$ $CD56^{dim}$ $CD16^+$ NK cells was significantly associated with a better OS in small cell lung cancer patients. Intratumoral NK cells were less cytotoxic in non-small cell lung cancer patients, as compared to circulating NK cells or those derived from normal lung tissues (53). Of note, NK cells were located in the tumor stroma not in direct contact with cancer cells. Further evidence indicate that NK cells are very rare within human non-small cell lung cancer and that those infiltrating tumor tissues resemble the circulating $CD56^{bright}$ NK cells (54). More recently, Lavin et al. found that NK cells are the least abundant immune cell population within lung adenocarcinomas and that those expressing CD16 are dramatically reduced in tumors as compared to normal tissues (55). This subset of NK cells is less cytotoxic, expressing low levels of granzyme B, IFN-γ, and CD57.

A reduced number of $CD56^{dim}CD16^{high}$ NK cells was detected in the liver tumor area as compared to non-neoplastic area (56). These cells also showed an impaired cytotoxic ability. In general, high density of NK cells within intratumoral region of hepatocellular carcinoma showed better OS and DFS. Conversely,

the few NK cells infiltrating advanced hepatocellular carcinoma exhibited attenuated capacities for cytokine productions. These evidence indicate that infiltration of functional NK cells in hepatocellular carcinoma tissues may represent the host reaction to cancer and that TME impairs NK cell function during disease progression (57).

Recently, a new subset of liver-resident NK cells characterized for expression of CD49a has been discovered (58). These cells are abundant in the peritumoral area of hepatocellular carcinoma and are characterized by the expression of PD1, CD96, and TIGIT. The accumulation of these cells in liver tumors was correlated with poor prognosis, thus suggesting a role of this NK cell subset in the hepatocellular carcinoma development (58).

In prostate cancer, a strong immunosuppressive microenvironment impairs NK cell function at multiple levels. Indeed, NK cells that infiltrated the cancer prostate tissues were mainly $CD56^{+}$ and displayed an immature, but activated phenotype with low or no cytotoxic potential. The authors found that TGF-β is highly secreted into the TME and provided an immunosuppressive effect on NK cells. Co-culture experiments revealed that tumor cells induced expression of inhibitory receptors downregulating that of the activating receptors NKp46, NKG2D, and CD16 on NK cells, thus preventing their recognition (46). Similarly, endometrial cancer was poorly infiltrated by NK cells. These cells, when present, expressed co-inhibitory molecules, such as TIGIT and TIM-3, proportionally with the severity of the disease, thus suggesting an important role of the TME in reducing recruitment of functional NK cells to the tumor site (59).

Overall, many evidences indicate that NK cells, although representing an extremely rare subset of immune cells, are able to invade some solid tumors and, when functionally activated, are associated with good prognosis. However, in several cases, the picture is much more complex, with contradictory results, and no or negative effects on patient's prognosis. Different factors contribute to attenuate the antitumoral properties of NK cells, thus providing the need to integrate the study of NK cell density, with equally important elements including phenotype and localization with respect to stroma and parenchyma tumor cells and other immune cell populations. Discordant results obtained in certain tumors could be derived from the methods used to unequivocally identify NK cells, the type of tissue, or the tumor phase (60). The inability of NK cells to limit tumor growth and improve patient survival may result from the presence of an immunosuppressive TME. Indeed, TME renders solid tumors particularly refractory to NK cell killing through a plethora of strategies ranging from preventing the recruitment of intratumoral NK cells, to confining them, when present, to the stromal part of the tissue, or rather attract the recruitment of non-cytotoxic NK cells ($CD56^{bright}CD16^{low/neg}$) in the tumor bed.

IMMUNOSUPPRESSIVE PROPERTIES OF THE TME ON NK CELLS

TME, composed of cancer cells, fibroblasts, endothelial cells, and immune cells, provides conditions that promote tumor progression. Accumulating evidences indicate that TME produced soluble modulators that negatively regulate maturation, proliferation, and effector function of NK cells (**Figure 1**). These immunosuppressive factors may act either directly on NK cells or indirectly by stimulating other immune cells, such as antigen-presenting cells (APC), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSC), to produce additional immunosuppressive molecules.

TGF-β is a cytokine produced in the TME by tumor cells, Tregs, MDSCs, and other stromal cells. This cytokine is known to inhibit both the expansion and function of effector cells and to promote the proliferation of Tregs (61). TGF-β impairs NK cell function directly or indirectly by cell–cell contact between NK and other cytokine-producing cells (62, 63). As a direct effect, TGF-β limits NK cell cytotoxicity and IFN-γ production by inhibiting the T-bet transcription factor (SMAD3) (64) and downregulating the expression of NKp30 and NKG2D, and its ligand MICA in cancer patients (65–67). In colorectal cancer and lung cancer, downregulation of NKG2D has been associated with increased serum levels of TGF-β (68). The levels of DNAX activating protein 12 kDa (DAP12), a crucial signaling adaptor of NKG2D in human NK cells, were downregulated by TGF-β-induced miR-183 (69). In an orthotopic liver cancer model, TGF-β was shown to bind on the cell surface of MDSCs and inhibits NK cell function (70). TGF-β is also able to inhibit NK cell function by targeting the serine and threonine kinase mTOR, a crucial signaling integrator of pro- and anti-inflammatory cytokines, such as interleukin-15 (IL-15), in both murine and human NK cells (71). Interestingly, TGF-β is able to transdifferentiate NK cells into ILC type 1 (ILC1), which are missing of cytotoxic functions (72, 73). TGF-β is also able to dampen $CD56^{dim}$ recruitment and favor that of $CD56^{bright}$ (74). It contributes to modulate chemokine repertoire reducing the expression of those attracting $CD56^{dim}$ NK cells (CXCL2, CX3CL1, CXCL1, and CXCL8), and increasing that of chemokines (CXCL9, CXCL10, CXCL19, and CCL5), driving migration of $CD56^{bright}$ NK cells (60, 74). In ovarian (75) and lung (76) cancer patients, $CD56^{bright}/CD16^{low}$ represents the predominant NK cell population in the TME. Moreover, the presence of intratumoral tertiary lymphoid structures (TLS) in lung cancer drives the expression of chemokines normally secreted in secondary lymphoid organs (CCL19, CCL21, etc.), thus preferentially attracting non-cytotoxic $CD56^{bright}$ NK cells at the tumor site (76). Based on these findings, TGF-β may be considered a target for enhancing NK cell-mediated antitumor immunity. Currently, inhibition of TGF-β signaling in preclinical studies (77–83), including those based on the combined use of immune checkpoint inhibitors, such as anti-PD-L1 (84–86), as well as in clinical trials (87–89), represents a promising antitumor approach supported mainly by the analysis on $CD8^{+}$ T cells. Interestingly, the use of anti-TGF-β in solid tumors allows the accumulation of NK cells, the increased production of IFN-γ, and the restoration of NKG2D (90).

Another crucial factor leading to dysfunctional NK cells in cancer is the impaired cellular metabolism (91–93). TME is known to be very poor in nutrients, such as glucose and glutamine, very important for NK cells (94–97). While

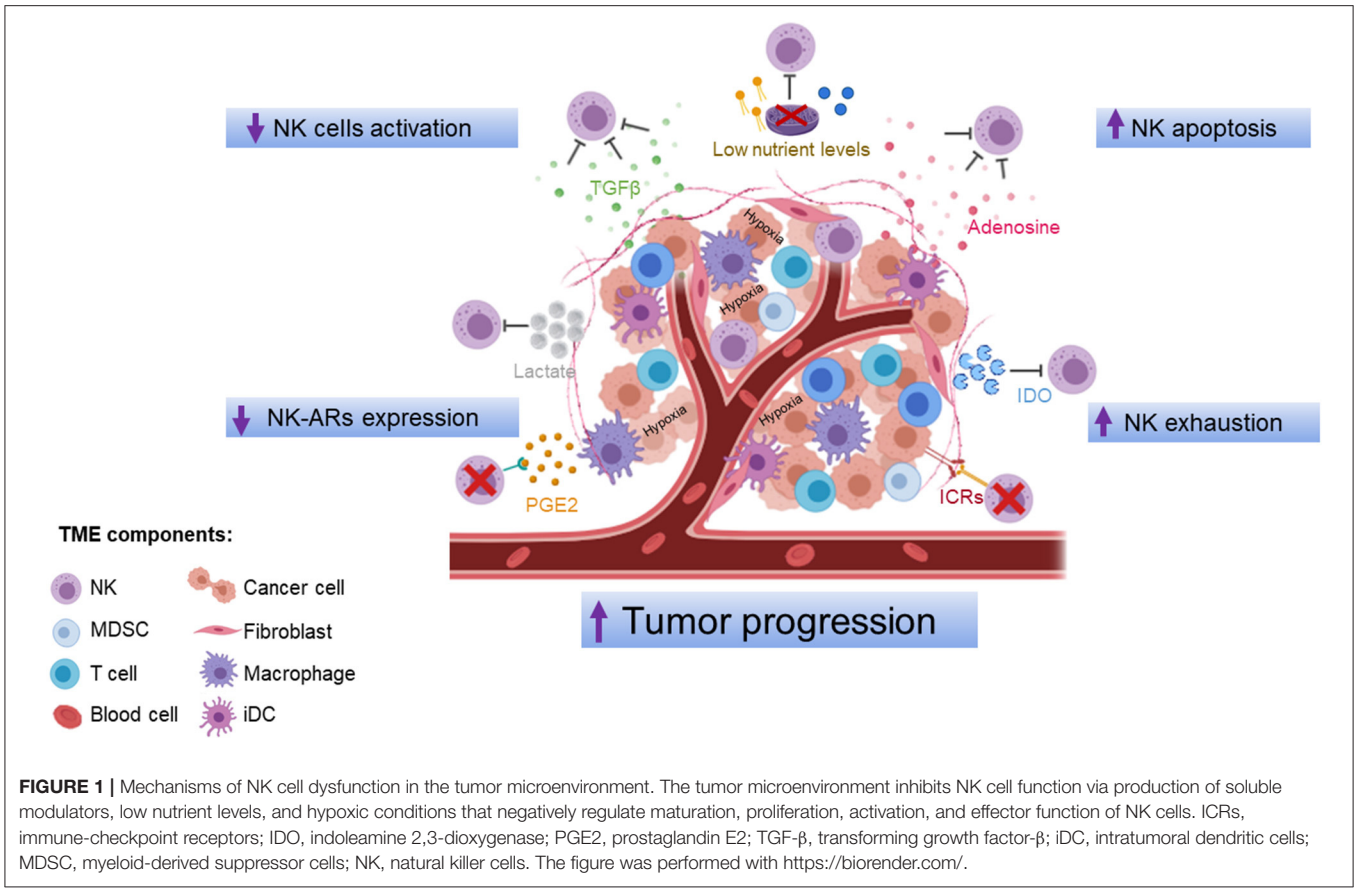

FIGURE 1 | Mechanisms of NK cell dysfunction in the tumor microenvironment. The tumor microenvironment inhibits NK cell function via production of soluble modulators, low nutrient levels, and hypoxic conditions that negatively regulate maturation, proliferation, activation, and effector function of NK cells. ICRs, immune-checkpoint receptors; IDO, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2; TGF-β, transforming growth factor-β; iDC, intratumoral dendritic cells; MDSC, myeloid-derived suppressor cells; NK, natural killer cells. The figure was performed with https://biorender.com/.

much attention has been focused on T cells and macrophages (98–101), little is known about the NK cell metabolism. In humans, increased glycolysis and oxidate phosphorylation (OXPHOS) stimulate $CD56^{bright}$ NK cells to produce IFN-γ (102). In mice, steady-state NK cells favor OXPHOS, rather than glycolysis, as the primary metabolic pathway. Conversely, activated NK cells induced by IL-2/IL-12 have a greater preference for glycolysis. Glycolysis is an important metabolic process for NK cell function, and in general, it is increased in activated NK cells. A recent study showed that lung tumors upregulate the expression of the gluconeogenesis enzyme fructose bisphosphatase 1 (FBP1) in tumor-infiltrating NK cells through a mechanism involving TGF-β (92). This leads to NK cell dysfunction by inhibiting both glycolysis and the viability of NK cells. The authors showed that pharmacological inhibition of FBP1 can revert the dysfunctional phenotype of NK cells during tumor promotion, but not during tumor progression. Indeed, similarly to tumor-infiltrating T cells (103, 104), NK cells in the mouse lung cancer are functional during tumor initiation, while assume a mild dysfunctional state during tumor promotion that can be reverted by pharmacological FBP1 inhibition. Subsequently, NK cells evolve into an irreversible dysfunctional state that can no longer be rescued by FBP1 inhibition (93). Other molecules that can potentially inhibit NK cell metabolism include two physiological products of enzymatic cholesterol oxidation, 25-hydroxycholesterol and 27-hydroxycholesterol, that inhibit the activation of SREBP transcription factors, key regulators of NK cell metabolism (105–107). Further evidence supporting the role of the impaired cellular metabolism in leading to dysfunctional NK cells come from studies on obesity in human and murine models. NK cells from obese mice and humans are less responsive to tumor target cells in terms of cytotoxicity and production of IFN-γ, granzyme B, and perforin (108). This is because NK cells fail to engage a metabolic response when stimulated with cytokines and have reduced metabolic rates, compared to lean counterparts (108). This metabolic dysfunction has been linked to peroxisome proliferator-activated receptor (PPAR)-driven lipid accumulation in NK cells leading to altered gene expression, downregulation of mTORC1 and MYC signaling, and decreased rates of glycolysis and OXPHOS (108). NK cells from obese mice and humans fail to kill tumor cells for their inability to form synapses with them, an event that is highly energy consuming (108).

The production of prostaglandin E2 (PGE2) in the TME is another mechanism adopted by tumors to selectively suppress the effector function of NK cells (109–112). PGE2 produced by tumor cells, tumor-associated macrophages, and stromal cells (113–115) represents a key regulator of the NK cell activity for mesenchymal stem cell (MSC)-mediated regulation of NK cell activity (116), tumor-derived MSCs (T-MSCs) (117), and MDSCs (118). In addition, PGE2 produced by

the thyroid cancer cell microenvironment suppresses NK cell cytotoxicity (119). PGE2 downregulates the expression of NKp30, NKp44, NKp46, and NKG2D by binding to E-prostanoid 2 (EP2) and EP4 receptors on NK cells (112), via a common cAMP-PKA signaling (120), thus resulting in the inhibition of cytotoxicity (116, 121). In PGE2-producing tumors, NK cells were less abundant and impaired in their ability to produce cytokines CCL5 and CXCL1 (109). Preclinical studies show that the blocking of PGE2 in a murine model of metastatic breast cancer (122) and in human gastric cancer cells (123) restores NK cell function against tumor. Consistently, the efficacy of celecoxib, a cyclooxygenase-2 (COX2) inhibitor able to blockade PGE2 signaling, has been demonstrated in several clinical studies in many solid tumors (https://ClinicalTrials.gov).

The intracellular enzyme indoleamine 2,3-dioxygenase (IDO) is a critical regulator of the TME converting tryptophan into a number of metabolites with immunosuppressive function, including L-Kynurenine (124). IDO overexpression was associated with tumor progression and growth arrest of tumor-infiltrating NK cells (124, 125). L-Kynurenine is known to affect NK cell activity by interfering with the IL-2-driven upregulation of NKp46 and NKG2D (126). The simultaneous use of PGE2 and IDO inhibitors completely restored NK cell proliferation (116). The NK cell-mediated IFN-γ production in response to leukemic cells upregulates IDO, rendering tumor cells more resistant to NK cell-mediated killing (127).

Adenosine, a purine metabolite present at high concentration in the TME, also acts by limiting the activity of protective immune infiltrates, including NK cells, and enhancing that of Tregs and MDSCs (128). Adenosine accumulated in the TME by CD39 and CD73, causing inhibition of tumor-infiltrating NK cells by binding to the purinergic adenosine A_{2A} receptor expressed on cell surface (129–133). Reduction of adenosine by blocking both CD73 and the A_{2A} receptor was shown to affect tumor growth and promote recruitment of tumor-infiltrating NK cells (134).

Another factor causing dysfunction of tumor-infiltrating NK cells is hypoxia. Hypoxia downregulates NKp46, NKp30, NKp44, NKG2D, perforin, and granzyme B (135–138). Treatment with IL-2 restores NK cell cytotoxicity in multiple myeloma by increasing NKG2D expression (139).

Tumor cells secrete a large amount of lactate in the TME, causing acidosis and immunosuppression (140). Lactate accumulation has been associated with reduced NK cell cytotoxicity and downregulation of NKp46 (141, 142). The acidification of the TME has been shown to induce apoptosis of liver-resident NK cells in colorectal cancer liver metastases (143). The neutralization of the TME restored the cytotoxic activity of NK cells enhancing NKG2D expression (144).

Additionally, NK cells may express a wide range of immune checkpoint receptors that inhibit their function, including KIRs, CD94/NKG2A, PD1, CTLA4, TIM3, TIGIT, CD96, KLRG-1, LAG3, and, recently discovered, IL-1R8 (145). The effect of these immune checkpoint molecules on NK cell function has been reviewed (146, 147).

NK CELL-BASED THERAPEUTIC STRATEGIES TO OVERCOME RESISTANCE IN SOLID TUMORS

NK cell-based immunotherapy has been successfully applied for hematological malignancies and represents an attractive strategy even for treating solid tumors (148). The therapeutic approaches currently adopted are reported below.

Use of Cytokines, Monoclonal Antibodies, and Immune Checkpoint Inhibitors

Therapeutic use of cytokines such as IL-12 and IL-18 in supporting ADCC (149–151), or IL-15 and IL-21 in triggering NK cell proliferation, NK-ARs induction, and cytokine production (152–156), resulted effective in increasing NK cell cytotoxicity against solid tumors. The cytokine agonists designed to improve the biological and pharmacokinetic activities of classical cytokines, including IL-15 superagonist ALT-803, showed promising results in both pre-clinical (157, 158) and clinical studies (159–161) (**Table 1**). Interestingly, encouraging results were obtained in combination with anti-cancer drugs, immune checkpoint inhibitors or vaccines, in terms of increased levels of circulating $CD56^{bright}$ NK cells in ALT-803-treated patients.

The secretion of ligands for NK-ARs by solid tumor cells has been associated to immune suppressive effects (162). In a murine model, the neutralization of soluble NKG2D ligands such as MICA and MICB with mAb B10G5 was effective against prostate carcinoma and metastasis, leading to the enhanced NK cell infiltration in the tumor parenchyma (163), and improving CTLA-4 blockade therapy (164).

Another approach to sensitize solid tumors to NK cell-mediated lysis is to improve ADCC with monoclonal antibodies (mAbs) specific for tumor antigens. Several clinical trials exploring the efficacy of various mAbs targeting tumor antigens are in phase I and II (**Table 1**). This therapeutic approach is very promising even in challenging pediatric tumors, such as neuroblastoma, in which amplification of the *MYCN* oncogene, clinically associated with poor prognosis, has been correlated with the reduced tumor susceptibility to NK cell-mediated killing (165).

The adoption of the chimeric mAb Ch14.18 recognizing the main tumor antigen GD2 on neuroblastoma cells was very successful (166) and presently under investigation in several clinical trials in combination with immune checkpoint inhibitors or in the form of chimeric antigen receptors (CARs) for both CAR-T (167) and CAR-NK cells (**Table 1**).

On the other hand, several approaches aimed to dampen the NK cell-inhibitory signals, such as those mediated by immune checkpoint molecules, have been explored, thus triggering directly immune effector functions including those mediated by NK cells. The initial adoption of anti-KIRs reported very few positive results (168), with potential benefits when used in combination with anti-PD1 for the cure of non-small cell lung cancer patients (169). Blocking of PD1/PD-L1 signaling enhances the production of cytokines and degranulation of

TABLE 1 | Some selected and most recent clinical trials (since January 1st 2016) on NK cell-based immunotherapies, in different forms of solid tumors, by using cytokines, monoclonal antibodies (mAbs), and immune checkpoint inhibitors.

CYTOKINES SUPPORTING NK CELL ACTIVITY				
Cytokines	**In combination with***	**Phase**	**ClinicalTrial.gov identifier**	**Tumor****
IL-15	–	I	NCT01946789	M, RCC, NSCLC, HNSCC
	–	I	NCT01572493	AST
	Ipilimumab and nivolumab	I	NCT03388632	MeM, RCC, NSCLC, HNSCC
IL-15 superagonist ALT-803	–	I	NCT02523469	NSCLC
	–	I	NCT03054909	OC
	Aldoxorubicin HCl	Ib/II	NCT03563157	CoC
		Ib/II	NCT03387098	PC
		Ib	NCT03586869	PC
		I/II	NCT03387085	TNIBC
	Bacillus Calmette-Guerin	II	NCT03022825	NMIBC
		I/II	NCT02138734	NMIBC
	Gemcitabine and nab-paclitaxel	I	NCT02559674	PC
	Anti cancer drugs	I/II	NCT03329248	PC
		I/II	NCT03175666	TNIBC
		I/II	NCT03136406	PC
	Nivolumab	I/II	NCT02523469	NSCLC
	Nivolumab or pembrolizumab or atezolizumab or avelumab	II	NCT03228667	NSCLC, SCLC, UC, HNSCC, MCC, M, RCC, GC, CeC, HC, MI, MRD, CoC
	ETBX-011 vaccine	Ib/II	NCT03127098	TC, CoC, OC, BC, LC, PC
ANTIBODIES REDIRECTING NK CELL ACTIVITY				
Antibodies	**In combination with**	**Phase**	**ClinicalTrial.gov identifier**	**Tumor**
Anti-ErbB3 (ISU104)	–	I	NCT03552406	AST, BC
Anti-globo H (OBI-888)	–	I/II	NCT03573544	LAMST
Anti-CD40 (JNJ-64457107)	–	I	NCT02829099	NSCLC, PC, M
Anti-CD47 (Hu5F9-G4)	–	I/II	NCT02953782	CoC
Anti-TAMUC1 (gatipotuzumab)	Anti-EGFR (tomuzotuximab)	Ib	NCT03360734	NSCLC, CoC, BC, GyC
Anti-semaforin 4D (pepinemab)	–	I/II	NCT03320330	OS
Anti-OX40 (INCAGNO1949)	–	I/II	NCT02923349	AST, EC, OC, RCC
Anti-GD2 (Ch14.18/CHO)	Nivolumab	I	NCT02914405	NB
	Nivolumab + ipilumumab + radiation	I/II	NCT03958383	M
IMMUNE CHECKPOINT INHIBITORS				
mAbs targeting PD-1				
Pembrolizumab	–	II	NCT03241927	M
	–	Ib	NCT03590054	MeM, HNSCC, UC, NSCLC, LC
	–	II	NCT02721732	AST
	–	II	NCT03428802	LAMST, OC, BC
	–	II	NCT03447678	NSCLC
	IL-12	I	NCT03030378	AST
	Autolous DC-CIK cells	I/II	NCT03190811	LC, RCC, GC, BlC, PC
	Lenalidomide	I	NCT02963610	Neoplams
	Enterococcus gallinarum (MRx0518)	I/II	NCT03637803	NSCLC, M, RCC, BlC
	Toll-like receptor (TLR) agonist (BDB001)	I/II	NCT03486301	AST
	AGEN1884	I/II	NCT02694822	AST

(Continued)

TABLE 1 | Continued

Antibodies	In combination with	Phase	ClinicalTrial.gov identifier	Tumor
	Nab-paclitaxel + anti cancer drugs	II	NCT03289819	BC
	TLR9 agonist (AST-008)	I/II	NCT03684785	MeM, HNSCC, SCC, MCC
	Poly ICLC	I	NCT02834052	CoC
Toripalimab	–	I	NCT02857166	Neoplasms
	–	I	NCT03713905	Neoplasms
	–	I	NCT03474640	GC, NC, HC, EsC, STS, CoS
	–	I	NCT02836795	UrC
	Anti-VEGFR (surufatinib)	I	NCT03879057	NeT, LiC, GC
GLS-010	–	II	NCT03704246	Neoplasms
HX008	–	I	NCT03468751	Neoplasms
HLX10	–	I	NCT02715284	NSCLC, EC, MSI-H
TSR-042, dostarlimab	–	I	NCT03286296	LAMST, NSCLC
	–	I	NCT02715284	NSCLC, EC, MSI-H
GB226	–	I	NCT03374007	M, NSCLC, RCC, HNSCC, EC, LC, BC
INCMGA00012	–	I	NCT03059823	LAMST
AK105	–	I	NCT03352531	AST
SG001	–	I	NCT03852823	AST
CS1003	–	I	NCT03809767	Neoplasms
SCT-I10A	–	I	NCT03821363	Neoplasms
Sym021	Anti-LAG3 (Sym022) or anti-TIM3 (Sym023)	I	NCT03311412	AST
Tislelizumab	–	II	NCT03736889	MSI-H
	Anti-TIM-3 (BGB-A425)	I/II	NCT03744468	AST
	Anti-TIGIT (BGB-A1217)	I	NCT04047862	AST
	Anti PD-L1 (BGB-A333)	I/II	NCT03379259	AST
	PARP inhibitor (BGB-290)	I	NCT02660034	AST
Nivolumab	Metformin + rosiglitazone	II	NCT04114136	RCC, NSCLC, HCC, MSI-H, UC, GC, HNSCC
	Ipilimumab or BMS-986218	I/II	NCT03110107	AST
	Ipilimumab	II	NCT02834013	AST
	Anti-TIGIT (BMS-986207)	I/IIa	NCT02913313	AST
	Anti-TIGIT (OMP-313M32)	I	NCT03119428	Neoplasms
	Anti-LAG3 (relatlimab)	I	NCT02966548	AST
	Anti-VEGF (axitinib)	II	NCT03595124	RCC
	Relatlimab + ipilimumab	II	NCT04080804	HNSCC
	Relatlimab or ipilimumab + IDO1 inhibitor (BMS-986205)	I/II	NCT03459222	LAST
	Pembrolizumab + a protein kinase C inhibitor (trigriluzole)	II	NCT03229278	AST, RCC, HNSCC, NSCLC, BIC, M
	Ipilimumab or RGX-104 or docetaxel or pembrolizumab or carboplatin or demetrexed	I	NCT02922764	AST, NSCLC
	Adenoviral product (Ad-p53) + pembrolizumab or capecitabine	I/II	NCT02842125	AST, HNSCC
	Genetically modified HSV for tumor lysis (RP1)	I/II	NCT03767348	M, BIC, MRD, MSI-H
Avelumab	DC1c (BDCA-1) + myeloid DC + ipilimumab	I	NCT03707808	Neoplasms
	Anti-EGFR (cetuximab) + irinotecan	II	NCT03608046	CoC
ABBV-181	Anti-delta-like 3 protein (rovalpituzumab) or venetoclax	I	NCT03000257	NSCLC, TNIBC, OC, HC, GC, SCLC, Me, Cho, MCC, HNSCC
Sintilimab	IBI310	I	NCT03545971	AST
Spartalizumab, PDR001	Adenosine A2A receptor antagonist (NIR178)	II	NCT03207867	NSCLC, RCC, PC, UC, HNSCC, MSCC, TNIBC, M
SHR-1210	Parp inhibitor (SHR3162)	I	NCT03182673	AST

(Continued)

TABLE 1 | Continued

Antibodies	In combination with	Phase	ClinicalTrial.gov identifier	Tumor
JNJ 63723283	Anti-FGFR1-4	I	NCT03547037	Neoplasms
AGEN2034	AGEN1884	II	NCT03894215	CeC
mAbs targeting PD-L1				
HLX20	–	I	NCT03588650	Neoplasms
KN035	–	I	NCT03248843	LAMST
	–	I	NCT03101488	HC
Atezolizumab	–	I	NCT02862275	Neoplasms
CS1001	–	Ia/Ib	NCT03312842	AST
MSB2311	–	I	NCT03463473	AST
SHR-1316	–	I	NCT03133247	AST
CK-301	–	I	NCT03212404	NSCLC, CC, HNSCC, M, RCC, UC, CoC, EC
LY3300054	CHK1 inhibitor (prexasertib)	I	NCT03495323	AST
Durvalumab	Tremelimumab	III	NCT03084471	Neoplasms
	Tremelimumab + fulvestrant	II	NCT03430466	BC
	Tremelimumab + azacitidine	Ib/II	NCT03019003	HNSCC
	Tremelimumab + radiation therapy	II	NCT03601455	BIC
	Tremelimumab + stereotactic body radiotherapy	Ib	NCT03275597	NSCLC
	Tremelimumab + metronomic vinorelbine	I/II	NCT03518606	BC, HNSCC, CeC, PrC
Avelumab	Anti-TNFRSF9 (utomilumab) + anti-OX40 (PF-04518600) + radiation therapy	I/II	NCT03217747	AST, PrC
mAbs targeting CTLA-4				
Fc-engineered IgG1 (AGEN1181)	–	I	NCT03860272	AST
Tremelimumab	PARP inhibitor (olaparib)	I/II	NCT02571725	OC
REGN4659	Cemiplimab	I	NCT03580694	NSCLC
INT230-6	Anti-PD-1	I/II	NCT03058289	M, HNSCC, BC, PC, LiC, CoC, LC, GB, BDC, OC, SCC
Ipilimumab	Oncolytic virus vaccine (pexa-Vec)	I	NCT02977156	Neoplasms
	SHR-1210	I	NCT03527251	NSCLC
	Nivolumab + anti cancer drugs	IIb	NCT03409198	BC
mAbs targeting TIM-3				
Sym023	–	I	NCT03489343	AST
INCAGNO02390	–	I	NCT03652077	CeC, GC, GC, EsC, HC, M, MCC, Me, NSCLC, Oc, HNSCC, RCC, MRD, UC
TSR-022	TSR-042 or anti-LAG3 (TRS-033)	I	NCT02817633	LAMST, CC, NSCLC
LY3321367	LY3300054	I	NCT03099109	AST
BGB-A425	Tislelizumab	I/II	NCT03744468	LAST
BISPECIFIC mAbs				
Anti-PD-1/anti-LAG3 (MDG013)	Anti-HER2 (margetuximab)	I	NCT03219268	Neoplasms
Anti-PD-1/anti-TIM3 (RO7121661)	–	I	NCT03708328	MeM, NSCLC, SCLC
Anti-PD-1/anti-CTLA-4 (AK104)	Oxaliplatin + capecitabine	I/II	NCT03852251	GC
Anti-PD-1/anti-CTLA-4 (MGD019 DART)	–	I	NCT03761017	AST
Anti-PD-L1/anti-TIM3 (LY3415244)	–	I	NCT03752177	AST
Anti-CTLA-4/anti-OX40 (ATOR-1015)	–	I	NCT03782467	AST

(Continued)

TABLE 1 | Continued

Antibodies	In combination with	Phase	ClinicalTrial.gov identifier	Tumor
Anti-CTLA-4/anti-LAG3 (XmAb®22841)	Pembrolizumab	I	NCT03849469	AST
Anti-HER2 (MBS301)	–	I	NCT03842085	BC, SC
Anti-GD2/anti-CD3 (hu3F8-BsAb)	–	I/II	NCT03860207	NB, OS
Fusion protein				
Anti-PD-L1/TGFβRII (M7824)	–	I/II	NCT03436563	MSI-H RC, CoC

*Immune checkpoint inhibitors reported in the column indicating the "combination" treatment: ipilimumab, pembrolizumab, AGEN1884, IBI310, tremelimumab, BMS-986218: anti-CTLA-4; nivolumab, pembrolizumab, TRS-042, tislelizumab, cemiplimab: anti-PD-1; atezolizumab, avelumab, durvalumab, LY3300054: anti-PD-L1.

**Abbreviation of solid tumors: AR, adenocarcinoma of rectum; AST, advanced solid tumors; BC, Breast Cancer; BlC, bladder cancer; BDC, bile duct cancer; BTC, biliary tract cancer; CeC, Cervical Cancer; Cho, cholangiocarcinoma; CoC, Colorectal Cancer; CoS, chondrosarcoma; EC, endometrial cancer; EsC, esophageal cancer; ES, Ewing's sarcoma; ESCC, esophageal squamous cell carcinoma; FTC, fallopian tube cancer; GC, Gastric Cancer; GyC, gynecological cancers; GB, glioblastoma, HC, Hepatocellular Carcinoma; HNSCC, Head and Neck Squamous Cell Carcinoma; HRM, high risk melanoma; LAMST, locally advanced or metastatic solid tumors; LaC, laryngeal cancer; LC, Lung Cancer; LiC, liver cancer; M, Melanoma; MCC, Merkel Cell Carcinoma; MGB, malignant glioma of brain; Me, mesothelioma; MEC, metastatic esophageal cancer; MeM, metastatic melanoma; MI, Microsatellite Instability; MRD, Mismatch Repair Deficiency; MSC, metastatic solid cancer; MSCC, microsatellite stable colon cancer; MSI-H, non-endometrial deficient mismatch repair (dMMR)/microsatellite instability-high; NC, nasopharyngeal carcinoma; NeT, neuroendocrine tumors; NB, neuroblastoma; NMIBC, non-muscle invasive bladder cancer; NSCLC, Non-Small Cell Lung Cancer; OC, Ovarian Cancer; OS, osteosarcoma; PC, Pancreatic Cancer; PDCC, pancreatic ductal cell carcinoma; PhC, pharyngeal cancer; PPC, primary peritoneal cancer; PrC, prostate carcinoma; RCC, Renal Cell Carcinoma; RS, rhabdomyosarcoma; SC, stomach cancer; SCC, squamous cell carcinoma; SCLC, Small Cell Lung Cancer; STS, soft tissue sarcoma; TC, Thyroid Cancer; TNIBC, triple-negative invasive breast carcinoma; ToC, tongue cancer; UC, Urothelial Carcinoma; UrC, urological cancer. Abbreviation of cells: DCs, dendritic cells; CIK, Cytokine-induced killer cells.*

NK cells *in vitro* by reducing their apoptosis (170). In a xenograft model, anti-PD1 suppressed the tumor growth of digestive cancers in an NK cell-dependent manner, suggesting a crucial role of PD1 in NK cell function (170). Moreover, anti-CTLA-4 combined with IL-15/IL-15Rα enhances the NK cell tumor infiltration, improving the tumor growth control in xenograft murine models of solid tumors. Melanoma patients treated with anti-CTLA-4 (ipilimumab) had higher intratumoral CD56 expression (171). Consistently, several phase I and II clinical trials are ongoing to test the efficacy of different anti-PD1, anti PD-L1, and anti-CTLA-4 mAbs, alone or in combination with other Abs, in multiple forms of advanced and metastatic solid tumors (**Table 1**). The competition of TIGIT and CD96 binding to DNAM-1 ligands PVR and Nectin-2 renders tumor-infiltrating NK cells exhausted (172–174). TIGIT-targeting therapy represents a promising cure for solid tumor (172, 173, 175). In preclinical studies, the murine model anti-TIGIT is able to improve the antitumor effect of anti-HER2 mAb (176) alone, and in combination with PD1/PD-L1 inhibitors (176). Preclinical studies showed that CD96 blocking combined with anti-PD1 or anti-CTLA-4 enhances NK cell infiltration and IFN-γ production, thus reducing tumor lung metastases (177). The blockade of TIM-3 was also found to enhance NK cell function against melanoma cells (178). The therapeutic efficacy of the combined use of different immune checkpoint inhibitors, such as anti-TIM-3 and anti-TIGIT, is currently tested in ongoing phase I and II clinical trials in solid tumor patients (**Table 1**).

Different bispecific mAbs for immune checkpoint inhibitors and for GD2–CD3 are in phase I and II clinical trials, respectively (**Table 1**). In addition, various bispecific NK cell engaging antibodies, such as ErbB2-CD16, EpCAM-CD16, and HER2-CD16, were promising for solid tumors in preclinical studies (179–181). Interestingly, the combined use of antibodies with cytokines, such as anti-GD2/GM-CSF/IL-2 (182, 183), as well as the adoption of the novel fusion proteins by linking antibodies to cytokines, such as anti-GD2 to IL-15 superagonist (184), or the TNF-targeting human IgG1 NHS76 to IL-12 (185), reported great therapeutic results. Currently, the fusion protein anti-PD-L1/TGFβRII is in phase I/II clinical trial (**Table 1**). The use of a novel monomeric carcinoembryonic-antigen (CEA)-targeted immunocytokine, or cergutuzumab amunaleukin (CEA-IL2v, RG7813), has shown success in preclinical models of colon cancer, resulting in a large expansion of NK cells and activation of T cells (186).

Adoptive Transfer of NK and CAR-NK Cells

Recently, great interest in the treatment of solid tumors is focused on the adoptive transfer of *ex vivo* expanded and activated NK cells that, for their peculiar innate features, relatively short lifespan, low risk of overexpression in infused patients, higher safety compared to infused T cells, and low costs, could represent an optimal therapeutic strategy (187, 188). Activated and expanded NK cells can be obtained by different sources, including NK cell lines, primary NK cells, umbilical cord blood (UCB)- and induced pluripotent stem cell (iPSC)-derived NK cells (189) (**Table 1**). Of note, NK cells are tolerant to health cells and sensible to tumor cells, mostly in alloreactive conditions (190). Thus, haploidentical allogenic NK cells represent an optimal cellular immunotherapy product, mainly for immunocompromising diseases, including solid tumors, whose patients can scantly count on their own cells and often need donor NK cells (191–193). Interestingly, there are several clinical trials in phase I and II in progress to treat solid tumors based on NK cell adoptive transfer combined with ALT-803, mAbs, anti-cancer drugs, irreversible electroporation, and

TABLE 2 | Some selected and most recent clinical trials (since January 1st 2016) on NK cell-based immunotherapies, in different forms of solid tumors, by using adoptive transfer of NK and CAR-NK cells.

ADOPTIVE TRANSFER OF *EX VIVO* EXPANDED AND ACTIVATED NK CELLS				
NK cell source	**In combination with**	**Phase**	**ClinicalTrial.gov identifier**	**Tumor**
NK-92	ALT-803	II	NCT02465957	MCC
Autologous vs. allogenic	–	II	NCT02853903	Neoplasms
Autologous	–	E I	NCT03662477	LC
	–	II	NCT03410368	NSCLC
	Bortezomib	I	NCT00720785	PC, CoC, NSCLC
	Anti cancer drugs	II	NCT02734524	NSCLC
	Anti-GD2 (Ch14.18) + lenalidomide	I	NCT02573896	NB
	Sintilimab	II	NCT03958097	NSCLC
Allogenic	–	I	NCT03358849	BTC
	ALT-803	I	NCT02890758	CoC, AR, STS, ES, RS
	Anti-GD2 (Hu14.18)-IL2 fusion protein	I	NCT03209869	NB
	Anti-GD2 + IL2	I/II	NCT03242603	NB
	Anti-GD2 (Hu3F8) + rIL2	I	NCT02650648	NB
	Pemetrexed	I	NCT03366064	NSCLC
NK cells *(not specified)*	–	I	NCT03619954	AST, OC
	Trastuzumab	I/II	NCT02843126	BC
	Cetuximab	I/II	NCT02845856	NSCLC
	Anti-VEGF (bevacizumab)	I/II	NCT02857920	Neoplasms
	Nivolumab	I/II	NCT02843204	Neoplasms
	Irreversible electroporation (IRE)	I/II	NCT02718859	PC
		I/II	NCT03008343	LiC
	Cryosurgery	I/II	NCT02843802	LiC
		I/II	NCT02844335	BC
		I/II	NCT02849379	ToC
		I/II	NCT02849314	LaC
		I/II	NCT02849327	PhC
		I/II	NCT02849340	CeC
		I/II	NCT02849015	LiC
		I/II	NCT02843581	MEC
		I/II	NCT02849353	OC
		I/II	NCT02843815	NSCLC
		I/II	NCT02843607	RCC
		I/II	NCT02849366	RS
	NKT cells	I	NCT03198923	NSCLC
CIK	DCs	I/II	NCT03047525	CcC, RC, NC, LC
UCB-derived	–	I/II	NCT03634501	LC, BC, CoC, PC, OC
	Anti cancer drugs	I	NCT03420963	AST
		I	NCT03539406	OC
iPSC-derived (FATE-NK100)	IL-2	I	NCT03213964	OC
	Anti-HER2/neu and anti-EGFR	I	NCT03319459	BC, CoC, HNSCC, HC, NSCLC, RCC, PC, M
iPSC-derived (FT500)	–	n. i.	NCT04106167	Neoplasms
	Immune checkpoint inhibitors	I	NCT03841110	Neoplasms
ADOPTIVE TRANSFER OF CAR-NK CELLS				
CAR NK cells	**In combination with**	**Phase state**	**ClinicalTrial.gov identifier**	**Tumor**
CD16A-IL2-NK-92 (haNK)	–	I	NCT03027128	LAST

(Continued)

TABLE 2 | Continued

CAR NK cells	In combination with	Phase state	ClinicalTrial.gov identifier	Tumor
	IL-15 superagonist (N-803) and avelumab	II	NCT03853317	MCC
	Anti-cancer drugs, vaccines and immune checkpoint inhibitors	I/II	NCT03387111	SCC, neoplasms
ROBO1-NK cells	–	I/II	NCT03940820	Neoplasms
BiCAR-NK cells (ROBO1 CAR-NK cells)	–	I/II	NCT03941457	PC
ErbB2/HER2-NK (NK-92/5.28z)	*Intracranial application*	I	NCT03383978	GB
NKG2D-NK	IL-2	I	NCT03415100	Neoplasms, OC
MUC1-NK	–	I/II	NCT02839954	HC, NSCLC, PC, TNIBC, MGB, CoC, GC

*Abbreviation of solid tumors and immune checkpoint inhibitors reported in the column indicating the "combination" treatment: see **Table 1** legend.*
Abbreviation of cells: DCs, dendritic cells; CIK, Cytokine-induced killer cells; NKT, natural killer-T cells; iPSC, induced pluripotent stem cells; UCB, umbilical cord blood. Other abbreviations: E I, early I phase; n. i., non-interventional, observational study.

cryosurgery (**Table 2**). This approach could overcome the effects of tumor immune evasion (194, 195) since mature and activated NK cells provided by current protocol of iPSC-derived NK cell expansion are able to infiltrate and kill solid tumors. This was firstly evaluated in a murine xenograft model of ovarian cancer (196), and more recently in subjects with advanced solid tumors as monotherapy and in combination with mAb (NCT03319459) or antitumor drugs (NCT03213964) (**Table 2**). Of interest, among most efficient immune-modulatory drugs able to induce ligands for NK-ARs on cancer cells (197), lenalidomide is used in a clinical setting in combination with expanded and activated NK cells in some hematopoietic malignancies and neuroblastoma patients (NCT02573896).

In order to potentiate both the specificity and activity of NK cells against solid tumors, genetically modified NK cells have been produced and clinically adopted with promising results. NK cells obtained by different sources have been engineered for CARs able to recognize specific tumor antigens or ligands for activating receptors (198–201). To avoid the need of exogenous IL-2 in culture, high-affinity NK (haNK) cells were obtained by engineering the NK-92 cell line for the expression of CD16A and IL-2 (202) and used in patients with solid tumors (NCT03027128). Several CAR-engineered NK cells recognizing EGFR for breast cancer brain metastases (203), both ErbB2/HER2 (204, 205) (NCT03383978) upon intracranial injection, and EGFRvIII (206) for glioblastoma, GD2 for neuroblastoma (207), EpCAM for breast carcinoma (208), NKG2D for ovarian cancer (201), and both MUC1 and ROBO1 for advanced refractory solid tumors, have been produced for preclinical studies and phase I and II clinical trials (**Table 2**). Finally, of emerging interest are the extracellular vesicles (EVs) secreted by large-scale *in vitro* expanded NK cells, that, containing lytic protein, showed antitumor efficacy not only in malignant hematopoietic cell lines but also in neuroblastoma and breast carcinoma cell lines, and in a xenograft murine model of glioblastoma (209, 210).

PERSPECTIVES

NK cells play a crucial role in triggering antitumor immune response. Although high levels of tumor infiltrating NK cells are associated with a better prognosis in certain human solid tumors, the immunosuppressive TME weakens their function in favor of neoplastic progression. Understanding the mechanisms adopted by the TME to hinder the NK cell function and how they can be neutralized is of fundamental importance to develop effective anti-cancer therapeutic protocols. In this regard, the use of NK cell-based immunotherapy, by the combined use of cytokines, mAbs, immune checkpoint inhibitors, and the adoptive transfer of NK cells and CAR-NK cells, appears to be promising in the treatment of solid tumors.

A significant number of cancer immunotherapies that involved engineering NK cells before adoptive transfer into patients have been developed. There is the possibility of engineering NK cells to make them resistant to the metabolically restrictive TME as well as to immunosuppressive molecules generated by the tumor and TME.

Overall, it is now clear that TME is crucial for the normal function of NK cells and that future investigations and pre-clinical studies in this area are likely needed to fully discern the biology of NK cells and reveal new and exciting anti-cancer therapeutic opportunities.

AUTHOR CONTRIBUTIONS

All the authors edited the manuscript and approved the final version for publication.

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High Cytotoxic Efficiency of Lentivirally and Alpharetrovirally Engineered CD19-Specific Chimeric Antigen Receptor Natural Killer Cells Against Acute Lymphoblastic Leukemia

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Autologous chimeric antigen receptor-modified (CAR) T cells with specificity for CD19 showed potent antitumor efficacy in clinical trials against relapsed and refractory B-cell acute lymphoblastic leukemia (B-ALL). Contrary to T cells, natural killer (NK) cells kill their targets in a non-antigen-specific manner and do not carry the risk of inducing graft vs. host disease (GvHD), allowing application of donor-derived cells in an allogenic setting. Hence, unlike autologous CAR-T cells, therapeutic CD19-CAR-NK cells can be generated as an off-the-shelf product from healthy donors. Nevertheless, genetic engineering of peripheral blood (PB) derived NK cells remains challenging and optimized protocols are needed. In our study, we aimed to optimize the generation of CD19-CAR-NK cells by retroviral transduction to improve the high antileukemic capacity of NK cells. We compared two different retroviral vector platforms, the lentiviral and alpharetroviral, both in combination with two different transduction enhancers (Retronectin and Vectofusin-1). We further explored different NK cell isolation techniques (NK cell enrichment and CD3/CD19 depletion) to identify the most efficacious methods for genetic engineering of NK cells. Our results demonstrated that transduction of NK cells with RD114-TR pseudotyped retroviral vectors, in combination with Vectofusin-1 was the most efficient method to generate CD19-CAR-NK cells. Retronectin was potent in enhancing lentiviral/VSV-G gene delivery to NK cells but not alpharetroviral/RD114-TR. Furthermore, the Vectofusin-based transduction of NK cells with CD19-CARs

delivered by alpharetroviral/RD114-TR and lentiviral/RD114-TR vectors outperformed lentiviral/VSV-G vectors. The final generated CD19-CAR-NK cells displayed superior cytotoxic activity against CD19-expressing target cells when compared to non-transduced NK cells achieving up to 90% specific killing activity. In summary, our findings present the use of RD114-TR pseudotyped retroviral particles in combination with Vectofusin-1 as a successful strategy to genetically modify PB-derived NK cells to achieve highly cytotoxic CD19-CAR-NK cells at high yield.

Keywords: chimeric antigen receptor, natural killer cells, acute lymphoblastic leukemia, alpharetroviral vector, lentiviral vector, gene therapy, CD19

INTRODUCTION

Treatment of refractory and relapsed B-cell acute lymphoblastic leukemia (B-ALL) is challenging and relies on therapies such as chemotherapy, monoclonal antibodies, and hematopoietic stem cell transplantation (1). Nevertheless, the outcome of these patients remains poor (2, 3). In clinical trials, autologous chimeric antigen receptor-modified (CAR) T cells with specificity for CD19 showed potent antitumor efficacy against relapsed and refractory B-ALL (4–8). However, the usage of CAR-T cells is restricted to an autologous setting as allogenic T cells, even if HLA-matched, carry the risk of inducing graft vs. host disease (GvHD) (9, 10). Additionally, generation of relevant doses of CAR-T cells for heavily pretreated patients suffering from lymphopenia may be impracticable. Hence, an allogenic product for those patients might offer a suitable alternative.

Natural killer (NK) cells are cytolytic lymphocytes that represent the first line of defense against aberrant cells caused by viral infections or malignancies (11, 12). Contrary to T cells, they do not cause GvHD, allowing application of donor-derived cells in an allogenic setting, which has been successfully demonstrated with cytokine expanded donor-derived NK cells (13–18). Therefore, CAR-NK cells generated as an off-the-shelf product from healthy donors, offer an alternative to CAR-T cells. Additionally, NK cells kill their targets in a non-antigen-specific manner using germ-line encoded receptors which recognize and kill aberrant cells (19, 20). Thereby, NK cells detect aberrant cells, which express elevated levels of stress induced ligands ("induced self") (21, 22), or cells, which downregulate or lose their human leukocytes antigen (HLA) I molecules ("missing self") (23, 24), a mechanism to escape T cell mediated killing (25). Furthermore, most NK cells express CD16 (FcγRIII), which allows them to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (11). Taken together, CD19-CAR-NK cells might attack tumor cells utilizing both the CAR-dependent and CAR-independent NK-cell intrinsic mechanisms, potentially killing a heterogeneous tumor cell population.

NK cells for genetic modification can be derived from different sources. The NK cell line NK-92 is widely used for CAR-NK cell engineering (26) and expressing CD19-specific CARs, they already showed high cytotoxic activity against leukemic cells (27). Nevertheless, as NK-92 cells are tumor derived cells (28), they must be irradiated prior clinical use to avoid permanent engraftment in patients. Additionally, they do not express CD16 and thus are unable to mediate ADCC (28, 29). Another possible source for CAR-NK cell engineering are NK cells enriched from peripheral blood (PB) (30). Compared to NK cell lines, they express a wider range of activating receptors (31) and do not require irradiation, making them a favorable NK cell source. First clinical trials using CAR-NK cells derived from a variety of sources (including PB-derived NK cells) have been started (32, 33).

To date, genetic engineering of PB-derived NK cells remains challenging, mainly as gene transfer efficiency into NK cells is low. Mostly retroviral vectors are used to genetically modify NK cells and a variety of protocols including different viral vector systems (alpharetroviral, gammaretroviral, or lentiviral), culture conditions and transduction enhancers are described [for review see (34)]. Often, genetically modified K562 cells, a tumor derived cell line, are used as feeder cells to enhance expansion [for review see (35)] and transduction of NK cells (36, 37). Previous approaches generated CD19-CAR-NK cells from PB-derived NK cells containing variable levels of CAR^+ cells depending on the used protocol. In a feeder cell-based protocol a high number of transduced cells was achieved (36). In contrast, PB-derived NK cells cultured and transduced in a feeder cell-free protocol underperformed, reaching only low amounts of transduced cells (38). Hence, optimized feeder cell-free protocols achieving transduced NK cells at high yield are lacking.

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; ASCT-2, acronym for Alanine, Serine, Cysteine Transporter 2. A sodium-dependent neutral amino acid transporter; BaEV, modified baboon envelope glycoprotein; B-ALL, B-cell lymphoblastic acute leukemia; BSA, bovine serum albumin; CAR, chimeric antigen receptor; CBA, cytometric bead array; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; E:T, effector to target ratio; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; GvHD, graft vs. host disease; HBSS/HEPES, Hank's balanced salt solution containing 2,5% of 1M HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; HLA, human leukocytes antigen; IFN-γ, interferon gamma; IL-15, interleukin 15; LV, lentiviral; MFI, mean fluorescence intensity; MIP-1α, macrophage inflammatory protein 1-alpha; MOI, multiplicity of infection, NK cell, natural killer cell; ns, not significant; NT-NK cell, non-transduced natural killer cell; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; Pen/Strep, Penicillin and Streptomycin; RD114-TR, modified version of the feline endogenous retrovirus (RD-114 virus) envelope; RPMI, Roswell Park Memorial Institute; scFv, single-chain variable fragment; SD, standard deviation; SFFV, Spleen Focus-Forming Virus; SIN, Self-Inactivating; SP, signal peptide; TNF-α, tumor necrosis factor alpha; VSV-G, vesicular stomatitis virus G glycoprotein; α, alpharetroviral.

In our study, we focused on optimizing the generation of PB-derived CD19-CAR-NK cells by viral transduction using a feeder cell-free culture. In addition to earlier studies, we compared lentiviral and alpharetroviral transduction, different transduction enhancers (Retronectin and Vectofusin-1) and NK cell isolation methods (NK cell enrichment and CD3/CD19 depletion) to identify the most efficient methods for genetic engineering of NK cells. Finally, we generated CD19-CAR-NK cells at high yield with an increased cell killing activity of leukemic cells.

MATERIALS AND METHODS

Cell Lines

Human embryonic kidney (HEK) 293T cells and human fibrosarcoma cell line HT1080 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (all Gibco). The cells were split every 2–3 days. Chronic myologenous erythroleukemia cell line K562 was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, GlutaMAX (Gibco) containing 10% FBS and 1% penicillin/streptomycin (Pen/Strep; Gibco). ALL cell line Sup-B15 was cultured in RPMI 1640 medium, GlutaMAX supplemented with 15% FBS and 1% Pen/Strep. Both leukemic cell lines were split every 3–4 days.

Isolation of NK Cells

This study was approved by the Ethics Committee of the Goethe University Frankfurt, Germany (approval no. 329/10). All participants gave written informed consent in accordance with the Declaration of Helsinki. NK cells were isolated from freshly generated, healthy and anonymous donor buffy coats provided by the German Red Cross Blood Donation Service (DRK-Blutspendedienst Baden-Württemberg-Hessen, Frankfurt, Germany) using Ficoll density gradient centrifugation (Biocoll, Biochrom) to isolate peripheral blood mononuclear cells (PBMCs) and finally enrich NK cells by immunomagnetic negative selection. Enrichment of NK cells were performed either using EasySep Human NK cell Enrichment Kit (in the following referred to as NK cell enrichment) or a combination of using EasySep Human CD3 Positive Selection Kit II and EasySep Human CD19 Positive Selection Kit II (in the following referred to as CD3/CD19 depletion) according to manufacturer's instructions (all StemCell Technologies). The CD3/CD19 depletion uses positive selection of T cells and B cells to deplete them from unbound PBMCs mainly $CD56^+CD3^-$ NK cells and other remaining lymphocytes (mainly $CD14^+$ dendritic cells, monocytes etc.). NK cell purity and characterization were determined by flow cytometry using fluorochrome-conjugated antibodies against CD56 BV421 (clone NCAM16.2), CD3 BUV395 (clone SK7), CD19 BB515 (clone HIB19), CD20 BUV737 (clone 2H7), CD45 BV510 (clone HI30) (all BD Biosciences), CD14 BV711 (clone M5E2), and CD16 PE (clone 3G8) (both Biolegend). Detailed gating strategy is shown in the **Supplementary Figure 1**. Freshly isolated cells were cultured in X-VIVO 10 medium (Lonza) supplemented with 5% heat-inactivated human plasma (DRK-Blutspendedienst), 1% Pen/Strep and 10 ng/ml IL-15 (Peprotech) at a concentration of 2×10^6 cells/ml (NK cell enrichment) or 3×10^6 cells/ml (CD3/CD19 depletion).

Retroviral SIN Vectors

The lentiviral EGFP vector (RRL.PPT.SF.GFPpre), the alpharetroviral EGFP vector (AlphaSIN.noTATA) and the lentiviral CD19-CAR vector (S-CD19-CAR-W) have been previously described (27, 39–42). Briefly, the vectors were equipped with a Spleen Focus-Forming Virus (SFFV) promotor to drive expression of the transgene cassettes (EGFP, second-generation CD19-CAR). The second-generation CD19-CAR consists of an immunoglobulin heavy-chain signal peptide (SP), the anti-CD19 single-chain variable fragment (scFv) domain of a murine monoclonal antibody (FMC63) (43), a myc-tag (Myc), a CD8α hinge region, the CD28 transmembrane domain, and a composite CD28-CD3ζ signaling domain (27, 41). The alpharetroviral CD19-CAR vector (Alpha-CD19-CAR) was generated by removing the EGFP transgene of the alpharetroviral EGFP vector and replacing it by the CD19-CAR transgene of the lentiviral CD19-CAR vector. More detailed architectures of vectors are described in the **Supplementary Figure 2**.

Production of Retroviral Vector Particles and Titer Estimation

Viral vector particles were produced by transfection of HEK 293T cells using the calcium phosphate precipitation method. Twenty-four hours prior to transfection $4–5 \times 10^6$ or $10–11 \times 10^6$ HEK 293T cells were seeded on a 10 or 15 cm culture dish in DMEM supplemented with 10% FBS and 1% L-glutamine (all Gibco). For transfection, the medium was exchanged with DMEM containing 10% FBS, 1% L-glutamine, 1% Pen/Strep, and 25 μM chloroquine (Sigma-Aldrich). To produce lentiviral particles, following amounts of plasmids were added: 10 or 23 μg of the transfer vector (RRL.PPT.SF.GFPpre or S-CD19-CAR-W), 10 or 23 μg of a lentiviral gag/pol plasmid (pcDNA3 g/p 4xCTE) (39), 5 or 11.5 μg of a Rev plasmid (pRSV-Rev) and 1.5 μg or 3.45 μg of VSV-G (pMD2.G) envelope plasmid or 4.5 μg or 9.2 μg of RD114-TR (44) envelope plasmid. For production of alpharetroviral particles 5 or 11.5 μg of the transfer vector (AlphaSIN.noTATA or Alpha-CD19-CAR), 2.5 or 5.75 μg of an alpharetroviral gag/pol plasmid (pcDNA3.ASLV gp co) (40) and 2 or 9.2 μg of RD114-TR envelope plasmid were added. Medium was changed after 12–18 h. Supernatants containing the viral particles were collected 24, 36, and 48 h after transfection. They were filtered through a 0.22 μm filter, concentrated by centrifugation at 50,000 × g for 1 h (VSV-G, RD114-TR) or overnight at 4,500–11,600 × g (RD114-TR), resuspended in X-VIVO 10 medium and stored at −80°C. To enhance purification of viral particles some supernatants were centrifuged with the addition of 20% Sucrose/PBS. Viral titers were estimated by transducing HT1080 cells with different volumes of viral supernatant. 50,000 HT1080 cells per well of 12-well plates were seeded. After adherence, serial dilutions of viral supernatant and 4 μg/ml protamine sulfate were added. Three days later the percentage of $EGFP^+$ or $CD19\text{-}CAR^+$ cells was quantified by flow cytometry. Titers were calculated using following formula: Titer (viral particles/ml) = 50,000 cells × transgene positive events (%)/volume of supernatant (ml).

Transduction of Primary Human NK Cells

On day four after isolation, NK cells were transduced with lentiviral and alpharetroviral vector particles. Prior to transduction, NK cell purity (percent amount of $CD56^+CD3^-$ cells) was determined by flow cytometry as described above. NK cell purity was 90.0% for NK cells isolated by NK cell enrichment and 90.9% for NK cells isolated by CD3/CD19 depletion. For Retronectin-mediated transduction, flat bottomed 96-well plates were pre-coated with 25 μg/ml Retronectin (Takara) for at least 2 h at room temperature or overnight at 4°C. In the next step, Retronectin was removed and wells were blocked for 30 min with PBS containing 2% BSA. The blocking solution was discarded, and wells were washed once with HBSS/HEPES. Viral supernatants were added onto the Retronectin coated wells and the plate was centrifuged at 1,000 × g for 30 min at 4°C. Afterwards, NK cells were added reaching cell densities of 2×10^6 cells/ml for transductions with EGFP vectors or 10^5 cells/ml for transductions with CD19-CAR vectors. Finally, 10 ng/ml IL-15 was added. For Vectofusin-1 (Miltenyi Biotech) based transduction, Vectofusin-1 and viral supernatant were diluted separately in X-VIVO 10 media supplemented with 5% heat-inactivated human plasma and 1% Pen/Strep. Both diluted solutions contained identical volumes, were finally mixed, shortly vortexed and incubated for 5–10 min. Next, the mixture was added to NK cells plated on flat bottomed 96-well plates reaching cell densities of 2×10^6 cells/ml for transductions with EGFP vectors or 10^5 cells/ml for transductions with CD19-CAR vectors. The final concentration of Vectofusin-1 was 10 μg/ml per well. Finally, 10 ng/ml IL-15 was added and the plate was centrifuged at 800 × g for 1.5 h at 32°C. Both protocols contained a change of medium 24 h post-transduction. Half of the medium was discarded and replaced by fresh medium containing 10 ng/ml IL-15. Non-transduced (NT) NK cells of the same donor were used as negative controls. The NT-NK cells were manipulated the exact same way as the transduced (either EGFP or CAR) NK cells except no pseudotyped viral particles were added.

Flow Cytometric Analysis of Transduced NK Cells

Transgene expression, CD16 distribution and contamination with CD3 and CD14 positive cells of gene modified NK cells were analyzed 3 days after transduction by using a BD FACSCelesta flow cytometer (BD Biosciences). Fluorochrome-conjugated antibodies against CD56 BV786 (clone NCAM16.2), CD16 PE-CF 594 (clone 3G8), CD3 BUV395 (clone Sk7) (all BD Biosciences) and CD14 BV711 (Biolegend) were used. CD19-CAR expression was confirmed with a PE conjugated Myc-tag-specific antibody (clone 9B11, Cell Signaling Technology). NT-NK cells of the same donor were used as negative controls. Data were analyzed using FlowJo (FlowJo LLC). Detailed gating strategy is shown in the **Supplementary Figure 3**.

CD19 Expression and Cytotoxicity Assay

CD19 expression on the surface of K562 and Sup-B15 cells was analyzed by flow cytometry using a fluorochrome-conjugated antibody against CD19 BB515 (clone HIB19; BD Biosciences). Cytotoxicity of NK cells against K562 and Sup-B15 cells was determined in a flow cytometry-based cytotoxicity assay. Assays were performed with CD19-CAR-NK cells and NT-NK cells of the same donors 3 days after transduction. Target cells were harvested and stained with Cell Trace CFSE proliferation kit (Invitrogen) in a final concentration of 5 μM. Afterwards, they were washed with PBS and resuspended in X-VIVO 10 media supplemented with 5% heat-inactivated human plasma and 1% Pen/Strep. Target cells and NK cells were combined in U-bottom shaped 96-well plates at effector to target (E:T) ratios of 1:1, 0.5:1, 0.25:1, and 0.1:1 for 4 h, using 25,000 target cells per well in a total volume of 100 μl. After a coincubation time of 4 h plates were centrifuged at 300 × g for 5 min, the supernatant was removed, and cells were finally harvested and resuspended in 400 μl DAPI solution (DAPI 1 mg/ml, diluted in PBS at 1:6,000) (AppliChem GmbH). From each well the same amount of target cells was acquired using a BD FACSCelesta flow cytometer (BD Biosciences). Dead target cells were identified as CFSE and DAPI double positive and samples of target cells only were used as controls for spontaneous cell lysis. Detailed gating strategy is shown in the **Supplementary Figures 5**, **6**.

Cytometric Bead Array

Cytokine release by NT-NK and CD19-CAR-NK cells was examined using BD Cytometric Bead Array (CBA) analyses (BD Bioscience). Supernatants of NT-NK cells, CD19-CAR-NK cells, and Sup-B15 cells in culture as well as after coculture in cytotoxicity assays (E:T ratio of 1:1) were frozen at −80°C. Cytokine concentrations in supernatants were measured using BD CBA Flex Sets for granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α; macrophage inflammatory protein (MIP)-1α and interferon (IFN)-γ (BD Biosciences). The tests were performed according to the manufacturer's instructions. Data were acquired with the BD FACSVerse Bioanalyzer and were quantitated using the FCAP Array software (v3.0.1; BD Biosciences).

Long Term Culture of NK Cells

For long term culture of CD19-CAR-NK and NT-NK cells, NK cells were cultured in NK MACS medium (Miltenyi Biotec) supplemented with 5% heat-inactivated human plasma, 1% Pen/Strep, and 10 ng/ml IL-15 at a starting concentration of 1×10^6 cells/ml on the day of transduction (day 0). On day 3, 7, 11, and 14 post-transduction, transduction efficiency was determined by flow cytometry. On these days, NK cells were additionally counted, and the cell number was adjusted to 2×10^6 cells/ml in fresh NK MACS medium (5% heat-inactivated human plasma, 1% Pen/Strep, and 10 ng/ml IL-15) for expansion analysis.

Statistical Analysis

For statistical analysis, under the assumption of normal distribution of the NK function in healthy donors, data were analyzed by two-tailed paired *t*-test. The resulting approximatively $p < 0.05$ were considered significant and are indicated in the results. Only data from experiments with three or more donors ($n \geq 3$) were considered for statistical analysis. Statistical calculations were performed with GraphPad PRISM version 8 (GraphPad Software, Inc.).

RESULTS

Vectofusin-1 Promotes Lentiviral Transduction of NK Cells as Efficient as Retronectin

To identify the most efficacious method for genetic engineering of NK cells, we first focused on optimizing transduction of NK cells with lentiviral vectors. For that purpose, NK cells isolated from PBMCs by NK cell enrichment or CD3/CD19 depletion were pre-activated for 4 days with low dose IL-15 (10 ng/ml) prior to lentiviral transduction comparing two transduction enhancers, afterwards the gene modified NK cells were continuously cultured in IL-15 for at least 3 days when finally phenotyping was performed (**Figure 1A**). NK cells isolated by NK cell enrichment were transduced with the VSV-G pseudotyped lentiviral EGFP vector comparing Retronectin and Vectofusin-1 for enhancement (**Figure 1B**). Overall transduction rates did not significantly differ between both protocols. Mean

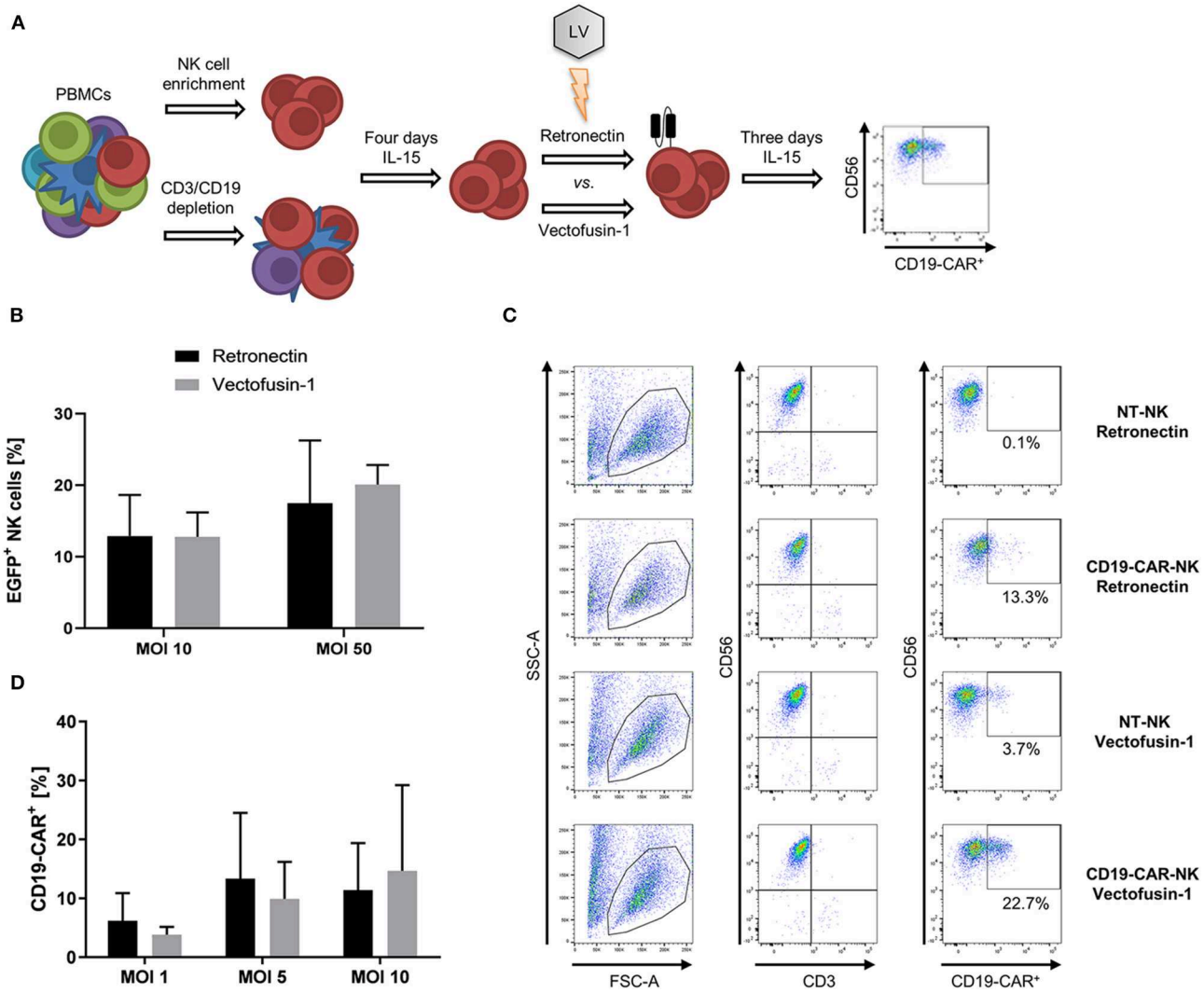

FIGURE 1 | Comparative lentiviral transduction of NK cells using Retronectin and Vectofusin-1. **(A)** Scheme of the transduction procedure. NK cells were isolated from PBMCs either by NK cell enrichment or CD3/CD19 depletion and cultivated for 4 days with IL-15 prior to transduction comparing the enhancers Retronectin and Vectofusin-1. After transduction, NK cells were cultivated for 3 days with IL-15 until transduction efficiency was analyzed by flow cytometry. **(B)** NK cells from three donors ($n = 3$) were transduced with VSV-G pseudotyped lentiviral EGFP particles at two different multiplicities of infection (MOI) and with two different transduction enhancers. **(C)** Gating strategy to estimate the transduction efficiency of NK cells transduced with VSV-G pseudotyped lentiviral CD19-CAR particles (e.g., for more detailed gating strategy see **Supplementary Material**). NK cells were identified as $CD56^+CD3^-$ leukocytes (first and second column). From those $CD19\text{-}CAR^+$ NK cells were estimated (third column). In the first and second row representative data of NK cells are depicted that were transduced with Retronectin at MOI 5 vs. non-transduced (NT) NK cells from NK cell preparations of the same donor. In the third and fourth row data from NK cells transduced with Vectofusin-1 at MOI 5 vs. NT-NK cells are shown. Percentage of false positive CD19-CAR events in NT-NK cells was subtracted from the percentages measured in the belonging transduced NK cells. Shown are the dot plots of one donor. **(D)** NK cells from four donors ($n = 4$) were transduced with VSV-G pseudotyped lentiviral CD19-CAR particles at shown MOIs and with two different transduction enhancers. Shown are mean values + SD. Statistical analysis was performed using two-tailed student's paired *t*-test. No significant differences were observed between analyzed groups in **(B,D)** as *p*-values were >0.05.

transduction rates of 12.9% with Retronectin and 12.8% with Vectofusin-1 were reached at MOI 10. A higher MOI of 50 could increase the rates up to 17.5% with Retronectin and 20.1% with Vectofusin-1. Next, we addressed generation of CD19-CAR-NK cells out of NK cells isolated by NK cell enrichment using the lentiviral second-generation CD19-CAR (lentiviral/VSV-G CD19-CAR) and compared the two transduction enhancers (**Figures 1C,D**). We could confirm the equality of Vectofusin-1 and Retronectin in their ability to enhance lentiviral gene delivery using the CD19-CAR. Transduction efficiencies of NK cells highly varied between different donors and, taken all MOIs together, a span of 0–34.0% transduced cells was reached. Using higher MOIs of 20 and 50 did not improve transduction efficiencies and a decline in total viable cells was observed with these MOIs (data not shown), so that the maximum used MOI was 10. The highest rates of transduced cells reached with Retronectin were 28.7% (MOI 5) and with Vectofusin-1 34.0% (MOI 10). Comparing mean transduction efficiencies, the best condition with the highest transduction rates was Vectofusin-1 combined with MOI 10 reaching 14.7% CD19-CAR-NK cells. Overall there was no significant difference between Retronectin or Vectofusin-1 based lentiviral transduction to generate CD19-CAR-NK cells.

Different Isolation Methods Are Suitable for the Generation of CD19-CAR-NK Cells

After we had established Vectofusin-1 based transduction of NK cells with VSV-G pseudotyped lentiviral vectors, we used this procedure in further lentiviral transduction experiments. Reason for this was the shorter and simpler usage of Vectofusin-1 compared to Retronectin. In the next step, we addressed whether NK cells isolated by CD3/CD19 depletion for genetic engineering may be more suitable for transduction with VSV-G pseudotyped second generation CD19-CAR lentiviral vectors as remaining bystander cells might support successful transduction. For that, NK cells isolated by different protocols from the same donors were compared. Overall, no significant differences on transduction efficiency could be observed between both isolation methods (**Figure 2A**, a representative dot plot is shown in **Supplementary Figure 4**). At MOI 10 highest rates of CD19-CAR-NK cells were reached for both isolation methods: 13.7% CD19-CAR-NK cells for CD3/CD19 depleted NK cells and 20.8% CD19-CAR-NK cells for NK cells isolated by NK cell enrichment. Mean fluorescence intensities (MFIs) of CD19-CAR-NK cells were on a same level for both isolation methods, regardless of used MOI (**Figure 2B**). Interestingly, highest MFIs for both isolation methods were reached with MOI 1. Furthermore, we analyzed CD19-CAR expression of NK cell subpopulations. Subpopulations are defined as $CD56^{high}CD16^{-}$ (hereinafter: $CD16^{-}$) and cytotoxic $CD56^{dim}CD16^{+}$ NK cells (hereinafter: $CD16^{+}$). In general, NK cell subpopulation distribution was not influenced by transduction (**Supplementary Figure 7**). Remarkably, CD19-CAR expression of $CD16^{+}$ and $CD16^{-}$ NK cell subpopulations did not significantly differ when compared within one isolation method, nor when one subgroup from one isolation method was compared with the belonging subgroup from the other isolation method (**Figure 2C**). Overall the relative CD19-CAR expression of the $CD16^{+}$ and $CD16^{-}$ NK cell subpopulations matched with the CD19-CAR expression of the total NK cell population (**Figures 2A,C**).

Alpharetroviral/RD114-TR Engineering of CD19-CAR-NK Cells Outperforms Lentiviral/VSV-G Engineering of CD19-CAR-NK Cells

Even though CD19-CAR-NK cells could be generated using lentiviral transduction, the resulting efficiencies remained on a moderate level independent of the used protocols. To further improve transduction efficiencies of NK cells to obtain genetically modified NK cells at high yield, we compared not only transduction efficiency, but also function of lentiviral and alpharetroviral CD19-CAR-vectors (**Figure 3A**). Previous work could show the feasibility of the alpharetroviral system for the genetic engineering of NK cells (45). For our study, alpharetroviral vectors were pseudotyped with RD114-TR because higher functional infectivity could be achieved with this envelope (own unpublished data) (40, 45). First, NK cells were transduced with the alpharetroviral EGFP vector comparing Retronectin and Vectofusin-1 based transduction. Interestingly, Vectofusin-1 promoted alpharetroviral transduction of NK cells better than Retronectin, reaching up to 70% transduced cells (**Figure 3B**). Next, we generated a CD19-CAR alpharetroviral vector by substituting the EGFP transgene cassette of the alpharetroviral EGFP vector for the second-generation CD19-CAR cassette of the lentiviral CD19-CAR vector. Vectofusin-1 based transduction of NK cells was performed using the new alpharetroviral CD19-CAR vector (alpharetroviral/RD114-TR CD19-CAR) and the lentiviral/VSV-G CD19-CAR vector. Across all MOIs the alpharetroviral/RD114-TR CD19-CAR vector outperformed the lentiviral/VSV-G CD19-CAR vector, reaching up to 82.9% transduced NK cells (**Figure 3C**). The lentiviral/VSV-G vector reached similar transduction rates of CD19-CAR-NK cells compared to previous results (**Figures 1D**, **2A**). In our study, MFIs of alpharetroviral/RD114-TR generated CD19-CAR-NK cells were significantly higher than those of lentiviral/VSV-G generated CD19-CAR-NK cells at MOI 5 and MOI 10 (**Figure 3D**), but did not differ at MOI 1. However, with higher MOI, the density of CAR-expression measured by MFI decreased following lentiviral/VSV-G CAR-transduction but increased following alpharetroviral/RD114-TR transduction (**Figure 3D**). Again, we analyzed the NK cell subpopulation distribution and their CD19-CAR expression. NK cell subpopulation distribution was neither influenced by lentiviral/VSV-G transduction nor by alpharetroviral/RD114-TR transduction (**Supplementary Figure 8**). CD19-CAR expression of $CD16^{+}$ and $CD16^{-}$ subpopulations did not significantly differ when compared within the viral vector system (**Figure 3E**) and matched with the CD19-CAR expression of the total NK cell population (**Figure 3C**). Overall, CD19-CAR expression of alpharetrovirally/RD114-TR transduced NK cell subpopulations outperformed those of lentiviral/VSV-G transduced NK cell subpopulations (**Figures 3C–E**).

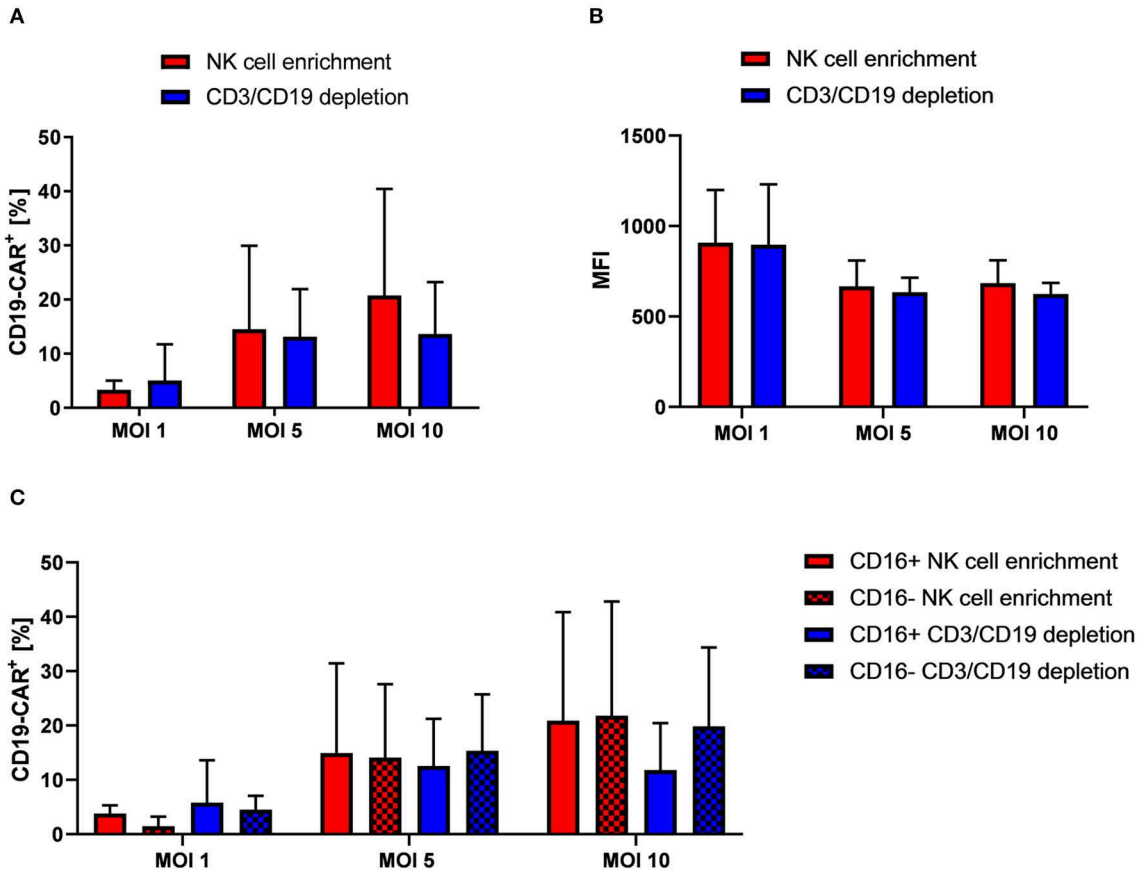

FIGURE 2 | NK cells isolated from PBMCs by NK cell enrichment or CD3/CD19 depletion show similar transduction efficiencies when transduced with VSV-G pseudotyped lentiviral CD19-CAR particles. **(A)** Vectofusin-1 based transduction of differently isolated NK cells was performed with VSV-G pseudotyped lentiviral CD19-CAR particles at shown MOIs. NK cells from four different donors were used *(n = 4)*. **(B)** Mean fluorescence intensities (MFI) of CD19-CAR in transduced cells. Data show average MFIs of CD19-CAR$^+$ cells transduced with depicted MOIs as shown in **(A)**. **(C)** CD19-CAR expression of CD16$^+$ and CD16$^-$ NK cell subpopulations. CD19-CAR expression of CD16$^+$ and CD16$^-$ NK cell subpopulations of transduced cells depicted in **(A)** are shown *(n = 4)*. Shown are mean values + SD. Statistical analysis was performed using two-tailed student's paired *t*-test. No significant differences were observed between analyzed groups transduced with the same MOI as *p*-values were >0.05.

CD19-CAR-NK Cell Products Produce High Levels of Inflammatory Cytokines

To further evaluate functional capacities of the CAR modified NK cells, cytokine production of GM-CSF, TNF-α, MIP-1α, and IFN-γ of lentivirally/VSV-G and alpharetrovirally/RD114-TR generated CD19-CAR-NK cells (both at MOI 5) was analyzed 3 days after transduction upon expansion in low dose IL-15 alone and in context of co-culturing with target-specific Sup-B15 ALL cells at an E:T ratio of 1:1 for 4 h. As controls, supernatant of Sup-B15 cells was analyzed. In general, CD19-CAR-NK cells tend to release more cytokines than NT-NK cells from the same donors regardless of target cell contact (**Figure 4**). This trend could be especially observed for CD19-CAR-NK cells transduced with lentiviral/VSV-G vectors (**Figure 4A**) for the release of MIP-1α and for CD19-CAR-NK cells transduced with alpharetroviral/RD114-TR vectors (**Figure 4B**) for the release of GM-CSF, TNF-α, MIP-1α, and IFN-γ. Of note, significant changes could only be observed for the release of MIP-1α of lentiviral/VSV-G CD19-CAR-NK cells upon contact with CAR specific target cells (**Figure 4A**) compared to NT-NK cells as well as compared to CD19-CAR-NK cells without target co-incubation. In the context of alpharetrovirally/RD114-TR transduced CD19-CAR-NK cells a slightly higher cytokine release of all analyzed cytokines could be shown, with significant changes only for GM-CSF (**Figure 4B**).

Alpharetrovirally/RD114-TR Generated CD19-CAR-NK Cell Products Surpass Killing Efficiencies of Lentivirally/VSV-G Generated CD19-CAR-NK Cell Products

Next, the cytotoxic potential of lentivirally/VSV-G and alpharetrovirally/RD114-TR generated CD19-CAR-NK cells needed further assessment (scheme **Figure 3A**). For that, CD19-negative K562 erythroleukemia cells and CD19-expressing Sup-B15 ALL cells (**Figure 5A**) were stained with CFSE and used as targets in a 4 h flow cytometry-based

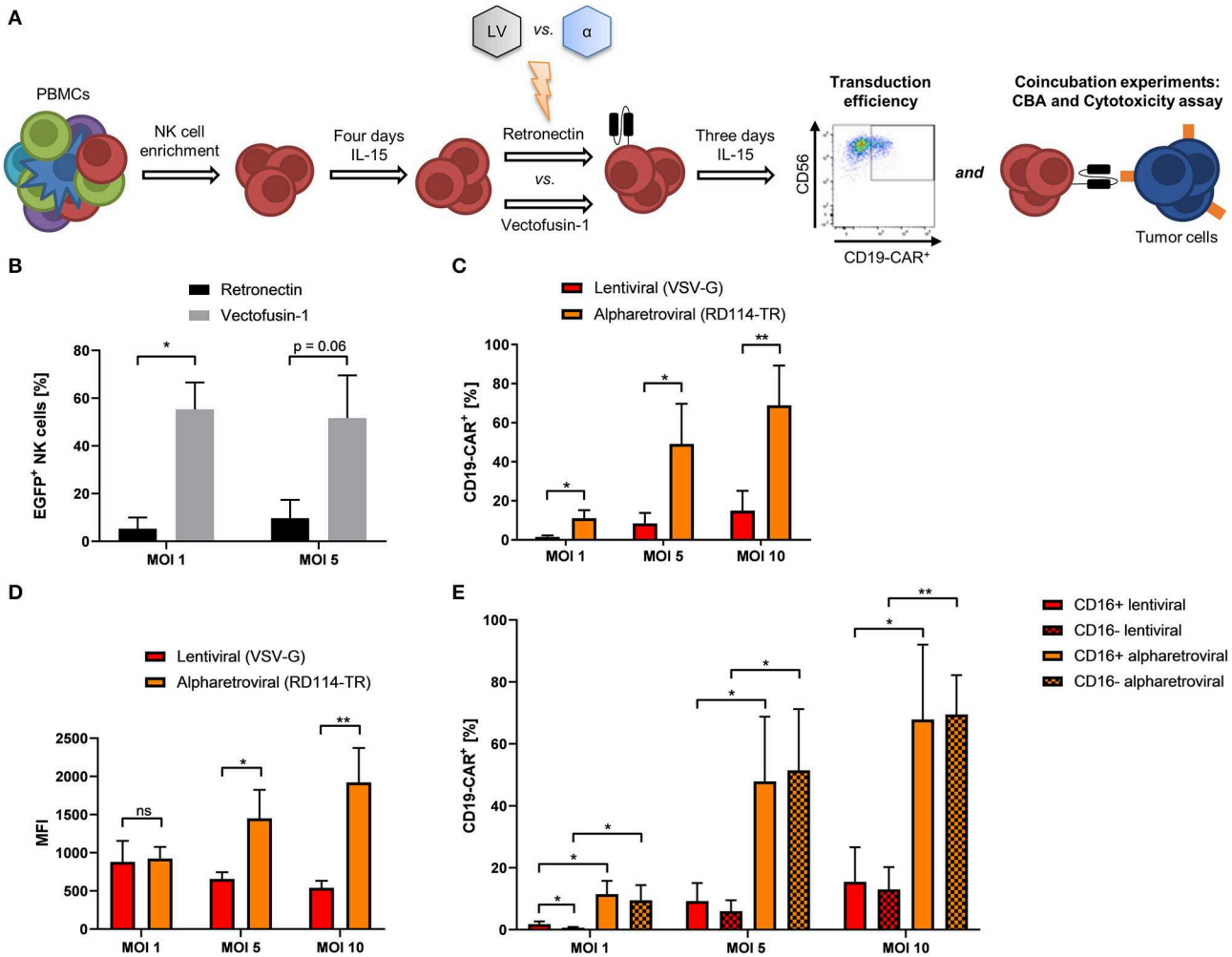

FIGURE 3 | Transduction rates of alpharetrovirally/RD114-TR modified NK cells outperform those of lentivirally/VSV-G modified NK cells. **(A)** Scheme of the comparative transduction procedure. NK cells were isolated from PBMCs by NK cell enrichment and cultivated for 4 days under the influence of IL-15 prior to transduction comparing the enhancers Retronectin and Vectofusin-1 and the lentiviral (LV) and alpharetroviral (α) vector system. After transduction, NK cells were cultivated for 3 days with IL-15 until transduction efficiency was analyzed by flow cytometry and functional assays were performed. **(B)** NK cells from three donors *(n = 3)* were transduced with RD114-TR pseudotyped alpharetroviral EGFP particles at shown MOIs. **(C)** Vectofusin-1 mediated transduction of NK cells from four donors *(n = 4)* was performed with RD114-TR pseudotyped alpharetroviral CD19-CAR particles or VSV-G pseudotyped lentiviral CD19-CAR particles at different MOIs. **(D)** MFI of CD19-CAR in transduced cells. Data show average MFIs of CD19-CAR$^+$ cells transduced with depicted MOIs as shown in **(B)**. **(E)** CD19-CAR expression of CD16$^+$ and CD16$^-$ NK cell subpopulations. CD19-CAR expression of CD16$^+$ and CD16$^-$ NK cell subpopulations of transduced cells depicted in **(B)** are shown *(n = 4)*. Shown are mean values + SD. Statistical analysis was performed using two-tailed student's paired *t*-test. $^{**}p < 0.01$; $^{*}p < 0.05$; ns, not significant.

cytotoxicity assay (for details see Materials and Methods). Lentivirally/VSV-G and alpharetrovirally/RD114-TR generated CD19-CAR-NK cells (both at MOI 5) and NT-NK cells were co-incubated with K562 or target-specific Sup-B15 for 4 h at E:T ratio of 1:1 (**Figure 5B**) or lower amount of effector cells (**Figures 5C,D**). In general, under assumption of normal distribution of NK cell function of healthy donors, CD19-CAR-NK cells showed a higher killing efficiency of CD19-expressing Sup-B15 cells than NT-NK cells (**Figures 5B–D**). At an E:T ratio of 1:1 the mean specific lysis of Sup-B15 was 90.5% for alpharetroviral/RD114-TR CD19-CAR-NK cells, 62.5% for lentiviral/VSV-G CD19-CAR-NK cells and 9.0% for NT-NK cells (**Figure 5B**). Interestingly, at an E:T ratio of 0.5:1 still comparable results were obtained (alpharetroviral/RD114-TR: 88.9%, lentiviral/VSV-G: 58.3%, NT-NK: 10.3%) (**Figure 5C**). As expected, lysis of CD19-negative K562 cells was on an equal level for all three NK cell preparations, without any significant difference between alpharetroviral/RD114-TR or lentiviral/VSV-G CD19-CAR-NK cells and NT-NK cells (**Figures 5B,C**). Mean transduction efficiencies of alpharetroviral/RD114-TR CD19-CAR-NK cells were 62.1% and 4.3% for lentiviral/VSV-G CD19-CAR-NK cells. In an additional assay, E:T ratios down to 0.1:1 were tested against Sup-B15 cells as targets. Even at low effector cell concentrations alpharetrovirally/RD114-TR

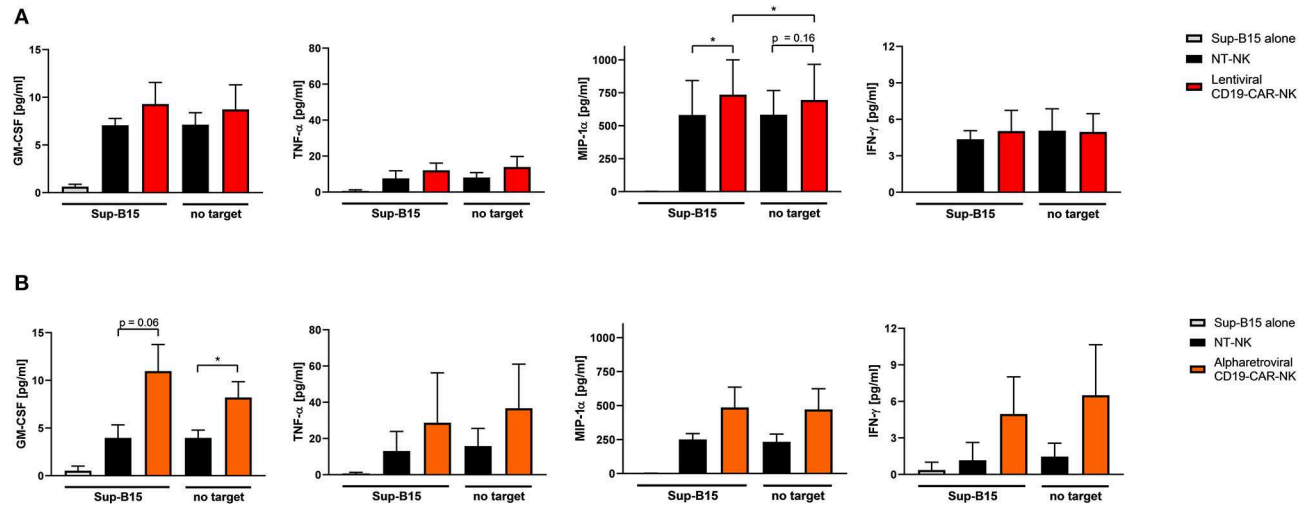

FIGURE 4 | Cytokine secretion of CD19-CAR-NK cells. NT-NK cells, lentiviral/VSV-G CD19-CAR-NK cells and alpharetroviral/RD114-TR CD19-CAR-NK cells were cocultured with Sup-B15 cells at an E:T ratio of 1:1 for 4 h. As controls, NK cells and Sup-B15 cells were cultured alone. Levels of GM-CSF, TNF-α, MIP-1α, and IFN-γ within the supernatants were measured using a cytometric bead array. **(A)** Shows cytokine secretion of lentiviral/VSV-G CD19-CAR-NK cells in comparison to NT-NK cells from three donors *(n = 3)*. Average transduction efficiency of used CD19-CAR-NK cells was 9.1% (range 7.3–11.6%). **(B)** Shows cytokine secretion of alpharetroviral/RD114-TR CD19-CAR-NK cells in comparison to NT-NK cells from three donors *(n = 3)*. Average transduction efficiency of used CD19-CAR-NK cells was 15.8% (range 8.1–20.3%). Experiments in **(A)** were performed with different donors than experiments in **(B)**. Shown are mean values + SD. Statistical analysis was performed using two-tailed student's paired *t*-test. $^*p < 0.05$. No significant differences were observed between analyzed groups without asterisks.

generated CD19-CAR-NK cells remained highly cytotoxic and induced 85.2% lysed cells at an E:T ratio of 0.1:1 (**Figure 5D**). Contrary, lentiviral/VSV-G generated CD19-CAR-NK cells showed reduced cytotoxicity levels at lower effector cell numbers (**Figure 5D**). In this experiment, transduction efficiency of alpharetroviral/RD114-TR CD19-CAR-NK cells was 60.5% and 6.9% of lentiviral/VSV-G CD19-CAR-NK cells. Calculating the total number of CD19-CAR-NK cells in the NK cell products, comparable values were obtained for lentiviral/VSV-G CD19-CAR-NK cells at E:T ratio of 1:1 (total of 1725 CD19-CAR-NK cells) and alpharetroviral/RD114-TR CD19-CAR-NK cells at E:T ratio of 0.1:1 (total of 1512 CD19-CAR-NK cells). Notably, the comparison of specific lysis of these two conditions (lentiviral/VSV-G E:T 1:1 specific lysis of 59.7% Sup-B15 cells; alpharetroviral/RD114-TR E:T 0.1:1 specific lysis of 85.2% Sup-B15 cells) revealed that alpharetrovirally/RD114-TR transduced CD19-CAR-NK cells showed a higher cytotoxicity than lentiviral/VSV-G CD19-CAR-NK cells.

Alpharetroviral and Lentiviral Vectors Perform Equally When Pseudotyped With RD114-TR

Finally, we investigated if the observed high transduction efficiencies using alpharetroviral vectors pseudotyped with RD114-TR were related to the alpharetroviral vector system or to the RD114-TR envelope. Prior transduction on day 4 we observed upregulation of the sodium-dependent neutral amino acid transporter 2 (ASCT2, also known as SLC1A5) in NK cells pre-activated with low dose IL-15 (10 ng/ml) (**Supplementary Figure 10**). ASCT2 serves as receptor for the feline endogenous retrovirus RD114, of which the RD114-TR envelope was derived (44, 46). As ASCT2 was upregulated, we hypothesized that RD114-TR may be the reason for high transduction rates using alpharetroviral/RD114-TR vectors and concluded a lentiviral vector pseudotyped with RD114-TR might perform equally well. Therefore, NK cells from the same donor were transduced with the lentiviral CD19-CAR vector pseudotyped with RD114-TR (lentiviral/RD114-TR CD19-CAR). NK cells transduced with either the alpharetroviral/RD114-TR CD19-CAR vector or the lentiviral/VSV-G CD19-CAR vector served as controls. Across all MOIs lentiviral/RD114-TR transduction performed equally compared to alpharetroviral/RD114-TR transduction, as no significant differences were observed between both groups (**Figure 6A**). Additionally, the lentiviral/RD114-TR CD19-CAR vector could also outperform the lentiviral/VSV-G CD19-CAR vector at MOI 1 and MOI 5. Highest transduction efficiencies reached with the lentiviral/RD114-TR transduction was 40.6% (MOI 10). The alpharetroviral/RD114-TR CD19-CAR vector performed similar and the highest efficiency it reached was 47.7% (MOI 10). Once more, we analyzed the NK cell subpopulation distribution and their CD19-CAR expression. NK cell subpopulation distribution was neither influenced by lentiviral transduction nor by alpharetroviral transduction (**Supplementary Figure 9**, data for lentiviral/VSV-G not shown). Interestingly, in the NK cell population transduced with the lentiviral/RD114-TR CD19-CAR vector, $CD16^+$ cells showed a higher CD19-CAR expression (**Figure 6B**). For NK cells

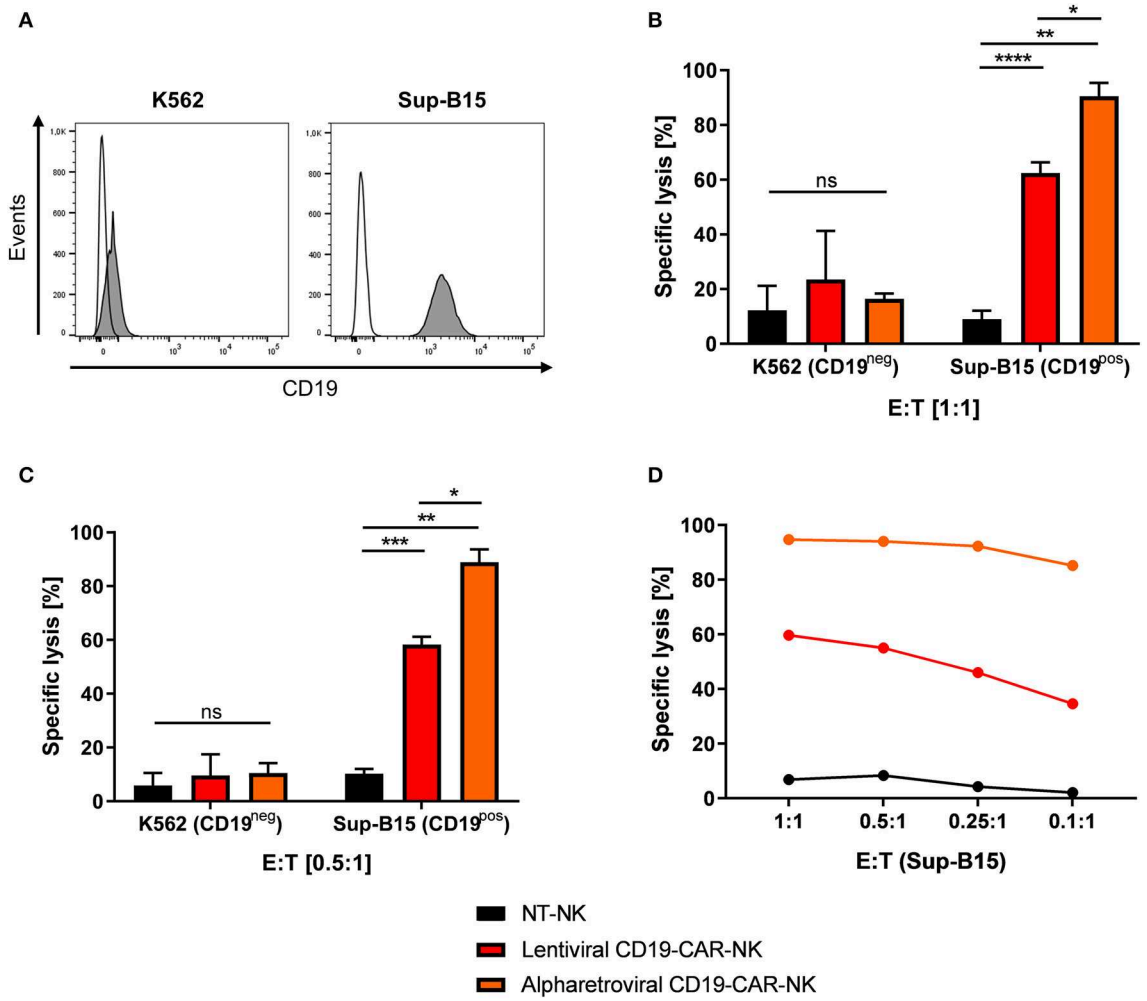

FIGURE 5 | Comparison of cytotoxic capacity of CD19-CAR-NK cells generated by lentiviral/VSV-G or alpharetroviral/RD114-TR transduction. **(A)** CD19 expression on the surface of K562 and Sup-B15 leukemia cells was analyzed by flow cytometry with a CD19-specific antibody (filled areas). Unstained cells were used as controls (empty areas). **(B,C)** Cytotoxic activity of NT-NK cells, lentiviral/VSV-G CD19-CAR-NK cells and alpharetroviral/RD114-TR CD19-CAR-NK cells against K562 and Sup-B15 cells was determined in a flow cytometry-based cytotoxicity assay after a coincubation time of 4 h and with different E:T ratios. Average transduction efficiency of used lentiviral/VSV-G CD19-CAR-NK cells was 4.3% (range 2.9–6.9%), of used alpharetroviral/RD114-TR CD19-CAR-NK cells was 62.1% (range 56.1–69.6%). Shown are mean values + SD from co-incubations using NK cells of three different donors *(n = 3)*. **(D)** Cytotoxic activity of NT-NK cells, lentiviral/VSV-G CD19-CAR-NK cells and alpharetroviral/RD114-TR CD19-CAR-NK cells against Sup-B15 at decreasing E:T ratios. Shown is a co-incubation using NK cells of one representative donor. Statistical analysis was performed using two-tailed student's paired *t*-test. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$, ns, not significant.

transduced with the alpharetroviral/RD114-TR CD19-CAR vector this could only be observed at MOI 1, as at higher MOIs comparable results to **Figure 3E** were achieved and CD19-CAR expression of $CD16^{+}$ and $CD16^{-}$ subpopulations did not significantly differ. Overall, CD19-CAR expression of alpharetrovirally/RD114-TR transduced NK cell populations were similar to those of lentivirally/RD114-TR transduced NK cell populations. Exceptions were observed for MOI 5 and MOI 10, as the alpharetroviral $CD16^{-}$ subpopulation expressed more CD19-CAR than the lentiviral $CD16^{-}$ subpopulation.

Next, we investigated transgene expression and expansion of generated CD19-CAR-NK cells over time. Therefore CD19-CAR-NK cells were generated using the lentiviral/RD114-TR CD19-CAR vector and the alpharetroviral/RD114-TR vector at MOIs 3–5, kept in culture with 10 ng/ml IL-15 and were analyzed by flow cytometry and counted every 3–4 days. First, we observed a decrease of the CD19-CAR expression for alpharetrovirally/RD114-TR generated CD19-CAR-NK cells (**Figure 6C**). But later on, CD19-CAR expression remained on a stable level for these cells. Lentivirally/RD114-TR generated CD19-CAR-NK cells expressed the CD19-CAR on an evenly level throughout the 14 days observation time (**Figure 6C**). Interestingly one donor could even increase the CD19-CAR expression level (from 15.3% on day 3 to 19.3% on day 14). Expansion of CD19-CAR-NK cells did not differ to NT-NK cells from the same donors, even though

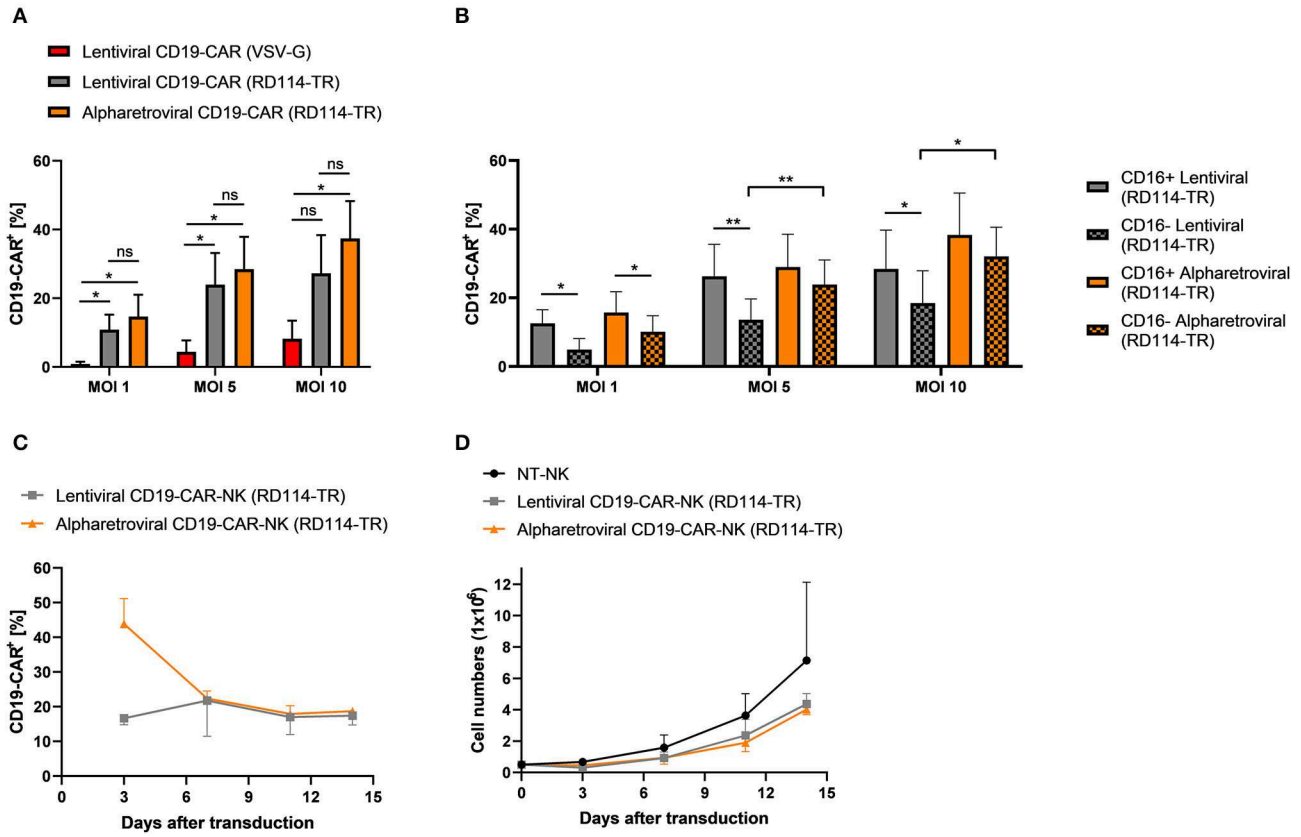

FIGURE 6 | Transduction rates of lentivirally/RD114-TR modified NK cells and alpharetrovirally/RD114-TR modified NK cells do not differ. **(A)** Vectofusin-1 mediated transduction of NK cells from four donors *(n = 4)* was performed with RD114-TR pseudotyped lentiviral CD19-CAR particles, RD114-TR pseudotyped alpharetroviral CD19-CAR particles or VSV-G pseudotyped lentiviral CD19-CAR particles at different MOIs. **(B)** CD19-CAR expression of $CD16^+$ and $CD16^-$ NK cell subpopulations. CD19-CAR expression of $CD16^+$ and $CD16^-$ NK cell subpopulations of lentiviral/RD114-TR and alpharetroviral/RD114-TR transduced cells depicted in **(A)** are shown *(n = 4)*. **(C)** After transduction (day 0), CD19-CAR expression of lentiviral/RD114-TR and alpharetroviral/RD114-TR CD19-CAR-NK cells was determined by flow cytometry on four serial time points. Data from two donors *(n = 2)* are shown. **(D)** Proliferation of NT-NK and CD19-CAR-NK cells was determined over a period of 14 days. Data from two donors *(n = 2)* are shown. Shown are mean values + SD. Statistical analysis for **(A)** and **(B)** was performed using two-tailed student's paired *t*-test. $^{**}p < 0.01$; $^{*}p < 0.05$; ns, not significant.

a slight better expansion could be observed for NT-NK cells (**Figure 6D**).

Functional and Cytotoxic Capacities of Lentivirally/RD114-TR Generated CD19-CAR-NK Cells Are Comparable to Alpharetrovirally/RD114-TR Generated CD19-CAR-NK Cells

To further evaluate functionality and cytotoxicity of lentivirally/RD114-TR generated CD19-CAR-NK cells (MOI 3–5), cytokine production and cytotoxicity of these cells was evaluated. Cytokine production of GM-CSF, TNF-α, MIP-1α, and IFN-γ of lentiviral/RD114-TR CD19-CAR-NK cells was analyzed 3 days after transduction upon expansion in low dose IL-15 alone and in context of co-culturing with target-specific Sup-B15 ALL cells at an E:T ratio of 1:1 for 4 h. As controls, supernatant of Sup-B15 cells was analyzed. In general, we observed similar results as shown in **Figure 4**: lentivirally/RD114-TR generated CD19-CAR-NK cells tend to release more cytokines than NT-NK cells from the same donors regardless of target cell contact (**Figure 7A**). To analyze cytotoxic capacities of lentiviral/RD114-TR generated CD19-CAR-NK cells (MOI 3–5), we performed cytotoxicity assays against CD19-negative K562 erythroleukemia cells and CD19-expressing Sup-B15 ALL cells as target cells. NT-NK cells and alpharetrovirally/RD114-TR generated CD19-CAR-NK cells (MOI 3–5) of the same donor served as controls and coincubations were performed at an E:T ratio of 1:1. Once more, CD19-CAR-NK cells showed a higher killing efficiency of CD19-expressing Sub-B15 cells than NT-NK cells (**Figure 7B**). Mean specific lysis of Sup-B15 cells was 74.7% for lentiviral/RD114-TR CD19-CAR-NK cells, 73.1% for alpharetroviral/RD114-TR CD19-CAR-NK cells, and 35.9% for NT-NK cells. Lysis of K562 cells was on an equal level for all three NK cell preparations. Mean transduction efficiencies of lentiviral/RD114-TR CD19-CAR-NK cells were 13.1% and 23.3% for alpharetroviral/RD114-TR CD19-CAR-NK cells. The lower transduction efficiencies might explain the lower observed

cytotoxic capacities compared to **Figure 5B**. Overall, in this setting lentivirally/RD114-TR generated CD19-CAR-NK cells performed equally compared to alpharetrovirally/RD114-TR generated CD19-CAR-NK cells.

DISCUSSION

NK cells modified to express a CD19-specific CAR represent an allogenic alternative to CD19-CAR-T cells for the therapy of relapsed and refractory B-ALL. However, genetic modification of PB-derived NK cells to express a CAR remains difficult. Here, we investigated different ways for genetic engineering of PB-derived NK cells to express a CD19-specific CAR by retroviral vectors. Highest transduction efficiencies were achieved with retroviral CD19-CAR vectors pseudotyped with RD114-TR envelope and combined with Vectofusin-1 as transduction enhancer, generating functional and highly cytotoxic CD19-CAR-NK cells.

Vectofusin-1, also known as LAH4-A4, is a cationic peptide derived from the LAH4 peptide family (47). Initially, LAH4 peptides were used as DNA transfection agents (48), but recent works showed the ability of Vectofusin-1 to promote transduction of human $CD34^{+}$ hematopoietic stem and

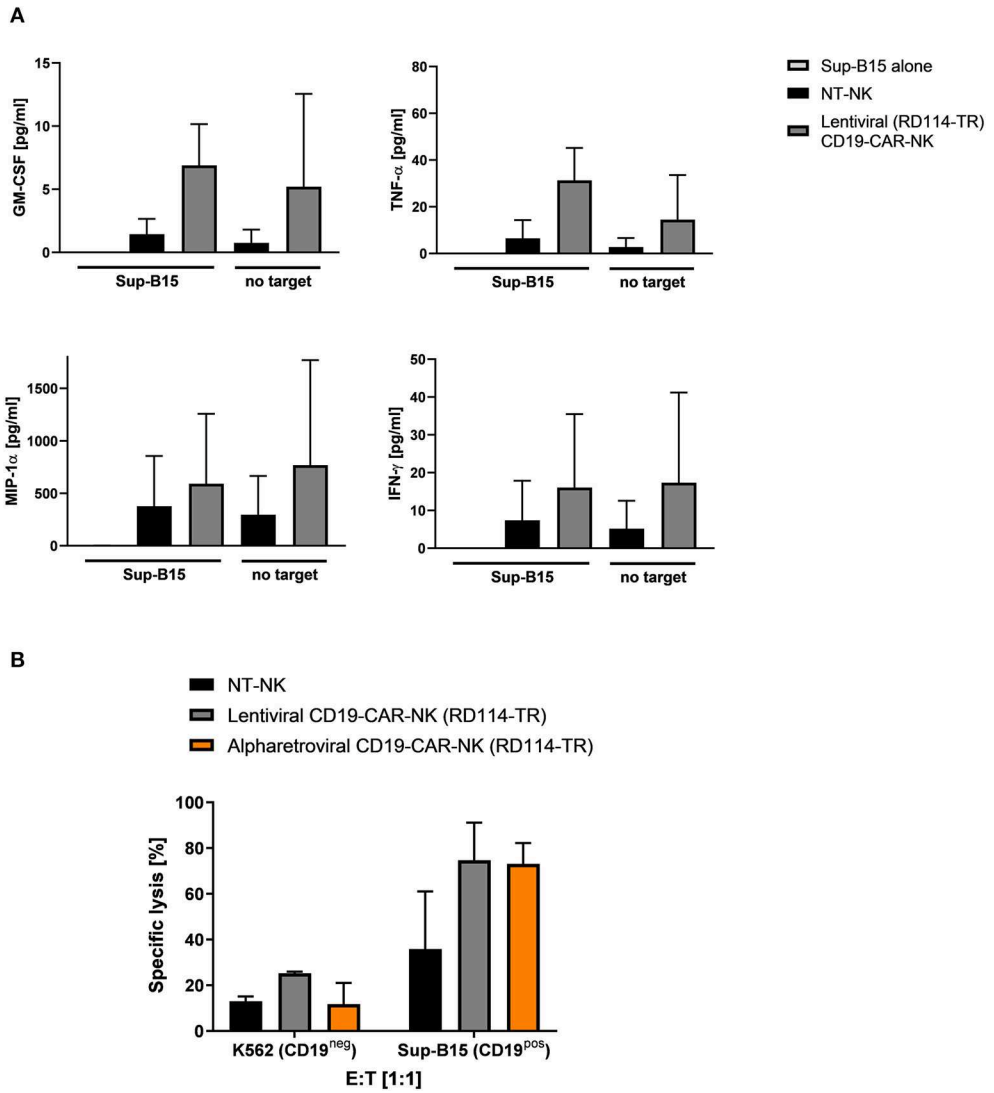

FIGURE 7 | Cytokine secretion and cytotoxic capacity of CD19-CAR-NK cells generated by RD114-TR pseudotyped lentiviral CD19-CAR particles. **(A)** NT-NK cells and lentiviral/RD114-TR CD19-CAR-NK cells were cocultured with Sup-B15 cells at an E:T ratio of 1:1 for 4 h. As controls, NK cells and Sup-B15 cells were cultured alone. Levels of GM-CSF, TNF-α, MIP-1α, and IFN-γ within the supernatants were measured using a cytometric bead array. Average transduction efficiency of used lentiviral/RD114-TR CD19-CAR-NK cells was 13.1% (range 11.6–14.5%). Cytokine secretion from two donors *(n = 2)* are shown. **(B)** Cytotoxic activity of NT-NK cells, lentiviral/RD114-TR CD19-CAR-NK cells and alpharetroviral/RD114-TR CD19-CAR-NK cells against K562 and Sup-B15 cells was determined in a flow cytometry-based cytotoxicity assay after a coincubation time of 4 h at an E:T: ratio of 1:1. Average transduction efficiency of used lentiviral/RD114-TR CD19-CAR-NK cells was 13.1% (range 11.6–14.5%), of used alpharetroviral/RD114-TR CD19-CAR-NK cells was 23.4% (range 22.6–23.9%). Shown are co-incubations using NK cells of two different donors *(n = 2)*. Shown are mean values + SD.

progenitor cells ($hCD34^+$ HSPCs) and human T cells using a broad range of lentiviral and gammaretroviral pseudotypes, including RD114-TR and at least for $hCD34^+$ HSPCs also VSV-G (47, 49, 50). Thereby, Vectofusin-1 was at least as effective as Retronectin in enhancing transduction, and in some cases more effective (47, 50). The two substances act differently enhancing gene delivery into target cells: Retronectin on the one hand is a fibronectin fragment that facilitates colocalization of viruses and target cells by binding viral particles and target cells with different domains (51). On the other hand the exact mechanism of Vectofusin-1 is not completely explored, but it was shown that Vectofusin-1 forms α-helical coiled-coil fibrils that sediment lentiviral particles and therefore increase the local virus concentration along the surface of target cells (52). Main advantage of using Vectofusin-1 instead of Retronectin is its simple usage, because Vectofusin-1 is added directly into the transduction mixture. In comparison, Retronectin needs a pre-coating step, which is more difficult to standardize and extends the transduction process. Comparing both substances in their ability to enhance the transduction of human NK cells, we could show that Vectofusin-1 is as effective as Retronectin in transduction of NK cells using VSV-G pseudotyped lentiviral vectors. Interestingly, in the context of alpharetroviral transduction using a RD114-TR pseudotyped EGFP vector, Vectofusin-1 promoted transduction of NK cells better than Retronectin. Overall, we demonstrated for the transduction of PB-derived NK cells that Vectofusin-1 serves as a promising potential alternative to the frequently used Retronectin.

The second component of the most successful protocol in this study was pseudotyping alpharetroviral and lentiviral vectors with RD114-TR. The alpharetroviral vector system is based on self-inactivating (SIN) alpharetroviral vectors with a split-packaging design (40). These alpharetroviral vectors were successfully used to genetically modify NK cells (45, 53, 54) and outperformed lentiviral and gammaretroviral transduction efficiencies of PB-derived NK cells using EGFP encoding vectors (45). Furthermore, due to its more random integration pattern with lower frequency of integrations in or close to gene coding regions, alpharetroviral vectors can be considered to be safer as insertional oncogenesis is less likely to occur (55, 56). Initially, we observed a superiority of alpharetroviral/RD114-TR transduction of NK cells compared to lentiviral/VSV-G transduction, using second-generation CD19-CAR encoding vectors. To confirm this, we compared alpharetroviral/RD114-TR transduction of NK cells to lentiviral/RD114-TR transduction. Thereby, no superiority of one system could be observed as both viral vector systems performed equally well. Additionally, lentiviral/RD114-TR transduction of NK cells also outperformed lentiviral/VSV-G transduction. Interestingly, these data suggest that the key for a high transduction rate was the pseudotyping of retroviral vectors with RD114-TR. Our findings are consistent with a recent publication by Bari et al., which showed that PB-derived NK cells were poorly transduced by lentiviral/VSV-G based transduction (57). But using a modified baboon envelope glycoprotein (BaEV) (58) to pseudotype lentiviral CD19-CAR particles, an average transduction efficiency of 70% could be reached and ASCT-2 has been proposed as the entry receptor for BaEV (59). Interestingly, ASCT-2 is also the receptor for feline endogenous retrovirus RD114 based envelopes (46). Indeed, we observed high expression of ASCT-2 in low dose IL-15 activated NK cells on the day of transduction. This may explain the success of the RD114-TR envelope and demonstrates how important the knowledge of receptor expression of NK cells is for an effective transduction.

Both CD19-CAR-NK cell preparations generated by RD114-TR pseudotyped retroviral vectors proliferated similar and no significant differences compared to NT-NK cells could be observed. Furthermore, 7–11 days after the transduction procedure, transduced NK cells showed still a stable expression of the CD19-CAR. Interestingly, CD19-CAR expression of alpharetrovirally transduced NK cells declined in the first 7–11 days, but afterwards a certain plateau was reached. Similar CD19-CAR expression could be observed for lentiviral/RD114-TR and alpharetroviral/RD114-TR transduced NK cells at the end of the observation period. Overall, these results indicate that both protocols are suitable to generate a sufficient number of CD19-CAR-NK cells for clinical applications, as the CD19-CAR is stable expressed and transduced cells proliferate as good as NT-NK cells.

Regarding functionality and killing activity, we addressed cytokine production and cytotoxic capacity of CD19-CAR-NK cells transduced with different methods. Independently of a direct contact with the CD19-expressing target cells, CD19-CAR-NK cells tend to release more cytokines than NT-NK cells. This tendency could be observed as a slight increase in production of MIP-1α in lentivirally/VSV-G transduced CD19-CAR-NK cells, and GM-CSF, TNF-α, MIP-1α, and IFN-γ in alpharetrovirally/RD114-TR and lentivirally/RD114-TR transduced CD19-CAR-NK cells. However, significant changes could only be observed for the release of MIP-1α of lentiviral/VSV-G CD19-CAR-NK cells and for GM-CSF in alpharetrovirally/RD114-TR transduced CD19-CAR-NK cells compared to NT-NK cells. These observations could be explained by possible influence of genetic engineering using retroviral vectors, as these changes occurred in transduced cells independent of direct contact with target-specific cells.

Concerning the direct cytotoxic potential against CD19-expressing Sup-B15 in comparison to the non-target specific cytotoxicity against K562 leukemia cells, alpharetrovirally/RD114-TR generated CD19-CAR-NK cells displayed higher cell killing activity than lentivirally/VSV-G generated CD19-CAR-NK cells. This is most likely in part due to the fact that more NK cells were transduced using the alpharetroviral vector, as lentivirally/RD114-TR generated CD19-CAR-NK cells with a similar transduction efficiency as alpharetrovirally/RD114-TR generated CD19-CAR-NK cells showed equally good cytotoxic activities against Sup-B15 cells. But if similar numbers of CD19-CAR-NK cells are applied independent of the used vector, CD19-CAR-NK cells generated with the alpharetroviral vector show a better killing performance than those generated by lentiviral/VSV-G transduction. Of note, lentivirally/VSV-G transduced CD19-CAR-NK cells with

a significantly lower mean transduction efficiency showed already around 60% killing of Sup-B15 cells. This might point either to an impressively high lytic capacity of each single lentivirally transduced NK cell or to a high and so far underestimated antigen-independent killing mechanism of NT-NK bystander cells. Overall our results demonstrate that, independent of the used retroviral vector for the genetic modification of NK cells, a relative low number of CD19-CAR-NK cells can kill a sufficient amount of target-specific tumor cells.

In general, the remarkably high killing capacity could be explained by serial killing activity of NK cells, as one NK cell is able to hit multiple targets (60, 61). It has been shown that serial killing of tumor cells by cytokine activated $CD16^+$ NK cells is increased in the presence of rituximab (62). Therefore, a combination of CD19-CAR-NK cells possessing ADCC with the anti-CD20 monoclonal antibody Rituximab may provide a treatment option for aggressive relapsed $CD19^+$ and $CD20^+$ B-ALL. Pre-clinical data have shown synergistic tumor suppressing action using CD19-CAR-T cells with rituximab on mice inoculated with B cell non-Hodgkin lymphoma (B-NHL) (63). Interestingly, the authors considered presence of contaminating NK cells in their setting as the cause for the synergistic effect, supporting our approach to utilize CAR-NK cells for immunotherapy of malignant diseases.

As CD16 expression is crucial for exerting ADCC, we investigated CD16 expression of transduced NK cells and their transducability. Consistent with previous other findings (own unpublished data), we observed a decline of the $CD16^+$ subpopulation in NK cells with advancing time of cell cultivation. However, our data here demonstrated that the CD16 proportion of transduced PB-derived NK cells is not influenced by the genetic engineering. Concerning lentiviral/VSV-G and alpharetroviral/RD114-TR transduced NK cells, CD19-CAR expression of $CD16^+$ and $CD16^-$ subpopulations did not differ, indicating that there is no favorable NK cell subpopulation for genetic engineering by retroviral vectors. Interestingly, the $CD16^+$ subpopulation was more susceptible to the lentiviral/RD114-TR CD19-CAR vector as their transduction rate was significantly higher than its $CD16^-$ counterpart.

In sum, our protocol to generate IL-15 activated CD19-CAR-NK cells led to increased cytotoxic activity against CD19-expressing target cells when compared to NT-NK cells. Alpharetrovirally/RD114-TR and lentivirally/RD114-TR transduced NK cells were highly cytotoxic, with alpharetrovirally/RD114-TR transduced NK cells achieving 90% specific killing activity. Moreover, our findings are the first ones that indicate the use of Vectofusin-1 in combination with alpharetroviral/RD114-TR vectors as a successful strategy to genetically modify PB-derived NK cells to achieve highly cytotoxic CD19-CAR-NK cells at high yields. Furthermore, we could show that lentiviral/RD114-TR vectors in combination with Vectofusin-1 act as a suitable alternative. With these newly developed NK cell expansion and transduction protocols, gene modified NK cell products can be prepared off-the-shelf that might open new avenues for cancer immunotherapy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Goethe University Frankfurt, Germany (approval no. 329/10). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EU designed the project. SM, TB, VG, FK, and ES performed experiments and analyzed data. AS and WW provided plasmids. JH, AS, UK, WW, and UM provided expert input. SM, TB, JH, AS, UK, WW, UM and EU discussed data. SM, UM, and EU wrote the manuscript with support from all other co-authors. All authors agree to be accountable for the content of the work.

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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.2019.03123/full#supplementary-material

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Conflict of Interest: WW (CD19-CAR sequence in the alpharetroviral and lentiviral CD19-CAR vector) is named as an inventor in patents and patent applications on CAR technology owned by Georg-Speyer-Haus. AS is named as an inventor on a patent describing alpharetroviral SIN vectors (alpharetroviral backbone sequence and used alpharetroviral helper plasmid).

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Boosting Natural Killer Cell-Mediated Targeting of Sarcoma Through DNAM-1 and NKG2D

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Sarcomas are malignancies of mesenchymal origin that occur in bone and soft tissues. Many are chemo- and radiotherapy resistant, thus conventional treatments fail to increase overall survival. Natural Killer (NK) cells exert anti-tumor activity upon detection of a complex array of tumor ligands, but this has not been thoroughly explored in the context of sarcoma immunotherapy. In this study, we investigated the NK cell receptor/ligand immune profile of primary human sarcoma explants. Analysis of tumors from 32 sarcoma patients identified the proliferative marker PCNA and DNAM-1 ligands CD112 and/or CD155 as commonly expressed antigens that could be efficiently targeted by genetically modified (GM) NK cells. Despite the strong expression of CD112 and CD155 on sarcoma cells, characterization of freshly dissociated sarcomas revealed a general decrease in tumor-infiltrating NK cells compared to the periphery, suggesting a defect in the endogenous NK cell response. We also applied a functional screening approach to identify relevant NK cell receptor/ligand interactions that induce efficient anti-tumor responses using a panel NK-92 cell lines GM to over-express 12 different activating receptors. Using GM NK-92 cells against primary sarcoma explants ($n = 12$) revealed that DNAM-1 over-expression on NK-92 cells led to efficient degranulation against all tested explants ($n = 12$). Additionally, NKG2D over-expression showed enhanced responses against 10 out of 12 explants. These results show that DNAM-1$^+$ or NKG2D$^+$ GM NK-92 cells may be an efficient approach in targeting sarcomas. The degranulation capacity of GM NK-92 cell lines was also tested against various established tumor cell lines, including neuroblastoma, Schwannoma, melanoma, myeloma, leukemia, prostate, pancreatic, colon, and lung cancer. Enhanced degranulation of DNAM-1$^+$ or NKG2D$^+$ GM NK-92

cells was observed against the majority of tumor cell lines tested. In conclusion, DNAM-1 or NKG2D over-expression elicited a dynamic increase in NK cell degranulation against all sarcoma explants and cancer cell lines tested, including those that failed to induce a notable response in WT NK-92 cells. These results support the broad therapeutic potential of DNAM-1$^+$ or NKG2D$^+$ GM NK-92 cells and GM human NK cells for the treatment of sarcomas and other malignancies.

Keywords: cancer immunotherapy, cancer immunology, sarcoma, natural killer (NK) cell, DNAM-1 (CD226), NKG2D (Natural killer group 2 member D), NK-92 cell line

INTRODUCTION

Sarcomas are a group of rare, heterogeneous, and aggressive tumors of mesenchymal origin that may arise in a range of different tissues, including bone, cartilage, connective tissue, muscle, fat, vasculature, and peripheral nerves. While the standard treatment options of chemotherapy, radiotherapy, and surgery efficiently control localized tumors, ∼40% of cases experience tumor relapse and metastatic spread with current treatments remaining ineffective in increasing overall survival (1–4). The heterogeneity of sarcoma subtypes and the fact that most therapies currently prove suboptimal, underline the importance of investigations aiming to develop novel targeted treatment approaches.

Both experimental and clinical data support the involvement of the immune system in sarcoma tumorigenesis as immunosuppressed individuals present with a higher risk (5). Moreover, there is evidence for both spontaneous regression as well-efficient immunosurveillance in sarcoma, prompting investigators to explore immunotherapy as a treatment modality (6, 7). Recently, several clinical trials have included sarcoma patients in the testing of various immunotherapy strategies, including: (i) immune checkpoint blockade (8–10); (ii) tumor-specific or tumor-associated peptide vaccines (11–14); and (iii) adoptive immune cell therapies with allogeneic NK cells (ongoing clinical trials), autologous T cells (15), CAR-T cells (ongoing clinical trials) and NY-ESO-1-specific T cells (16) to name a few (17). However, these studies aim to target general tumor markers found in a variety of solid tumors and hematological malignancies, not specifically targeting sarcomas. This highlights the need for more precise tumor characterization, targeted immunomodulation of the individual tumor microenvironment and targeting of sarcoma-specific molecules.

NK cells are promising candidates for cancer immunotherapy, as engagement of their activating receptors with ligands expressed on targets leads to rapid response and efficient elimination of tumor cells (18). In general, NK cell infusions have proven to be well-tolerated and safe with minimal side effects (19). However, modulation of ligand expression in order to avoid NK cell detection is a well-known strategy employed by tumors to escape immune recognition (20). Known ligands for NK cell receptors are expressed on several tumors, including some subtypes of sarcomas. More specifically, proliferating cell nuclear antigen (PCNA) has been shown to be an inhibitory ligand of natural cytotoxicity receptor NKp44 (21, 22). It is also associated with poor prognosis in an analysis covering 16 studies with a total of 691 osteosarcoma patients (23). Osteosarcomas have been previously shown to express CD155 (Poliovirus Receptor, PVR) (24), which is one of the ligands for DNAX accessory molecule-1 (DNAM-1 or CD226), an activating receptor expressed on NK cells, monocytes and a subset of T cells. Rhabdomyosarcoma (RMS) cell lines have been shown to express CD112 (Nectin-2) along with CD155, both ligands of DNAM-1 (25, 26). They also expressed KIR-ligand HLA-I but had low expression of NKG2D ligands MICA/MICB (27). However, these studies focused on a limited group of sarcoma subtypes and NK cell receptors, thus, better characterization of the individual sarcoma tumor's expression of various NK cell ligands could identify relevant receptor/ligand interactions that could mediate efficient NK cell cytotoxic activity.

In this study, we demonstrate an inherent immune defect in the tumor infiltrating NK cell compartment of sarcoma tumors. This prompted the investigation of NK cell ligand expression on fresh and *in vitro* propagated primary sarcoma explants, which identified the presence of DNAM-1 ligands CD112 and CD155. We developed a novel cell-based screening platform which allowed the identification of tumor-specific NK cell receptor engagements. This platform, together with extensive flow cytometry-based characterization of rapidly processed fresh sarcoma surgical material and respective short-term cultured primary human sarcoma explants, were used to identify targetable NK cell receptor/ligand interactions in sarcoma.

Our results show that over-expression of the activating receptor DNAM-1 or NKG2D on NK-92 cells induces efficient anti-sarcoma responses *in vitro* by amplifying the interaction with prevalent ligands CD112 and CD155 or MICA/B and ULBP1-5, respectively, on sarcoma and other tumor cells. This way of arming NK cells against tumor targets that they would otherwise remain inert against, provides a promising novel cellular immunotherapy strategy that can easily be translated to the clinic and has the potential to significantly improve sarcoma treatment.

MATERIALS AND METHODS

Patient Material

Primary sarcoma tumors and blood were collected at the Center for Orthopedic Innovations of the Mercy Miami Hospital,

Florida according to rules and regulation specified under Nova Southeastern University Institutional Review Board (protocol # 2017-304).

Primary Sarcoma Explant Generation From Patient Material

Sarcoma tumor samples were processed within 12 h of surgical excision with the Miltenyi Tumor Dissociation Kit to obtain homogenous cell suspensions in RPMI medium (Gibco) using the Miltenyi GentleMACS Octo Dissociator with heaters. Homogenous cell suspensions were seeded in complete DMEM medium [DMEM (high glucose, GlutaMAX, Gibco) 10% FBS (Gemini Bio-Products), supplemented with 1X non-essential amino acids (NEAA), 1X Antibiotic-Antimycotic and 25 mM HEPES (all from Gibco)] which was changed every 4 days during the first 2 weeks. After 2 weeks in culture, serial passaging is performed based on confluency for the selection of adherent cells. Multiple passages were vitally frozen along the process of explant generation, which was considered complete at passage 12.

Cell Culture

Primary sarcoma explants were cultured in complete DMEM as explained above. Culture media was renewed once a week, splits were done based on confluency, predictably every 7–10 days. All cell lines except for 293FT (Thermo Scientific), A375 and DM6 were obtained from ATCC. DM6 cells were a kind gift from Dr. Hilliard F. Seigler (Department of Immunology, Duke University Medical Center) and A375 cells were a kind gift from Prof. Michael Nishimura (Loyola University Chicago). U-2 OS and Saos-2 cell lines used during the revision of the manuscript were kind gifts provided by Uygar Tazebay (Gebze Technical University) and Mehmet Öztürk (Izmir Biomedicine and Genome Center). NK-92 cells (ATCC CRL-2407) were cultured in CellGro SCGM (Cellgenix) supplied with 20% FBS and 1,000 U/ml IL-2 (Miltenyi Biotech). K562, THP-1, LNCaP, PC-3, ARH-77, RPMI8226, DM6, and A375 cells were cultured in RPMI (GlutaMAX, Gibco) supplied with 10% FBS. U266 cells were cultured in RPMI (GlutaMAX, Gibco), supplied with 15% FBS. 293FT (Thermo Fisher Scientific), MeWo, SK-MEL-28, sNF02.2, Capan-2 cells were cultured in DMEM (GlutaMAX, Gibco) supplied with 10% FBS. Saos-2 and U-2 OS cells were cultured in McCoy's 5A Medium (ATCC) supplied with 15% and 10% FBS, respectively. SH-SY5Y cells were cultured in EMEM (ATCC) supplied with 10% FBS. Caco-2 cells were cultured in EMEM (ATCC) supplied with 20% FBS. A549 cells were cultured in F-12K medium (ATCC) supplied with 10% FBS.

Flow Cytometry-Based Phenotyping of Sarcoma Primary Explants and Other Cell Lines

Single cell suspensions of the generated primary sarcoma explants were dissociated using 0.05% trypsin at 37°C, and manual scraping when necessary. The cells were stained with Live/Dead fixable Aqua in PBS for dead cell discrimination (Invitrogen). Surface stainings were performed in BD Horizon Brilliant Stain Buffer (BD Biosciences) according to the manufacturer's instructions. Stained cells were washed in FACS buffer (PBS, 2% FBS, 2 mM EDTA) twice before assessed. Data were acquired on a BD LSR Fortessa X-20 flow cytometer and analyzed using the FlowJo software v10.1 (BD Biosciences). The antibodies from Biolegend and BD Biosciences that were used for stainings are listed in **Table S2**.

Intracellular Cytokine Staining

The ability of WT, DNAM-1, and NKG2D modified NK-92 cells to produce IFNγ and TNFα upon 4 h of co-culture of 200,000 NK-92 cells with 200,000 primary sarcoma explant lines or the cell line Saos-2, was assessed by intracellular cytokine staining using the BD cytofix/cytoperm kit according to the manufacturer's instructions. The following antibodies from Biolegend were used at the recommended amounts: IFNγ PE (B27), TNFα APC (MAb11), PE mouse IgG1, κ isotype control (MOPC-21) in PE and APC. BD biosciences: CD56 BV421 (NCAM16.2). For data acquisition the BD LSR Fortessa X-20 flow cytometer were used. Data were analyzed with the FlowJo software v10.1 (BD Biosciences).

Ligand Expression Characterization Using NKG2D-Fc Chimeric Protein

In order to assess the presence of NKG2D ligands on the surface of primary sarcoma explant lines, we used an NKG2D-Fc chimeric protein where the receptor was fused to the Fc of a human IgG1 (1299-NK, R&D Systems). NKG2D-Fc was reconstituted at 100 μg/ml in PBS. 2 μg/ml of NKG2D-Fc were pre-complexed with 4 μg/ml of mouse-anti-human IgG secondary antibody PE (clone HP6017, Biolegend) in FACS buffer for 1 h on ice. The cells were prepared at 2×10^5 cells per well in a 96-well plate, washed and resuspended in 200 μl of the precomplexed mix (or just secondary antibody as control) and incubated on ice for 30 min. The samples were washed twice with FACS buffer and acquired on the BD LSR Fortessa X-20 flow cytometer. Data were analyzed using FlowJo software v10.1 (BD Biosciences) and normalized to unstained control.

qPCR-Based Expression Analysis

Primers were selected from the Harvard Medical School PrimerBank (https://pga.mgh.harvard.edu/primerbank/) using the Entrez gene numbers obtained from the Human Protein Atlas. Entrez No's: *ULBP1* #80329, *ULBP2* #80328, *ULBP3* #79465 (https://www.proteinatlas.org/). Primer IDs: *ULBP1* #56181385c1, *ULBP2* #13376824a3, *ULBP3* #13375655c1. Exon-spanning primers capable of detecting multiple transcript variants were selected when possible. Primers were synthesized by Midlands Certified Reagents (Midland, TX).

ULBP1

Forward: TAAGTCCAGACCTGAACCACA
Reverse: TCCACCACGTCTCTTAGTGTT

ULBP2

Forward: GTGGTGGACATACTTACAGAGC
Reverse: CTGCCCATCGAAACTGAACTG

ULBP3

Forward: TCTATGGGTCACCTAGAAGAGC
Reverse: TCCACTGGGTGTGAAATCCTC

RNA was collected and purified with the E.Z.N.A. HP Total RNA Kit (OmegaBioTek R6812-02) and eluted to 40 µl. The cDNA was synthesized with qScript cDNA SuperMix (Quantabio 95048-025). Briefly, 100 ng of RNA was combined with 8 µl qScript cDNA SuperMix and water added to 20 µl total reaction volume. The reaction was thermal-cycled at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and held at 4°C. Using the PerfeCTa SYBR Green FastMix Low ROX (QuantaBio 95074-250), RT-PCR reactions were carried out in triplicate on 96-well plates (VWR 82006-664) using an AriaMX Real-Time PCR system (Agilent). Each reaction contained: 10 µl of SYBR Green master mix, 5 µl of 100 ng/µl template cDNA, 10 µM of primers, then water added for a 20 µl total reaction volume. Reactions were heated to 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and 76°C for 15 s. Following cycling, the reactions were held at 4°C until use. The melting curve at 95°C for 30 s, 65°C for 30 s, and 95°C for 30 s showed no primer dimers. Housekeeping: *GAPDH* in triplicate. Calibrators: *ULBP1- THP1, ULBP2 & 3- U2OS, ULBP4 & 5- Capan-2*.

The Cancer Genome Atlas Database (TCGA) Analysis

We retrieved TPM table of sarcoma data from TCGA (28) to validate the gene expression of the ligands of NK cell receptors DNAM-1 and NKG2D. Based on the TPM value of each gene, we classified the patients into two groups and examined their prognoses. The prognosis of each group of patients was examined by Kaplan-Meier survival estimators, and the survival outcomes of the two groups were compared by log-rank tests. To choose the best TPM cut-offs for grouping the patients most significantly, all TPM values from the 20th to 80th percentiles were used to group the patients, significant differences in the survival outcomes of the groups were examined and the value yielding the lowest log-rank P-value is selected (28).

Generation of Genetically Modified NK-92 Cells

For all NK-92 receptor transgenes, codon-optimized cDNA was cloned upstream of IRES in LeGO-iG2 vector under the control of the SFFV promoter (LeGO-iG2 was a kind gift from Boris Fehse, Addgene #27341) (29). Lentiviral vector production and transduction were performed as before (30). Briefly, NK-92 cells were seeded at 5×10^5 cells/ml in viral supernatant containing the gene-of-interest-iG2 virus for 6 h in the presence of 6 µM BX795 (Sigma-Aldrich). Next, the cells were washed and put back into culture with fresh media. GFP percentage was checked by flow cytometry 3 days later. The GFP^+ $CD56\text{-}APC^+$ cells were sorted using BD FACS AriaFusion.

Degranulation Assay

2×10^5 NK-92 cells were co-incubated with 2×10^5 target cells in a final volume of 200 µl in V-bottomed 96-well plate at 37°C and 5% CO_2 for 4 h. In blocking experiments, target cells were pre-incubated with the corresponding 25 µg/mL blocking antibodies [anti-CD155 (clone SKII.4, Biolegend), anti-DNAM1 (clone DX11, BD Pharmingen) and anti-NKG2D (clone 1D11, Biolegend)] for 15 min on ice prior to co-culture. Fluorochrome-conjugated anti-CD107a-PE (H4A3, BD Biosciences) mAb was added at the initiation of the assay. After 1 h of co-incubation, GolgiStop (BD Biosciences) was added at a 1:300 dilution. The cells were then washed, resuspended in ice-cold PBS and stained with surface anti-CD56 (NCAM 16.2, APC, or BV421, BD Biosciences or Biolegend) to be analyzed by flow cytometry. For flow cytometry, cells were first gated on FSC-A vs. SSC-A, followed by gating on single cells via FSC-A vs. FSC-H, then GFP^+CD56^+ cells were selected and lastly $CD107a^+CD56^+$ percentage was recorded for analysis. All flow cytometry analysis was performed with FlowJo software v10.5 (BD Biosciences).

Microscopy-Based Cellular Cytotoxicity

Image cytometry evaluation of NK cell cytotoxicity using calcein-acetoxymethylester (calcein-AM) fluorescent dyes has been previously reported to provide comparable sensitivity to traditional Cr^{51}-release assays, while simultaneously providing morphological information and avoiding the use of radioactive materials (31, 32).

Tumor cells were seeded in flat-bottom 96-well CellBind plates (Corning) at a density of 1×10^4-2×10^4 cells/per well and cultured for 24–48 h. When the desired confluence was reached, the assay was initiated and cells were washed with HBSS and stained with 40 µl of 10 µM Calcein Red-AM (Thermo Fisher Scientific) and 1 µg/ml Hoescht-33342 (Thermo Fisher Scientific) for 30 min at 37°C. Cells were washed twice with HBSS before adding 50 µl fresh phenol red-free DMEM assay media. Probenecid (2 µM) was added to staining and assay medias to reduce dye efflux. NK-92 cells were additionally counted and resuspended in the same assay media. NK-92 cells (50 µl) were added to target cells at a 10:1 or 5:1 E:T ratio based on the number of seeded tumor cells and plates were incubated at 37°C for 4–6 h. Images were then collected using the CellInsight CX7 High-Content Imaging System (Thermo Fisher Scientific). Using a 10X objective, four fields of view were collected per well, with conditions run in triplicate. Images were analyzed using HCS Studio Software (Thermo Fisher Scientific). Briefly, tumor cells were identified as DAPI positive nuclei bounded by a Calcein Red positive cytoplasmic border. Apoptotic bodies were size-excluded from the analysis. Tumor nuclei were further distinguished based on their size and shape compared to NK-92 cell nuclei. Parameters were validated across all patient samples for all conditions and at 4 or 6 h timepoints. Tumor cell viability was assessed by determining the average fluorescent intensity (AFI) of individual tumor cells within each well. Percent viability was calculated by comparing the AFI of each condition to non-treated controls. Results are reported as the mean viability of two independent experiments. Results were analyzed using a one-way ANOVA with Tukey's *post-hoc* analysis.

Analysis of NK Cells Cytotoxicity by Xcelligence RTCA

Real-time cell viability experiments were performed using the xCELLigence RTCA DP device (ACEA Biosciences) placed in a

humidified incubator at 37°C and 5% CO_2. The E-16 plates were incubated with 100 μL of cell-free growth medium [10% and 15% FBS containing McCoy's 5A medium (GE)] at room temperature for 15 min. After incubation, background impedance signal was measured to control all the connections. The target cells were seeded into plates at 5×10^3 in 100 μL for U-2 OS and Saos-2 cell lines. The plates were mounted to the device after incubation at room temperature for 30 min before starting the experiment. The target cells were allowed to settle for 15–17 h before adding effector cells. The following day, the effector cells were added onto the target cells at an E:T ratio of 1:1. Real time measurements were performed by recording the Cell index (CI) every 15 min for a period of 40 h. Data analysis was carried out with the RTCA software (version 1.2, Roche Diagnostics).

Statistical Analysis

For preparation of graphs and statistical analysis, GraphPad Prism v.7.0 (GraphPad Software Inc.) was used.

RESULTS

Lymphocyte Characterization in Human Sarcomas and Matched Peripheral Blood Reveals a Defect in the Tumor-Infiltrating NK Cell Compartment

In order to understand the immunological landscape of sarcomas and to assess possible immune defects that may contribute to the poor clinical responses observed, we characterized the major tumor-infiltrating leukocyte (TIL) populations and compared their frequencies to matched PBMCs from the same patients (**Figure 1** and **Table 1**). A decrease in the overall percentage of TILs was observed in all patients compared to matched PBMCs ($n = 14$, Wilcoxon test, $p = 0.0078$) (**Figure 1B**). This was primarily reflected by a significant decrease in the $CD3^-CD56^+$ NK cell compartment ($p = 0.0005$), while no clear trend was observed between tumor-infiltrating and peripheral $CD3^+$ bulk T cells, $CD3^+CD4^+$ and $CD3^+$ $CD8^+$ T cells (**Figure 1B**).

Since this pointed toward a general NK cell defect in sarcomas, we performed more detailed analysis of the surface receptor expression profile of the $CD3^-CD56^+$ population in tumors vs. PBMC. When assessing CD16 (FcγRIII) and collectively KIR2DL1 (CD158a), KIR2DL2/L3 (CD158b), and KIR3DL1(CD158e) in seven sarcoma patients, we observed a generalized decrease in the $CD16^+KIR^+$ and $CD16^+KIR^-$ NK cell population in the TILs compared to NK cells in matched PBMC ($n = 14$, $p = 0.0002$ and 0.0107, respectively) (**Figure 1C**; **Figure S1A**). A similar comparison was performed on the expression of activating receptors DNAM-1 (CD226) and NKG2D on $CD3^-CD56^+$ NK cells in TILs and PBMC of the same seven patients. DNAM-1 expression was decreased in 10 out of 14 TIL NK cells compared to PBMC, while the remaining four patients had increased expression of DNAM-1 on TIL NK cells (**Figure 1D**; **Figure S1B**). Lastly, 12 patients out of 14, exhibited decreased or unchanged expression of NKG2D on NK cells in the tumor compared to in PBMC ($p = 0.040$) (**Figure 1D**; **Figure S1B**). Overall, we observed minimal NK cell infiltration in sarcomas and the expression of activating receptors DNAM-1 and NKG2D was very low in both peripheral and TIL NK cells.

NK Cell Ligand Expression Profiling of Fresh and *in vitro* Propagated Sarcoma Explants Identifies Common Expression of PCNA, CD112, and CD155

Next, we attempted to identify possible interaction partners between sarcoma tumor cells and NK cells which could account for the observed decrease in tumor-infiltrating NK cells. To do that, we performed detailed phenotyping of the lymphocyte ligands expressed on freshly isolated as well as *in vitro* propagated primary sarcoma explants, which we generated by serial passaging of cells from the freshly dissociated sarcomas (**Table 1**). Using a flow cytometry-based phenotyping approach, we assessed the expression of PCNA (NKp44 ligand), CD112 (DNAM-1 ligand), CD155 (DNAM-1 ligand), MICA/B (NKG2D ligand), CD48 (BLAST-1) (2B4 ligand), NTBA (NTBA ligand), MUC1 (Mucin 1) (Siglec-7 and−9 ligand), MHC Class I molecules HLA-ABC, and HLA-C (KIR2DL2/3 ligand), as well as MHC Class II molecules HLA-DR/DP/DQ (**Figure 2A**). Freshly isolated primary sarcomas expressed activating ligands CD112 and low levels of CD155 and CD48 as well as the inhibitory ligands PCNA, MUC1, HLA-ABC, and HLA-DR/DP/DQ ($n = 13$) (**Figures 2B,C**). Consistent with the analysis of the fresh sarcoma explants, the *in vitro* propagated primary sarcoma explants robustly expressed a similar but enhanced signature including the markers PCNA, CD112, CD155 to a lesser extent and HLA-ABC ($n = 32$) (**Figures 2D,E**). The phenotypes observed in sarcoma explants reveal that, despite the fact that NK cell infiltration in the tumor is limited and DNAM-1 expression on tumor infiltrating NK cells is low, CD112 and CD155 stand out as tangible targets for immunotherapies (**Figures 1A,D**).

GM NK-92 Cells Overexpressing DNAM-1 or NKG2D Efficiently Degranulate Against Primary Sarcoma Explants

The presence of several ligands for activating NK cell receptors prompted the analysis of functional NK cell responses against sarcoma cells. Therefore, we developed a screening platform that assesses the capacity of different NK cell activating receptors to efficiently trigger degranulation against target cells without prior knowledge of which ligands are present on the target cell surface. Genetically modified (GM) NK-92 cells over-expressing one NK receptor at a time were generated by introducing the gene-of-interest-IRES-GFP lentiviral constructs to NK-92 cells and sorting GFP^+ cells containing the receptors depicted in **Figure 3A** and **Figure S3**. Sorted cells were expanded and used in a functional, cell-based screening approach to test which of them could be optimal candidates to target each primary sarcoma explant. GM NK-92 cells were co-cultured at 1:1 (E:T) ratio with 12 selected primary sarcoma explants (**Table 1**) and two well-established sarcoma cell lines (Saos-2 and U-2 OS) side by side with control NK-92 cells for comparative analysis of degranulation (**Figure 3B**; **Figure S4**). In

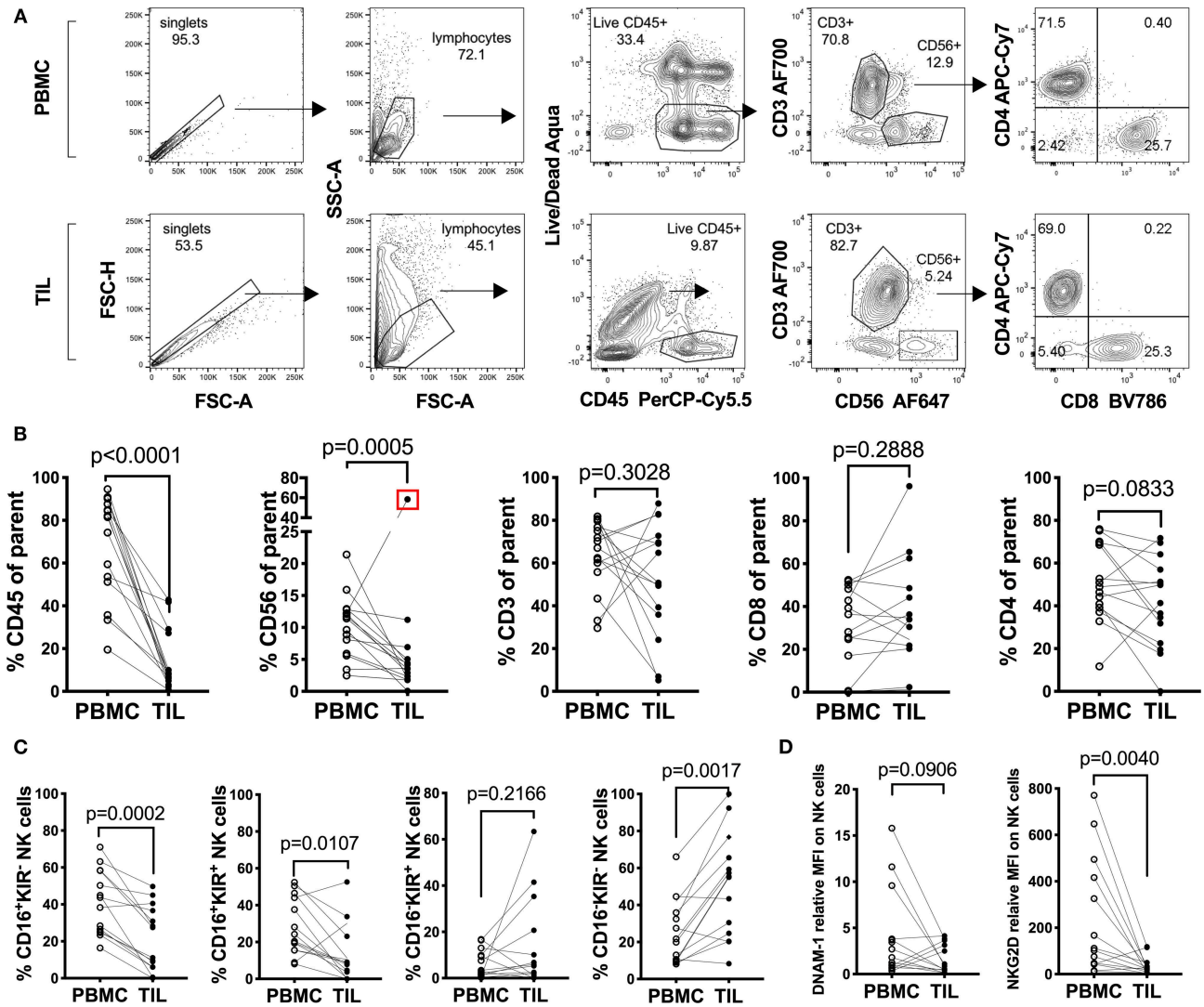

FIGURE 1 | Characterization of peripheral and tumor-infiltrating T and NK cell populations from freshly isolated sarcoma patient material. **(A)** Representative gating strategy and **(B)** percentages of leukocytes: $CD45^+$, $T_{helper/regulatory}$ cells: $CD3^+CD4^+$, $T_{cytotoxic}$ cells: $CD3^+CD8^+$ and NK cells: $CD3^-CD56^+$ among fresh PBMC and TIL of sarcoma patients ($n = 14$). The Live/Dead exclusion gate also includes markers CD14-V500 and CD19-V500 as a dump channel **(C)** Percentages of the Q1: $CD16^+KIR^-$, Q2: $CD16^+KIR^+$, Q3: $CD16^-KIR^+$, and Q4: $CD16^-KIR^-$ of NK cells from PBMC and TIL of sarcoma patients from **(C)** ($n = 14$). **(D)** Relative Median Fluorescence Intensity of DNAM-1 and NKG2D expression on NK cells in PBMC and TIL of sarcoma patients ($n = 14$). Statistical analysis was performed by Wilcoxon matched pairs signed rank non-parametric *t*-test and the Grubb's outlier test. In **(B,C)** the outlier is marked with a red square and the *p*-value is based on statistical analysis upon exclusion of the outlier.

line with the observed expression of DNAM-1 receptor ligands CD112 and CD155 (**Figure 2C**), DNAM-1^+ GM NK-92 cells degranulated significantly against all 12 sarcoma explants and cell lines, while NKG2D$^+$ GM NK-92 cells degranulated more than 20% to 10 out of 12 (*p*-values in **Table S1**). We further confirmed the triggering of cytokine production by DNAM-1^+ and NKG2D$^+$ NK-92 cells (**Figure S6**) upon culture with primary sarcoma explants (HTT12, HTT25, HTT31). Compared to WT NK-92 cells, DNAM-1^+ and NKG2D$^+$ NK-92 cells displayed enhanced production of TNFα and IFNγ upon co-culture with Saos-2 cells indicating that the GM NK cells have the capacity to trigger a wide range of effector functions. We also observed strong involvement of DNAM-1-mediated anti-tumor responses with 6 independent *in vitro* propagated sarcoma explants (HTT15, HTT53, HTT55, HTT73, HTT79, and HTT82) and 5 healthy donor PBMCs (**Figure S5**). As expected, tumors expressing CD112 and/or CD155 triggered a high degranulation response from only DNAM-1^+ NK-92 cells while these only low/moderately responded in the case of healthy donor PBMCs. Thus, it can be concluded that overexpression of DNAM-1 on NK-92 cells directly increased degranulation and cytokine prodcution against *in vitro* propagated primary sarcoma explants bearing CD112 and/or CD155, while healthy PBMCs were minimally affected.

TABLE 1 | List of sarcomas used in this study.

Tumor code	Tumor type	Age	NK cell ligand profiling		PBMC vs. TILs info	NK cell screening
			Fresh	Propagated		
HTT10	Synovial Sarcoma	40–45		+		+
HTT12	Extraoseos Osteosarcoma	85–90		+		+
HTT15	Rhabdomyosarcoma	5–10		+		+*
HTT16	Pleomorphic Myxofibrosarcoma	75–80		+		+
HTT17	Osteosarcoma	15–20		+		+
HTT21	Myxofibrosarcoma	35–40		+		+
HTT25	Pleaomorphic Spindle Cell Sarcoma	80–85		+		+
HTT26	Chondrosarcoma	70–75		+		+
HTT29	Chondrosarcoma	25–30		+		+
HTT30	Chondrosarcoma	55–60		+		+
HTT31	High Grade Pleomorphic Fibrosarcoma	70–75		+		+
HTT38	ST sarcoma	85–90		+		+
HTT39	Osteosarcoma	20–25		+		+
HTT41	Fibrosarcoma	40–45		+		
HTT42	Fibrosarcoma	70–75		+		
HTT45	Chondrosarcoma	75–80		+		
HTT46	Chindrosarcoma	75–80		+		
HTT47	Ewing's sarcoma	30–35		+		
HTT48	Pleomorphic Myxofibrosarcoma	75–80		+		
HTT49	Unknown subtype	65–70		+		
HTT50	Ewing's sarcoma	10–15	+	+	+	
HTT51	Myxofibrosarcoma	70–75	+			
HTT52	Myxofibrosarcoma	70–75	+	+	+	
HTT53	Myofibrosarcoma, High Grade	55–60	+	+		+*
HTT54	Myxoliposarcoma	50–55		+		
HTT55	Myxoid liposarcoma	70–75	+	+	+	+*
HTT57	Osteosarcoma	55–60	+	+	+	
HTT58	Leiomyosarcoma	60–65	+	+	+	
HTT61	Osteosarcoma	5–10		+	+	
HTT62	Chondrosarcoma	25–30		+	+	
HTT64	Synovial Sarcoma	30–35			+	
HTT67	Fibrosarcoma	0–5	+	+		
HTT71	Renal cell carcinoma	35–40			+	
HTT73	Ewing's sarcoma	75–80		+		+*
HTT77	Leiomyosarcoma	70–75			+	
HTT78	Osteosarcoma	5–10	+	+		
HTT79	Chondrosarcoma	65–70	+	+		+*
HTT80	Fibrosarcoma	80–85		+	+	
HTT81	Chondrosarcoma	55–60		+	+	
HTT82	Osteosarcoma	5–10	+	+	+	+*
HTT85	Chondrosarcoma	50–55			+	
HTT86	Pleomorphic fibroblastic sarcoma	50–55			+	

**Only tested with WT and DNAM-1$^+$ GM NK-92s.*

DNAM-1 and NKG2D Are Responsible for Degranulation Against Primary Sarcoma Explants

To verify the direct contribution of the respective activating NK cell receptors in mediating the observed degranulation, we used blocking antibodies to the receptors on the NK-92 cells or the corresponding ligand on the target cells (CD155 in the case of DNAM-1), in order to interfere with their specific interaction.

When DNAM-1$^+$ GM NK-92 cells were treated with a blocking antibody against DNAM-1, degranulation responses against HTT12, HTT17, HTT25, and HTT26 were completely abolished (**Figure 4A**). When CD155 was blocked on the sarcoma explants, a residual response could still be observed by the DNAM-1$^+$ GM NK-92 cells which is possibly due to the presence of the other ligand, CD112, on the surface of tumors. However, when DNAM-1 was blocked, all

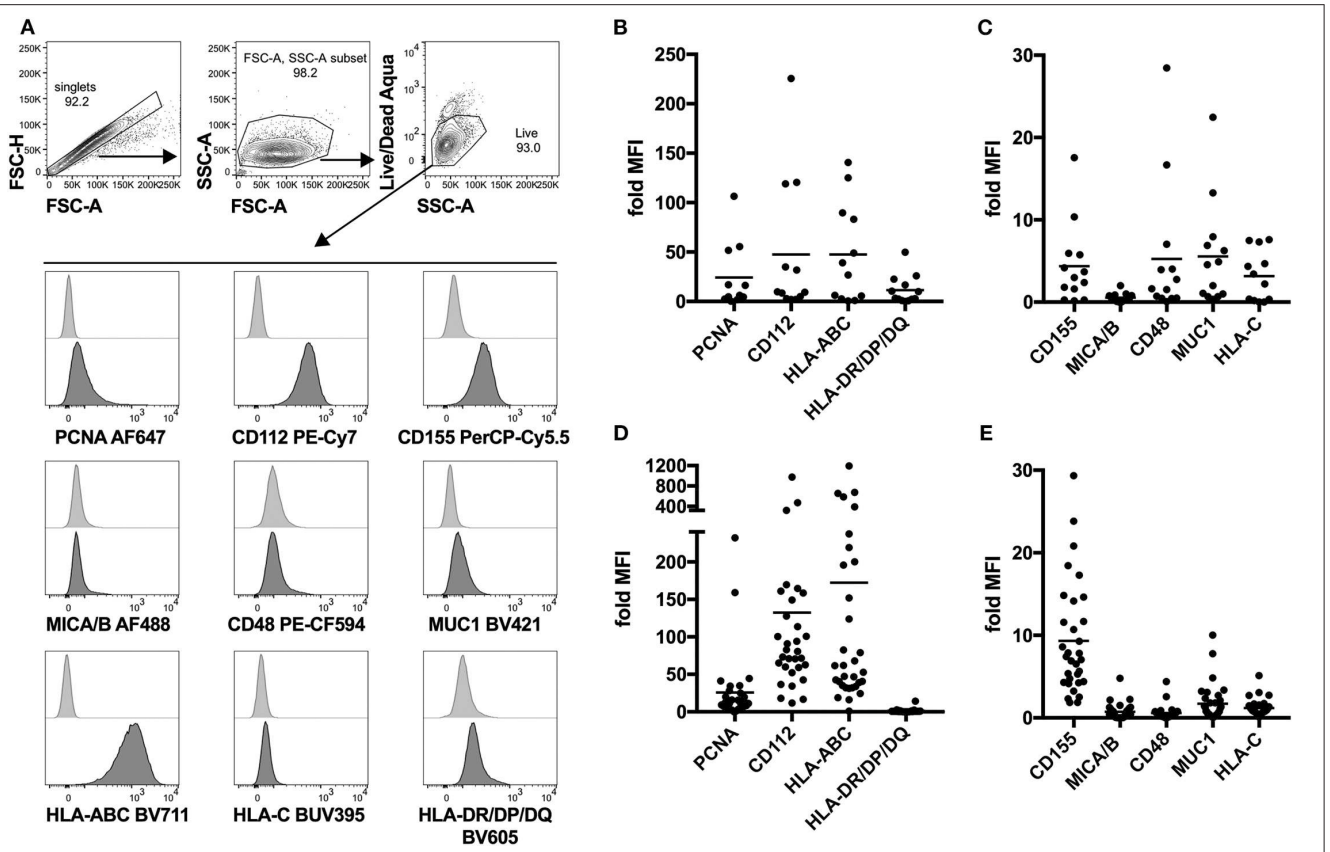

FIGURE 2 | Lymphocyte ligand expression profile of fresh and *in vitro* propagated sarcoma explants. **(A)** Representative gating strategy for flow cytometry-based analysis of lymphocyte ligand surface expression on *in vitro* propagated sarcoma explants. In the case of fresh sarcoma explants, CD45-BV510 is included in the same channel as Live/Dead Aqua as an exclusion marker. **(B,C)** Fold MFI of lymphocyte ligands on fresh sarcoma explants. Normalization: (sample MFI – unstained MFI)/unstained MFI ($n = 13$). **(D,E)** Fold MFI of lymphocyte ligands on *in vitro* propagated sarcoma explants. Normalization: (sample MFI – unstained MFI)/unstained MFI ($n = 32$).

degranulation was abrogated. Hence, it was confirmed that the degranulation of GM NK-92 cells was only mediated through the engagement of DNAM-1 with either of its ligands on the sarcoma explants.

Similarly, degranulation of NK-92 cells overexpressing NKG2D was assessed against HTT12, HTT17, HTT25, and HTT26, in blocking experiments where the effector cells were incubated with a blocking antibody against NKG2D prior to co-incubation (**Figure 4C**). As expected, blocking of NKG2D abrogated the response to levels similar to that of no target controls for samples HTT12, HTT25, and HTT26. As expected, HTT17 which was not a target of NKG2D$^+$ GM NK-92 cells, was not affected by the blocking.

Likewise, the blocking of DNAM-1 and NKG2D on NK-92 cells prior to co-incubation with targets, caused the respective responses of DNAM-1$^+$ and NKG2D$^+$ GM NK-92 cells to decrease to background levels when tested against Saos-2 and U-2 OS sarcoma cell lines (**Figures 4B,D**). Thus, it can be concluded that the degranulation responses of GM NK-92s over-expressing DNAM-1 and NKG2D are solely due to the abundant and functional interaction between the respective receptors on the NK-92 cells and the corresponding ligands on the sarcoma cells.

Having observed a relevant role for both DNAM-1 and NKG2D, we further analyzed whether the co-expression of the two receptors would synergize in triggering activity against sarcoma cells. For this purpose, we carried out a second genetic modification on NKG2D$^+$ GM NK-92 cells using the DNAM-1 expression vector and used FACS sorting to enrich the NKG2D$^+$/DNAM-1$^+$ GM NK-92 cells. Post-sorting analysis of DNAM-1 and NKG2D single or co-expressing cells confirmed similar levels of receptor expression and assured that any observed functional differences would not be merely due to this (**Figure S7**). Analysis of degranulation against U-2 OS and Saos-2 cell lines by co-expressing NK-92 cells revealed a slight increase in degranulation activity (**Figure 4E**) in comparison to DNAM-1 or NKG2D single positive cells. Due to lack of synergistic effects with co-expression of both DNAM-1 and NKG2D, we did not proceed further with tests against primary sarcoma explants.

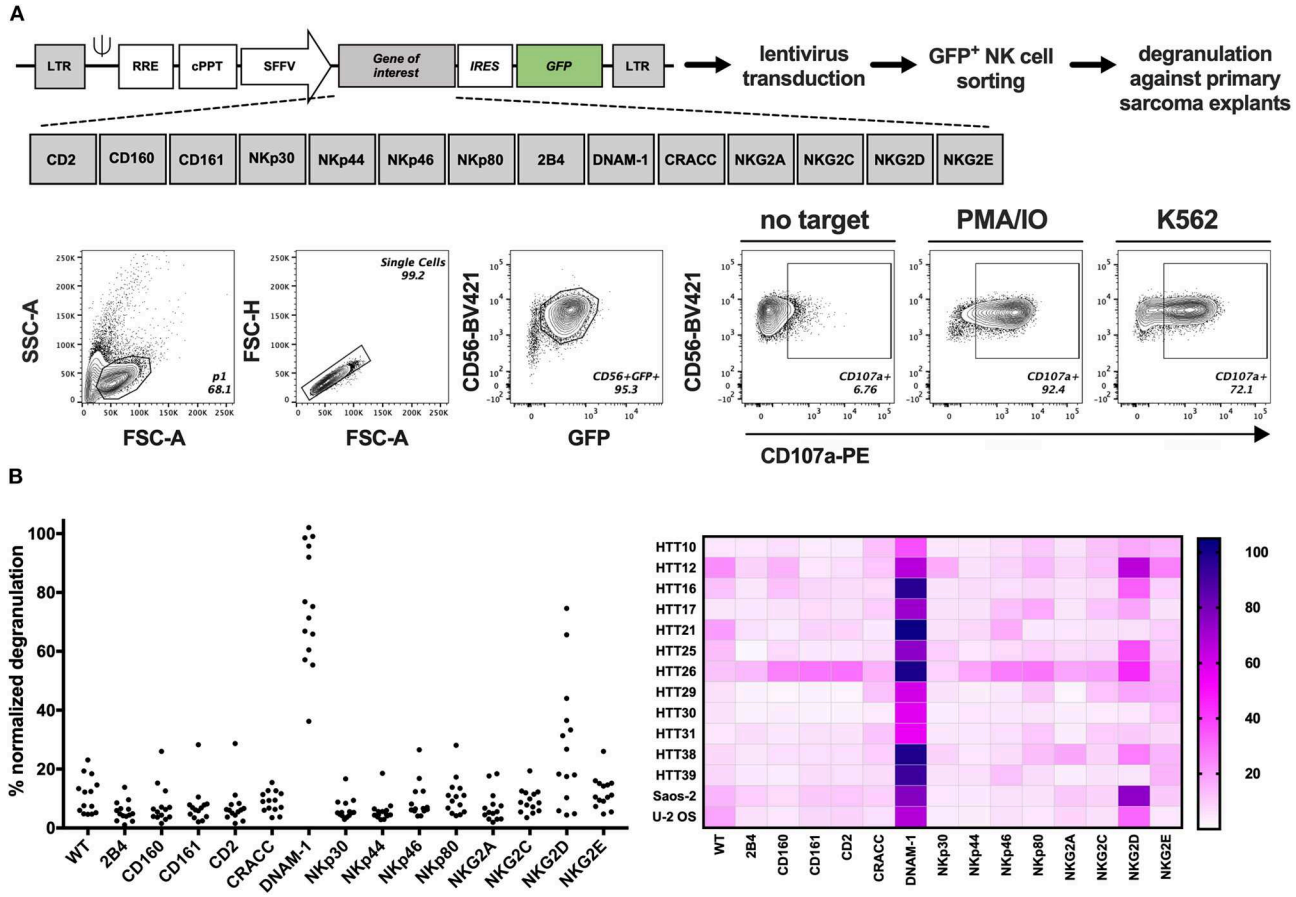

FIGURE 3 | GM NK-92 cell-based screening platform tested against 12 different primary sarcoma explants. WT or GM NK-92 cells were co-cultured with target cells at 1:1 (E:T) ratio for 4 h. %CD56$^+$CD107a$^+$ NK-92 cells were analyzed by flow cytometry. PMA and ionomycin (PMA/IO) were used as positive stimulators of degranulation and K562 as the validated target of NK-92 cells. **(A)** Generation of GM NK-92 cells and gating strategy for all degranulation flow cytometry analysis. **(B)** Dot plots and heatmap showing normalized %CD56$^+$CD107a$^+$ WT or GM NK-92 cells against each sarcoma explant and sarcoma cell lines Saos-2 and U-2 OS (PMA/IO responses for each GM NK-92 cell line was set as 100% for the normalization of the data; results from one representative experiment, plotted as means of technical replicates).

DNAM-1$^+$ GM NK-92 Cells Demonstrate Enhanced Cytotoxic Activity Against Primary Sarcoma Explants

While NK cell degranulation is a prerequisite for direct NK cell-mediated cytotoxicity, it does not necessarily correlate with target cell lysis. In order to observe the functional consequences of the slightly increased levels of degranulation in NK-92 cells co-expressing DNAM-1 and NKG2D, we performed electrical impedance-based cytotoxicity assays against U-2 OS and Saos-2 cells (**Figure 5A**). Quantification of cytotoxic activity at 4 h revealed that DNAM-1 single positive NK-92 cells are the most efficient in killing U-2 OS and Saos-2 cells. While both other NK-92 cell lines also rapidly killed target cells at a much higher rate compared WT NK-92 cells, the co-expression of the two receptors did not significantly enhance cytotoxic activity (**Figure 5A**). Rather, we observed that co-expressing NK-92 cells do not show as high cytotoxicity as DNAM-1 single positive NK-92 cells. Therefore, we conclude that the co-expression of DNAM-1 and NKG2D does not seem to be a feasible approach in further enhancing the anti-sarcoma activity of NK cells.

Furthermore, we used a calcein-AM image cytometry assay to visually validate target cell killing by WT or GM NK-92 cells. While testing of NKG2D$^+$ GM NK-92 cells against sarcoma explants did not yield any significant results (**Figure S8**), DNAM-1$^+$ GM NK-92 cells exhibited significantly increased cytotoxicity compared to WT-NK-92 cells against all three sarcoma explants (HTT12, HTT25, and HTT26), which were simultaneously tested in the same assay (**Figure 5A**). Representative images from three primary sarcoma explants co-cultured with WT and DNAM-1$^+$ GM NK-92 cells for 4 h (E:T ratio 10:1) show that target cells in the spontaneous control (without effector cells) exhibited brightly fluorescent live cells. While there is some variability between samples, fewer fluorescent

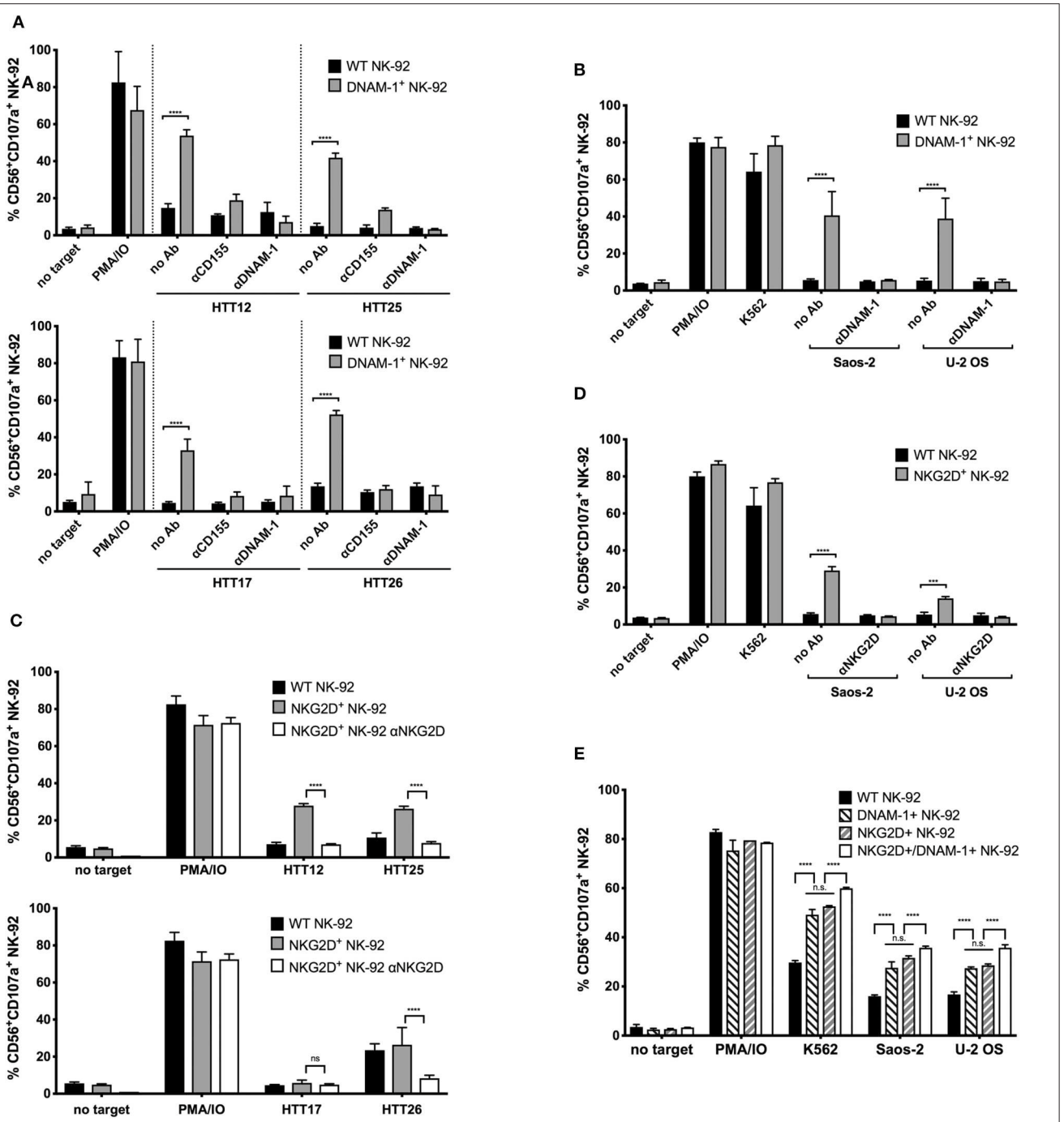

FIGURE 4 | Enhanced degranulation of NKG2D$^+$ and DNAM-1$^+$ GM NK-92 cells against selected sarcoma explants and Saos-2 and U-2 OS cell lines is receptor mediated. CD155, DNAM-1, or NKG2D blocking was done 15 min prior to degranulation set up. The assay was set as previously described, at E:T ratio 1:1. **(A)** Anti-CD155 on tumors and anti-DNAM-1 blocking of WT or DNAM-1$^+$ GM NK-92 cells against primary sarcoma explants and **(B)** anti-DNAM-1 blocking of WT or DNAM-1$^+$ GM NK-92 cells against Saos-2 and U-2 OS cell lines. **(C)** Anti-NKG2D blocking of NKG2D$^+$ GM NK-92 cells against primary sarcoma explants and **(D)** anti-NKG2D blocking of WT or NKG2D$^+$ GM NK-92 cells against Saos-2 and U-2 OS cell lines (results from two independent experiments, means plotted with error bars indicating SD). **(E)** Comparison of degranulation by DNAM-1 or NKG2D GM NK-92 cells with NK-92 cells co-expressing both receptors against Saos-2 and U-2 OS cells (**** indicates $p < 0.0001$ with 2-way ANOVA analysis).

cells with diminished intensity were observed when co-cultured with WT-NK-92 cells. Overall, DNAM-1$^+$ GM NK-92 cells exerted increased cytotoxic activity compared to WT controls in the imaging-based assays, supporting the degranulation results obtained and putting forward DNAM-1 as a prominent candidate in sarcoma immunotherapy.

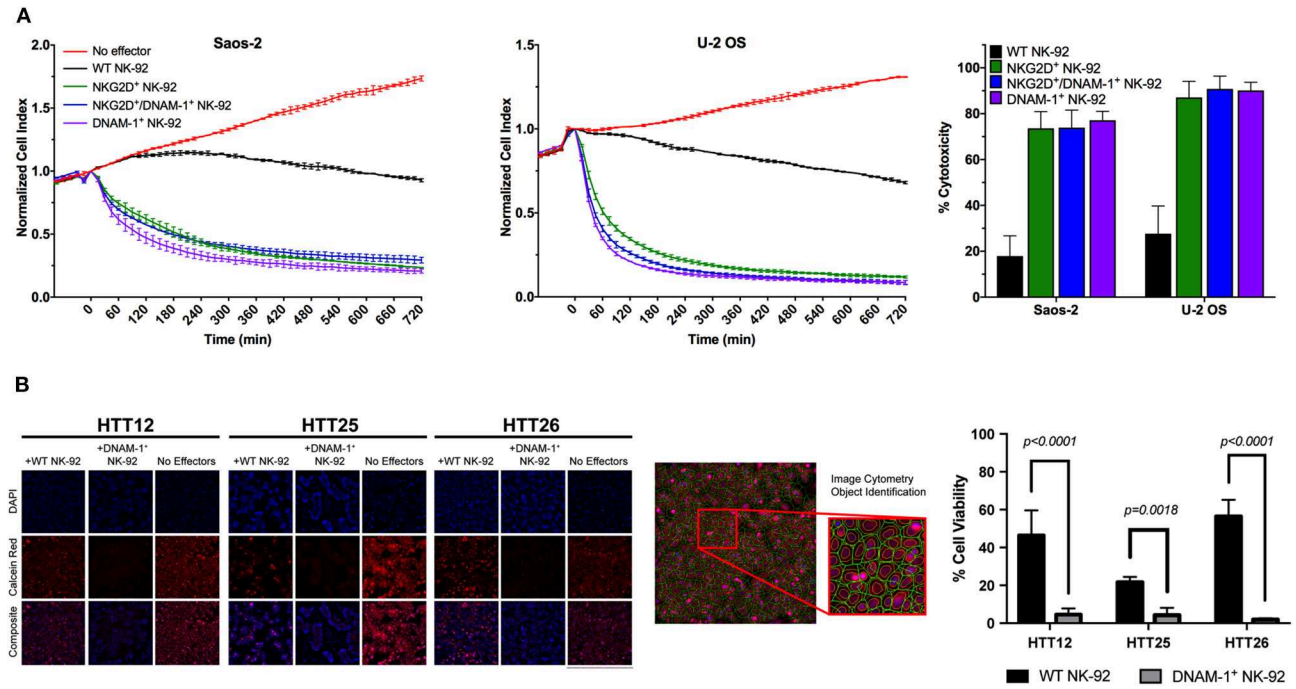

FIGURE 5 | Evaluation of NK cell cytotoxicity. **(A)** Representative results from cytotoxicity assays on Saos-2 (left panel) and U-2 OS (middle panel) cell lines using the xCelligence RTCA platform. Calculation of cytotoxic activity after 4 h of co-culture from two independent experiments (right panel) shows enhanced cytotoxic activity by GM NK cells. **(B)** Live tumor cells were loaded with Calcein-AM dye and co-cultured with effector cells at a 10:1 E/T ratio and incubated for 4 h. Live tumor cells were identified as Hoescht positive nuclei bounded by a Calcein Red positive cytoplasmic border. Apoptotic bodies were size excluded from the analysis. Tumor cell viability was assessed by determining the average fluorescent intensity (AFI) of individual tumor cells within each well. Percent viability was calculated by comparing the AFI of each condition to non-treated controls. Results are reported as the mean viability of two independent experiments, with three technical replicates per experiment and four fields of view per well. Results were analyzed using a two-way ANOVA with Tukey's *post-hoc* analysis in GraphPad Prism. DNAM-1$^+$ NK-92 cells were found to significantly reduce the viability of primary sarcoma patient tumor cells, $p < 0.0001$ (HTT12, HTT26); $p = 0.0018$ (HTT25).

DNAM-1 and NKG2D Ligand Expression Is Associated With Poor Survival in Sarcoma

In order to investigate the individual roles of NKG2D ligands in the response of GM NK-92 cells, we primarily determined mRNA level expression of ULBP1, ULBP2, ULBP3, ULBP4, and ULBP5 molecules in the sarcoma explants used in **Figure 3** and complemented this data with MICA/B cell surface expression and NKG2D-Fc staining of each explant (**Figure S2**). Overall, we observe that the tumors differentially express various NKG2D ligands as was also confirmed with NKG2D-Fc staining. Interestingly, mRNA expression did not always correlate with cell surface NKG2D-Fc staining which may be due to a deficiency in membrane trafficking or shedding of ULBP molecules (33). Given the current data, it is difficult to speculate which one of the NKG2D ligands is the most potent engager of anti-tumor responses.

To investigate the potential clinical significance of individual NKG2D and DNAM-1 ligands, we analyzed their expression levels in 259 sarcoma samples available in The Cancer Genome Atlas (TCGA) database of the National Cancer Institute (**Figure 6**). We observed that the high expression of DNAM-1 ligand CD155 as well as the high expression of NKG2D ligands ULBP1, ULBP2, and ULBP3 are negatively associated with survival in sarcoma patients. Taken together with our observation of decreased DNAM-1 and NKG2D expression on tumor-infiltrating NK cells in sarcoma and the enhanced cytotoxic activity of NK-92 cells expressing DNAM-1 or NKG2D against sarcoma explants, these results put forward DNAM-1- or NKG2D-based immunotherapy as a potentially effective approach in sarcoma treatment.

DNAM-1$^+$ and NKG2D$^+$ GM NK-92 Cells Provide a Novel Approach for Efficiently Targeting a Wide Range of Solid and Hematological Malignancies

Identification of the role of DNAM-1 and NKG2D in boosting NK cell responses against sarcoma using functional screening with GM NK-92 cells (**Figures 3**, **4**; **Figure S4**), encouraged us to further evaluate the use of this approach against other solid and hematological malignancies (**Figure 7A**; **Table 2**). We assessed the degranulation capacity of all 14 GM NK-92 cell lines against various well-established cancer cell lines, including the metastatic prostate carcinomas PC-3 and LNCaP, primary pancreatic ductal adenocarcinoma Capan-2, primary colorectal adenocarcinoma Caco-2, primary lung alveolar basal epithelial adenocarcinoma A549, metastatic neuroblastoma SH-Sy5y, metastatic nerve sheath tumor SNF02.2, melanomas SK-MEL-28, MeWo, A375 and DM6, myelomas U266, ARH-77 and

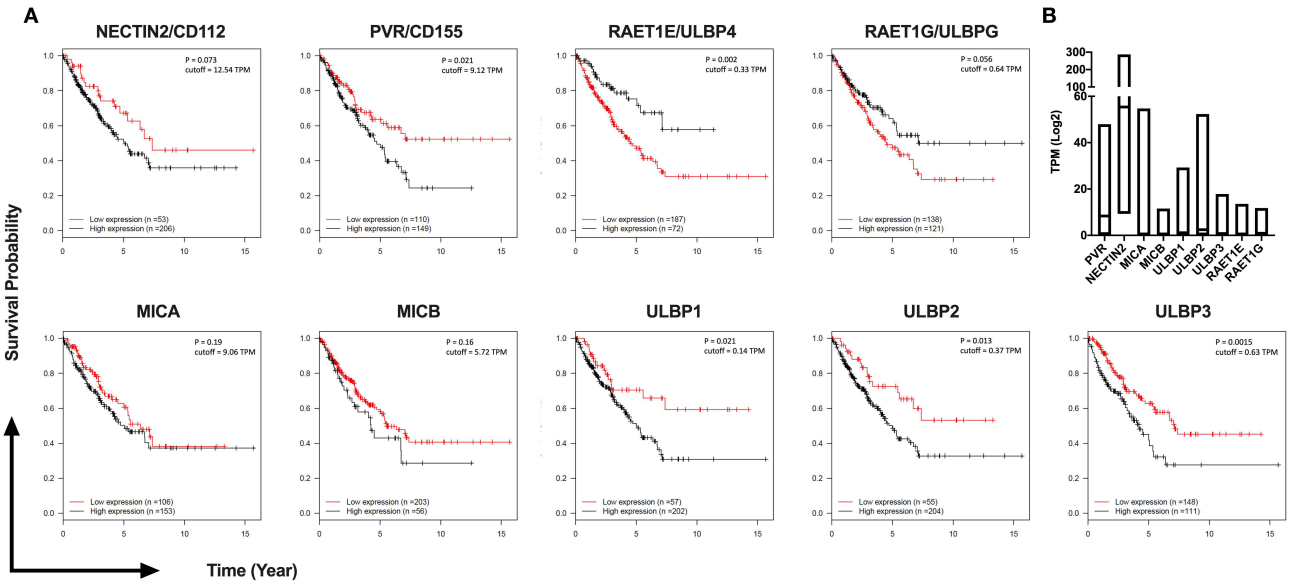

FIGURE 6 | TCGA analysis of 259 Sarcoma patients reveals high expression of DNAM-1 and NKG2D ligands are associated with poor survival. **(A)** Kaplan-Meier survival estimation analysis results from 259 TCGA Sarcoma TPM values based on expression of DNAM-1 ligands (Nectin 2 or CD112, PVR or CD155) and NKG2D ligands (MICA, MICB, ULPB1, ULPB2, ULBP3, RAET1E, or ULBP4 and RAET1G or ULBP5). Data from 20th to 80th percentile was compared. **(B)** Log2 TPM value of ligands of NKG2D and DNAM-1 from TCGA Sarcoma expression data.

RPMI 8226 and leukemias K562 (CML), and THP-1 (AMoL) (**Figure 7A**; **Table 2**). Assessment of degranulation responses with the GM NK-92 cell-based screening platform showed that NKG2D$^+$ GM NK-92 cells had enhanced degranulation against majority of the cell lines except for SH-SY5Y (**Figure 7A**). In line with this observation, previous studies and the human protein atlas database demonstrated moderate/high expression of at least one NKG2D ligand in all cell lines used except in the neuroblastoma cell line SH-SY5Y (35–41). Importantly, identification of differential receptor-mediated responses against tested cells lines such as DM6, THP-1, and ARH-77 cell lines, validates the functionality of each receptor introduced to NK-92 cells, and further demonstrates the applicability of the NK cell-based screening platform for the identification of tumor type- and most importantly patient-specific targetable NK cell receptor/ligand interactions (**Figure 7A**).

Moreover, DNAM-1$^+$ GM NK-92 cells also showed overall enhanced responses compared to WT control except against RPMI 8226, while the range of the response varied greatly depending on the different target cell types (**Figure 7A**). Further validation of the superior degranulation capacity of DNAM-1$^+$ GM NK-92 cells compared to WT controls, was independently assessed for all other types of cancers and showed that all cell lines induced significantly enhanced degranulation responses upon engagement of the DNAM-1$^+$ GM NK-92 cells, except for RPMI 8226 (**Figure 7B**).

Since CD112 and/or CD155 expression profiles of some of the cell lines used in this study have not been fully identified previously, we next assessed the surface expression of CD112 and CD155 by flow cytometry (**Figure 7C**; **Table 2**). The general increase observed in anti-tumor responses exerted by DNAM-1$^+$ GM NK-92 cells was in line with surface expression of at least one of the ligands on the surface of the cancer cell lines. With the exception of target cell line RPMI 8226, where surface expression of CD112 and CD155 were very low, all degranulation responses by DNAM-1$^+$ GM NK-92 cells were significantly enhanced compared to WT NK-92 ($p < 0.0001$).

Overall, our results suggest that arming NK-92 cells with activating receptors such as DNAM-1 and NKG2D can boost anti-tumor responses against various different malignancies. Likewise, identification of the unique NK cell receptor response profile of various tumors highlights the potential importance of the cell-based screening in the identification of targetable patient-specific NK cell/tumor interactions.

DISCUSSION

In this study, our primary aim was to identify potential NK cell/sarcoma interactions that can be modulated or targeted to enhance NK cell anti-tumor responses. To achieve this, we have assessed the immune profile of freshly isolated sarcoma explants, TILs and matched PBMCs, and developed a GM NK-92 cell line-based screening platform to identify the potential functional effects of different NK cell receptors in anti-sarcoma responses. Using this platform for the first time, we have characterized tumor-specific functional NK cell receptor signatures of primary human sarcomas as well as well-established cancer cell lines. Briefly, we have observed that NK cells minimally infiltrated sarcomas and expression of DNAM-1 and NKG2D were very low in both peripheral and sarcoma-infiltrating NK cells (**Figure 1**), even though ligand expression was observed in

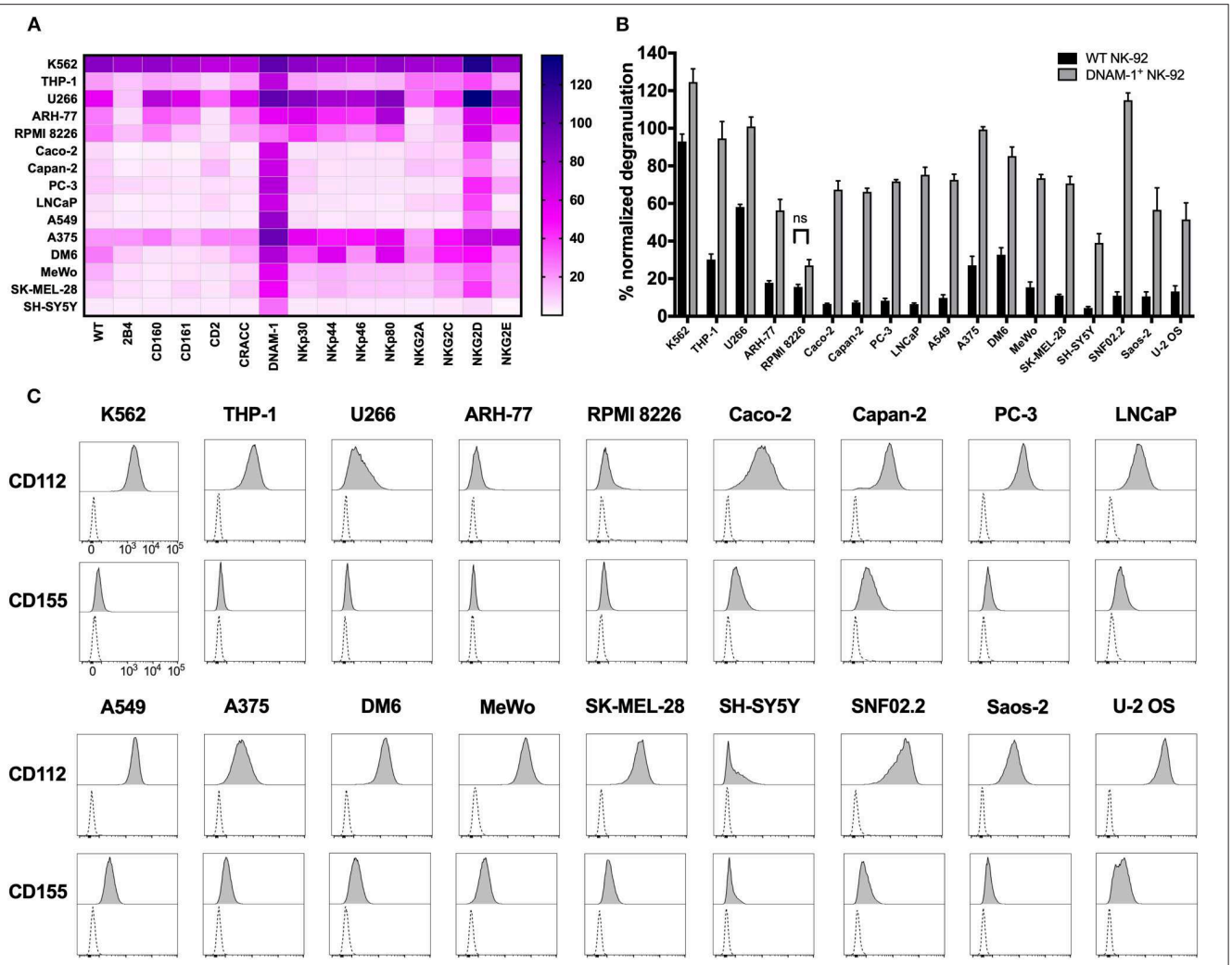

FIGURE 7 | Cancer cell lines expressing CD112 and/or CD155 are targeted by DNAM-1$^+$ NK-92 cells. **(A)** Degranulation response of all GM NK-92 cells and **(B)** of WT or DNAM-1$^+$ NK-92 cells in 4 h at 1:1 (E:T) with indicated cell lines were normalized according to the PMA/ionomycin response of effectors in each run [**A**: a single representative, **B**: Means of two independent experiments, run in duplicates are depicted with error bars indicating SEM. All except for RPMI 8226 response give significance ($p < 0.0001$ with 2-way ANOVA analysis.)] **(C)** Cancer cell lines were stained for surface expression of CD112 and CD155 and analyzed by flow cytometry (dashed line: unstained, filled histogram: stained). ns, not significant.

freshly dissociated tumors (**Figure 2**) and the expression profiles of DNAM-1 and NKG2D ligands were associated with poor survival in sarcoma patients (**Figure 6**). On the other hand, GM NK-92 cells that overexpress DNAM-1 or NKG2D efficiently targeted sarcoma explants (**Figure 3**) and various established tumor cell lines (**Figure 7**), while WT NK-92 cells failed to respond in general. The response of GM NK-92 cells was strictly dependent on the corresponding receptor and ligands (**Figure 4**) and cytotoxicity was further confirmed with imaging-based cytotoxicity assays (**Figure 5**).

Our results provide an essential insight into how to enhance NK cell mediated anti-sarcoma responses, while confirming for the first time the feasibility of a GM NK-92 cell-based functional screening approach in developing and predicting personalized immunotherapies for cancer patients. Given the clinical applicability of NK-92 cells (42, 43), it is also possible to generate off-the shelf GM therapeutic NK-92 libraries and predict the most efficient treatment option for each individual using such an *in vitro* functional screen carried out on tumor biopsies. This rapidly personalized adoptive immunotherapy regimen can then be used as monotherapy as well as in combination with other already available approaches, such as checkpoint inhibition.

Compelling evidence supports an important role for the immune system in the disease pathogenesis as well as in anti-tumor responses against sarcomas. This also suggests that immunotherapies would be a promising treatment alternative for sarcoma patients, but surprisingly they have been inadequately explored. Clinical trials using T cell receptor (TCR) modified T cells seem to focus on NY-ESO-I on subtypes of sarcomas, such as liposarcoma and synovial sarcoma (NCT03450122,

TABLE 2 | List of cell lines used in this study.

Cell line	Origin	CD112 (fold MFI*)	CD155 (fold MFI*)	WT NK-92 (%CD56$^+$CD107a$^+$)	DNAM-1$^+$NK-92 (%CD56$^+$CD107a$^+$)
K562 (ATCC® CCL-243™)	Chronic myelogenous leukemia	99.05	1.36	71.7 (±5.9)	81.3 (±6.5)
THP-1 (ATCC® TIB-202™)	Acute monocytic leukemia	64.83	0.94	23.4 (±6.7)	60.7 (±11.7)
U266 (ATCC® TIB-196™)	Myeloma; plasmacytoma	10.82	2.14	38.65 (±5)	69.8 (±9.3)
RPMI8226 (ATCC® CCL-155™)	Myeloma; plasmacytoma	1.16	1.24	10.5 (±2.9)	18.5 (±3.6)
ARH-77 (ATCC® CRL-1621™)	Plasma cell leukemia	3.22	0.59	11.7 (±0.4)	39.1 (±9.4)
Caco-2 (ATCC® HTB-37™)	Primary colorectal adenocarcinoma	59.73	4.06	4.7 (±1.1)	46 (±10.3)
Capan-2 (ATCC® HTB-80™)	Primary pancreatic ductal adenocarcinoma	50.92	4.73	5.3 (±1.9)	45.4 (±9.4)
PC-3 (ATCC® CRL-1435™)	Metastatic prostate adenocarcinoma	60.7	1.44	6.8 (±2.8)	52.9 (±0.7)
LNCaP (ATCC® CRL-1740™)	Metastatic prostate carcinoma	18.60	2.25	4.6 (±1.2)	47.5 (±7.3)
A549 (ATCC® CCL-185™)	Primary lung alveolar basal cell epithelial adenocarcinoma	85.31	6.69	7.7 (±3.6)	41.5 (±7.9)
A375 (ATCC® CRL-1619™)	Malignant melanoma	18.28	2.71	17.6 (±5.8)	59.9 (±4)
DM6 (34)	Malignant melanoma	51.67	2.76	23.3 (±7.5)	53.5 (±7)
SK-MEL-28 (ATCC® HTB-72™)	Malignant melanoma	60.45	4.75	7.9 (±2.1)	36.8 (±8.3)
MeWo (ATCC® HTB-65™)	Malignant melanoma	120.91	5.58	11.4 (±5.8)	47.4 (±16.9)
SH-SY5Y (ATCC® CRL-2266™)	Metastatic neuroblastoma	7.89	3.64	3.2 (±0.7)	21.7 (±3.1)
sNF02.2 (ATCC® CRL-2885™)	Metastatic malignant peripheral nerve sheath tumor (MPNST)	106.87	4.40	11 (±4)	115 (±7.7)
Saos-2 (ATCC® HTB-85™)	Osteosarcoma	59.46	5.28	9.4 (±4.9)	43.8 (±16.4)
U-2 OS (ATCC® HTB-96™)	Osteosarcoma	425.32	15.59	11.8 (±6.1)	39.9 (±12.2)

*$Calculated\ as\ (MFI_{stained} - MFI_{unstained})/MFI_{unstained}$.

NCT01477021, NCT01343043, NCT03399448) (16, 44) while applications of Chimeric Antigen Receptor (CAR) technology in sarcoma include HER2 (NCT00902044), EGFR (NCT03618381), CD44v6 and GD2 (45) directed CARs. However, downregulation of tumor-associated antigens is a common mechanism that tumors use to escape immune recognition, which has been shown to occur in both soft tissue and bone sarcomas (46–48), highlighting the potential importance of NK cell-based immunotherapies in the cases where antigen-specific responses fail (49, 50).

While NK cells are one of the promising candidates in the development of advanced cancer immunotherapies (51–54), very few clinical trials are currently exploring NK cells as a therapeutic option for sarcomas and none are exclusively designed to be sarcoma-specific (NCT02100891, NCT01875601). Nevertheless, sarcomas comprise over 100 different subtypes (55) and this diversity has made it very difficult to predict or customize efficient immunotherapies. This study provides a novel perspective for the development of efficient sarcoma-specific immunotherapies through the identification of DNAM-1 and NKG2D as well as their respective ligands as potential therapeutic targets for various subtypes of sarcomas and also enhancing NK cell mediated anti-sarcoma responses by DNAM-1$^+$ and NKG2D$^+$ GM NK cells.

Additionally, as a new angle to introduce antigen-specific recognition to NK cells, we and others recently published a novel approach to arm an NK cell line with a TCR in order to target tumors with high specificity while at the same time overcoming the problem of endogenous TCR mispairing (56, 57). Combining expression of antigen-specific receptors and tumor-specific NK cell receptors to further enhance anti-tumor responses by dual arming stands out as one of the future reflections of this study. NK cells that can be armed to enhance adaptive and innate anti-tumor responses are also inherently equipped to detect loss of MHC-I expression and get activated through disengagement of KIR/MHC-I mediated inhibitory signaling, thus potentially providing a back-up plan in case tumors downregulate MHC-I due to the TCR-mediated immune pressure. The persistence and multi-targeting potential of genetically enhanced NK cells can be further fine-tuned by inhibition of the tumor and the suppressive tumor microenvironment using, for example, checkpoint blockade therapy and inducing antigen-specific anti-tumor responses using antigen-specific monoclonal antibodies that induce CD16-mediated NK cell activation and antibody-dependent cellular cytotoxicity (ADCC).

DNAM-1 and NKG2D are two activating NK cell receptors which recognize stress-induced ligands that are commonly

over-expressed by tumors. Thus, they have been implicated as key players in immunity against human tumors and have been extensively explored in multiple approaches to cancer immunotherapy (58). Many studies have shown reduced expression of activating receptors like DNAM-1 and NKG2D on TILs from cancer patients (59, 60) or shedding of their respective ligands from the tumor cells (61, 62). In line with this, we observed very low expression of DNAM-1 and NKG2D both on peripheral and on tumor-infiltrating NK cells (**Figure 1D**). The downregulation of NK cell activating receptors in the periphery may result in diminished anti-tumor responses and can be reversed by *ex vivo* activation of NK cells expressing DNAM-1 and NKG2D, that have been shown to efficiently target and kill Ewing sarcoma (EWS), rhabdomyosarcoma (RMS) and osteosarcoma cell lines (derived from patient tumor samples) *in vitro* (27, 63, 64). On the other hand, here we demonstrate that primary human sarcomas (**Figure 3**) and other tumors (**Figure 7**) can be efficiently targeted by genetically modified NK-92 cells overexpressing NKG2D or DNAM-1 receptors.

Moreover, while previous studies have demonstrated expression and clinical relevance of CD112 and CD155 mostly from a sarcoma subset-oriented perspective (24, 63, 65), here we simultaneously assessed the expression of CD112 and CD155 in a diverse group of primary human sarcomas. The restricted expression of these molecules in healthy tissues, combined with the expression on all sarcoma explants provides valuable information regarding potential new targets for the development of targeted immunotherapies for sarcoma (**Figure 2**). DNAM-1$^+$ GM NK-92 cells degranulated against all primary sarcoma explants as well as against the majority of the established cell lines, proving the potential of CD112 and CD155 as tumor-specific markers for targeted immunotherapies (**Figures 3**, **7**). This, along with the absence of the degranulation response of NK cells against healthy donor PBMCs, showed that the use of DNAM-1$^+$ GM NK-92 cells could be restricted/directed only to targets that had elevated CD112 and/or CD155 expression (**Figures 2**, **7**), which is observed in tumors from various origins (65–83), including osteosarcomas (24).

As previously mentioned, NKG2D expression was very low on TIL NK cells but also on PBMC of sarcoma patients (**Figure 1D**), as was the expression of NKG2D ligands MICA/B on both fresh and propagated sarcoma explants (**Figures 2C,E**). This could be a result of peripheral immunosuppression induced by the tumor through NKG2D ligand shedding as has been observed in colon adenocarcinoma patients (84). While NKG2D is expressed on both T and NK cells, few attempts have been made to sensitize T cells to tumors through the NKG2D signaling axis (85, 86). However, NKG2D plays a significant role in tumor cell immune recognition and in particular in the perforin-mediated cytolytic response of NK cells (87). Moreover, NKG2D ligands are abundantly overexpressed in several human malignancies (88–93) and sensitize tumors to NK cell-mediated killing (94–99). Thus, it comes as no surprise that tumors employ a wide array of mechanisms to modulate NKG2D ligand expression to escape NK cell immune surveillance (100–106). Additional approaches to the one we describe here, have focused on developing therapeutics that increase the expression of NKG2D on NK cells (27, 107, 108) or enhance the expression levels of NKG2D ligands, as has been tested for Ewing sarcoma cell lines (109, 110).

Similar to the DNAM-1$^+$ GM NK-92 cells, using NKG2D$^+$ GM NK-92 cells, we were able to identify a subset of sarcoma explants that also induced efficient degranulation albeit to a lower degree (**Figure 3B**). NKG2D$^+$ GM NK-92 cells also showed efficient degranulation responses against several of the well-established tumor cell lines of various types of malignancies (**Figure 7**). Degranulation was NKG2D-specific as the use of blocking antibodies to the receptor abrogated the responses (**Figures 4C,D**). This could be explained by the presence of other NKG2D ligands such as the ULBP family of proteins which are also expressed by the U-2 OS and Saos-2 sarcoma cell lines (40). The approach described here, identifies NKG2D as an additional receptor that can arm NK cells to successfully target patient tumor cells expressing the relevant ligands.

We also addressed whether the co-expression of DNAM-1 and NKG2D would further enhance the anti-sarcoma activity of NK-92 cells. NKG2D and DNAM-1 are known to use similar activation motifs and downstream signaling of the receptors overlap significantly, sharing involvement of essential molecules to exert their effector functions (111–113). Thus, it is possible to argue that the co-triggering of DNAM-1 and NKG2D may result in the enhancement of these signals but also has the risk of the two signals stumbling upon a bottleneck of either ligand engagement or signaling intermediates that dictates the amount of activation possible. Our results indicate that while the co-expression of the two receptors seems to slightly affect degranulation, it did not provide a significant enhancement in cytotoxic activity (**Figures 4E**, **5A**). A third scenario including synergistic effects would theoretically be more possible if the second receptor made use of distinct signaling pathways, as in the case of 2B4, TRAIL, and FASL, but such an approach remains to be analyzed in further studies.

Overall, while tumor-infiltrating NK cells have decreased expression of DNAM-1 and NKG2D, sarcoma tumors highly express DNAM-1 and NKG2D ligands. Thus, we propose that genetically modified NK cells overexpressing DNAM-1 and/or NKG2D can be used to target these tumors efficiently and overcome the observed NK cell deficiency in sarcomas. Moreover, we have also demonstrated that the higher expression of many DNAM-1 or NKG2D ligands significantly associate with poor survival of sarcoma patients (**Figure 6**). Taking this information into account, it is possible to suggest that treatment with DNAM-1 and/or NKG2D expressing NK cells would especially benefit patients with bad prognosis.

In an era when personalized medicine is continuously gaining momentum, the use of biomarkers to identify the most efficient course of treatment is becoming increasingly necessary. To that end, efforts to develop new approaches that enable quick and efficient generation of important information regarding a patient's individual tumor phenotype have intensified in recent years. The functional screening platform described in this study enables the identification of NK cell receptor reactivities against

a variety of different types of sarcomas, as well as many other cancer cell lines. Consequently, the NK-92 cell-based screening platform could function as a tool to perform parallel assessment of several activating NK cell receptors and identify the ones with the ability to arm NK-92 cells for cytotoxic activity against the individual patients' tumor cells. This would provide important information that could contribute to making immunotherapy treatments patient-customized and more efficient.

While the *in vivo* anti-tumor activity and treatment efficiency of DNAM-1$^+$ or NKG2D$^+$ GM NK-92 cells yet remains to be investigated in xenograft models of sarcoma, we and others have demonstrated that WT and GM NK-92 cells have the ability to exert potent anti-tumor responses both in pre-clinical animal models and clinical trials including various solid tumors (114–118) as well as several hematological malignancies (119–122). Both, modified and unmodified NK-92 cells have shown successful treatment responses and are currently under investigation for numerous indications. Quick and robust expansion of the relevant GM NK-92 cells to large numbers facilitates their use as a standardized off-the-shelf therapeutic that is safe, efficient and highly specific to the patient's tumor. Ultimately, the described approach can provide prognostic value through the identification of potent tumor/NK cell interactions, but also GM NK-92 cells that overexpress DNAM-1 or NKG2D can also be used in the clinical setting to treat cancer patients.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MC, EA, TS, and AD contributed to the conception and design of the NK screening platform. TS designed lentiviral constructs based on LeGO vectors. MC and RK produced virus and did transductions. KK sorted genetically modified cells. ES, MC, DO, and BJ maintained cell culture of all NK and target cell lines. HT provided deidentified surgical material. AD, A-MG, and HT designed the clinic-to-bench pipeline. ES, RK, MH, AD, and A-MG did tumor dissociation and PBMC isolations. ES, RK, and MH maintained sarcoma serial passaging and stocking. A-MG, AD, and TC ran flow cytometry of sarcoma (fresh/propagated) and patient blood samples. MC, ES, and DO ran degranulation assays with GM NK-92 cells. DO and CP has generated GM NK cell lines, sorted, and performed Xcelligence assays. RP performed qPCR. MA, AM, and CZ performed analysis of TCGA data. BJ and A-MG ran imaging-based cellular cytotoxicity assays. A-MG performed co-culture experiments for intracellular cytokine detection in NK-92 cells upon tumor co-culture. ES and A-MG analyzed data, prepared figures, and wrote the first draft of the manuscript. BJ, DO, and AD analyzed data and prepared figures. ES, A-MG, AD, TS, BJ, and MC edited and finalized the manuscript. All other authors contributed to the final editing of 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.2020.00040/full#supplementary-material

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REVIEW
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Targeting Natural Killer Cells for Tumor Immunotherapy

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Natural killer (NK) cells are important innate cytotoxic lymphocytes with a rapid and efficient capacity to recognize and kill tumor cells. In recent years, adoptive transfer of autologous- or allogeneic-activated NK cells has become a promising cellular therapy for cancer. However, the therapeutic efficiency is encouraging in hematopoietic malignancies, but disappointing in solid tumors, for which the use of NK-cell-based therapies presents considerable challenges. It is difficult for NK cells to traffic to, and infiltrate into, tumor sites. NK cell function, phenotype, activation, and persistence are impaired by the tumor microenvironment, even leading to NK cell dysfunction or exhaustion. Many strategies focusing on improving NK cells' durable persistence, activation, and cytolytic activity, including activation with cytokines or analogs, have been attempted. Modifying them with chimeric antigen receptors further increases the targeting specificity of NK cells. Checkpoint blockades can relieve the exhausted state of NK cells. In this review, we discuss how the cytolytic and effector functions of NK cells are affected by the tumor microenvironment and summarize the various immunotherapeutic strategies based on NK cells. In particular, we discuss recent advances in overcoming the suppressive effect of the tumor microenvironment with the aim of enhancing the clinical outcome in solid tumors treated with NK-cell-based immunotherapy.

Keywords: natural killer cells, tumor microenvironment, cytokine, CAR-NK, checkpoint blockade, tumor immunotherapy

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INTRODUCTION

The important innate cytotoxic lymphocytes, natural killer (NK) cells, show rapid, and efficient cytolytic activity to recognize and kill both virally infected and transformed cells. They exert effector functions relying on germline-encoded receptors for their activation, without the need for prior exposure to the antigen. NK cells possess an extensive repertoire of receptors (activating and inhibitory) that recognize altered protein expression on target cells, thereby controlling the cytolytic function. Uniquely, these receptors can distinguish normal from transformed cells via "missing-self" or "induced self" recognition models (1, 2). The balance between activating and inhibitory receptors tightly governs the inhibition or activation of NK cells, and inhibitory signals from inhibitory receptors usually predominate over activation signals for the maintenance of homeostasis (3, 4). NK-cell-activating receptors recognize stress-induced molecules on target cells to prime NK cell activation. The downregulation of inhibitory ligands and the expression of ligands that activate receptors on tumor cells trigger the activation of NK cells to kill these abnormal cells. Both human and mouse NK cells express a CD16-activating receptor, which mediates antibody-dependent cellular cytotoxicity (ADCC) upon binding to the Fc portion of antibodies.

To stimulate responses to target cells, NK-cell-activating receptors, such as NK group 2D (NKG2D), CD226, and the natural cytotoxicity receptors NKp30, NKp44, and NKp46, are considered the most relevant receptors. Once activated, NK cells lyse tumor cells through the release of the cytotoxic molecules perforin and granzyme, upregulating the expression of the Fas ligand and tumor necrosis factor-related apoptosis inducing ligand, and the secretion of cytokines such as interferon gamma (IFN-γ) and TNF alpha (TNF-α).

In addition to their direct cytotoxic potential against target cells, NK cells also exhibit immunoregulatory functions. NK cells contribute to the homeostasis of the immune system and initiate or promote the activation and effector functions of other innate and adaptive immune cells via secretion of cytokines and chemokines, or through direct cell–cell contact (5, 6). NK cells can shape adaptive immune responses through cross-talk with other immune cells such as T cells, B cells, and dendritic cells (DCs). NK cells promote Th1 polarization by producing IFN-γ. They also indirectly enhance the immune responses of adaptive T cells by promoting DC maturation (6, 7). Emerging evidence suggests that NK cells enhance $CD8^+$ T cells function and/or ameliorate their exhaustion (8–10). NK cell function is proven to be critical for the antitumor or antiviral effects of $CD8^+$ T cells, even with checkpoint blockade therapy (11).

Recently, it was found that NK cells have features of adaptive immune cells, particularly the memory-like responses (12–18). A long-lived murine NK cell population had been reported to be able to self-renew and survive in a lymphopenic environment for more than 6 months (19). To date, three types of long-lived memory NK cells have been identified: hepatic-liver-resident NK cells, cytomegalovirus (CMV)-specific NK cells, and cytokine-induced memory-like NK cells (20–22). They usually express high levels of NKG2C and have the capacity for long-term *in vivo* proliferation and persistence. Upon restimulation with antigens or cytokines, memory-like NK cells undergo clonal-like expansion followed by longevity, self-renewal, and recall responses (13, 23, 24). Recently, the transcription factor interferon regulatory factor 8 has been found to orchestrate the adaptive NK cell response against CMV infection (25). A recent study showed that naive NK cells could be induced to functionally convert into tumor-induced memory-like NK cells by priming using acute myeloid leukemia or pediatric acute B-cell leukemia specimens (14). These tumor-induced memory-like NK cells exhibit certain similarities to cytokine-induced memory-like NK cells and CMV-specific NK cells; however, more importantly, they show significant differences, such as higher tumor-specific cytotoxicity and increased synthesis of perforins, but not IFN-γ secretion. These NK cell adaptive features are promising for the future use of immunotherapy to treat cancers and infective diseases.

NK cells' critical role in immunosurveillance and their powerful antitumor efficacy have prompted their use in many clinical trials to control tumor growth via their effector capacity. However, although the results have been encouraging in hematological malignancies, there has been less success for solid tumors. Indeed, solid tumors present considerable challenges to the application of NK-cell-based therapies. For example, it is difficult for NK cells to traffic and infiltrate into the tumor sites. NK cell function, activation, and phenotype are impaired by the tumor microenvironment, even rendering NK cells dysfunctional or exhausted. Thus, strategies to improve the cytolytic activity, durable persistence, and activation of NK cells have been developed. In the present review, we discuss how the cytolytic and effector functions of NK cells are affected by the tumor microenvironment. We also summarize the various immunotherapeutic strategies based on NK cells, especially the recent attempts to improve NK-cell-based immunotherapy clinical outcomes against solid tumors by overcoming the suppressive effect of the tumor microenvironment.

EFFECT OF THE TUMOR MICROENVIRONMENT ON NK CELLS' CYTOLYTIC FUNCTION

NK-cell-based immunotherapies, particularly the adoptive transfer of autologous or allogeneic NK cells, or gene-modified NK cells, have been used widely in clinical trials and have shown great promise for different hematological malignancies (26, 27). However, for patients with solid tumors, the outcomes of adoptive NK cell infusions have been disappointing. There are considerable challenges for NK cell therapy to treat patients with solid tumors. One of the major challenges is the difficulty of NK cells to traffic to the tumor location and infiltrate into the tumor. This poor ability of NK cells to infiltrate into solid tumors limits the clinical outcome of adoptive NK cell infusion. Enhanced infiltration of NK cells into tumor lesions has been associated with good prognosis for patients with diverse types of solid cancer (28, 29).

Another major challenge comes from the tumor microenvironment, which impairs the phenotype, activation, persistence, and function of NK cells. Accumulating data have shown that tumor-infiltrating NK cells exhibit poor cytotoxic capacity, accompanied by downregulation of activating receptors and upregulation of inhibitory receptors, compared with NK cells in non-tumor tissues (4, 30, 31). The tumor microenvironment is a complex network comprising regulatory T cells (Tregs), tumor-associated macrophages (TAMs), regulatory γδT cells, myeloid-derived suppressor cells (MDSCs), soluble factors, the extracellular matrix, and suppressive molecules expressed on tumor cells (32–35). NK cell proliferation and antitumor activity are suppressed by tumor cell secretion of various immunosuppressive factors, including prostaglandin E2, indoleamine 2,3-dioxygenase (IDO), interleukin 10 (IL-10), transforming growth factor-β (TGF-β), and vascular endothelial growth factor. The growth of many types of solid tumors promotes the expansion of immunosuppressive cells, including Tregs, MDSCs, and TAMs. Tumor cells also secrete chemokines, such as C–X–C motif chemokine ligand 8 or C–C motif chemokine ligand 2, to promote Tregs, TAM, or MDSCs accumulation at the tumor sites. By producing TGF-β and IL-10, or via direct cell-to-cell interaction, these immunosuppressive cells inhibit intratumoral NK cell cytotoxicity (36–38). Tregs directly inhibit NK cell

cytolytic functions via the production of TGF-β and also reduce the expression of activating receptors NKG2D and natural cytotoxicity triggering receptor 3 (NKp30) through membrane bound TGF-β (39, 40). MDSCs suppress NK cell cytotoxicity and cytokine secretion via membrane-bound TGF-β on MDSCs in a cell-contact-dependent manner or dependent on NKp30 on NK cells (39, 41). Several proinflammatory cytokines have also been reported to contribute to MDSC-mediated NK cell inhibition, resulting in reduced NK cytotoxicity, decreased expression of activating receptor NKGD or NKR, and limited release of IFN-γ (42). These soluble factors include TGF-β, IDO, nitric oxide (NO), and adenosine (38, 43–45). Adenosine is an important immunosuppressive molecule that is released at high levels by MDSCs in response to hypoxia and inflammation of the tumor microenvironment. In tumors sites, high levels of ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and 5′-nucleotidase ecto (CD73) are expressed by MDSCs, which results in markedly increased levels of adenosine secretion. NK cell antitumor activities are inhibited by adenosine via limiting IFN-γ/TNF-α release, inhibiting Fas ligand and perforin-mediated cytotoxic activity and blocking granzyme exocytosis (38, 46). In patients with hepatocellular carcinoma, a substantial proportion of $CD11b^{-}CD27^{-}$NK subsets with an inactive and immature phenotype was reported to accumulate in tumor tissues and render tumor-infiltrating NK cells less tumoricidal, which was also associated with tumor progression (47). High levels of inhibitory molecules, including programmed death ligand 1 (PD-L1) or PD-L2, are expressed on tumor cells, antigen-presenting cells, immunosuppressive cells, and stromal cells in the tumor microenvironment, thus preventing the activation of NK cells through binding with their respective inhibitory receptors on NK cells, which results in exhaustion and dysfunction of NK cells (48, 49). Among the stromal cells, cancer-associated fibroblasts (CAFs) are the major cells that affect the antitumor capacity of NK cells. CAFs secrete IDO or prostaglandin E2 to decrease NK cell expression of NKG2D, and secrete TGF-β to reduce the expression of NKG2D, NKp30, and NKp44 (50, 51). CAFs also inhibit the killing activity of NK cells by downregulating the level of the poliovirus receptor (poliovirus receptor/CD155) (52).

NK-CELL-BASED TUMOR IMMUNOTHERAPY

Cytokine-Activated NK Cells in Tumor Immunotherapy

NK cells' unique capability to distinguish normal from transformed cells, and their rapid and efficient killing capacity, have led to them becoming an attractive option for tumor immunotherapy and have shown great promise recently. The initial attempts comprised adoptive transfer of peripheral blood-derived autologous or allogeneic NK cells or those stimulated and expanded with cytokines, before injection into patients. In most cases, autologous or allogeneic NK cells were stimulated to activate and proliferate using cytokines (IL-2 or IL-15), in the presence of feeder cells genetically engineered to express costimulatory molecules or cytokines (IL-21 and IL-15) in the culture. The NK cells were then transferred into the patient, followed by the administration of IL-2 to maintain the expansion and function of the infused NK cells (20, 26). Encouraging results were achieved using adoptive transfer of haploidentical NK cells or allogeneic NK cells from killer cell immunoglobulin like receptor (KIR) mismatched donors, leading to graft vs. host disease (GVHD) in patients with hematological cancers. However, these approaches are undesirable to treat patients with solid tumors. The safety of adoptive transfer of autologous or allogeneic NK cells has been proved; however, efficacy is limited by their poor proliferation and persistence *in vivo* and the increase in Tregs related to IL-2 administration (53). Administration of exogenous IL-15 has been shown to improve survival, *in vivo* persistence, and therapeutic efficacy without inducing the survival and expansion of Tregs (54, 55). A human NKG2D–IL-15 fusion protein could efficiently bind to major histocompatibility complex class I polypeptide-related sequence^{+} tumor cells and stimulated NK cell activity by transpresentation of IL-15, thus exhibiting higher control efficiency of the growth of xenografted human gastric cancer via the NK cell recruitment and activation (55). In addition, membrane-bound IL-15 expressed on NK cells (mbIL-15 NK) or *IL15* gene-modified NK cells have been investigated and displayed the ability to survive and proliferate without exogenous cytokines, as well as superior cytotoxicity against both hematological neoplasms and solid tumors (56–59). Moreover, an IL15-IL15Rα-Sushi-Fc fusion protein (ALT-803), termed a superagonist, in which the IL-15Ra sushi domain is complexed with IL-15, potently enhances NK cell survival and cytotoxic activity compared with that induced by native IL-15. In preclinical studies, ALT-803 enhanced memory $CD8^{+}$ T cell subpopulations and specific NK expansion, promoted the secretion of IFN-γ, and improved NK cell function in multiple animal models, including B cell lymphoma, glioblastoma, colon cancer, and ovarian cancer (60–62). Phase I or II clinical trial evaluating its safety and efficacy in patients with both hematological neoplasms (e.g., relapsed or refractory multiple myeloma, acute myelogenous leukemia, acute lymphoblastic leukemia, and myelodysplastic syndromes) and solid tumors (NCT01885897, NCT01946789, NCT02099539, NCT03054909) (63, 64) or in combination with NK cell adoptive therapy (NCT02465957, NCT02890758), or with nivolumab (NCT02523469) (65) are currently ongoing. New expansion methods are being exploited to obtain large numbers of NK cells with enhanced cytotoxic activity (66). Notably, pre-activation of human peripheral blood-derived NK cells (PB-NK) using cytokine combinations, such as IL-18, IL-15, and IL-13, were observed to induce human NK cell differentiation into memory-like NK cells (67–69). These human memory-like NK cells expressed NKG2C, NKG2A, and killer cell lectin like receptor D1 (CD94); however, they had reduced expression of inhibitory KIRs. The pre-activated NK cells express the high-affinity IL-2 receptor and thus are more sensitive to low concentrations of IL-2, such that in IL-2 culture, they proliferate more rapidly and NK cell recovery is higher. The peculiar features of cytokine-induced memory-like NK cells are antigen unspecific with

increased proliferative capacity, long-term survival, and *in vivo* persistence, enhanced production of IFN-γ and higher cytotoxicity during *ex vivo* restimulation. Thus, cytokine-induced memory-like NK cells are becoming an attractive tool for antitumor immunotherapy. Recently, several phase I and II clinical trials of cytokine-induced memory-like NK cells have been performed in patients with relapsed or refractory acute myeloid leukemia (AML) after allogeneic hematopoietic cell transplant (NCT03068819, NCT02782546) or myelodysplastic syndrome (NCT01898793) sponsored by Washington University School of Medicine and have shown robust antitumor capacity (67). In addition to PB-NK, human embryonic stem cells, induced pluripotent stem cells (iPSCs), and bone marrow or umbilical-cord blood are being studied as alternative sources of therapeutic NK cells and are showing great promise (70).

CAR-Modified NK Cells in Tumor Immunotherapy

Recently, the development of chimeric antigen receptor (CAR)-T cells represents a breakthrough in cancer immunotherapy, particularly to treat hematological malignancies. Transfusion of CAR-T cells targeting CD19 to treat relapsed B-cell acute lymphoblastic leukemia ALL (B-ALL) and certain types of relapsed non-Hodgkin's lymphoma obtain as high as 70–90% clinical complete response rates. Thus, CD19-CAR-T cells have been approved by the Food and Drug Administration (FDA) to treat refractory ALL and diffuse large B-cell lymphoma. Currently, multiple CAR-T cells targeting different surface tumor antigens are undergoing clinical development to treat other hematological malignancies and several solid tumors (71, 72). However, there are still a number of obstacles that limit their clinical application. The main problems with CAR-T cell therapy are cytokine release syndrome, neurotoxicity, and on-target/off-tumor effects. To prevent GVHD, CAR-T cells are usually prepared from autologous peripheral blood, which is costly, time consuming, and personalized. NK cells modified with CAR (CAR-NK) are potentially safer than CAR-T cells in that infusion of CAR-NK cells seldom results in cytokine release syndrome because the NK cells secrete mainly restricted levels of granulocyte-macrophage colony-stimulating factor and IFN-γ, while the pro-inflammatory cytokines IL-1 and IL-6 are seldom secreted. The low likelihood of triggering GVHD upon allogeneic infusion means that CAR-NK cells may be prepared as an "off-the-shelf" product and are not restricted to autologous cells. Moreover, natural recognition receptors that recognize stress-induced ligands independent of CARs are retained on CAR-NK cells, including CD226, NKp30, NKp46, NKG2D, and NKp44. NK cells express FcγRIII (CD16) that can mediate ADCC. Therefore, CAR-NK cells are able to induce tumor cell lysis in CAR-dependent and CAR-independent manners, not only further promoting their killing activity but also decreasing the possibility of loss of CAR-targeting antigen-related relapse (73, 74). Intriguingly, activating signaling mediated by CAR is able to overcome the uneducated NK cell hyporesponsiveness, thereby mediating target cell killing. Indeed, CAR-mediated activation is able to overcome the dominant inhibition associated with NKG2A-mediated signaling, thereby enhancing NK cells' antitumor responses (75, 76).

To date, CAR-NK cells are being studied in ongoing clinical trials to evaluate their safety and efficacy for both hematological malignancies and solid tumors, which have shown promising results (**Table 1**). The main targets for hematological malignancy include CD19, CD22, CD33, and CD7 for relapsed and refractory leukemia and lymphoma (NCT01974479, NCT02742727, NCT02892695, NCT02944162, NCT03056339, NCT03579927, NCT03690310, NCT03692767, and NCT03824951) and B-cell maturation antigen for multiple myeloma (NCT03940833). The targets of current ongoing CAR-NK cells for solid cancer in clinical trials include mucin 1, human epidermal growth factor receptor 2, NKG2D ligands, roundabout guidance receptor 1 (ROBO1), and Mesothelin (NCT02839954, NCT03383978, NCT03415100, NCT03692637, NCT03692663, NCT03931720, NCT03940820, NCT03941457).

The main challenges to the clinical applications of CAR-NK cells include their low transfection efficiency, low *in vivo* persistence, difficulty to infiltrate into the solid tumor sites, and suppression from the tumor microenvironment. Although the viral transduction technology for NK cells has been improved recently, the transfection efficiency for primary NK cells remains unsatisfactory (77, 78). Non-viral vectors, an alternative gene transfer systems, particularly the *Sleeping Beauty* (SB) transposon system, could mediate stable transgene expression (79). CAR transgenes have been successfully transfected into T cells via electroporation using the SB transposon system (80, 81). Several phase I clinical trials have been sponsored by MD Anderson Cancer for the treatment of patients with advanced non-Hodgkin's lymphoma, ALL, and chronic lymphocytic leukemia by infusion of SB-modified CD19-specific CAR-T cells following hematopoietic stem cell transplantation (HSCT) (NCT00968760, NCT01362452, NCT01497184, NCT01653717, and NCT02529813). The results identified 84% CAR expression as well as the feasibility and safety of the SB system (81). An UltraCAR-T co-expressing CAR, mbIL15, and a kill switch introduced by SB transposon system using electroporation was approved by FDA for investigational new drug (IND) application to initiate a phase I study for the treatment of patients with advanced platinum-resistant ovarian cancer. The piggyBac transposon system has also been utilized recently to introduce the *CAR* gene into T or NK cells. Nanoparticle delivery technology has been harnessed to improve the transduction efficiency and augment the antitumor efficacy of immunotherapy (82). Using a T-cell-targeted nanoparticle to deliver the *CAR* gene, Smith et al. report one potential novel and interesting technology to establish *in situ* programming of CD19-CAR-T cells to treat leukemia (83). This approach contains several features: anti-CD3e F(ab′)2 fragments targeting T cells were coupled on the surface of nanoparticles; efficient nuclear delivery was achieved by biodegradable polymer functionalized with nuclear localization signals and microtubule-associated sequences; and stable chromosomal integration was facilitated by the piggyBac transposon system. The authors demonstrated that the nanoparticle-reprogrammed CAR-T cells have similar

TABLE 1 | Current clinical trials of CAR-NK cells.

ClinicalTrials.gov Identifier	Malignancy	Phase	NK source	Target	Signaling domain of CAR
NCT02944162	$CD33^+$ acute myeloid leukemia	I/II	NK-92 cell	CD33	CD28-4-1 BB-CD3ζ
NCT01974479	B-cell acute lymphoblastic leukemia	II	Primary NK cells	CD19	CD8αTM-4-1 BB-CD3ζ
NCT02742727	Leukemia and Lymphoma	I/II	NK-92 cell	CD7	CD28-4-1 BB-CD3ζ
NCT02892695	Leukemia and Lymphoma	I/II	NK-92 cell	CD19	CD28-4-1 BB-CD3ζ
NCT03056339	B lymphoid malignancies	I/II	Cord blood NK cells	CD19	CD28-CD3ζ
NCT03579927	B lymphoid malignancies	I/II	Cord blood NK cells	CD19	CD28-CD3ζ
NCT01974479	B-cell acute lymphoblastic leukemia	I	Primary NK cells	CD19	4-1 BB-CD3ζ
NCT03940833	Multiple myeloma	I/II	NK-92 cell	BCMA	CD8αTM-4-1 BB-CD3ζ
NCT03692767	Refractory B-cell lymphoma	I	Unknown	CD22	CD244
NCT03690310	Refractory B-cell lymphoma	I	NK-92 cell	CD19	CD244
NCT03824951	Refractory B-cell lymphoma	I	iPS derived NK cells	CD19	CD244
NCT03824964	Refractory B-cell lymphoma	I	Unknown	CD19/CD22	CD244
NCT02839954	MUC1 positive relapsed or refractory solid tumor	I/II	NK-92 cell	MUC1	CD28-4-1 BB-CD3ζ
NCT03383978	Glioblastoma	I	NK-92 cell	HER2	CD28-CD3ζ
NCT03415100	Solid tumors	I	Primary NK cells	NKG2D-Ligand	Unknown
NCT03940820	Solid tumors	I/II	NK-92 cell	ROBO1	CD8αTM-4-1 BB-CD3ζ
NCT03931720	Malignant tumor	I/II	NK-92 cell	ROBO1	CD8αTM-4-1 BB-CD3ζ
NCT03941457	Pancreatic cancer	I/II	NK-92 cell	ROBO1	CD8αTM-4-1 BB-CD3ζ
NCT03692663	Castration-resistant prostate cancer	I	NK-92 cell	PSMA	CD244
NCT03692637	Epithelial ovarian cancer	I	Primary NK cells	Mesothelin	CD244

antitumor therapeutic efficacy to conventional lentiviral-based CAR-T therapy in a B-cell ALL mouse model. Moreover, the particle synthesis is relatively simple, the product is stable, and can be lyophilized for extended storage, with low cost (84, 85). The piggyBac transposon system was also used to introduce NKG2D. CAR-presenting vectors were transfected into NK92 cells to generate NKG2D CAR-NK cells, and efficacy was evaluated in a human lung cancer xenograft model. These non-viral engineered NKG2D CAR-NK cells in combination with CD73 blockade showed significant synergistic therapeutic efficacy (86). The SB transposon system to generate CAR-T cells was found to be safe in recent clinical trials (NCT00968760, NCT01362452, NCT01497184, NCT01653717, and NCT02529813). Therefore, it might be feasible to use the SB or piggyBac transposon system combined with nanoparticle delivery technology to establish CAR-NK cells for clinical therapy, although the potential risks and benefits need to be carefully evaluated.

The CAR constructs in most CAR-NK are similar to those in CAR-T cells, except for the unique signaling, and activation features of NK cells. Thus, designing optimized CAR structures suitable for NK cells has been pursued. The NK-cell-activating receptor NKG2D recognizes multiple ligands, such as UL-16-binding proteins, major histocompatibility complex class I chain-related A, and MICB, which are present in increased abundance on the surface of viral-infected cells and many tumor cells. The first NK-cell-based CAR were NKG2D-expressing CAR-NK cells containing a NKG2D–DNAX activation protein 10 (DAP10)–CD3ζ construct, which have shown encouraging results *in vitro* and in a mouse model of osteosarcoma (87). This NKG2D CAR can recognize ∼90% of human tumor types that express NKG2D ligands. Notably, several studies have demonstrated that NKG2D ligands are expressed on immunosuppressive cells, such as MDSCs and Tregs; therefore, NKG2D-expressing CAR-NK cells not only directly kill $NKG2D^+$ tumor cells but also could eradicate immunosuppressive cells in the tumor environment (88). Other NK-activating receptors (NKp30 and DNAM-1)-based CAR could be designed using a similar strategy, having advantage that one CAR could target several of tumor types that express the corresponding ligands. Prostate stem cell antigen (PSCA)-DAP12 CAR, targeting the prostate stem cell antigen, was expressed in primary NK cells and the YTS-NK cell line and improved the specific cytotoxicity compared with PSCA-CD3ζ-based CAR NK cells (89). Interestingly, no additional costimulatory signaling molecules for *in vitro* cytotoxicity and activation were required by PSCA-DAP12 CAR. A panel of NK-cell-specific CAR constructs comprising various combinations of NK-cell-specific activating domains were screened for NK cell activation recently (90). The results showed that for optimal antigen-specific NK cell signaling and NK-cell-mediated cytotoxicity, the intracellular 2B4 domain, the CD3ζ signaling domain, and the transmembrane NKG2D are necessary. Based on these CAR constructs, mesothelin-targeted CAR-NK cells were constructed using human-induced pluripotent stem cells, and the antitumor activity was examined *in vitro* and using a murine ovarian cancer xenograft model. The NK-specific CAR-NK cells exhibited a markedly augmented cytolytic capacity, a significantly lower tumor burden, and prolonged survival compared with a traditional T-cell CAR construct expressing CAR-NK cells (90). Notably, the *in vivo* expansion and survival

of the NK cells was improved as a result of the NK-cell-specific CAR-mediated signaling.

To increase the specificity, efficacy, and safety of CAR-NK cells, as well as to overcome suppression by the tumor microenvironment, the strategies and experience from CAR-T studies could be applied to CAR-NK research. The targets used for CAR-NK research in preclinical studies include CD19, CD20, CD138, CD5, CD2 subset 1 (CS1), NKG2D ligand, glucosylceramidase beta (GD2), HER-2, epidermal growth factor receptor (EGFR), EGFRvIII, epithelial cell adhesion molecule 1, glypican 3, and guanine nucleotide-binding protein alpha-7 (91). For example, IL15 was introduced into CAR design to increase CAR-NK cells' antitumor capacity and persistence (92). CAR-NK cells can be engineered with chemokine receptors or chemokines to enhance NK cell homing and infiltrating to tumor sites.

The sources of NK cells include PB-NK cells, umbilical cord blood NK cells, and the NK cell line NK-92. However, the use of PB-NK cells is limited because primary NK cells are generally hard to genetically modify; therefore, the transduction efficacy is very low, even with viral vectors, and in the adoptive transfer setting, the allogeneic NK cells can survive for only a few weeks, limiting their antitumor efficacy (93). Recently, stem-cell-derived NK cells, such as those induced from human embryonic stem cells or iPSCs, have showed promise as candidates to develop off-the-shelf adoptive NK cell therapy. They have a similar phenotype and function to primary NK cells with an efficient capacity to kill both hematological malignancies and solid tumor cells. More importantly, they are homogenous, reproducible, and amenable to genetic modification, and can be easily expanded on the clinical scale (70, 94–96).

Notably, mesothelin-targeted CAR-NK cells prepared from human iPSCs showed significantly inhibited tumor growth, prolonged survival, and markedly augmented cytotoxicity in a xenograft model of ovarian cancer (90). Thus, iPSC-derived CAR-NK cells could be a promising resource to generate standardized, off-the-shelf, and clinical-scale adoptive immunotherapeutic products. Moreover, human iPSC-NK cells could be genetically modified to further improve their *in vivo* persistence, overcome the immunosuppressive tumor microenvironment, and enhance their antitumor capacity. iPSC-NK cells could also be harnessed in combination with other therapies (such as checkpoint blockade). Clinical trials treating cancer with human iPSC-NK cells are currently under IND application. The safety concerns for iPSC-NK cells include possible immune responses against allogenic iPSCs and their potential for malignant transformation. HLA-E-expressing or HLA knockout together with overexpression of CD47 hypoimmunogenic iPSCs have been generated recently to evade immune rejection by host cells (97, 98).

Antibody-Based NK Cell Therapy

ADCC of NK Cells Mediated by Tumor-Specific Antibodies

One of the main killing mechanism for antitumor effects of NK cells is ADCC, which involves the binding of the Fc portion of an antibody (Ab) to CD16 on NK cells. Most tumor-targeting Ab drugs exert their antitumor effect by promoting NK-cell ADCC. Studies have tried to increase the affinity of Abs to Fc receptors on NK cells to enhance ADCC by modifying Abs via mutagenesis or glycosylation (99). Expression high-affinity CD16 FcγRIII in NK cells is also a strategy used to enhance ADCC-mediated antitumor effects. To enhance NK-mediated ADCC using Cetuximab, Trastuzumab, and Pertuzumab, IL-2 and the high-affinity CD16 FcγRIIIa (158V) allele were engineered into NK-92 cells (named haNK) (100). Phase I and II clinical trials of haNK, alone, or in combination with anti-PD-L1 Ab (avelumab), a cancer vaccine, or super-IL-15, are ongoing for the treatment of triple negative breast cancer, squamous cell carcinoma, Merkel cell carcinoma, pancreatic cancer, and other types of cancers (NCT03027128, NCT03387085, NCT03387111, NCT03853317, and NCT03586869). CAR-modified haNK (also named target-activated NK-92) cells targeting CD19 and PD-L1 are currently under investigation and have recently been approved for IND by the FDA.

Potentiation of NK Cell Activity Using Bi- or Trispecific Killer Engagers

Molecules comprising single-chain variable fragments against activating receptors on NK cells and tumor-associated antigen, termed bi- or trispecific killer cell engagers (BiKEs or TriKEs), have been developed to create an immune connection between NK and tumor cells, thus promoting NK-mediated killing of tumor cells. Several NK-based BiKEs or TriKEs are currently in preclinical and clinical development. CD16-directed BiKEs CD16 × 19 and CD16 × 33 and TriKE CD16 × 19 × 22 were shown to specifically stimulate NK cell activation via CD16, which triggers NK cell cytolytic activity and cytokine secretion to fight lymphoma and leukemia (101, 102). A combination comprising an ADAM metallopeptidase domain 17 inhibitor further enhanced the function of NK cells by preventing CD16 shedding from the membrane of NK cells (102). Multiple other CD16-directed BiKEs, such as CD16 × Erb-B2 receptor tyrosine kinase 2, CD16 × human epidermal growth factor receptor 2/neu, CD16 × EGFR, CD16 × carcinoembryonic antigen, and CD16 × EpCAM, are under investigation (103). A bi-specific Ab-recognizing CS1-NKG2D, which comprises an anti-NKG2D scFv and an anti-CS1 scFv, showed enhanced cytotoxicity and cytokine production from NK cells and significantly prolonged their survival in a multiple myeloma xenograft NOD-SCID$^{IL2\gamma c-/-}$ (NSG) mouse model (104). A novel CD30/CD16A tetravalent bispecific Ab (AFM13), which has a longer half-life compared with that of other bi-/trispecific Abs, is currently under evaluation in a phase II clinical trial to treat patients with relapsed/refractory Hodgkin's lymphomas (NCT03192202) (105, 106).

To augment the *in vivo* expansion and survival of NK cells, a TriKE was constructed having a modified IL-15 cross-linker (107, 108). CD16 × IL-15 × CD33 displayed markedly enhanced NK cytotoxicity against AML and better cell persistence *in vivo* compared with those of BiKEs. Importantly, it is expected to target both malignant cells and CD33$^+$ MDSCs, thus exerting more efficient antitumor effect (107). Currently, CD16 × IL-15 × CD33 TriKE is being evaluated in phase I and II clinical trials in patients with advanced systemic mastocytosis, refractory/relapsed acute myeloid leukemia, or CD33-expressing high-risk myelodysplastic syndromes (NCT03214666). CD16 ×

IL-15 × Epcam TriKE, CD16 × IL-15 × CD133 TriKEs, and CD16 × IL-15 × CD133 × Epcam TetraKE are under investigation to treat colorectal cancer and have shown significantly enhanced cytokine secretion, NK cell proliferation, and lytic degranulation (108–110). More recently, trifunctional NK cell engagers targeting two activating receptors, NKp46 and CD16, on NK cells and a tumor antigen (CD19, CD20, or EGFR) on cancer cells were generated by Prof. Vivier and his colleagues (111). By cotargeting NKp46 and CD16, which led to full NK cell activation, the trifunctional NK cell engagers showed more efficient promotion of NK cell activation and cytotoxicity *in vitro* and more effective control of tumor growth in mouse models of solid and invasive tumors compared with those of current clinically available therapeutic antibodies. Remarkably, TriKEs represent a cost-effective and versatile platform for the incorporation of novel targeting molecules and could potentially boost NK cell function. For example, introducing Abs against checkpoint receptors or TGF-β to construct TriKEs might enhance NK cell efficacy by reversing the immunosuppression from the tumor microenvironment (TME) (112).

Releasing the Inhibition on NK Cells by Targeting the Immune Checkpoints

Recently, monoclonal antibodies (mAbs) targeting immune checkpoint ligands or receptors have been applied to markedly enhance the tumor-suppressed T-cell immune response, leading to the improved control of several types of cancer. Similarly, a variety of immune checkpoints receptors are expressed in NK cells, including KIRs, NKG2A, T-cell immunoglobulin- and mucin-domain-containing molecule 3, programmed cell death 1 (PD-1), lymphocyte activation gene-3, and T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibition motif domains (TIGIT). These inhibitory receptors are often induced or upregulated on tumor-infiltrating NK cells and affect the antitumor function of NK cells upon interaction with their respective ligands, even leading to dysfunction or exhaustion of NK cells (113). Functionally exhausted NK cells show less proliferation, reduced cytolytic activity, and downregulated cytokine secretion, thus losing the ability to attack tumor cells. Therapeutic blockade of the immune checkpoint receptors or ligands with mAbs can restore the antitumor function of NK cells, which have shown great promise for NK-cell-based tumor immunotherapy (114).

The first attempt was blockade of HLA-KIR interactions to ameliorate NK cell inhibition and enhance NK cell activity using the anti-KIR antibody, IPH2101, to treat leukemia, lymphoma, and multiple myeloma, which demonstrated efficacy and safety (115). The second generation, fully human immunoglobulin G4 anti-KIR antibody, IPH2102, was well-tolerated in patients with hematological malignancies and solid tumors (116). Anti-KIR antibodies combined with other immunotherapies, for example, anti-PD-1 and anticytotoxic T-lymphocyte-associated protein 4 Abs, to treat solid tumors are currently ongoing (NCT03203876, NCT03347123).

In various cancers, such as cervical cancer, breast cancer, hepatocellular carcinoma, and lung cancer, high NKG2A expression was identified inn tumor-infiltrating NK and T cells, which contributes to exhaustion of these cells and predicts a poor prognosis (49). Therapeutic blockade of NKG2A could improve NK cell dysfunction in both hematological malignancies and solid tumors (117, 118). In particular, a humanized anti-NKG2A Ab, Monalizumab (IPH2201), was reported to augment NK cell activity against certain types of tumor cell (B-cell lymphoma, solid tumors, and T-cell lymphoma) and rescue the effector and activation functions $CD8^+$ T and NK cells, especially in combination with PD-L1 blockade (118). A phase I and II clinical trial of IPH2201 and Cetuximab, with or without anti-PD-L1 Abs, in patients with human papillomavirus (+) and (–) recurrent or metastatic squamous cell carcinoma of the head and neck is ongoing (NCT02643550). Its safety was evaluated as well-tolerated for the combination therapy. The interim results of the phase II trial for treatment efficacy showed that combination therapy comprising IPH2201 and Cetuximab resulted in 31% partial response, 54% stable disease, and 11% progressive disease (118). Therefore, anti-NKG2A mAbs are proposed as promising checkpoint inhibitors that could enhance antitumor immunity via unleashing the potential of both T and NK cells (113, 119).

Tumor-infiltrating NK cells from patients with various cancers induce PD-1, which causes inducing functional failure of activated NK cells, which is associated with poor prognosis (120, 121). Blocking PD-1/PD-L1 signaling markedly increased the cytotoxicity and cytokine production of NK cells and significantly suppressed tumor growth *in vivo* (121). Several antibodies targeting PD-1 and PD-L1 have been used in clinical practice, and several others are under investigation to treat different solid and hematological malignancies (122). Various combination therapies of anti-PD-1/PD-L1 Abs with other checkpoint blockades, antiangiogenic bevacizumab, or chemotherapy have been explored recently in clinical trials. A current phase II clinical trial is assessing the effect of Pembrolizumab (an anti-PD-1 Ab) to induce changes to NK cell exhaustion and function in patients with unresectable melanoma at stage III or stage IV (NCT03241927).

Inhibitory receptors TIGIT and CD96 are regarded as new checkpoint receptors for NK cells and T cells. TIGIT competes with CD226 (DNAM-1) (an NK activating receptor) for the same set of ligands CD112 [Poliovirus receptor-related 2 (Herpesvirus entry mediator B)] and CD155 (poliovirus receptor), while CD96 shares binding of CD155 with CD226 and TIGIT but also binds to CD111 to directly inhibit NK cell function. Similar to the CD28/cytotoxic T-lymphocyte-associated protein 4 pathway, TIGIT and CD96, together with CD226, form a pathway in which CD226 functions as a costimulatory receptor, whereas TIGIT and CD96 act as coinhibitory receptors (123). The balance among the three receptors fine tunes the immune response against tumors. In patients with cancer, TIGIT and CD96 are upregulated in tumor-associated NK cells and promote NK cell functional exhaustion, accompanied by poor cytolytic potential and impaired cytokine production (124, 125). Blockade of TIGIT with Abs could effectively reverse NK cell exhaustion and enhance NK-cell-dependent antitumor immune responses in several tumor-bearing mouse models. Intriguingly, blockade of TIGIT could further promote tumor-specific T-cell immune responses and improve memory responses to tumor rechallenge (11). Blocking CD96–CD155

TABLE 2 | Current clinical trials of checkpoint blockade molecules.

Checkpoint Receptor	Ab	Combination	Malignancy	Phase	ClinicalTrials.gov Identifier
KIR	IPH4102		Cutaneous T-cell lymphoma	I	NCT02593045
	Lirilumab	Nivolumab (anti-PD1 Ab)	Bladder cancer	I	NCT03532451
	Lirilumab	Nivolumab (anti-PD1 Ab) Ipilimumab (anti-CTLA-4 Ab)	Advanced and/or metastatic solid tumors	I	NCT03203876
	Lirilumab		Advanced solid tumors	I/II	NCT01714739
	Lirilumab	Nivolumab (anti-PD1 Ab) Ipilimumab (anti-CTLA-4 Ab)	Advanced (metastatic and/or unresectable) Refractory Solid Tumors	I/II	NCT01714739
	Lirilumab	Nivolumab (anti-PD1 Ab) Ipilimumab (anti-CTLA-4 Ab)	Solid tumors	I/II	NCT03347123
	Lirilumab	Nivolumab (anti-PD1 Ab) Ipilimumab (anti-CTLA-4 Ab)Daratumumab (anti-CD38 Ab)	Multiple myeloma	I/II	NCT01592370
	Lirilumab	Nivolumab (anti-PD1 Ab)	Squamous cell carcinoma of the head and neck	II	NCT03341936
	Lirilumab	Rituximab (anti-CD20 Ab)	Lymphocytic leukemia	II	NCT02481297
NKG2A	Monalizumab		Hematologic malignancies	I	NCT02921685
	Monalizumab		Gynecologic cancer	I	NCT02459301
	Monalizumab		Chronic lymphocytic leukemia	I/II	NCT02557516
	Monalizumab	Cetuximab (anti-EGFR Ab)	Head and neck neoplasms	I/II	NCT02643550
	Monalizumab	Durvalumab (anti-PD-L1 Ab)	Advanced solid tumors	I/II	NCT02671435
	Monalizumab	Durvalumab (anti-PD-L1 Ab) Oleclumab (anti-CD73 Ab)	Non-small cell lung cancer	II	NCT03833440
	Monalizumab	Durvalumab (anti-PD-L1 Ab) Oleclumab (anti-CD73 Ab)	Stage III non-small cell lung cancer	II	NCT03822351
TIGIT	OMP-313M32		Locally advanced cancer, metastatic cancer	I	NCT03119428
	AB154	AB122 (anti-PD1 Ab)	Advanced malignancies	I	NCT03628677
	MTIG7192A	Atezolizumab (anti-PD1 Ab)	Non-small cell lung cancer	II	NCT03563716
TIM-3	Sym023		Metastatic cancer, solid tumor, lymphoma	I	NCT03489343
	TSR-022		Advanced or metastatic solid tumors	I	NCT02817633
	RO7121661		Solid tumors, metastatic melanoma, non-small cell lung cancer	I	NCT03708328
	LY3321367		Solid tumor	I	NCT03099109
	MBG453	Spartalizumab (anti-PD1 Ab)	Glioblastoma multiforme	I	NCT03961971
	MBG453		Acute myeloid leukemia	I	NCT03940352
	MBG453	PDR001 (anti-PD1 Ab)	Leukemia	I	NCT03066648
	INCAGN02390		Advanced malignancies	I	NCT03652077
	BGB-A425	Tislelizumab (anti-PD1 Ab)	Locally advanced or metastatic solid tumors	I/II	NCT03744468
	MBG453	PDR001 (anti-PD1 Ab)	Advanced malignancies	I/II	NCT02608268
	TSR-022	TSR-042 (anti-PD1 Ab)	Liver cancer	II	NCT03680508
PD-1	Nivolumab Pembrolizumab Atezolizumab	iPSC-derived NK Cells	Advanced solid tumors	I	NCT03841110
	Pembrolizumab	DC-NK Cells	Solid tumor	I	NCT03815084
	Nivolumab	NK Cells	Renal cell carcinoma	I	NCT03891485
	Pembrolizumab	Allogeneic NK Cells	Biliary tract cancer	I/II	NCT03937895
	Pembrolizumab		Lymphoma	I/II	NCT02535247
	PD-1 Ab	NK Cells	Non-small cell lung cancer	II	NCT03958097
	Avelumab	haNK^{TM}	Merkel cell carcinoma	II	NCT03853317
LAG-3	Sym022		Metastatic cancer, solid tumor, lymphoma	I	NCT03489369
	TSR-033		Advanced solid tumors	I	NCT03250832
	BMS-986016	Nivolumab (anti-PD1 Ab)	Glioblastoma	I	NCT02658981

(Continued)

TABLE 2 | Continued

Checkpoint Receptor	Ab	Combination	Malignancy	Phase	ClinicalTrials.gov Identifier
	REGN3767	REGN2810 (anti-PD1 Ab)	Malignancies	I	NCT03005782
	MGD013		Advanced solid tumors, hematologic neoplasms	I	NCT03219268
	IMP321		Solid tumors	I	NCT03252938
	Relatlimab	Nivolumab (anti-PD1 Ab)	Advanced solid tumors	I	NCT02966548
	Relatlimab	Nivolumab (anti-PD1 Ab)	Gastric cancer, cancer of the stomach	I	NCT03662659
	FS118		Advanced cancer, metastatic cancer	I	NCT03440437
	BMS-986016		Hematologic neoplasms	I/II	NCT02061761
	Relatlimab		Solid tumors	I/II	NCT01968109
	IMP321	Pembrolizumab (anti-PD1 Ab)	Non-small cell lung carcinoma, head and heck carcinoma	II	NCT03625323
	Relatlimab	Nivolumab (anti-PD1 Ab)	Melanoma	II	NCT03743766
	Relatlimab	Nivolumab (anti-PD1 Ab)	Advanced chordoma	II	NCT03623854

interaction could reverse NK- and T-cell exhaustion, and restore both NK- and T-cell-mediated antitumor immunity (125, 126). Recently, IL-1 receptor 8 long isoform (also known as single immunoglobulin IL-1R-related receptor) was identified as an NK cell checkpoint protein that regulates NK cell maturation and antitumor activity. Genetic blockade of IL-1 receptor 8 long isoform induces NK-cell-mediated resistance in hepatic carcinogenesis and liver or lung metastasis (127). Currently, the safety and efficacy of anti-TIGIT Ab, alone or in combination with anti-PD-1 or anti-PD-L1 Abs, are being evaluated in phase I and II clinical trials in patients with locally advanced or metastatic solid tumors, e.g., renal cell carcinoma, non-small cell lung cancer, breast cancer, squamous cell carcinoma of the head and neck, melanoma, and colorectal cancer (NCT03119428, NCT03563716, NCT03628677). Blocking Abs for other checkpoint proteins, including T-cell immunoglobulin- and mucin-domain-containing molecule 3 and lymphocyte activation gene-3, alone or in combination with other therapeutic approaches, are also currently in progress in patients in clinical trials or are under investigation for therapy of both hematological neoplasms and various solid tumors (e.g., NCT02060188, NCT02817633, NCT03307785, NCT03680508, NCT03250832, NCT03489369, NCT03625323, NCT03662659). Current clinical trials of checkpoint blockade antibodies to enhance NK cell antitumor efficacy are summarized in **Table 2**.

Other Strategies to Overcome the Suppression by the Tumor Microenvironment

The TME is a major obstacle for ensuring the optimum antitumor activity of NK cells, in which immunosuppressive cells and molecules limit NK cell activity. In addition, to overcome the suppression from inhibitory receptors or ligands by checkpoint blockade, neutralizing, or blocking suppressive cytokines secreted by tumor cells, immunosuppressive cells, or stromal cells in the TME is another key strategy. One of the most important immunosuppressive cytokine is TGF-β, which is secreted by tumor cells, Tregs, MDSCs, and other stromal cells in the TME to hamper the antitumor immune response. NK cell antitumor function is inhibited by TGF-β-mediated downregulation of the expression of NK-activating receptors NKp30 and NKG2D and also reduces the expression of NKG2D ligands on tumor cells, thus suppressing NK-mediated cytotoxic capacity and IFN-γ secretion (103). TGF-β also affects the development and differentiation of human NK cell subsets (128). Blockade of TGF-β signaling or neutralization of TGF-β could prevent NKG2D downregulation and restore the antitumor function of NK cells. Therapies that interrupt TGF-β signaling to enhance NK cell antitumor capacity are currently in clinical trials or are under investigation. The safety and efficacy of human TGF-β neutralizing mAb Fresolimumab (GC1008) and TGFβR1 inhibitor Galunisertib (LY2157299) have been evaluated in a phase I clinical trial to treat patients with advanced malignant melanoma, renal cell carcinoma, and other advanced solid tumors, and have obtained acceptable tolerability and safety results (NCT00356460, NCT01722825). Importantly, Galunisertib therapy could restore NKG2D and NKp30 expression on activated NK cells and enhanced NK cell cytotoxicity. Knockdown of *TGFBR2* or *SMAD3* in NK cells, engineering CB NK cells to express TGF-β dominant negative receptor II, or modifying NK cells using CAR containing TGF-β type II receptor extracellular and transmembrane domains, and the intracellular domain of NKG2D, are all under investigation and have shown great promise for the recovery of tumor-suppressed NK cell antitumor activity to treat patients with solid tumors (129–131).

CONCLUSION AND PERSPECTIVES

Although there are still some challenges that limit the widespread use of NK-cell-based therapies, particular for solid tumors, advances in *ex vivo* expansion and activation technologies, genetic modification, and nanoparticle delivery technology will lead to novel therapeutic strategies to overcome the immune suppression from the TME of solid tumors, indicating that that NK cell therapy is achievable and promises to become a powerful method to treat cancers. In certain respects, NK cells have

unique advantages over conventional T cells (73, 132). Notably, the use of NK cells could result in off-the-shelf allogeneic products to treat patients, thereby eliminating the necessity for personalized and patient-specific products in current CAR-T cell therapies. NK-cell-based therapy provides an alternative or complementary immunotherapy approach to T-cell therapy. It is worth exploring the utility of the selective expansion of suitable NK cell subsets and their delivery to the corresponding type of tumor. The longer lifespan of memory-like NK cells compared with that of PB-derived NK cells may make them ideal sources for NK cell therapy. Recently, CMV-induced adaptive memory NK cells were demonstrated as relatively resistant to Tregs and MDSCs' suppressive effects, which might have important clinical implications (133, 134). Remarkable benefits might be achieved by engineering the CAR protein into memory-like NK cells or a specific NK cell subset. In fact, CD19-specific CAR-engineered $NKG2C^{+}CD57^{+}$ adaptive NK cells showed an enhanced ability to kill $CD19^{+}$ tumor cells compared with that of other NK subsets (75). Different combinations of multiple strategies could play an increasingly prominent role in clinical practice. For example, the use of checkpoint blockade to overcome the immunosuppressive effect within the TME could increase the endogenous NK cell antitumor ability and the function of adoptive transferred NK cells. Therefore, it is reasonable to combine checkpoint inhibition with adoptive transfer of allogeneic NK cells or CAR-transduced NK cells. Checkpoint receptor blockade in conjunction with BiKEs or TriKEs could increase antigen specificity and reverse immunosuppression, thus further enhancing the antitumor responses of NK cells.

AUTHOR CONTRIBUTIONS

CZ and YH designed and conceived the review. YH and CS performed the literature search and analysis. CZ wrote the initial draft of the manuscript together with YH and CS. All authors revised and approved the final submitted version of the manuscript.

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