THE SECOND LIFE OF NATURAL KILLER (NK) CELLS

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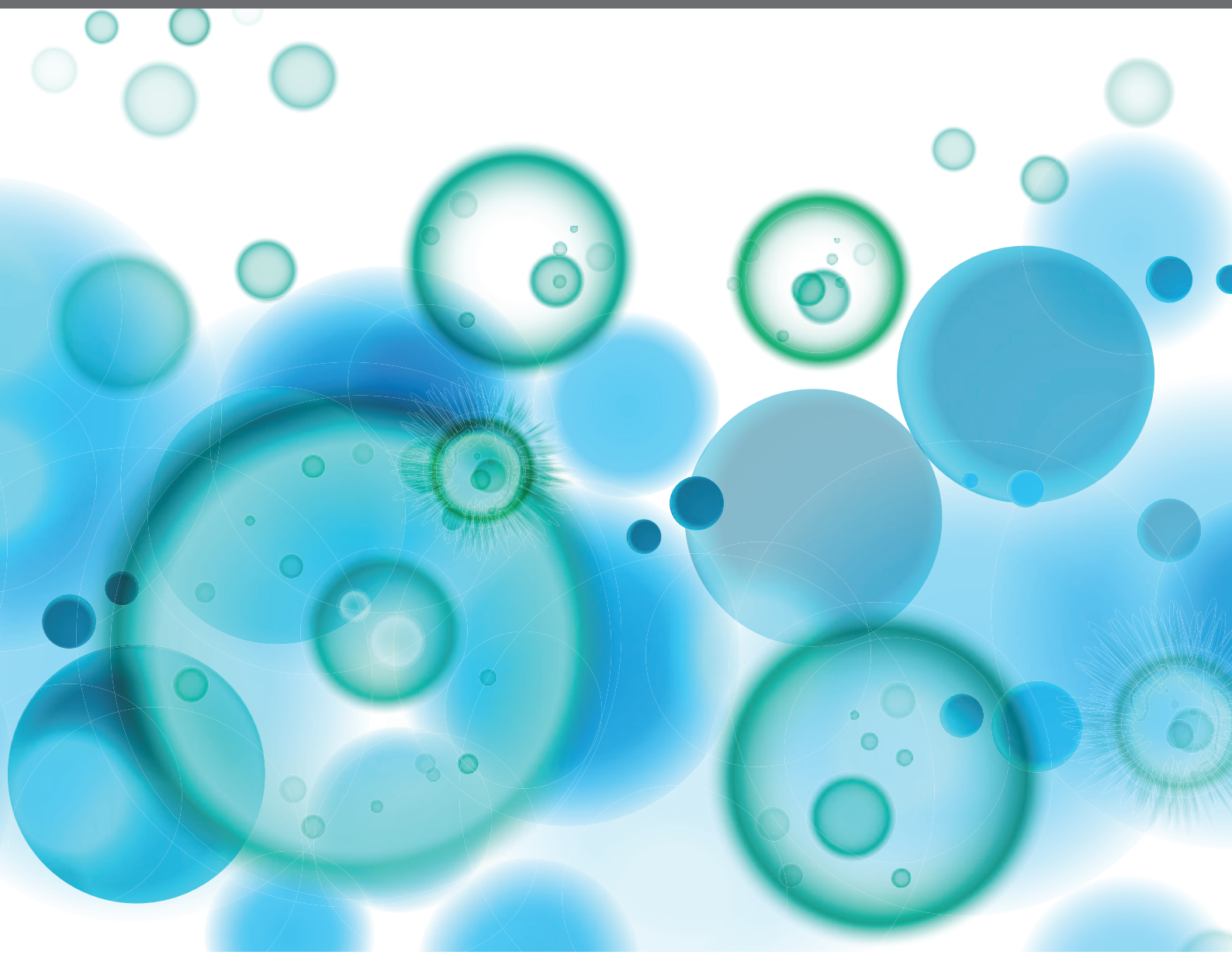

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THE SECOND LIFE OF NATURAL KILLER (NK) CELLS

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Natural Killer (NK) cells are innate lymphocytes, now recognized as members of a larger family of "Innate lymphoid cells" (ILCs). Both murine and human NK cells are well characterized effector cells with cytotoxic as well as cytokine production ability which mainly react in response to microbial and cell stress stimuli, thus playing a central role in the defense against pathogen infection, in tumor surveillance and in regulating immune homeostasis. Despite these established concepts, our understanding of the complexity of NK cells, also in view of their developmental and functional relationship with other ILC subsets, is only recently emerging.
This Research Topic highlights the recent advances in NK cell (and ILC) research in human and mouse from basic research to clinical applications.

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MINI REVIEW
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"Natural Regulators": NK Cells as Modulators of T Cell Immunity

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Natural killer (NK) cells are known as frontline responders capable of rapidly mediating a response upon encountering transformed or infected cells. Recent findings indicate that NK cells, in addition to acting as innate effectors, can also regulate adaptive immune responses. Here, we review recent studies on the immunoregulatory function of NK cells with a specific focus on their ability to affect the generation of early, as well as long-term antiviral T cell responses, and their role in modulating immune pathology and disease. In addition, we summarize the current knowledge of the factors governing regulatory NK cell responses and discuss origin, tissue specificity, and open questions about the classification of regulatory NK cells as classical NK cells versus group 1 innate lymphoid cells.

Keywords: NK cells, immune regulation, adaptive immunity, viral infection, ILC

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INTRODUCTION

Natural killer (NK) cells were identified in the 1970s as natural effector cells with the capacity to lyse tumor cells (1, 2). The role of NK cells as effectors against transformed and virally infected cells has been well established (3). Regulated through a range of activating and inhibitory receptors (4), NK cell activation results in cytotoxic degranulation and the production of inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (5). While the importance of NK cells as frontline responders remains undisputed, regulatory functions of NK cells are being increasingly appreciated in several settings and especially in the regulation of immune responses during viral infections (6–12). A regulatory role of NK cells is also emerging in the context of reproduction [reviewed in Ref. (13)]. A major issue that is still to be resolved is the nature of these "natural immune regulators," and whether they represent classical NK cells or a population of the recently defined innate lymphoid cells (ILCs).

IMPACT THROUGH CYTOKINE AND CHEMOKINE PRODUCTION

Activated NK cells can express a number of secreted molecules that may augment or dampen immune responses. NK cell-derived IFN-γ and TNF-α have antiviral effects and play a role in the activation and/or maturation of dendritic cells (DCs) (14–16), macrophages, and T cells (5, 17). Moreover, NK cells can produce granulocyte-macrophage colony-stimulating factor (18), IL-5, IL-13 (19), and IL-10 (6), thereby impacting various downstream immune responses. NK cells can also affect immune responses through the production of chemokines, such as CCL3, CCL4, and CCL5, which recruit other effector cells to sites of inflammation (20).

IMPACT ON DCs AND THE PRIMING OF ADAPTIVE IMMUNE RESPONSES

In addition to cytokine- and chemokine-mediated effects, NK cells can affect the priming of adaptive immune responses through killing of infected or transformed cells (7, 16, 21, 22). Early elimination of murine cytomegalovirus (MCMV)-infected cells through recognition of the viral protein m157 by Ly49H$^+$ NK cells has been associated with decreased levels of type I IFN production by plasmacytoid DCs (21). As a result, DCs were maintained in higher numbers, augmenting the very early priming of antiviral CD8$^+$ T cell responses. However, this was a transient effect, as Ly49H$^+$ NK cells also eliminate MCMV-infected DCs, ultimately reducing T cell priming and thereby curbing ongoing T cell responses (7).

Similarly, early depletion of NK cells in a mouse model of lymphocytic choriomeningitis virus (LCMV) infection improved DC priming of virus-specific T cells (11). Together, these studies establish a role for NK cells in the regulation of DCs and, as a consequence, the priming of adaptive T cell responses.

NK CELLS AS DIRECT MODULATORS OF ADAPTIVE T CELL-MEDIATED IMMUNITY

As mentioned above, NK cells can contribute to the activation of T cells through the production of cytokines that promote T cell priming and differentiation (5). In addition, NK cells can express costimulatory molecules, such as OX40 ligand, allowing them to directly promote T cell proliferation (23). Direct NK cell-mediated inhibition of T cell responses has also been reported. In this regard, a number of different *in vitro* and *in vivo* studies in human and mouse models have shown that NK cells are capable of directly killing T cells. An *in vitro* approach studying the role of human NK cells in the response to intracellular antigen revealed that activated NK cells can eliminate regulatory T cells (24). Direct elimination of effector CD4$^+$ T cells by NK cells has been reported in mouse models of chronic inflammation (25) and graft-versus-host disease (26). Similarly, human NK cells have been reported capable of killing activated T cells (27, 28). *In vitro* experiments investigating the sensitivity of T cells to NK cell-mediated killing revealed that, while resting T cells were resistant to lysis by NK cells, recently primed T cells were susceptible (29). Susceptibility to killing was limited to the first few days following T cell activation, and declined thereafter, with T cells regaining sensitivity to NK cell killing upon re-encountering cognate antigen. The sensitivity of activated T cells was associated with the upregulation of ligands for the NK cell activating receptor NKG2D. The role of NKG2D-mediated activities in the killing of T cells by NK cells *in vivo* is more complicated, with reports that NKG2D is both important (8, 9) or irrelevant (10, 30) being available. Furthermore, blocking inhibitory signals delivered upon engagement of the non-classical MHC I molecule Qa-1 has been shown to allow the NK cell-mediated elimination of autoreactive T cells, resulting in reduced pathology in a model of experimental autoimmune encephalomyelitis (31). Although published studies mostly report NK cell-mediated effects on CD4$^+$ T cells, suggesting that CD4$^+$ T cells may be more susceptible to NK cell-mediated elimination, both CD4$^+$ and CD8$^+$ T cells can be eliminated by NK cells, and this primarily involves perforin-dependent activities (8–10, 32). Elimination of T cells involving the TNF-related apoptosis-inducing ligand (TRAIL) (12, 27, 33) and Fas (26) has also been reported.

NK CELLS IN THE REGULATION OF ANTIVIRAL T CELL RESPONSES

Even though the interactions between NK cells and T cells have been studied in a number of different models, the most extensive investigations have been undertaken in the setting of viral infections. In this context, it is increasingly evident that, in addition to contributing to the elimination of infected cells, NK cells can also limit antiviral immunity (7, 9–12). Specifically, NK cells have been shown to impair both initial and ongoing antiviral T cell responses.

NK Cells as Regulators of Early Antiviral T Cell Immunity

A frequently used model to investigate the role of NK cells in viral infection is MCMV. The first indication that NK cells could modulate T cell responses came from studies showing that their depletion in C57BL/6 mice at the time of MCMV infection resulted in increased T cell proliferation and higher numbers of T cells expressing IFN-γ (34). This important finding was extended by our studies showing that NK cells limited the function and longevity of antiviral T cell responses *via* the elimination of MCMV-infected DCs (7). Notably, the more robust antiviral T cell activities generated in the absence of early NK cell responses expedited the control of chronic MCMV infection (7). Furthermore, it has been reported that a mutation in the gene encoding the NK cell activating receptor NKp46, which leads to hyperreactivity of NK cells, results in suboptimal anti-MCMV T cell responses (35). Most recently, our studies have revealed an immune-regulatory function for NK cells in the salivary glands, a site of chronic MCMV infection (12). Viral control in the salivary glands is directly dependent on CD4$^+$ T cells, with more robust antiviral CD4$^+$ T cell responses resulting in accelerated viral control. Notably, CD4$^+$ T cells accumulating in the salivary glands during chronic MCMV infection upregulate the receptor for TRAIL, rendering them susceptible to TRAIL-mediated activities. These activities were found to be mediated by TRAIL on NK cells (12), with timed NK cell depletion or deficiency in TRAIL resulting in elevated numbers of antiviral CD4$^+$ T cells and enhanced viral control. The specific upregulation of NKG2D ligands on CD4$^+$, but not CD8$^+$ T cells, and the NKG2D expression on TRAIL$^+$ NK cells provided strong support for an important role being mediated by these additional interactions in determining the fate of TRAIL-R$^+$ CD4$^+$ T cells upon encounter with TRAIL$^+$ NK cells.

Similar to observations made for MCMV infection, there is accumulating evidence supporting a role for NK cells in the regulation of antiviral T cell responses to LCMV. A study investigating the role of the NK cell receptor 2B4, revealed that

2B4-deficient mice had reduced numbers of activated $CD8^+$ T cells and consequently prolonged viral persistence (32). This phenotype could be reverted by depletion of NK cells, indicating that the absence of 2B4 leads to NK cell-mediated elimination of LCMV-specific $CD8^+$ T cells.

Further studies have supported the hypothesis that NK cells regulate T cell responses during both an acute and chronic LCMV infection. Following both types of LCMV infection, NK cells showed enhanced cytotoxicity and were capable of eliminating T cells that had been activated *in vitro* (9). Notably, in the absence of NK cells, LCMV infection resulted in increased frequencies of LCMV-specific $CD8^+$ T cells, significantly reduced viral titers, and less virus-induced hepatitis, suggesting that NK cells promote the development of chronic infection (9). Protection against T cell exhaustion was also reported in the absence of NK cells (11). A concurrent study confirmed the regulatory effect of NK cells on the generation of antiviral T cell responses, immune pathology, and viral control (10). Here, however, the authors extended previous findings and reported that viral dose had a significant impact on how NK cells affected antiviral immune responses. The varying effects of NK cells in infections with different LCMV viral doses were due to changes in the patterns of T cell-mediated pathology. In mice infected with a high viral dose, NK cells prevented mortality but promoted viral persistence. Here, NK cell depletion resulted in elevated T cell numbers, reduced T cell exhaustion, but increased mortality, indicating a protective effect of NK cells in this setting. In contrast, the severe immune pathology associated with infection with a medium LCMV viral dose was abrogated by depletion of NK cells, and complete viral clearance was achieved with 100% survival. Although ultimately NK cells suppressed $CD8^+$ T cell responses, the authors propose that this is mediated indirectly *via* the elimination of $CD4^+$ T cells (10).

Together, these studies clearly outline a role for NK cells in the regulation of primary T cell responses to viral infection and open the possibility that the regulatory effects of NK cells may also modulate long-term antiviral responses and the generation of memory.

Regulatory NK Affect Ongoing Antiviral T Cell Immunity and Memory Generation

In MCMV infection, mice lacking the capacity to eliminate MCMV-infected DCs through $Ly49H^+$ NK cells generated more robust T cell responses, with significant differences in both kinetics and functionality (7). Importantly, these differences are maintained in the long term, with stronger cytokine and chemokine expression and increased retention of leukocytes in tissues observed in mice lacking early Ly49H-mediated NK cell activities (unpublished observations, S. V. VanDommelen, Iona S. Schuster, Christopher E. Andoniou, Mariapia A. Degli-Esposti).

An effect on ongoing antiviral responses was also noted in MCMV-infected mice lacking $TRAIL^+$ NK cells in the salivary glands (12). The lack of $TRAIL^+$ NK cells resulted in increased accumulation of antiviral T cells and improved viral control in the salivary glands. However, the failure to restrain $CD4^+$ T cell responses was associated with an increase in autoantibody production, the formation of tertiary lymphoid-like structures, and, most importantly, the loss of tissue function in the affected exocrine glands indicating the development of an autoimmune disease with the characteristics of human Sjogren's syndrome (12). These observations are of particular interest as this study provided the first evidence for a physiological effect of NK cell immune regulation, with NK cells playing a crucial role in maintaining a balance between protective immunity and excessive pathologic or autoreactive immune responses.

Similar to MCMV infection, the immunomodulatory functions of NK cells in LCMV infected mice not only affect early antiviral immune responses but also modulate ongoing antiviral immunity and the generation of immunological memory (11, 30, 36). First indicators of the long-term effects of NK cells on anti-LCMV T cell responses were the reduced frequencies of virus-specific T cells in mice lacking the NK cell inhibitory receptor 2B4 observed up to 100 days post-LCMV infection (32), and the observation that T cell exhaustion, as defined by PD-1 and Lag-3 expression, was decreased in NK-cell depleted mice (11). Further studies demonstrated that NK cell depletion in the initial stages of acute LCMV infection, as well as Pichinde virus infection, specifically altered the generation of memory precursor effector cells and resulted in T cell memory with improved functional capacity (30). Furthermore, NK cell-depleted mice had elevated numbers of T follicular helper cells (Tfh) and germinal center B cells, resulting in more sustained antibody responses (30). The latter results were corroborated by data demonstrating that mice depleted of NK cells just before LCMV infection generated a more robust Tfh response facilitating greater germinal center B cell and plasmablast formation, elevated concentration of anti-LCMV antibodies, and improved control of chronic infection (36).

Overall, these studies form a strong body of evidence supporting the hypothesis that NK cells not only affect the generation of primary T cell responses to viral pathogens but also alter the quantity and quality of ongoing responses, including T and B cell memory responses and long-term protection. Although the NK cell-mediated moderation of both primary antiviral T cell responses and the ensuing generation of memory may at face value appear detrimental to the host, the development of autoimmunity observed in mice lacking regulatory NK cell functions indicates that, in physiological settings, NK cells contribute to the balancing of immune responses (**Figure 1**).

Much remains to be understood about the immune-regulatory role of NK cells, including their impact on adaptive immunity at late stages of persistent viral infections. Interestingly, in a model of Friend retrovirus infection, NK cell depletion in the acute phase of infection impaired viral control, but depletion of NK cells during chronic infection led to increased numbers of activated $CD8^+$ T cells and expedited viral control (37). A study by Peppa et al. (33) outlined a role for NK cells in limiting hepatitis B virus (HBV)-specific $CD8^+$ T cell numbers. Analysis of PBMCs from chronically infected patients cultured in the presence or absence of NK cells showed increased numbers of activated $CD8^+$ T cells in NK cell-depleted cultures. A significant upregulation of TRAIL receptor 2 (TRAIL-R2) was noted on hepatic $CD8^+$ T cells from chronically HBV-infected patients, compared with

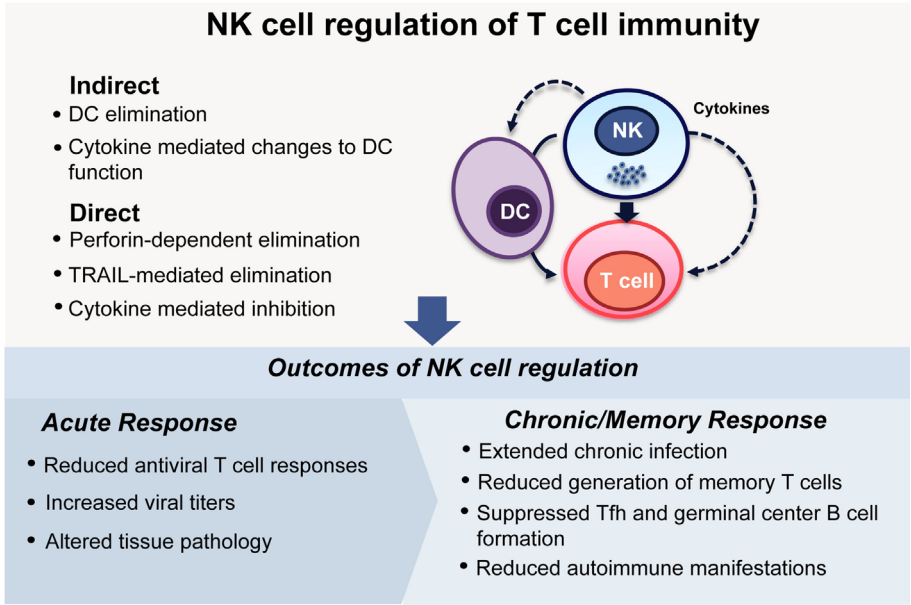

FIGURE 1 | NK cell regulation of T cell immunity. NK cells can modulate the activities of T cells either indirectly, by eliminating, or affecting the functions of antigen-presenting cells, especially DCs, or through direct interactions with T cells themselves. During viral infections, these regulatory interactions can lead to differing outcomes depending on the infecting viral pathogen and the phase of the infection. During acute infection, NK cell regulation can dampen antiviral T cell responses (7, 9–11), lead to increased viral loads (9–11), and ultimately determine the extent of pathology in tissues (9, 10). In infections that have a chronic phase, NK cells can determine the longevity of the infection and indeed extend it (7, 11, 12, 33, 36). Memory T cell responses are generated at reduced frequencies (30), and antibody responses can be compromised due to impaired formation of germinal centers (30, 36). This apparently incongruous dampening of immune response is reconciled by the finding that curbing chronic immune responses following viral infections is critical to reducing the development of autoimmunity (12).

healthy controls, and blocking TRAIL augmented the survival of hepatic $CD8^+$ T cells cultured with PBMCs (33). These results indicate that the immune-regulatory functions of NK cells are not only restricted to mouse models of infection but are also observed in the human system. Whether NK cells play similar immunoregulatory activities in other settings of chronic disease remains to be described.

REGULATING THE REGULATORS

Factors that modulate the regulatory functions of NK cells in inflammatory settings have recently been identified. NK cells from mice with a mutation in the gene for the NK cell activating receptor NKp46 failed to downregulate the transcription factor *Helios*, resulting in overly responsive NK cells, which in turn weakened T cell responses in inflammatory settings, such as viral infection (35). Other factors regulating the susceptibility of T cells to NK cell-mediated elimination include the transcription regulator NOD-like receptor caspase recruitment domain containing protein 5 (NLRC5) (38). The absence of NLRC5 in activated T cells responding to different inflammatory stimuli resulted in significantly reduced MHC I expression and rendered these cells susceptible to NK cell killing. The susceptibility of T cells to NK cell killing can also be affected by type I IFN, and *Ifnar1*$^{-/-}$ T cells were found to have reduced expression of the NK cell inhibitory ligands MHC I and Qa-1b (39) and elevated expression of ligands for the NK cell activating receptor NKp46 (40), leading to increased NK cell killing of LCMV-specific T cells.

REGULATORY NK CELLS: ILC1 VERSUS CLASSICAL NK CELLS

Even though immune-regulatory effects of NK cells are slowly being unraveled, many questions remain unanswered regarding the generation, phenotype, and tissue specificity of NK cells with immune-regulatory capacity. Addressing these questions has been complicated by the recent identification of novel populations of ILCs. NK cells have been classified as being a subclass of group 1 ILCs (41). The group 1 ILCs are characterized by their expression of the T-box transcription factor T-bet, an ability to produce IFN-γ, and an inability to produce T_H2- and T_H17-associated cytokines (41). The group 1 ILC can be subdivided into NK cells and ILC1, but the distinction between the subsets is hardly straightforward and appears to be tissue and activation dependent (42). In liver, at steady state, for example, ILC1 can be distinguished from NK cells by the expression of CD49a and TRAIL and a lack of CD49b expression and the transcription factor Eomesodermin (Eomes) (43, 44). NK cells are strongly cytotoxic and express high levels of perforin and granzymes, whereas ILC1 express low levels of perforin, but show increased expression of cytokines, including IFN-γ and especially TNF-α (43, 45). Despite reduced perforin expression, hepatic $TRAIL^+$ NK cells were reported to kill tumor

cells in both a TRAIL-dependent and -independent manner (46) and appear to contribute to liver pathology in chronic HBV sufferers by eliminating hepatocytes (47, 48). Together, these data suggest that hepatic ILC1 cells may have a greater cytotoxic potential than currently assumed.

In addition to being found in the liver, ILC1 cells have been described in other tissues, including the gut, skin, uterus, and salivary glands (45, 49). Differentiating between NK cells and ILC1 is of particular interest in the salivary glands, where TRAIL$^+$ NK cells limit anti-MCMV CD4$^+$ T cell responses (12). Tessmer et al. (50) described steady-state salivary gland NK cells as a phenotypically distinct population, based on their integrin and activation marker profile, with these cells unable to mount efficient cytotoxic responses to various stimuli. Following MCMV infection, a lack of NK cell recruitment was noted, suggesting that the NK cell regulatory response in the salivary glands is mediated by proliferation of tissue-resident cells. This characteristic of salivary gland NK cells is shared with ILC1 cells that are thought to be primarily tissue resident (45). These results differ from our own, where regulatory TRAIL$^+$ NK cells seem to be recruited into the salivary glands in response to MCMV infection (12). Possible explanations for this disparity include differences in the timing of NK cell transfer, the strain of mouse, and/or the strain of MCMV used in the different experiments.

The ILC populations found in the salivary glands have phenotypic profiles consistent with both NK cells and ILC1 (49). Interestingly, the development of these cells under homeostatic conditions was found to be independent of the transcription factor Nfil3, suggesting that NK cells present in the salivary glands at steady state are ILC1 (49, 51). However, salivary gland NK cells express both T-bet and Eomes, consistent with an NK cell phenotype. Furthermore, under inflammatory conditions, such as viral infection, it appears that NK cells can be recruited to the salivary glands, and their phenotype does not conform to that of ILC1 (12). Thus, the regulatory NK cell population that balances protective antiviral immunity with potentially pathologic autoreactive immune responses appears to be classical NK cells. However, in their entirety, the available data suggest that a distinction between classical NK cells versus ILC1s may be very difficult especially in inflamed tissues, where cytokines can drastically alter the phenotype of these populations.

CONCLUSION

The collective data reviewed here establish that NK cells regulate adaptive responses both during priming and at later stages. Importantly, the regulatory functions of NK cells are not limited to interactions in lymphoid tissues but have been observed in non-lymphoid organs. An intriguing question yet to be answered is whether the regulation of adaptive immune responses in tissues is mediated by ILC1, NK cells or both subsets, and if there is any plasticity between these cell populations.

AUTHOR CONTRIBUTIONS

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

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A New Biological Feature of Natural Killer Cells: The Recognition of Solid Tumor-Derived Cancer Stem Cells

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Natural killer (NK) cells are classified as a member of the innate lymphoid cells (ILCs) group 1. ILCs have been recently identified and grouped on the basis of their phenotypical and functional characteristics. They are effectors of innate immunity and are involved in secondary lymphoid organ generation and tissue remodeling. NK cells are powerful cytotoxic lymphocytes able to recognize and eliminate tumor- and virus-infected cells by limiting their spread and tissue damage. The recognition of tumor cells is mediated by both activating and inhibitory receptors. While in hematological malignancies the role played by NK cells is widely known, their role in recognizing solid tumors remains unclear. Recently, tumor cell populations have been divided into two compartments: cancer-initiating cells (CICs) or cancer stem cells (CSCs) and senescent tumor cells. Here, CSC will be used. CSCs are a small subset of malignant cells with stem-like properties that are involved in tumor maintenance and recurrence due to their ability to survive to traditional therapies; they are, moreover, poorly recognized by T lymphocytes. Recent data showed that NK cells recognize *in vitro* cancer-initiating cells derived from colon cancer, glioblastoma, and melanoma. However, more *in vivo* studies are urgently required to fully understand whether these new antitumor NK cells with cytotoxic capability may be considered in the design of new immunotherapeutic interventions.

Keywords: NK cells, CSCs, MHC class I, immunotherapy, solid tumor

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NK CELLS

The large granular innate lymphoid cells (ILCs) are named as natural killer (NK) cells for their protective and cytotoxic role in infectious and tumor diseases. They play an active role in transplant rejection and in pathological conditions. The main effectors mechanisms of NK cells are lymphocytotoxicity and proinflammatory cytokine production. Recently, they were classified in Group 1 of the ILCs (1).

In human, NK cells are phenotypically characterized by the surface expression of molecules like CD56 and CD16 (FcγRIII) and by the absence of CD3, while in mice, they are defined as cells expressing NK1.1, DX5 and not expressing CD3. NK cells rely on a set of germ-line encoded activating and inhibitory receptors. The activating receptors include CD16, NKG2D, DNAM-1, and NKp46, all common to both human and mouse, while the other NK receptors (NCRs: NKp44 and NKp30) are expressed solely by human NK cells. Apart from the CD16 receptor recognizing Fc domain

antibodies, the other receptors interact with ligands on infected and transformed cells. Molecules associated with a "stress signature," such as, MICA, MICB, ULBPs, and Hsp90 in human and Rae-1, MULT-1, and H60 in mouse, have been identified as major activating ligands (2). Other activating NK ligands are PVR and NECTIN-2 bound by DNAM-1, expressed both in human and mice. NCR ligands are still unknown, except for NKp30, whose ligands are B7-H6, BAG-6, and Gal-3 (3, 4).

The inhibitory receptors of NK cells interact with major histocompatibility complex (MHC-I) molecules. In human, most of these receptors belong to the family of killer cell immunoglobulin like receptors (KIRs) that directly bind to certain HLA-A, -B, and -C alleles. KIRs are highly polymorphic receptors that interact with MHC-I molecules and elicit inhibitory or activating signals. Their name is correlated with the number of Ig-like domains in their extracellular regions and with the presence of a long (inhibitory) or short (activating) cytoplasmic tail. Other types of inhibitory and activating NK cell receptors are the lectin-like heterodimers of NKG2 family. The best-known members are the inhibitory heterodimer CD94/NKG2A and its activating counterpart CD94/NKG2C, both recognizing the non-classical MHC class I molecule, HLA-E. In mice, the inhibitory receptors belong to the superfamily of lectin-like type II integral membrane proteins, known as Ly49 and CD94/NKG2A receptors.

The lack or reduced expression of MHC-I on the target cells' surface leads to "missing self" recognition. There are clinical observations and ongoing clinical trials based on the idea that NK cells transferred from one individual to a non-MHC-I-matched patient can exert antileukemic effects based on "missing self" recognition (5).

In both species, the fully matured highly cytotoxic NK phenotype is acquired after a, still poorly understood, MHC class I molecule-dependent ontogenic process. The NK cells ontogeny process has been termed "education – licensing – arming/disarming" (6, 7). Recently, Kärre and coworkers have proposed to explain the NK cells' education in the "Rheostat Model" (8). The "Rheostat Model" postulates that education of NK cells represents a tuning mechanism that goes on continuously and reversibly accordingly to the MHC environment, even after NK cell maturation. This model has implications for immunotherapy based on exploiting "missing self" reactivity when NK cells are transferred to a cancer patient from a healthy, non-MHC-matched individual. If the NK cells are "re-tuned" in the new environment, as proposed in the rheostat model, this may affect their efficacy to react against the recipient's leukemia cells and can influence the reactivity of NK cells against tumor cells in different situations. It has been shown that NK cells can exert antitumor effects in the new host environment. Recently, Wagner and coworkers provided further support to the rheostat model demonstrating that NK cells infused in a recipient become tolerant to healthy cells representing "missing self" in the new environment, while the re-tuned NK cells will retain activity against tumor cells representing "missing self," due to a higher expression of ligands for activating NK receptors on tumor cells (9).

Another new concept that has contributed to change our knowledge about the NK cells biology derives from the observations made during human and mice CMV infection, where NK cell subsets recognizing CMV-infected cells expanded. The expanded CMV-specific population has been proposed to be a relevant NK cell memory subset able to provide immunity in the second infection round (10–12).

Therefore, the information that are now available on the NK cells biology are pushing the field toward their use in clinical settings.

NK AND CANCER

The history of NK cells has evolved closely related with the history of cancer immunotherapy since they were discovered in the 1970s for their capability to target and kill cancer cells. NK cells preventing leukemia relapse in mismatched hematopoietic stem cell transplant (HSCT) patients stirred a renewed interest in the use of NK cells in the fight against cancer. They are the most efficient effectors against tumors and are considered suitable candidates for adoptive immunotherapy of both hematological and non-hematological malignancies (13–15).

Nearly 50 years after their discovery, we are now starting to realize the potential of these NK cells in tumor immune therapy trials.

CANCER STEM CELLS

The processes underlying the etiology of cancer have been the source of several theories for a century (16, 17).

It was demonstrated that any cell exposed to genotoxic stress acquires multiple genetic mutations and may give rise to a tumor (18). More recently, Tomasetti and Vogelstein demonstrated that stochastic influences are the major contributors to cancer overall, often even more than hereditary or external environmental factors. Many genomic changes occur simply by chance during DNA replication rather than as a result of carcinogenic factors. Considering the number of mutations needed for a malignant transformation, it is conceivable that these can only accumulate in long-lived cells (stem cells) (19).

Therefore, cancer stem cell hypothesis postulates that only a small subset of cells, termed cancer-initiating cells (CICs) or cancer stem cells (CSCs), is able to give rise to and maintain tumors (20–22), even though for transplanted murine tumors, such as melanoma, the relevance of CSCs has been questioned (23).

Cancer stem cells constitute a self-renewing stem cell-like population, having an asymmetric proliferating pattern responsible for the progression, metastasis, resistance to treatment, and recurrence of several tumors (24–26).

Compelling data support the cancer stem cell model in various human cancers including malignant embryonic tissues cell cancers (27, 28), leukemias (29, 30), breast cancers (31), brain cancers (32), and colon cancers (33–35). Based on the different tumor types, the CSCs were identified by the expression of different markers, such as $CD133^+$ $CD166^+$, $CD44^+$, $CD29^+$, $CD24^+$, EpCAM, Lgr5, aldehyde dehydrogenase, and a few others (36).

The main feature of the CSCs is that a small amount of them (up to 100 cells) may transfer disease upon transplantation into immune-compromised NOD/SCID mice (34, 35). Some markers have been identified that distinguish the

leukemogenic/tumorigenic cancer cells (leukemogenic CSC) from the bulk populations of non-leukemogenic/tumorigenic cells (non-leukemogenic CSC). The ability to predict which cells are tumorigenic based on marker expression indicates that the tumorigenic cells are intrinsically different from non-tumorigenic cancer cells.

NK AND CSC

Cancer stem cells have been suggested to be responsible for the hematological metastatic spreading of solid tumors (37). The asymmetrical growth and their low rate of proliferation make them resistant to conventional radiotherapy and chemotherapy. Therefore, to prevent CSCs contribution in sustaining tumor progression, several groups have evaluated whether the effector immune cytotoxic cells (NK cells, CD8 T cells, and γδ T cells) could eliminate the tumor stem compartment. However, the low level of MHC class I expression reported for CSCs predicts a low efficiency of CSCs targeting by $CD8^+$ cytotoxic T lymphocytes, while recent data demonstrate that once the CSC is sensitized with bisphosphonate zoledronate, the human γδ T cells could efficiently target CSCs *in vitro* (38). The cytotoxic interaction was regulated *via* TCR and to a lesser extent by NKG2D receptors (38). On the other hand, data have shown that NK cells, mainly *in vitro*, selectively kill human colon-derived CSCs without any pharmacological pretreatment (39), melanoma (40), and glioblastoma (41). The molecular mechanisms leading the NK cell recognition of CSC have been partially elucidated.

In our laboratory, we demonstrate that freshly purified autologous and allogeneic NK cells show a robust cytotoxic effect on CIC derived from colorectal carcinoma cells (CRC), whereas the CRCs were less susceptible to the NK lysis, Tallerico et al. (39). The capability to preferentially recognize and kill colorectal-CIC is correlated with a higher expression of NCR ligands (in particular, NKp30 and NKp44) and a lower expression of MHC class I on the surface of CSCs compared to their tumor counterparts (39).

In another pathological setting, Pietra and colleagues (40) analyzed different melanoma cells derived from metastatic primary cultures or from established cell lines. These cells have a highly heterogeneous expression of different stem cell-associated markers including CD133, c-kit/CD117, and p75 neurotrophin receptor/CD271. IL-2-activated NK cells are able to recognize and lyse melanoma cells enriched CSC through the involvement of a different combination of activating NK receptors. Indeed, NK cells efficiently kill melanoma cells resistant to radiotherapy, either with ability to form melanospheres *in vitro* or having the stem cell-associated marker CD133 expression, underlying the possibility to use a novel NK-based immunotherapeutic strategy to eliminate CSC (42).

This was demonstrated also by Castriconi and colleagues (41) in glioblastoma patients, in which CSCs were isolated and characterized for typical markers of neural stem cells. They were capable of partial multilineage differentiation *in vitro* and gave origin to infiltrating tumors when orthotopically injected in NOD/SCID mice. These cells, characterized by stem cell-like properties, are well killed by allogeneic and autologous NK cells activated by IL-2 or IL-15. The NK-mediated killing of glioblastoma cells (GBM) has been identified by the low levels of HLA class I both classical (HLA-A, -B, -C) and non-classical (HLA-E) molecules and by the expression of DNAM-1 and NKp46 activating NK receptors. Moreover, most of GBM cells express different amounts of NKG2D ligands, while all GBM express PVR and NECTIN-2 (DNAM-1 ligands) (41).

The Pietra and the Castriconi studies provide novel therapeutic approaches based on the use of activated NK cells useful to eradicate tumor cells residuals after surgery or a traditional therapy.

Thus, these combined studies confirm that the molecular mechanisms behind the NK cell-mediated recognition of CSC rely on their loss or low MHC class I expression and increased amounts of activating NK ligands, on their cell surfaces. It should be noted that the low expression of MHC class I is not always the main mechanism by which NK cells recognize tumor cells. Tumors could lose or not their MHC class I expression, however, they could acquire NK receptor activating ligands on the cell membrane leading to a specific NK cell recognition.

A crucial issue to exploit the NK cells to target CSC is to validate these *in vitro* observations in experimental *in vivo* models.

So far, very few *in vivo* studies address the potential of NK cells to ablate the CSC compartment from the tumor population.

Recently, Ames and colleagues (42) showed that NK cells kill CSCs from different kinds of tumors, through the interaction of the NKG2D activating receptor with its ligand (MICA/B). They have shown, *ex vivo* and *in vivo*, the efficacy of the NK cell therapy to target CSCs. They implanted human tumor cell lines in NOD SCID gamma (NSG) mice and then injected activated NK cells. After NK infusion, they sacrificed mice and observed the number of tumor colonies when homogenized lung cells were plated in a lung colony formation assay. The colonies that did develop in NK cell-treated mice showed an impressive reduction in size, consistent with NK cell targeting of stem-like tumor cells. In mice with established orthotopic pancreatic tumors that were treated with NK infusion, Ames observed by bioluminescence, the reduction of the tumor size, demonstrating the therapeutic efficacy of NK cells (42). All the discussed data are summarized in **Table 1**.

CONCLUSION

Different promising strategies to directly attack CSCs have been proposed using antibody-based therapies (43) by blocking pathways important for homing and engraftment (CD44), self-renewal (CD27), protection against phagocytosis (CD47), or by strongly activating $CD8^+$ T cells (CTLs) specific for CSC antigens or simply by forcing CSC into cell cycle. However, their therapeutic success has been limited. This calls for new approaches to target the CSCs compartment as a valid anticancer therapy.

The recent findings discussed here showed that the immune system, in particular the innate part (dendritic, NK, and γδ T cells), can efficiently cooperate to eliminate CSC; this allow us to hypothesize that a promising avenue to eradicate CSC might lie in harnessing NK and γδ T cells. However, the *in vivo* functional outcome of NK–stem cell interplay may also result

TABLE 1 | Phenotypes and NK cells recognition pattern of cancer stem cells and related tumors.

Human tumor	Type	PVR	NECTIN-2	MICA	MICB	ULBPs	NCR ligands	HLA	NK cell recognition
Colorectal carcinoma (39)	Tumor	NC	NC	NC	NC	NC	+/– NKp30L NKp44L	+ HLA-A, -B, -C	+/–
	Cancer stem cells	NC	NC	NC	NC	NC	+ NKp30L NKp44L	+/– HLA-A, -B, -C	++
Melanoma (40)	Tumor	+/–	+/–	+/–	ND	+/– ULBP2	ND	+ HLA-A, -B, -C	+
	Cancer stem cells	+/–	+/–	+/–	ND	+/– ULBP3	ND	– HLA-A, -B, -C	+
Glioblastoma (41)	Tumor	–	–	–	–	–	ND	+ HLA-E	+/–
	Cancer stem cells	+	+	+/–	+/–	+/–	ND	+ HLA-A, -B, -C HLA-E	++
Pancreatic adenocarcinoma (42)	Tumor	ND	ND	–	–	ND	–	NC	+/–
	Cancer stem cells	ND	ND	+	+	ND	–	NC	++
Sarcoma Ewing (42)	Tumor	ND	ND	–	–	ND	–	NC	+/–
	Cancer stem cells	ND	ND	+	+	ND	–	NC	++
Liposarcoma (42)	Tumor	ND	ND	ND	ND	ND	ND	ND	+/–
	Cancer stem cells	ND	ND	ND	ND	ND	ND	ND	++
Breast carcinoma (42)	Tumor	ND	ND	ND	ND	ND	ND	ND	+/–
	Cancer stem cells	ND	ND	ND	ND	ND	ND	ND	++
Glioblastoma (42)	Tumor	ND	ND	ND	ND	ND	ND	ND	+/–
	Cancer stem cells	ND	ND	ND	ND	ND	ND	ND	++

NC, no change between tumor and CSC; ND, not detected.

in NK anergy (44, 45). Such NK cell dysfunctional plasticity is believed to have a major impact in NK cell-based immunotherapeutic approaches and deserves a deeper understanding through *in vivo* models.

Several studies (46) have demonstrated that chemotherapy either induce or increase the CSCs susceptibility to NK- and γδ T cell-mediated killing. Therefore, combination of immune-based therapies with chemotherapy could be beneficial in the treatment of many cancers.

The current failure of standard therapies is attributed to a small fraction of the primary cell population with stem-like characteristics (CSC), such as self-renewal and differentiation. So, it is imperative to target all CSCs within the tumor to prevent relapse. Even though different aspects of CSCs have been explored in recent targeting strategies, their success has been very limited probably because an exhaustive comprehension of their basic biology and evolution is far from being clarified.

In order to eradicate the CSCs, we need to act at multiple levels: increasing their sensitivity to chemotherapy and to novel compounds, stimulating or reactivating a tumor-specific immune response selectively directed against the CSCs, enhancing the efficacy of therapies currently in use with a selective induction of the immune response, and testing the antitumor and immune-stimulating properties of new compounds. Further investigations are necessary to better understand the basic biology of immune recognition of CSC, which may be rapidly translated into innovative therapeutic approaches for the treatment of different forms of cancer.

Moreover, in accordance with our and Pietra, Castriconi, and Ames studies, NK-mediated killing is a possible candidate for targeting CSCs following the depletion of non-CSCs by anti-proliferative therapies. These studies focused on possible strategies to eradicate CSCs from established tumors; taking together the data produced, it is conceivable to think that NK cells can selectively recognize CSC compartments of several solid tumors, *in vitro*. At this stage, however, only a few studies are available on the *in vivo* NK cell-mediated CSC eradication and tumor control.

Therefore, more *in vivo* studies are necessary to address the hypothesis that NK cells can eradicate the CSCs population in a pathophysiological setting and to establish new immune-intervention strategies in solid tumors that can be readily transferred in new clinical trials.

AUTHOR CONTRIBUTIONS

All the authors contributed in the writing of this review.

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REVIEW
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Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control

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Natural killer (NK) cells are known for their well characterized ability to control viral infections and eliminate tumor cells. Through their repertoire of activating and inhibitory receptors, NK cells are able to survey different potential target cells for various surface markers, such as MHC-I – which signals to the NK cell that the target is healthy – as well as stress ligands or viral proteins, which alert the NK cell to the aberrant state of the target and initiate a response. According to the "licensing" hypothesis, interactions between self-specific MHC-I receptors – Ly49 in mice and KIR in humans – and self-MHC-I molecules during NK cell development is crucial for NK cell functionality. However, there also exists a large proportion of NK cells in mice and humans, which lack self-specific MHC-I receptors and are consequentially "unlicensed." While the licensed NK cell subset plays a major role in the control of MHC-I-deficient tumors, this review will go on to highlight the important role of the unlicensed NK cell subset in the control of MHC-I-expressing tumors, as well as in viral control. Unlike the licensed NK cells, unlicensed NK cells seem to benefit from the lack of self-specific inhibitory receptors, which could otherwise be exploited by some aberrant cells for immunoevasion by upregulating the expression of ligands or mimic ligands for these receptors.

Keywords: NK cells, licensing, Ly49, immunity, MHC-I

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INTRODUCTION

Natural killer (NK) cells are part of the innate immune system and were originally identified due to their unique ability to kill tumor cells without prior sensitization, which greatly differed from the defined functions of other major lymphocyte subsets (1, 2). The ability of an NK cell to recognize tumor or virus-infected cells is due to the expression of various activating and inhibitory receptors on its cell surface; such receptors include Ly49, CD94/NKG2, and NKp46, among others (3). While NK cell deficiencies are rare – possibly highlighting the necessity of NK cells in immunity – the few documented cases of NK cell deficiencies further support their important role. Individuals who exhibit reduced NK cell numbers, cytotoxicity, and/or cytokine production are characteristically more susceptible to certain viral infections (4–12).

Target cells can express ligands that bind to a variety of activating and inhibitory receptors on NK cells; it is this interplay between inhibitory and activating signals, which determines the NK cell response to the target (3). In addition, upon recognition of pathogen-associated molecular patterns, sentinels of the immune system, such as dendritic cells and macrophages, secrete a range of

inflammatory cytokines in order to recruit and activate NK cells. Macrophages and dendritic cells are known producers of IL-2, IL-15, IL-18, and IL-21, all of which stimulate production of both type I and II IFNs by NK cells, as well as direct cytotoxicity of NK cells (13–17).

Members of the Ly49 receptor family, the murine functional homolog of the human killer-cell Ig-like receptor (KIR) family, can be either activating or inhibiting and interacting with class I major histocompatibility complex (MHC-I) molecules (18). Almost all adult nucleated cells constitutively express MHC-I molecules on their surface (19, 20). Both NK and $CD8^+$ T cells depend on MHC-I recognition for their function. While the engagement of T cell receptors by MHC-I molecules is required for $CD8^+$ T cell activation, the opposite is true for NK cells (21, 22). According to the "missing-self" hypothesis, NK cells preferentially target cells lacking MHC-I expression, which is recognized by self Ly49 receptors (23, 24). MHC-I expression on the target cell surface acts as a health marker for NK cells, signaling to the NK cell to spare the target. Conversely, aberrant cells often downregulate the surface expression of MHC-I to avoid detection and killing by cytotoxic T cells, but become a target for NK cells (25, 26).

The classical characterization of an NK cell is its ability to recognize and eliminate cells, which have decreased surface expression levels of MHC-I, a common phenomenon in cancer cells (2, 27, 28). Varied expression levels of human MHC-I – human leukocyte antigen (HLA) – on cells shows an inverse correlation with their susceptibility to killing by NK cells: variants with decreased levels of HLA were more susceptible to NK cell killing than the parental cell line, and accordingly, variants with higher levels of HLA benefited from protection from NK cells compared to the original cell line (29). While other inhibitory receptors, such as NKG2A, have been shown to play a role in NK cell licensing as well (30), the focus of our review will be on the dual role of the Ly49 family receptors on NK cells during target cell recognition and immune evasion.

MHC-I-MEDIATED NK CELL EDUCATION/LICENSING

Natural killer cell functionality depends on the presence of self-MHC-I molecules as proposed by the "licensing" hypothesis, in which a self-specific Ly49 receptor must interact with self-MHC-I in order for the NK cell to become functional (30, 31). Consequently, NK cells from *B2m*$^{-/-}$ mice, in which MHC-I expression is abrogated, exhibit a diminished ability to kill MHC-I-deficient target cells that are normally readily killed by NK cells from WT mice, and exhibit defective cytokine production (28, 32). Similarly, our studies have demonstrated that the NK cells from Ly49-deficient mice are unlicensed and show impaired recognition of MHC-I-deficient target cells (33). NK cell responsiveness has also been shown to be proportional to the number of self-MHC-I-specific inhibitory receptors they express (34, 35). NK cells that express a higher number of self-MHC-I-specific Ly49 receptors are more responsive to stimuli than those that express fewer of these receptors. Human NK cells undergo a similar licensing process, which requires the interaction of HLA and KIR (36, 37). NK cells expressing self-MHC-I-specific KIRs exhibited a more robust responsiveness and cytokine production than self-KIR-negative NK cells.

While the mechanics of NK cell licensing are still unclear, whether it be *via* the arming, disarming, or rheostat model, or through *cis* interactions with self-MHC-I [as previously reviewed in Ref. (38)], the process has been shown to be an ongoing and fluid process, wherein an environmental change can alter the licensed state of even fully mature NK cells (34, 39–41). Contrary to the original school of thought that NK cell education occurs during NK cell development in the bone marrow, when unlicensed mature NK cells from MHC-I-deficient mice were adoptively transferred into WT mice, their function was restored showing that mature NK cell can also acquire licensing through the interaction of their inhibitory Ly49 receptors with the host MHC-I molecules (30, 31, 39, 40). Although licensing is important for NK cells to acquire effector functions, the unlicensed NK cells appear to respond effectively against target cells under specific conditions, such as when the target cells express high levels of MHC-I to evade NK detection by interacting with the inhibitory Ly49 receptors. This ability to detect MHC-I-expressing tumors and viruses may be the reason why up to 50% of NK cells are unlicensed with respect to self-Ly49 expression, but are still maintained in immune-competent mice (31, 42).

CANCER IMMUNOSURVEILLANCE BY NK CELLS

The importance of the immune system in tumor control is highlighted by the increased cancer risk in immune-compromised individuals. Those with human immunodeficiency virus (HIV) infection, including individuals who have progressed to acquired immunodeficiency syndrome (AIDS), are at notably greater risk of developing lung cancer independent of smoking (43). Immunosuppressed renal transplant patients have increased incidence of skin cancer over the general population (44). Those having undergone heart transplants are particularly at increased risk for non-Hodgkin's lymphoma, oral, and lung cancers (45). Moreover, in human cross-sectional studies, the presence of tumor infiltrating lymphocytes is a strong predictor of positive patient outcome (46), indicating a correlation between the immune system and cancer protection or recovery.

In support of the importance of NK cells in cancer immunity, NK-compromised *beige* mice – a model for human Chediak–Higashi syndrome – exhibit defective cytotoxic activity against tumor cells, and are more susceptible to spontaneous fatal tumor development, possibly due to ineffective immunosurveillance (47, 48). Chediak–Higashi syndrome is caused by a homozygous or compound heterozygous mutation in the lysosomal trafficking regulator gene. Affected individuals present with a host of immunodeficiency disorders such as granular anomalies in their lymphocytes, defective chemotactic and bactericidal activity of their neutrophils, defective NK cell function, and defective peptide loading and antigen presentation (49–52). Antibody-mediated depletion of NK cells prior to tumor cell injection in various mouse strains results in prolonged tumor survival, as well as an increased number of artificial lung metastases and

spontaneous metastases (53). In humans, NK cells comprise up to 15% of the blood lymphocytes (54). In a clinical setting, low NK cell activity in cancer-diagnosed individuals has been associated with poor prognosis, and those with advanced stage cancer often possess minimally cytotoxic NK cells (55). High cytotoxic activity of peripheral blood NK cells is correlated with up to 10% reduced incidence of cancer (56). As well, in a clinical case of childhood-onset Hodgkin's lymphoma, this individual was observed to have non-functional NK cells (57).

RECOGNITION OF MHC-I-DEFICIENT TUMOR CELLS BY LICENSED NK CELLS

Tumors have developed multiple mechanisms for evading host immune recognition. One well-documented escape mechanism, the downregulation of MHC-I expression, is effective against T cells, but renders the tumor more susceptible to NK cells. Reduced expression levels of MHC-I has been documented in bladder, breast, cervical, colorectal, and ovarian human cancers (58–63).

The classic tumor model, in which missing-self was first discovered retrospectively, also helps to highlight the importance of the MHC-I status of the target cell (23). Mutagenesis of RBL-5, a Rauscher virus-induced leukemia, led to the derivation of the MHC-I-deficient RMA-S and MHC-I-expressing RMA cell lines (23). The difference in MHC-I expression levels of these two cell lines leads to differential recognition by NK cells (23). The RMA-S induced flank tumors are much better controlled compared to the accelerated growth of the RMA tumors (23). With the use of $B2m^{-/-}$ mutant mice, it has been shown that a MHC-I deficiency renders the NK cells defective at killing traditionally well recognized MHC-I-deficient NK tumor cell line targets (32).

Our group has shown that mice lacking Ly49-mediated NK licensing also exhibit reduced activity against MHC-I-deficient tumor cells both *in vitro* and *in vivo* (**Figure 1**) (33). In the *in vivo*

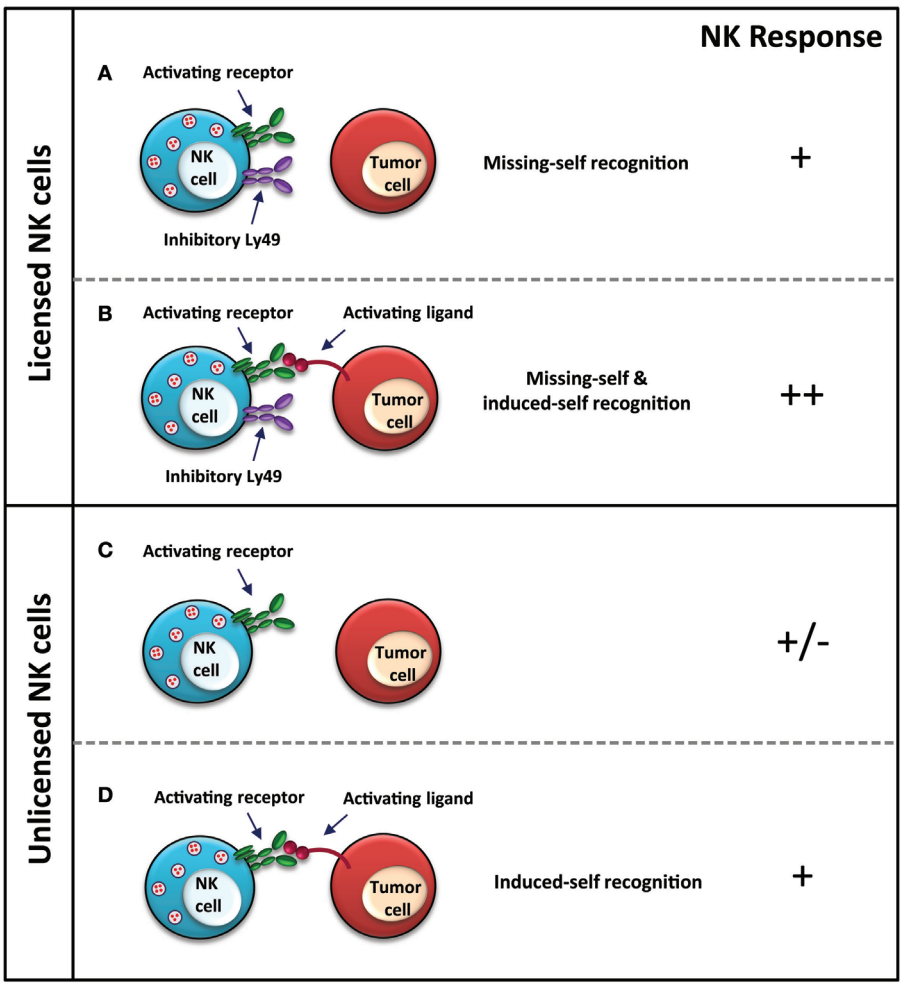

FIGURE 1 | NK cell response to MHC-I-deficient tumors. Licensed NK cell recognition MHC-I-deficient tumors through "missing-self" due to a lack of MHC-I expression on the tumor cell **(A)**, as well as through "induced-self" *via* stress ligands, which are recognized by activating receptors on NK cells **(B)**. Unlicensed NK cells are unable to recognize MHC-I-deficient tumors through "missing-self" **(C)**, however, are still functional upon activating receptor–ligand interaction for "induced-self" recognition **(D)**. NK cell response "–" represents no activation, and "+" represents activation. In the case of **(C)**, +/– represents that the response of the NK cell, whether it is activated or not, also depends upon other immune cells, receptor–ligand interactions, and cytokines in the tumor microenvironment, which are not depicted in the figure for brevity.

rejection assays spanning up to 18 h, these mice exhibited reduced capacity at eliminating the MHC-I-deficient variants of RMA and C1498 compared to wild-type mice (33). Ly49-mediated NK cell education plays a major role in NK cell-mediated cancer immunosurveillance with tumor cell-induced flank tumors, experimental tumor metastases, methylcholanthrene-induced sarcoma, and spontaneous B cell lymphoma, with observations of increased and earlier onset tumor incidence in each model (64). Interestingly, the tumors, which developed in the mice with reduced levels of licensed NK cells, exhibited MHC-I-directed tumor immunoediting, wherein levels of both H-2K^b and H-2D^b were reduced, possibly as a mechanism of escape from cytotoxic T cells in an environment where evasion from NK cells is no longer a priority for survival due to their unlicensed nature (64).

INDUCED-SELF RECOGNITION OF TUMOR CELLS BY UNLICENSED NK CELLS

While the self-specific inhibitory Ly49 receptors have been shown to be the mediators of NK cell licensing required for effective missing-self recognition (65), blockade of these receptors also helps to elicit a stronger NK cell response. Antibody-mediated blockade of self-specific Ly49 receptors improved *B2m*$^{-/-}$ bone marrow allograft success similar to results seen with complete NK cell depletion; suggesting the importance of the inhibitory self-specific Ly49C/I subset in the effector functions of NK cells (66). This study shows successful grafting of MHC-I-deficient bone marrow cells in WT mice when Ly49C/I$^+$ NK cells are depleted, thus providing evidence for missing self-recognition of MHC-I-deficient bone marrow cells by licensed NK cells.

Treatment of NK cells with monoclonal blocking antibody to Ly49C/I led to inhibited *in vitro* growth of MHC-I-expressing C1498, a murine leukemia cell line, and EL4, a T cell lymphoma cell line (65). *In vivo* antibody blockade prior to leukemia induction with C1498 led to increased survival (65). Considering everything we know about NK cell education and the importance of the Ly49C/I subset in licensing, in this case, the inhibitory nature of these receptors takes precedence over their educating role. However, one thing of note is the MHC-I status of the tumor cell lines utilized, with both lines expressing moderate to high levels of MHC-I, suggesting that missing-self recognition by licensed NK cell subsets is not a key requirement in this case. Self MHC-licensed NK cells are much less efficient than unlicensed NK cells at responding to the MHC class I-expressing target RMA cells (67). The requirement for licensed NK cells is not as imperative in the event that the tumor cells express MHC-I, since these cells would not traditionally be recognized through missing-self. Various cancer cells, which maintain expression of MHC-I on their cell surface, while escaping immune recognition through a missing-self response can also dampen NK cell activity through engagement of inhibitory KIRs (68–70). Antibody-mediated blockade of all KIR2D receptors elicits a heightened NK cell response with respect to cell cytotoxicity and has proven its efficacy in stage 1 clinical trials against acute myeloid leukemia and multiple myeloma (69–71).

In some instances, it appears that the unlicensed NK cells are more efficient at eliminating MHC-I-expressing aberrant cells (**Figure 2**). Unlicensed human NK cells, which lack inhibitory self-KIR expression, are more effective at killing neuroblastoma cells through antibody-dependent cell-mediated cytotoxicity (ADCC), following treatment with an antibody, which targets the disialoganglioside surface antigen GD2 on tumor cells (72). The activated NK cells recognize MHC-I on the cell surface as a health marker, thus sparing the MHC-I-expressing neuroblastoma cells and concurrently selecting for the MHC-I-expressing subset. MHC-I-expressing tumor cells can inhibit licensed NK cells through the engagement of inhibitory KIRs. Unlicensed NK cells, on the other hand, are not inhibited and are better mediators of neuroblastoma cell killing *via* ADCC, which is particularly relevant in the absence of tumor-expressed NK activating ligands (72). Therefore, unlicensed NK cells appear to be the better mediators of an anti-tumor response when the tumor cells express ligands for self-specific inhibitory NK cell receptors.

Natural killer cells can also kill certain virus-infected and tumor cells despite their expression of MHC-I, as explained by the "induced-self" model (73–75). The licensed status of the NK cell, in this case, does not wholly dictate its response. Several studies have shown that unlicensed NK cells can recognize aberrant cells through the recognition of activating ligands, similar to licensed NK cells (**Figures 1** and **2**). The NKG2D activating receptor plays a major role in the control of both lymphoid and non-lymphoid cancers; loss of this receptor leads to increased susceptibility of oncogene-driven cancer development (76). Expression of Rae1, the ligand for the activating NKG2D receptor, on RMA cells elicits a strong *in vivo* rejection response by unlicensed NK cells in Ly49-deficient mice (33). *In vitro* killing of splenocytes from Rae1ε transgenic mice is comparable between licensed and unlicensed NK cells, indicating no effect of licensing in this model (33). Additionally, *in vitro* and *in vivo* killing of Rae1β-expressing RMA-S cells is comparable between NK cells from wild-type mice and unlicensed NK cells from *B2m*$^{-/-}$ and Ly49-deficient mice, suggesting that other signals, such as those from activating receptors, are able to compensate for the hyporesponsiveness of unlicensed NK cells to the loss of MHC-I expression (33, 64). As well, stimulation with the double-stranded RNA viral mimic, polyinosinic:polycytidylic acid (poly I:C), induces a strong immune response in the otherwise hyporesponsive Ly49-deficient mice against MHC-I-deficient B16 F10 tumor cells. Prior treatment with poly I:C improves tumor rejection, reducing the number of pulmonary metastases in Ly49-deficient mice to wild-type levels (64). While the unlicensed NK cells are hyporesponsive in an Ly49-dependent manner, NK activation can be achieved through other means, which can bypass the hyporesponsiveness of these cells.

NK CELL-MEDIATED RECOGNITION OF VIRUS-INFECTED CELLS

The importance of NK cells in an immune response against a pathogen challenge can be seen in various clinical case studies

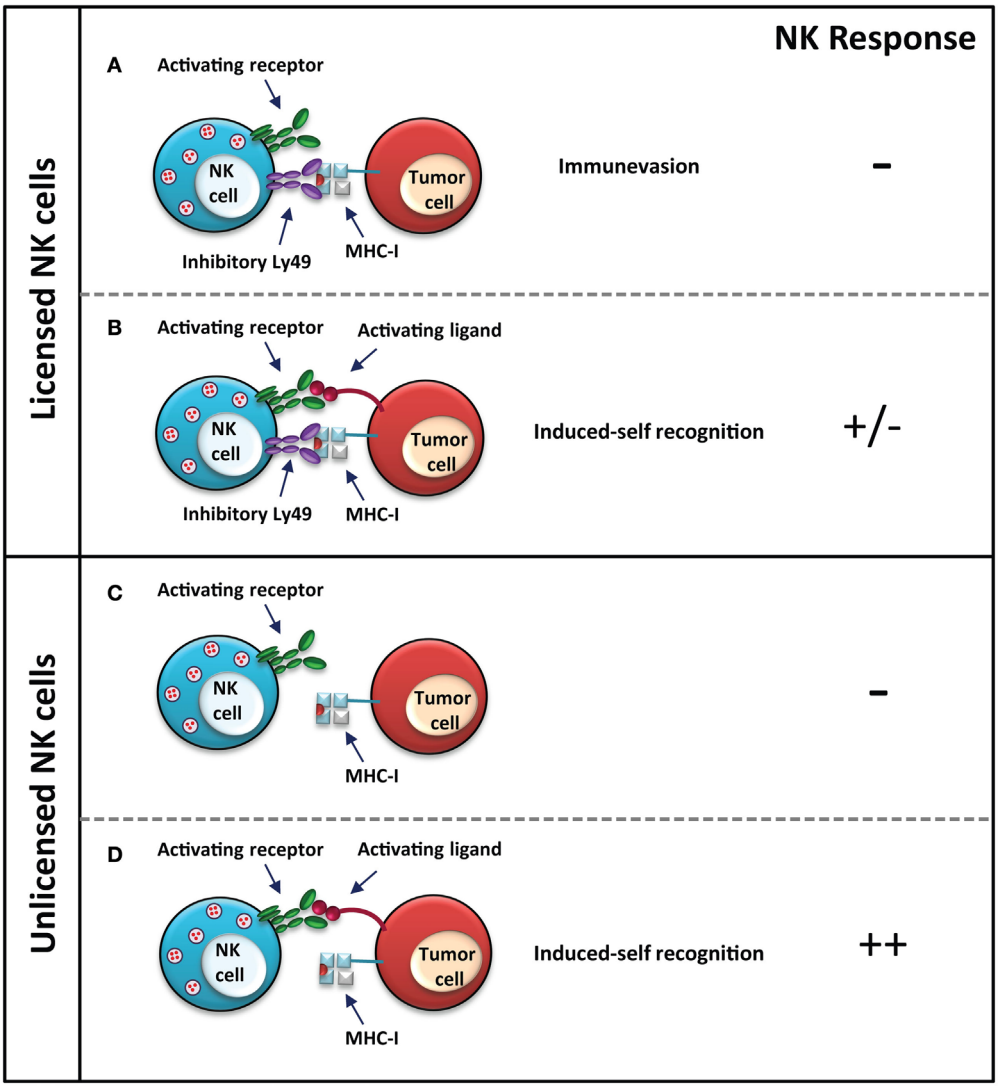

FIGURE 2 | **NK control of MHC-I-sufficient tumors**. Licensed NK cells do not elicit a response against MHC-I-expressing tumor cells, since the presence of MHC-I is recognized as a health marker. Tumor cells have evolved various immune evasion mechanisms, such as in this case, to make them appear as a healthy "normal" cell to NK cells by presenting MHC-I; however, this may render them susceptible still to cytotoxic T cells **(A)**. The response to both activating and inhibitory signals, such as expression of both MHC-I and an activating stress ligand is dependent on a balance of activating and inhibitory signals, which will determine the response **(B)**. Unlicensed NK cells do not respond strongly to tumor cells, which express MHC-I **(C)**; however, a stronger response is elicited if the tumor cell expresses stress ligands recognized by the activating receptor **(D)**. In the case of unlicensed NK cells, the response is stronger since the activating signal is not dampened by inhibitory signals *via* inhibitory Ly49–MHC-I interaction as in the licensed NK cells. NK cell response "–" represents no activation, and "+" represents activation.

of individuals lacking functional NK cells, leading to recurrent, life-threatening infections by otherwise non-consequential pathogens (4, 77). In humans, NK cells have been shown to play a seminal role in the control of viruses from the herpesvirus, poxvirus, and papillomavirus families (4, 77). Murine studies have further established the importance of NK cells in protection against vaccinia, hepatitis, and cytomegalovirus infections (78). In one study, anti-asialo GM1 antibody-mediated depletion of NK cells followed by viral infection lead to increased viral titer and interferon (IFN) production, as well as increased viral-associated hepatitis and liver damage (78).

Viruses have evolved mechanisms to evade recognition by immune cells. The MHC-I antigen presentation machinery appears to be a major target for immunoevasion by viruses. Murine cytomegalovirus (MCMV) gene products, such as m06, m152, and m04, have the ability to alter MHC-I function (79–82). While *m06* and *m152* gene products interfere with the expression of assembled MHC-I molecules on the cell surface, *m04* gene product does not interfere with cell surface expression, but rather prevents MHC-I recognition by T cell receptors (79–82). These strategies as well as the expression of an MHC-I mimic (described below) prevent recognition of infected cells by T cells

and at the same time maintain sufficient expression of ligands for inhibitory Ly49 receptors to prevent "missing-self" recognition by NK cells. Similarly, an increased binding of inhibitory KIR by MHC-I ligands on infected cells is reported during influenza virus infection (83, 84). In these studies, a redistribution of MHC-I molecules on the infected cells was proposed to allow better recognition by the inhibitory KIR and subsequent NK cell inhibition (84). Infection of a human pulmonary epithelial cell line by influenza virus was also shown to activate p53 and cause MHC-I upregulation on the surface of infected cells (85).

Viruses have been shown to also utilize other mechanisms, such as mimicry of host proteins on their cell surface, to evade host immune recognition. Often these viral mimics engage inhibitory receptors on immune cells to cause inhibition of immune responses. A classical example of this is the product of MCMV *m157* gene, a viral MHC-I-like protein expressed on the surface of infected cells (86, 87). While similar to MHC-I in structure, expression of the viral mimic is independent of the host's MHC-I antigen processing machinery (88). Engagement of inhibitory Ly49 receptors by m157 molecules on the surface of infected cells causes NK cell inhibition in MCMV-susceptible mouse strains (**Figure 3**) (89). Leukocyte Ig-like receptor 1, also known as ILT2, is a cell surface receptor expressed on human immune cell subsets including T and NK cells, and is capable of binding classical MHC-I and HLA-G (90). A mechanism of immune evasion by human cytomegalovirus involves the expression a glycoprotein human homolog of MHC-I, UL18, which can inhibit NK cell function by binding to its ILT2 receptor (91, 92).

Nevertheless, NK cells are able to recognize virus-infected cells through the engagement of their activating receptors. Activating receptors on NK cells are thought to have arisen as a result of selective pressure exerted by continuous pathogen challenge. Following Epstein–Barr infection, increased expression of a MHC-I ligand, which interacts with the activating KIR2DS1, is detected on the cells surface (93). In MCMV-resistant mouse strains, such as C57BL/6 (B6), the activating Ly49H receptor is able to recognize the MCMV m157 glycoprotein (86, 87). The presence of the activating Ly49H receptor in the Ly49 receptor repertoire of B6 mice confers resistance from MCMV infection. In contrast, strains, which lack genes encoding for the activating Ly49H, such as 129 and BALB/c, or the genetically manipulated Ly49H-deficient B6, are highly susceptible to MCMV infection (86, 87, 94). In the 129 mouse strain, m157 binds the inhibitory Ly49I receptor (86). Sequence variants of m157 are known to also interact with the inhibitory Ly49C receptor from B6 mice, which could inhibit NK cell activation. However, since most Ly49H$^+$ NK cells do not express Ly49C, MCMV clearance is not disrupted in B6 mice (95, 96). Similarly, the activating Ly49P receptor confers resistance in MA/My mice to MCMV infection through the recognition of MCMV m04 protein in association with H-2D^k MHC-I haplotype (97, 98). In both cases, viral gene products aimed at evading immune recognition have become the target for activating Ly49 receptors.

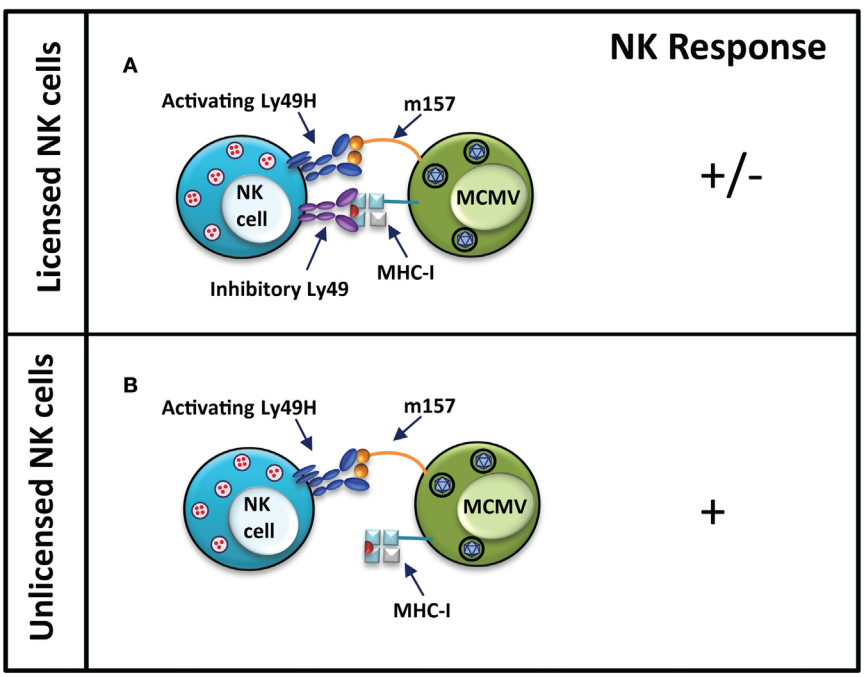

FIGURE 3 | Unlicensed NK cells dominate responses to MCMV infection in MCMV-resistant mouse strains. In MCMV-resistant mouse strain, NK cells activation occurs when the activating Ly49H receptor is engaged by m157 protein on the infected cells. Simultaneous engagement of inhibitory Ly49 receptors by MHC-I molecules can inhibit activation of licensed NK cells **(A)**. In unlicensed NK cells, the interaction of Ly49H and m157 induces a strong activating response due to the lack of self-MHC-I-specific inhibitory Ly49 receptors on these cells **(B)**. NK cell response "–" represents no activation, and "+" represents activation.

IS NK CELL LICENSING ALWAYS REQUIRED FOR THE RECOGNITION OF VIRUS-INFECTED CELLS?

The fact that the same inhibitory receptors, which are exploited by viruses to evade immune recognition, are also involved in NK cell licensing raises the question of whether NK cell licensing is always required for the recognition of virus-infected cells. As it appears, licensed NK cells, bearing inhibitory Ly49 receptors, are at a disadvantage during certain virus infections. It has been shown during MCMV infection that the NK-mediated response is dominated by the unlicensed (Ly49C/I$^-$) NK cell subset (67). While the antibody used to deplete the licensed NK cells, 5E6, is expected to deplete both Ly49C and Ly49I, there is also evidence that 5E6 is only capable of recognizing Ly49I on NK cells (99, 100).

In contrast to licensed NK cells, which are the primary mediators of an anti-tumor response through missing-self recognition, it is the unlicensed cell subset, which confers protection from MCMV infection. Selective depletion of the Ly49C/I$^+$ licensed NK cell subset rendered minimal viral titer increase, while depletion of Ly49C/I$^-$ unlicenced NK cells led to significant viral titer increase in the infected mice. Moreover, adoptive transfer of the Ly49H$^+$ Ly49C/I$^-$ NK cell subset into neonatal mice was sufficient to protect them against an MCMV infection, and was more effective than the Ly49H$^+$ Ly49C/I$^+$ NK cell subset. The interaction between Ly49C/I and its ligand limits the ability of licensed NK cells to control the infection. These findings indicated that unlicensed NK cells are responsive and play a major role in an immune response during a viral infection, the activity of unlicensed NK cells could be due to the pro-inflammatory cytokine environment of viral infection, which is known to enhance NK cell function. As a further confirmation, the classical unlicensed NK cell model – *B2m*$^{-/-}$ mice – which possess only unlicensed NK cells due to their lack of MHC-I surface expression, was better able to control MCMV infection than their wild-type counterparts (67). NK cell depletion in *B2m*$^{-/-}$ during MCMV infection leads to enhanced virus titer in the salivary gland (80). Our group has also shown that Ly49-deficient and *B2m*$^{-/-}$ mice, which possess unlicensed NK cells, fare better following influenza infection than their wild-type counterparts in an NK cell-dependent manner (101). *B2m*$^{-/-}$ and Ly49-deficient mice treated with NK cell-depleting monoclonal antibody, as well as perforin-deficient Ly49-deficient mice, are more susceptible to influenza virus infection, demonstrating that the improved survival of these mice is due do the functional activity of unlicensed NK cells. The Ly49-deficient mice exhibit reduced viral titer and lung pathology. Our study also provides evidence for influenza virus infection-driven immune evasion. Following influenza virus infection, upregulation of MHC-I is observed on pulmonary epithelial cells, possibly as a mechanism to evade detection by licensed NK cells. This provides Ly49-deficient mice with an advantage since their unlicensed NK cells will not be affected by the increased levels of MHC-I, which would traditionally be recognized by members of the Ly49 receptor family leading to NK cell inhibition. Moreover, blockade of the interaction between Ly49:MHC-I rendered licensed NK cells in wild-type mice better at controlling influenza virus infection. It is suggested that the unlicensed NK cells are better effectors in viral control due to their ability to surpass inhibition mediated by MHC-I or MHC-I-like viral ligands expressed on the surface of infected cells.

In a hematopoietic stem cell transplantation (HSCT) mouse model for NK cell licensing, depletion of licensed NK cells resulted in higher viral titers in the liver of MCMV-infected mice at early time points but not at later time points after infection (102). In the same study, the licensed NK cells also expanded and produced IFNγ upon infection but were suppressed by regulatory T (Treg) cells and TGF-β (102). It was proposed that the licensed NK cells mount a strong early response against MCMV infection, but become inhibited or exhausted. The unlicensed NK cells, on the other hand, show a strong late response due to the presence of activation stimuli and the absence of inhibition from the inhibitory Ly49:MHC-I binding. This is also corroborated by results showing that prior activation by stimuli, such as poly I:C, results in an efficient anti-virus and anti-tumor response by the unlicensed NK cells (64, 66, 102).

Epidemiological human studies have shown that HIV-infected individuals who have KIR3DS1, an activating NK cell receptor, and its ligand, HLA-B Bw4-80I, exhibit slow progression to AIDS, compared to other HIV-infected individuals (103). In support of this finding, an *in vitro* study has shown that NK cells derived from individuals with the KIR3DS1/HLA-B Bw4-80I compound genotype were able to mediate inhibition of HIV-1 replication in a contact-dependent manner (104). Interestingly, early after HIV infection, the frequencies of the activating KIR3DS1$^+$ and the inhibitory KIR3DL1$^+$ NK cells are specifically increased in patients with acute HIV-1 infection in the presence of HLA-B Bw480I. Unfortunately, this expansion is not associated with reduction in HIV levels in the blood. Engagement of the inhibitory KIR3DL1 receptor on these NK cells with its ligand on the target cells could result in the inhibition of NK cell cytotoxicity toward the HIV-infected cells, explaining the maintained level of HIV in those patients in comparison to KIR3DL1-deficient patients (105). Similarly, studies have shown that CD56$^-$ CD16$^+$ NK cells, which are greatly expanded in HIV-viremic individuals, have impaired function. Characterization of this NK cell subset revealed that the expression of inhibitory KIR2DL2 and KIR2DL3 receptors were high on these cells, which would explain their defective lytic capability toward HIV-infected cells (106). Taken together, these reports indicate that HIV-infected cells may augment NK cell inhibition through interactions between inhibitory KIR and HLA receptors.

Therefore, under circumstances where inhibitory Ly49 receptors are engaged strongly by MHC-I or viral mimic ligands on the infected cells, NK cells that lack inhibitory receptors for MHC-I seem to exhibit better effector functions. This may also explain why a large proportion of unlicenced NK cells, which do not express receptors for self-MHC-I, are maintained in both mice and humans. In mice, up to half of the NK cells are "unlicensed" with respect to self-Ly49 expression (31, 42). In human studies, almost 25% of CD56^{dim} and over 60% of CD56^{bright} NK cells do not express KIR, as assessed by their negative staining for

KIR2DL1, KIR2DS1, KIR2DL2, KIR2DL3, KIR2DS2, KIR3DL1, and KIR2DS4 (107).

CONCLUSION

These non-conventional observations possibly underlie a different approach to understanding NK cell function dependent on its licensed or unlicensed status. The licensed status of the cell is of biological importance during rejection of MHC-I-deficient cancer cells, MHC-mismatched bone marrow transplants, and other target cells which exhibit MHC-I downregulation. Various studies suggest that licensed NK cells excel at tumor cell recognition and, while still controversial, the unlicensed NK cells preferentially protect from viral infections. The roles of these NK cell subsets may not be so clearly defined as either preferential to viral or cancer control, but may be due to more nuances of the disease. Viral infection induces robust cytokine secretion, which may reactivate unlicensed NK cells to respond to the infection. Additionally, the status of NK cell control may not be dependent on the cells being licensed or unlicensed, as it has been shown that activation can happen in the absence of licensing through other means such as through engagement of activating receptors such as NKG2D, ADCC, or through immunostimulants such as poly I:C. Rather, NK recognition may be dependent on the characteristics of the aberrant cell, including its MHC-I expression levels and the microenvironment where they are encountered. The role for unlicensed NK cells in the control of cancers and virally infected cells, which have mediated upregulation of MHC-I helps to explain why the unlicensed NK cell subset is still present in modern day mice and humans, and has not been evolutionarily selected against.

AUTHOR CONTRIBUTIONS

MMT and ABM wrote the manuscript. APM supervised and reviewed the manuscript.

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REVIEW
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Targeting NK Cells for Anticancer Immunotherapy: Clinical and Preclinical Approaches

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The recent success of checkpoint blockade has highlighted the potential of immunotherapy approaches for cancer treatment. Although the majority of approved immunotherapy drugs target T cell subsets, it is appreciated that other components of the immune system have important roles in tumor immune surveillance as well and thus represent promising additional targets for immunotherapy. Natural killer (NK) cells are the body's first line of defense against infected or transformed cells, as they kill target cells in an antigen-independent manner. Although several studies have clearly demonstrated the active role of NK cells in cancer immune surveillance, only few clinically approved therapies currently exist that harness their potential. Our increased understanding of NK cell biology over the past few years has renewed the interest in NK cell-based anticancer therapies, which has lead to a steady increase of NK cell-based clinical and preclinical trials. Here, the role of NK cells in cancer immune surveillance is summarized, and several novel approaches to enhance NK cell cytotoxicity against cancer are discussed.

Keywords: natural killer cells, checkpoint inhibitors, immunotherapy, cancer, immune therapy

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OVERVIEW

Natural killer (NK) cells were identified in 1975 as a unique lymphocyte population that is clearly distinct from the other lymphoid lineages, such as T- and B-cells. NK cells were shown to differ from adaptive lymphocytes in respect to their morphology as well as in their capability to kill tumor cells without prior sensitization (1, 2). Since their discovery, research over the past 40 years significantly improved our understanding of the regulation of NK cells and has established several essential roles of NK cells during development in healthy individuals and during disease that can be therapeutically utilized.

Natural killer cells are the founding member of the innate lymphoid cell (ILC) family and are generally grouped based on their organ of development and tissue localization: we distinguish bone marrow-derived or adult conventional NK (cNK) cells, thymic-derived, fetal liver-derived, liver-resident, uterine-resident, and intestinal-resident NK cells. Adult cNK cells develop from the common lymphoid progenitor in the bone marrow in mice and humans and are considered the major NK cell subset responsible for tumor immune surveillance, albeit a role of the other subsets cannot completely be ruled out. During murine adult hematopoietic development, NK cell precursors are thought to be derived from a common innate lymphoid progenitor (CILP) and then mature through several progenitor stages into mature NK cells and migrate to several lymphoid and non-lymphoid tissues (3, 4). Peripheral NK cell maturation is then defined by the differential

expression of CD11b, CD27, and KLRG1. Immature NK cells are defined as $CD11b^{-}CD27^{+}KLRG1^{-}$ and mature NK cells as $CD11b^{+}CD27^{+}KLRG1^{-}$ (M1) or $CD11b^{+}CD27^{-}KLRG1^{+}$ (M2) (4–6). These different subsets differ in their ability to lyse target cells and their ability to secrete cytokines (5, 6). The mature $CD11b^{high}CD27^{-}KLRG1^{+}$ NK cells are the dominant population in non-lymphoid organs except for the liver, where a distinct TNF-related apoptosis-inducing ligand (TRAIL)$^{+}$CD49b^{-}CD11 b^{low} expressing population exists (7–9).

In contrast to murine NK cell development, the NK cell precursor populations in humans are currently not as well defined (10). Mature NK cells make up around 5–20% of peripheral blood lymphocytes. They are usually defined as $CD3^{-}CD56^{+}$ lymphoid cells and are subdivided into two major subpopulations, $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ cells. $CD56^{dim}$ NK cells are the dominant subset in peripheral blood and spleen, express perforin, and are the most potent one in killing cancer cells (11–13). $CD56^{bright}$ NK cells represent the main NK cell subset in lymph nodes and tonsils, lack perforin expression but are efficient producers of cytokines, such as IFN-γ, in response to the interleukins (IL)-12, IL-15, and IL-18. Thus, this subset is considered to be one of the key regulators of immune responses.

A major difference between NK and T cells is that NK cells can kill target cells instantly without needing prior sensitization, giving the adaptive immune reaction enough time to mount an antigen-specific immune response. Although the speed in which NK cells can kill infected or malignant cells is a big advantage when fast immune reactions are required, the ready-to-kill status of NK cells could be potentially dangerous for the body. Thus, NK cell activation is tightly regulated by activating the inhibitory receptors and the balance of the signaling through these receptors dictates if NK cells kill their target cells or remain inactive (14, 15). To prevent autoreactivity, NK cells express MHC class-I-specific receptors such as the killer cell immunoglobulin-like receptors (KIRs) in human, the lectin-like Ly49 dimers in the mouse, and the CD94–NKG2A heterodimers which exist on both, mice and humans. Binding of MHC-I molecules to these inhibitory receptors prevents cytolytic activity against healthy cells. During cancer progression, cancerous cells often decrease or even loose the expression of MHC-I on the surface, which allows them to evade T cell recognition and killing. However, loss of the MHC-I-mediated inhibitory signal on NK cells results in NK cell activation and cancer cell killing if no other inhibitory signals are active. In addition to the loss of inhibitory receptor signaling, NK cells can be directly activated by activating receptors, such as NKG2D, NKp30, NKp44, NKp46, 2B4, DNAM-1 (CD226), or CD16 (4, 6, 14, 16, 17). Although the ligands for some activating receptors have not yet been identified, it is currently believed that activating ligands are not expressed on healthy cells but are upregulated on diseased cells and that signaling through the activating receptors will dominate over the MHC-I-mediated inhibitory signaling. Besides the direct ligand–receptor interaction, NK cell functions are as well modulated by several cytokines. NK cells can be activated through type I interferons, IL-2, IL-12, IL-15, IL-18, and IL-21, whereas suppressive cytokines, such as transforming growth factor (TGF)-β or IL-10, can render NK cells inactive (18).

Several different pathways exist through which NK cells kill their target cells. On the one hand, NK cells induce apoptosis in their target cells by releasing lytic granules, such as granzyme B and perforin, *via* the formation of a lytic immunological synapse between the NK and target cells (19). Released perforin induces membrane perforation allowing the secretion of granzymes into the intracellular space inducing either caspase-dependent or -independent apoptosis. Another mechanism to kill is the induction of the death receptor-mediated apoptosis pathway. Here, FasL and TRAIL expressed on NK cells bind to Fas and TRAIL receptor triggering target cell apoptosis. In addition, NK cell-derived TNF-α can as well induce target cell apoptosis.

Despite the majority of current NK cell-mediated anticancer therapies focus on the lytic capability of NK cells, the indirect antitumor immunity capacity of NK cells should not be disregarded. NK cells are known to regulate the innate and adaptive immune response through the secretion of various cytokines, chemokines, adenosine, and growth factors (20, 21). NK cell-derived IFN-γ induces dendritic cell (DC) maturation leading to increased IL-12 production. IFN-γ as well induces the differentiation of $CD8^{+}$ T cells into cytotoxic T cells (CTLs) and promotes the differentiation of $CD4^{+}$ cells into Th1 T cells, which in turn promote the CTL response. NK cells not only enhance immune responses but also dampen T cell responses by either killing DC or inhibiting $CD8^{+}$ T cell responses directly through IL-10 secretion. Our current understanding of the immune modulatory role of NK cells is, however, still limited and a better understanding will certainly open the door to novel NK cell-based immunotherapy approaches.

Evidence for the Importance of NK Cells in Anticancer Immunosurveillance

An essential role for NK cells in human immune surveillance has been clearly established. Defects in human NK cell development or effector functions result in recurrent viral infections and in an increased risk of cancer development (22). Probably, the best evidence for the role of NK cells in anticancer immune surveillance comes from an epidemiological 11-year follow-up cohort study among a Japanese general population: the study demonstrated that high cytotoxic activity in peripheral blood lymphocytes is associated with reduced cancer risk, whereas low activity is associated with increased risk to develop various types of cancer (23). Subsequently, several other studies found that high levels of tumor infiltrating NK cells are associated with favorable outcome in patients with colorectal carcinoma, gastric cancer, and squamous cell lung cancer (24). Indicative of an important role of NK cells in tumor control, cancer cells have developed several strategies to escape from NK cell recognition. Tumor cells can upregulate ligands for inhibitory receptors or secrete immune suppressive factors, including TGF-β, IL-10, prostaglandin E2, indoleamine 2,3-dioxygenase (Ido), and adenosine (25–29). Shedding of ligands for activating receptors represents another potential strategy by tumor cells to reduce the amount of activating ligands on the surface of tumor cells and/or induce NK cell desensitization (30–33). However, a recent report questioned the shedding mechanism as a way to invade the immune surveillance.

In the mouse model, Deng et al. demonstrated that a shed form of the mouse NKG2D ligand MULT1 can lead to boosting of NK cell activity (34).

Despite ample evidence that NK cells participate in the fight against cancerous cells, very few therapeutical approaches currently exist that are targeting NK cells. However, support for the potential of NK cells as therapeutic targets is coming from approved cancer cell-targeting therapies as several drugs have been recently demonstrated to additionally modulate NK cell activity. In the next section, I will review the effect of a few of such therapies.

Cancer Cell-Targeting Drugs with NK Cell-Modulating Activity

Noteworthy, many targets of current cancer therapies are expressed in cancer cells and immune cells. It is therefore not surprising that few cancer therapies not only impact on cancer cell survival and proliferation but also influence the immune system. But because the majority of cancer-targeting drugs is generally tested preclinically for their efficacy and safety in xenograft models that lack a functional immune system, this effect is often not apparent. Indeed, recent studies have shown that radiotherapy or chemotherapies, such as Ara-C, cisplantin, or 5-FU, can lead to increased expression of NK cell activating ligands and thus enhance NK cell recognition and killing (35). More recently, several precision medicine drugs have additionally been demonstrated to increase NK cell-mediated tumor killing (36, 37). The proteasome inhibitor bortezomib, currently successfully used in the treatment of multiple myeloma, can induce the expression of ligands of NK cell activating receptors. Another example is the immunomodulatory (IMiD) drug lenalidomide, which is approved for the treatment of multiple myeloma and myelodysplastic syndromes (MDS). Besides having a direct effect on cancer cells and angiogenesis, lenalidomide modulates the immune response by increasing the NK cell number in the periphery. The exact mode of action of lenalidomide on NK cells is currently not clear. Several modes of actions have been proposed. Lenalidomide might increase NK cell activation indirectly by upregulating ligands on tumor cells and induce the expression of NK cell stimulatory cytokines such as T cell-derived IL-2 or directly by lowering the threshold for NK cell activation (38, 39). A better understanding of the mode of actions of lenalidomide on NK cells will be certainly crucial to design rational combination therapies. This is highlighted by the fact that lenalidomide in combination with the anti-CD20 antibody rituximab can lead to increased efficacy in B cell malignancies by enhancing the antibody-dependent cell-mediated cytotoxicity (ADCC) effect, but the combination with dexamethasone inhibits the immune-stimulatory effect of lenalidomide on NK cells, potentially *via* suppressing IL-2 production in $CD4^+$ T cells (40–42).

However, cancer-targeting drugs not always enhance the activity of immune cells, but in some cases, have been reported to exert detrimental effects on the immune system. Ibrutinib is a novel irreversible inhibitor of Bruton's tyrosine kinase that shows promising effects in the treatment of mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL). Rituximab in combination with chemotherapy is currently standard of care in $CD20^+$ B-cell malignancies and thus a potential combination of ibrutinib with rituximab is attractive. However, recent studies demonstrated that ibrutinib actually antagonizes the ADCC effect of rituximab in $CD20^+$ B-cell lymphoma due to Ibrutinib irreversible binding to IL-2 inducible tyrosine kinase (ITK), which is required for FcR-stimulated NK cell function (43, 44).

Another example is ruxolitinib, a small molecule inhibitor of the JAK 1/2/3 signaling pathway. Ruxolitinib is currently approved for the treatment of myelofibrosis (MPN). As several cytokines regulate NK cell development and function *via* the JAK/STAT signaling pathway, patients who were treated with ruxolitinib had drastically reduced circulating NK cell numbers. *In vitro* studies further demonstrated that ruxolitinib potently inhibited the cytokine-induced cytolytic activity of NK cells (45). However, importantly, NK cell depletion by ruxolitinib was reversible as the NK cell levels rose back to normal values in patients who stopped ruxolitinib treatment. Thus, when combined with NK cell-based immunotherapies, proper scheduling of therapeutic drugs will be crucial.

Clinical or Preclinical Therapies Augmenting NK Cell Function

Checkpoint Inhibitors

PD-1

Checkpoint inhibitors are currently the most promising approaches among immunotherapies. Treatment with anti-CTLA4 or anti-PD-1 antibodies restores T cell activity in cancer patients and has resulted in durable tumor regression in some patients. And the combination of both checkpoint inhibitors was able to further enhance the therapeutic benefit significantly (46, 47). The expression of PD-1, however, is not restricted to activated and exhausted T cells but can be detected on subsets of other immune cells and even on melanoma cells (48–51). A recent report demonstrated that NK cells from multiple myeloma and renal carcinoma patients expressed PD-1 on their surface and engagement of PD-1 signaling reduced their cytolytic potential (**Figure 1**) (50, 51). Treatment of patient-derived $PD\text{-}1^+$ NK cells with an anti-PD-1 antibody (pidilizumab, CT-011) was able to increase NK cell-mediated killing of autologous cancer cells *in vitro* (50). A recent phase II trial tested the efficacy of pidilizumab with rituximab in patients with relapsed follicular lymphoma and found that the combination is well tolerated and indicated favorable therapeutic effects when compared to rituximab single treatment (52). The therapeutic benefit of re-invigorating $PD\text{-}1^+$ NK cells in cancer patients is currently not well understood, and the major therapeutical effect is certainly due to re-activation of exhausted T cells. However, a contribution of NK cells to the observed therapeutic benefit cannot be excluded, especially in hematological malignancies, and thus warrants further investigation.

TIM-3

TIM-3, also known as HAVCR2, is another immune checkpoint receptor that is currently being tested in preclinical models for its potential to re-invigorate exhausted T cells in cancer patients (53).

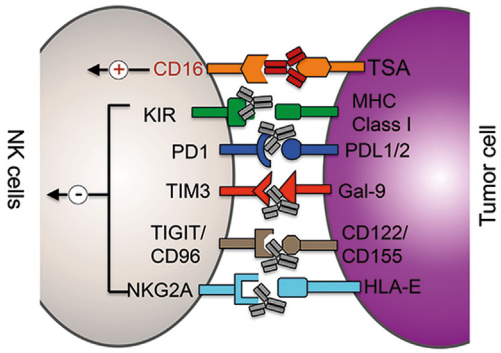

FIGURE 1 | Clinical and preclinical therapies augmenting NK cells function. mAb (gray)-mediated blockade of the interaction between inhibitor receptors (in black) expressed on NK cells with the respective ligands on tumor cells (or suppressor cells) results in increased cytolytic potential of NK cells. ADCC therapy: binding of mAbs (red) to tumor-specific antigens (TSA) results in the activation of NK cells *via* the activation of the activating receptor CD16 (red).

Resting T cells express low levels of TIM-3, and its expression is strongly upregulated in activated and exhausted T cells. Antibody-mediated blockade of TIM-3 signaling was able to reverse the exhausted phenotype of $CD4^+$ and $CD8^+$ T cells in melanoma patients proving the inhibitory function of TIM-3 in T cells (54). Human tumor-derived $CD8^+$ T cells often coexpress TIM-3 and PD-1, and preclinical studies in several murine tumor models demonstrated that the combination of TIM-3 and PD1 blocking antibodies can significantly increase the reversal of T-cell exhaustion. Like PD-1, TIM-3 expression is not restricted to T cells but can also be detected on murine and human NK cells (55, 56). In contrast to T cells, where TIM-3 surface expression marks dysfunctional T cells, TIM-3 is expressed on virtually all human NK cells and is further upregulated on cytokine-activated NK cells (**Figure 1**). Thus, TIM-3 expression is regarded as a marker for mature NK cells. Currently, the functional role of TIM-3 on NK cells is highly controversial. Ndhlovu et al. recently demonstrated that crosslinking of TIM-3 *via* anti-TIM-3 antibodies on the human NK cell line NKL or on human PBMC-derived NK cells significantly decreased their cytolytic ability (57). In stark contrast to these findings, Gleason et al. showed that activation of TIM-3 through the ligand Gal-9 actually increased the production of IFN-γ in NK cells (58). A more recent report suggested that the discrepancy between these two studies might origin from the different experimental layout as well as by the fact that NK cell lines and NK cells from healthy donors have been analyzed (56). Therefore, they tested the effect of TIM-3 blockade on NK cells derived from advanced melanoma patients. da Silva et al. found that TIM-3 surface expression increases with the progression of the cancer, $TIM\text{-}3^+$ NK cells display an exhausted phenotype and that high expression levels correlated with poor prognosis. More importantly, when $TIM\text{-}3^+$ NK cells derived from melanoma patients were incubated with anti-TIM-3-coated beads, TIM-3 activation resulted in modest, but statistically significant decrease in IFN-γ secretion and degranulation. In summary, the function of TIM-3 on NK cells is currently controversial, and more detailed studies on the role of TIM-3 on NK cells derived from cancer patients are required to fully understand the role and therapeutic potential of TIM-3 blockade in NK cell therapy.

NKG2A

The heterodimer CD94/NKG2A is another checkpoint inhibitor complex whose expression is shared between T and NK cells (**Figure 1**). Human CD94–NKG2A/C/E heterodimers recognize the non-classical MHC class-I molecule HLA-E in humans and Qa-1 in mice, which is expressed on many lymphoid cells (59). The NKG2A chain of the CD94/NKG2A receptor contains two immunoreceptor Tyr-based inhibitory motifs (ITIMs) in its cytoplasmic tail and HLA-E/NKG2A interaction results in a dominant inhibitory signaling event that causes a strong decrease in NK cell effector functions. Several solid cancer and hematological malignancies use the upregulation of HLA-E expression as an immune escape mechanism in order to evade killing by NK cells and T cells (60, 61). Therefore, the use of a blocking NKG2A antibody could be another useful addition to the steadily growing list of T/NK cell-targeting immunotherapy approaches. Monalizumab (previously IPH2201) represents such an anti-NKG2A checkpoint inhibitor and is currently under clinical investigation. In a phase I/II trial, monalizumab is currently being evaluated in head and neck cancer and ovarian cancer. Furthermore, the effects of the combination of monalizumab with ibrutinib (CLL, phase I/II), cetuximab (head and neck, phase I/II), and duvalumab (solid tumors, phase I/II) are currently investigated.

TIGIT and CD96

TIGIT, CD96, and CD226 (DNAM-1) belong to the same immunoglobulin family of receptors that interact with nectin and nectin-like proteins (16). Although all three receptors are expressed on NK cells and can bind CD155 and CD112, ligand binding is triggering different responses (**Figure 1**). CD226 is an activating receptor that is important for NK cell-mediated tumor surveillance and ligand binding increases the cytotoxic potential of NK cells against target cells (16). On the other hand, TIGIT and CD96 contain ITIM motifs in their cytoplasmic domains and are inhibitory receptors. Although CD155 present on tumor cells can induce CD226-dependent immunosurveillance, the expression of CD96 and TIGIT on the same cell can counterbalance CD226 activity. While activation of TIGIT on human NK cells inhibited *in vitro* cell killing of target cells, antibody-mediated blocking of TIGIT significantly increased the cytolytic activity (62). Using $CD96^{-/-}$ mice, Chan et al. recently demonstrated that loss of CD96 expression resulted in improved tumor control of methylcholanthrene (MCA)-induced fibrosarcoma and lung metastasis (63). Although blockade of TIGIT *in vitro* increased the cytolytic activity of NK cells, the improved antitumor response in *CD96*-deficient mice was dependent on IFN-γ production by NK cells. It is currently not clear why NK cells express simultaneously two inhibitory receptors on the same cell to counteract CD226 activation, but data from the Smyth group indicate that the two receptors control different NK cell effector functions: TIGIT may predominantly inhibit the cytolytic potential of NK cells, whereas CD96 regulates the production of IFN-γ (16, 63). Future research

will unravel which of these two receptors should be inhibited to increase tumor surveillance in human patients or if inhibition of both receptors simultaneously will result in improved NK/T cell-mediated cancer cell control.

Killer Cell Immunoglobulin-Like Receptors

Natural killer cells express inhibitory KIRs that recognize self-MHC class-I molecules to prevent cytotoxicity against host cells (**Figure 1**). As tumor cells express the same MHC class-I molecules than healthy tissue, the interaction between self-HLA on cancer cells with KIRs on NK cells reduces the cytolytic activity of NK cells against tumor cells (64–68). Therefore, KIRs represent an interesting class of targets for NK cell-specific checkpoint inhibition. Following this reasoning, a humanized KIR-blocking monoclonal antibody (mAb), IPH2101, has been generated and is currently tested in clinical trials. IPH2101 is specific against three inhibitory KIRs, namely, KIR2DL-1, -2, and -3, that are specific for all HLA-C molecules. Preclinical *in vitro* and *in vivo* studies demonstrated that IPH2101-mediated blockade of KIRs on human NK cells significantly increased cytolytic activity against tumor cells (69–71). Importantly, no sign of autoimmunity was observed in treated mice. Confirming the results of the preclinical studies, no severe side effects were observed in clinical phase I and phase II trials in patients with acute lymphoblastic leukemia or multiple myeloma (72–74). Although the current clinical trials using IPH2101 as a monotherapy did not demonstrate significant antitumor efficacy, based on the encouraging preclinical data of IPH2101 and the recent success of combining checkpoint inhibitors, there is still hope that the rational combination with other drugs can lead to improved clinical antitumor responses. Potential combination partners could be the above described checkpoint inhibitors and other IMiD drugs, such as lenalidomide or NK cell activating cytokines.

Redirection of NK Cell Cytotoxicity via Biologics

Antibodies recognizing tumor-specific epitopes represent a highly efficient strategy to direct the cytolytic activity of NK cells against malignant cells. One approach that is currently successfully used in the clinics is *ADCC-based therapies*. NK cells express the activating surface receptor CD16 (FcγRIIIA), which specifically binds the constant region (Fc) of immunoglobulin G (IgG) antibodies. The interaction between CD16 on NK cells and the Fc portion of a tumor-specific IgG antibody bound on cancer cells results in the activation of NK cells and subsequently killing of respective tumor cells (**Figure 1**). Currently, several ADCC therapies are tested in clinical trials or are already successfully used in the clinics, such as α-CD20, α-GD2, α-Her2, and α-EGFR mAbs. The current status of ADCC therapies were summarized in recent review (75). However, it is important to mention that CD16 is expressed not only on NK cells but also on activated myeloid subsets. Therefore, several hematopoietic lineages are likely to contribute to the observed therapeutic effects of ADCC (75). Besides mAbs, bispecific or trispecific killer engagers (BiKEs and TriKEs) are currently developed. These antibodies are able to target either one (BiKE) or two (TriKE) different antigens on the tumor cell and bind to another epitope of the CD16 receptor leading to improved NK cell-mediated ADCC effect [for a review on BiKEs and TriKEs, please see Wang et al. (75) and Kontermann and Brinkmann (76)].

Targeting Immune Suppressive Signaling

Transforming Growth Factor-β

Secretion of TGF-β by tumor cells or the tumor microenvironment has copious effects on tumor progression and on the immune system (77). During cancer progression, TGF-β can play a key role in tumor immune escape. TGF-β levels are often increased in the serum of cancer patients and elevated levels correlate with systemic inhibition of the immune system and poor prognosis (78, 79). Like $CD8^+$ T cells, NK cells from patients with elevated TGF-β levels displayed reduced cytotoxicity and had reduced expression levels of the activation markers, NKG2D, NKp46, or increased expression of NKG2A (**Figure 2**) (28, 80). *Ex vivo* treatment of patient-derived NK cells with neutralizing anti-TGF-β mAbs was able to restore activating receptor expression, proliferation, and cytokine secretion (29). Coculture of healthy human NK cells with human ALL blasts reduced their cytolytic activity and IFN-γ production. This effect was mediated by ALL-derived TGF-β as an anti-TGF-β blocking antibody was able to rescue NK cell functions (28). In line with a direct effect of TGF-β signaling on NK cell receptor expression and NK cell function, *in vitro* incubation of human NK cells with TGF-β resulted in downregulation of NKp30 and NKG2D, inhibition of IL-15 induced NK cell proliferation and IFN-γ secretion (81). Therefore, targeting TGF-β signaling in NK cells represents an attractive immunotherapy approach in cancer patients with elevated TGF-β levels. However, due to the many functions of TGF-β in normal tissue, cancer cells, tumor microenvironment, and immune cells, developing potent inhibitors with a low toxicity profile is challenging (82, 83). Currently, several approaches

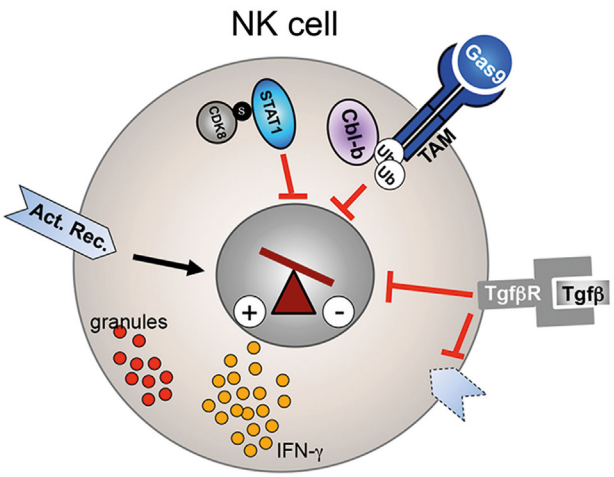

FIGURE 2 | Regulation of NK cell activity. CDK8-mediated STAT1–Ser727 phosphorylation inhibits the cytolytic activity of NK cells. Similarly, Cbl-b-mediated ubiquitinylation of the TAM receptor results in reduced perforin, granzyme B (red granules), and IFN-γ secretion. TGF-β signaling reduces as well NK cell effector function and leads to downregulation of activating receptors (dotted receptor).

to inhibit TGF-β signaling are pursued to increase efficacy and limit toxicity in preclinical and in clinical trials with various successes. Approaches include ligand traps, antisense oligonucleotides, receptor kinase inhibitors, and peptide aptamers (84). In summary, while it is currently to early to judge if anti-TGF-β immunotherapies will become reality, preclinical studies yielded enough convincing results that interference with the TGF-β pathway is able to increase NK cell (and T cell) effector functions to warrant further new drug development.

EXPLORATORY TARGETS – THE NEXT WAVE?

Intracellular Targets

The majority of approved immunotherapies target surface receptors on immune cells *via* mAbs that either inhibit protein–protein interactions between immune cells and other cell types (antagonistic antibodies) or activate target receptor on certain immune cells (agonistic antibodies). Whereas in cancer-targeting therapeutic approaches small molecule drugs dominate, this class of therapies is conspicuously missing or at least under-represented in current anticancer immunotherapy approaches. Targeting intracellular proteins *via* small molecules significantly extends the pool of potential novel immunotherapy targets. Different inhibitory receptors often use the same intracellular pathways to relay their inhibitory signal into the nucleus. Thus, by inhibiting such pathways, it might be possible to therapeutically affect several inhibitory receptors at the same time *via* inhibiting one molecule. The phosphatases Src homology region 2 domain-containing phosphatase (SHP)-1 (PTPN6) and SHP-2 (PTPN11) are two good examples as several inhibitory receptors on T cells and NK cells have been shown to recruit SHP-1 and/or SHP-2 after activation (85). Of course, targeting intracellular pathways with such broad activity can come with a cost, in this case, the potential increase of toxicity. Other advantages of small molecules over biologicals are excellently summarized in a recent review (86). In this next section, I will discuss a few recently published molecules that play a role in the regulation of NK cell function and might represent potential future targets for NK cell-mediated immunotherapy.

Casitas B-Lineage Lymphoma Proto-Oncogene-b

Post-transcriptional modification of proteins, such as ubiquitination, is an important regulatory mechanism for the fine-tuning of several pathways. Recent studies demonstrated that the E3 ligase Casitas B-lineage lymphoma proto-oncogene-b (Cbl-b) is a key regulator of the immune response against cancer (87). Cbl-b is highly expressed in most murine and human immune cells, including T and NK cells. The importance of Cbl-b in antitumor immune response was discovered when *Cbl-b*-deficient mice spontaneously rejected several different tumors (88, 89). Tumor rejection was first considered to be mainly mediated by $CD8^+$ T cells. However, a recent study elegantly demonstrated that Cbl-b also plays a key role in NK cell-mediated tumor control. Deletion or pharmacological inhibition of Cbl-b increased the cytolytic potential, proliferative capacity, and IFN-γ secretion of NK cells *in vitro* (**Figure 2**) (90). More importantly, tumor growth and metastasis were significantly decreased in $Rag2^{-/-}Cbl\text{-}b^{-/-}$ mice when compared to $Rag2^{-/-}Cbl\text{-}b^{wt}$ mice. Antibody-mediated NK cell depletion and inactivation *via* anti-NK1.1 and anti-NKG2D antibodies, respectively, abrogated this antitumor response, identifying the NK cell lineage as the main mediator of the observed antitumor effect in these mice. Furthermore, NK cell activity was completely dependent on the catalytic domain of the E3 ligase of Cbl-b. Through an *in vitro* ubiquitinylation screen of 9000 human proteins, the authors then identified the TAM receptor tyrosine kinases AXL, TYRO3, and MER as targets of Cbl-b. Indeed, activation of TAM receptors on wild-type NK cells *via* the natural ligand Gas6 suppressed IFN-γ secretion *in vitro*, whereas *Cbl-b*-deficient NK cells were resistant to this inhibition. These date therefore indicate that TAM or Cbl-b is potentially suitable targets for NK cell-mediated immunotherapy. Indeed, Paolino et al. developed a highly selective TAM kinase inhibitor, LDC1267, which increased the lytic activity of NK cell against B16F10 melanoma cells *in vitro* and *in vivo* in an adoptive transfer mouse model. Furthermore, intra-peritoneal injection of LDC1267 resulted in a decrease of micro-metastases in mice that were injected with the syngenic tumor cell line 4T1. In summary, interference of TAM receptor activity represents an interesting novel immunotherapy approach. Alternatively, a small molecule inhibitor of Cbl-b potentially could increase NK cell and T cell effector function against cancer cells (87). It is currently unclear how toxic such a small molecule would be as Cbl-b is expressed in many hematopoietic cell types. However, *Cbl-b*-deficient mice are viable and do not show signs of severe autoimmunity, thus a therapeutic window might exist.

CDK8

Natural killer cell development and functions are tightly regulated by several cytokines, such as IL-2, IL-12, IL-15, or type I interferons. The JAK/STAT pathway is playing a central role in relaying the effect of these different cytokines into the nucleus. IL-2 and IL-15 promote NK cell development and homeostasis mainly *via* activation of the transcription factor signal transducer and activator of transcription protein 5 (STAT5) (91, 92). On the other hand, STAT1 plays an essential role on the regulation of NK cell effector function (93). Type I interferon and IL12 induce STAT1 activation, resulting in increased cytotoxicity and IFN-γ secretion. Not surprisingly, therefore, deletion of STAT1 results in impaired NK cytolytic activity *in vitro* and reduced tumor rejection *in vivo*, despite normal numbers of NK cells, whereas *STAT5*-deficient mice lack NK cells completely (94–96). The activity of STAT1 is mainly regulated post-transcriptionally. For activation and translocation of STAT1 into the nucleus, STAT1 has to be phosphorylated at tyrosine 701 (Y701) by the Janus kinase JAK. A recent report demonstrated a role of STAT1–Ser727 phosphorylation in the regulation of the lytic potential of NK cells (96). Resting NK cells showed a basal level of STAT1–Ser727 phosphorylation, which increased after *in vitro* stimulation with either IFN-β or IL-12. Interestingly, in contrast to Y701 phosphorylation, Ser727 phosphorylation resulted in an inhibitory effect on NK cell activity, indicating that phosphorylation of STAT1–Ser727 represents a negative feedback in activated NK cells loop

to prevent over-stimulation. *Ex vivo* isolated STAT1–Ser727A mutant NK cells had increased lytic potential against a range of tumor cell lines *in vitro* and secreted increased levels of granzyme B and perforin. *In vivo*, STAT1–Ser727A mutant mice showed increased anticancer immunosurveillance against the murine tumor lines B16F10, 4T1, and a v-abl transformed leukemic cell line. Through the generation of *Rag1*$^{-/-}$ *STAT1–Ser727A* mice that lack B and T cells but have NK cells, Putz et al. demonstrated that the antitumor effect was strictly dependent on NK cell activity. Interestingly, By contrast, the molecules mentioned above which limited both, the production of lytic granules and IFN-γ secretion, STAT–1Ser727 repressed perforin and granzyme B but induced IFN-γ secretion slightly. The authors then identified the cyclin-dependent kinase 8 (CDK8) as the kinase responsible for the phosphorylation of STAT1 as Ser727 (**Figure 2**). Knock-down of CDK8 reduced STAT-1–Ser727 phosphorylation and slightly increased target cell lysis in an *in vitro* killing assay. Thus, pharmacological inhibition of CDK8 kinase activity might represent an attractive approach to augment NK cell-mediated anticancer immunosurveillance. Currently, the open questions are if the same effect will be seen in human NK cells and how toxic a CDK8 inhibitor will be given the broad expression of CDK8. However, CDK8 therapy could potentially have another positive anticancer effect: CDK8 has been previously shown to be an oncogenic driver in colorectal cancer, breast cancer, and melanoma (97–99). Therefore, one could envision that CDK8 inhibitors, on the one hand, induce tumor cell death and, on the other hand, stimulate NK cell activity.

EZH2

The H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2) is essential for many biological processes, including the regulation of immune responses, and is overexpressed in several cancers. Therefore, the pharmacological targeting of EZH2 is an interesting approach for future immunotherapies (100, 101). A recent study demonstrated a role of EZH2 in NK cell development (102). Absence of EZH2 in human and murine hematopoietic progenitors resulted in an increased commitment to the NK cell lineage. In addition, *EZH2*$^{-/-}$ NK cells expressed higher levels of NKG2D, IL2Rα, IL7Rα, and the lytic proteases granzyme A and B. The negative regulation of NK cell development and function by EZH2 was dependent on its methyltransferase activity as pharmacological inhibition of EZH2 resulted in a similar phenotype when compared to *EZH2*$^{-/-}$ NK cells. These data suggest that EZH2 inhibitors may not only have an effect on cancer cell growth and survival but potentially can augment NK cell number and function in patients. However, this remains to be tested as the above-mentioned study mainly focused on the effect of EZH2 on *in vitro* NK cell differentiation, and little data are currently available on the effect on mature NK cells. Nevertheless, several studies are testing the efficacy of adoptive transfer of *ex vivo* expanded NK cells as an immunotherapy approach. Thus, it will be of interest if the inhibition of EZH2 during the NK cell differentiation/expansion phase can lead to an increase in cell number and augment the activity of NK cells.

CONCLUSION

Although the ability of NK cells to kill malignant cells efficiently has been demonstrated several decades ago, the potential of NK cell-based immunotherapy is often questioned due to modest clinical responses of current therapies. Recent advances in our understanding of NK cell biology yielded already in promising new therapeutic approaches and continuous investigation of the mechanisms that regulate NK cell function will result in improved and more efficacious therapies in the future.

Many of the above described targets are not specific to NK cells, but often also function in other therapeutically interesting immune cells, such as T cells. Although the close relationship between NK and T cells makes if often difficult to identify how much of the therapeutic effect is due to NK cell activity, therapies that activate both effector cells are highly interesting as they are able to combine the therapeutic effects of both cell types (103). Until recently, only few experimental approaches existed to test the potential of NK cells in antitumor therapy. The most common and most feasible approach represented the antibody-mediated depletion of NK cells to investigate tumor growth in the presence or absence of NK cells. However, due to the lack of specific NK cell markers that can be targeted for depletion, it is often unclear if other cell types, such as T cells, have been affected as well. Recently, a novel NK cell-less mouse model has been established *via* the conditionally deletion of Mcl1 in NK cells (Mcl1$^{fl/fl}$ NCR1^{Cre}) (104). As MCL1 expression is essential for NK cell survival, virtually no residual NK cell subsets in all anatomical locations tested have been detected. As NCR1 is expressed as well on a subset of ILC3 cells in the gut, this mouse models lacked all NK cells and NCR1^{+} ILC3 cells. Nevertheless, this genetically engineered mouse represents an attractive model to test the specific role of NK cells in various disease settings.

Natural killer cell therapy as a monotherapy is unlikely to be curative for most, if not all, cancer types and a critical parameter for successful NK cell therapies will be the choice of combination partners. Therefore, future studies that investigate the interaction of NK cells with the other components of the immune system will be crucial for the optimal design of combination therapies.

In summary, while the potential of NK cell therapy is currently still not entirely clear, the recent advances in our understanding of NK cells certainly have resulted in novel, promising approaches, and it is very likely that future discoveries will continue to improve the efficacy of NK cell-based therapies.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Functional Reconstitution of Natural Killer Cells in Allogeneic Hematopoietic Stem Cell Transplantation

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Natural killer (NK) cells are the first lymphocyte population to reconstitute following allogeneic hematopoietic stem cell transplantation (HSCT) and are important in mediating immunity against both leukemia and pathogens. Although NK cell numbers generally reconstitute within a month, the acquisition of mature NK cell phenotype and full functional competency can take 6 months or more, and is influenced by graft composition, concurrent pharmacologic immunosuppression, graft-versus-host disease, and other clinical factors. In addition, cytomegalovirus infection and reactivation have a dominant effect on NK cell memory imprinting following allogeneic HSCT just as it does in healthy individuals. Our understanding of NK cell education and licensing has evolved in the years since the "missing self" hypothesis for NK-mediated graft-versus-leukemia effect was first put forward. For example, we now know that NK cell "re-education" can occur, and that unlicensed NK cells can be more protective than licensed NK cells in certain settings, thus raising new questions about how best to harness graft-versus-leukemia effect. Here, we review current understanding of the functional reconstitution of NK cells and NK cell education following allogeneic HSCT, highlighting a conceptual framework for future research.

Keywords: NK cell, allogeneic HSCT, NK cell education, memory NK cell, cytomegalovirus

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BACKGROUND

Allogeneic hematopoietic stem cell transplantation (HSCT) can be curative of otherwise incurable leukemia through its ability to mediate an immunological graft-versus-leukemia effect. Its main limitations are graft-versus-host disease (GVHD), infections, and leukemia relapse, all of which are critically dependent on immune reconstitution. Natural killer (NK) cells are well-established mediators of anti-leukemic and anti-viral responses (1). Donor NK cells can also attenuate GVHD, possibly by lysing alloreactive donor T cells and recipient antigen-presenting cells (2–5). There is much interest surrounding the importance or otherwise of NK cells on clinical outcome, particularly in regard to NK cell-mediated GVL effects (5–8). In this review, we will present the current state of knowledge on the functional reconstitution of NK cells to provide a framework to the debate.

KINETICS OF NK CELL RECONSTITUTION

Natural Killer cells are the first donor-derived lymphocyte population to reconstitute numerically following allogeneic HSCT. Normal NK cell numbers are generally observed within the first month post-transplant irrespective of the graft source: bone marrow (9), granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cell (PBSC) (9–11), or umbilical cord blood (12–14). It is generally thought that the reconstituting NK cells are primarily derived from the differentiation and maturation of progenitor cells rather than the expansion of mature NK cells within the graft. This concept is supported by two observations. First, the rate of NK cell reconstitution is largely independent of the type of graft and its NK cell content: side-by-side comparisons have found similar reconstitution kinetics following unmanipulated PBSC transplant, $CD34^+$-selected PBSC transplant, and bone marrow transplant despite log-fold differences in NK cell content (median 20–70 $\times$ 10^6/kg, 0.2–0.7 $\times$ 10^6/kg, and 5–7 $\times$ 10^6/kg, respectively) (9, 15–17), Second, the early reconstituting NK cells have an immature $CD56^{bright}$ phenotype and do not acquire the predominantly $CD56^{dim}$ donor NK phenotype for several months (17–19). Although NK cell development from progenitors is likely dominant, the *in vivo* expansion of transferred NK cells can also contribute. In a comparison of two different methods of T-cell depletion (CD3/CD19-depletion versus CD34-selection), NK cell reconstitution and acquisition of mature NK cell phenotype were more rapid in recipients of CD3/CD19-depleted grafts, which contained 3-log more mature NK cells than CD34-selected grafts (20). The impact of T cells on NK cell reconstitution is difficult to cleanly define as it is also linked to the use of post-graft immunosuppressive therapy. In haploidentical transplantation using extensively T-cell-depleted graft without post-transplant immunosuppression, NK cell reconstitution is particularly brisk (8) but in other settings where cyclosporine-based immunosuppression is used in both T-cell-deplete and T-cell-replete arms, the reconstitution of NK cell numbers was generally found to be similar between the groups (15, 17, 18).

ACQUISITION OF NK CELL FUNCTIONALITY

Although NK cells reconstitute numbers by around 1 month post-transplant, they take several months to acquire the immunophenotypic and functional characteristics found in healthy donors. $CD56^{bright}$ NK cells, which are the precursors of $CD56^{dim}$ NK cells (21), account for 40–50% of the NK cells in the first 3 months post-transplant as compared to only 5–10% in healthy donors (17, 19, 22–25). These early reconstituting NK cells also express higher levels of the inhibitory receptor, NKG2A, at around 90% compared to around 50% in healthy donors (17, 22–25). During NK maturation, the $CD56^{dim}$ NK cells lose NKG2A expression and express the activating NKG2C receptor, killer cell inhibitory immunoglobulin-like receptors (KIRs), and CD57 (26, 27). The acquisition of full donor surface phenotype takes 3–6 months, sometimes longer (17, 24–26, 28). Full NK cell functionality is similarly not achieved for at least 6 months post-transplant (17, 24, 29). In healthy individuals, $CD56^{bright}$ NK cells are adapted to produce cytokines, particularly interferon-γ (IFN-γ) and tumor necrosis factor (TNF), whereas $CD56^{dim}$ NK cells are enriched for perforin and granzymes, and thus adapted for cytotoxicity (30, 31). Following allogeneic HSCT, however, there is a dissociation between the recovery of cytokine production and cytotoxic function (29). Despite the high proportion of $CD56^{bright}$ NK cells in the first 6 months post-transplant, IFN-γ production in response to the MHC class I-deficient K562 cell line or primary acute myeloid leukemia cells is more severely and consistently impaired than NK cell degranulation and cytotoxicity (24, 27, 29). This somewhat contradictory finding is nonetheless consistent with the reduced expression of T-bet, a key inducer of IFN-γ production (32), at all stages of NK cell differentiation post-transplant (27). Furthermore, NK cell expression of T-cell immunoglobulin and mucin-containing domain-3 (Tim-3) is also lower post-transplant (33). In healthy individuals, Tim-3 is expressed on nearly all mature $CD56^{dim}$ NK cells and a majority of immature $CD56^{bright}$ NK cells (33, 34). It is upregulated by IL-15 or IL-12 and IL-18 *in vitro* (33, 34), and has been shown to both enhance IFN-γ secretion (33) and suppress cytotoxicity (34). As the level of Tim-3 expression at 3–6 months post-transplant is only half that of healthy controls, this may partly account for the discordant recovery of cytokine production and cytotoxic function (29).

The influence of graft T cell content on NK cell development and function is of clinical interest because the NK cell-mediated GVL effect is most evident in T-cell-depleted transplantation (5–8). While T-cell graft content does not have a significant influence on the numerical reconstitution of NK cells (15, 17, 18), there is a general trend towards enhanced functional NK cell maturation in T-cell-replete versus T-cell-deplete transplants, which is contrary to the relative importance of NK cells in T-cell-deplete transplants. In a study comparing HLA-matched T-cell-replete transplant with immunosuppression versus HLA-partially matched T-cell-deplete transplant without immunosuppression, target cell-induced IFN-γ secretion and degranulation were relatively attenuated in the T-cell-deplete group (29). This is consistent with an earlier study by the same group that found that NK cells in partially T-cell-deplete transplants had attenuated IFN-γ production compared to T-cell-replete transplants, with a similar proportion in both groups receiving cyclosporin A for GVHD prophylaxis (70 versus 81%) (18). Similarly, in a study comparing partial T-cell-depleted transplant (median 54 $\times$ 10^4 T cells/kg) versus extensive T-cell-depleted transplant (median 3.7 $\times$ 10^4 T cells/kg), with neither group receiving post-transplant immunosuppressive therapy, the reconstituting NK cells in the extensively T-cell-depleted group had higher proportions of $CD56^{bright}$ and $NKG2A^+$ immature NK cells and diminished cytotoxicity, although IFN-γ secretion was enhanced (19, 22). The mechanism by which T cells facilitate NK cell functional maturation is unclear: it may include the direct activation of $CD56^{bright}$ NK cells by T cell-derived IL-2 (35), or indirectly through IL-12 and IL-18 produced by activated macrophages during acute GVHD (36), and this effect was observed irrespective of the use of post-transplant immunosuppression. It is difficult to isolate the effect of pharmaceutical immunosuppression on NK cell reconsitution because it is tightly linked to the graft

T-cell content and subsequent risk of GVHD, both of which can influence NK cell reconstitution. Cyclosporin A does not have any impact on NK cell function in short-term cultures (37) but it has been shown to suppress the *in vitro* proliferation of NK cells, especially the $CD56^{dim}CD16^{+}KIR^{+}$ NK cells, resulting in a relative increase in the number of immature $CD56^{bright}CD16^{-}KIR^{-}$ NK cells (38). Hence, cyclosporin A can have a direct effect on NK cells in addition to any indirect effect through modulation of GVHD although more studies, including *in vivo* studies, will be required.

NK CELL EDUCATION FOLLOWING ALLOGENEIC HSCT

Natural killer cells sense and respond to cellular transformation, stress, and infection via an array of germ-line encoded activating and inhibitory receptors (39). The inhibitory receptors recognizing self-MHC class I are considered the predominant mediators of self-tolerance and the engagement of these receptors with their cognate MHC during NK cell development results in "licensed" NK cells that have functional competency, whereas failure of receptor engagement results in hyporesponsiveness (40–42). In HLA-mismatched transplantation where there is a mismatch in the inhibitory KIR-ligands located on HLA-B and HLA-C loci, there is a potential for donor NK cells that are licensed through the non-shared donor HLA to recognize and attack recipient leukemic cells that lack the cognate inhibitory HLA ligand. This "missing self" NK alloreactivity can be very potent and is associated with decreased risk of relapse in T-cell-depleted haploidentical transplantation for acute myeloid leukemia (5, 6, 43). The importance of MHC class I-mediated NK cell licensing is, however, not entirely clear-cut, particularly in allogeneic HSCT that are HLA-matched or HLA-mismatched but KIR-ligand matched. In both healthy donors and patients post-transplant, there is a hierarchy of target cell-induced NK cell degranulation response: (i) $NKG2A^{-}KIR^{-}$ and $NKG2A^{-}$Non-self-KIR^{+} NK cells are hyporesponsive (where non-self KIR recognizes an HLA ligand that is not expressed by the individual), (ii) $NKG2A^{+}KIR^{-}$ NK cells and $NKG2A^{-}$Self-KIR^{+} NK cells have similar degrees of responsiveness (where self-KIR is a KIR that recognizes a self HLA ligand), and (iii) $NKG2A^{+}$Self-KIR^{+} NK cells have the highest level of responsiveness (17, 29, 44). Hence, NKG2A have a role in NK cell education post-transplant that is additive to that of inhibitory KIRs. Since KIR expression is reduced for at least 3–6 months post-transplant (17–19, 22, 23, 44), NK cell degranulation response during this time is dominated by $NKG2A^{+}KIR^{-}$ NK cells rather than KIR^{+} NK cells (17). The extent to which these $NKG2A^{+}KIR^{-}$ NK cells can mediate GVL effect is likely context dependent. The ligand for NKG2A, HLA-E, is often expressed on leukemic cells, and its immune evasive capacity is underscored by the demonstration that antibodies against NKG2A can enhance NK cell-mediated lysis of leukemic cells both *in vitro* and *in vivo* (22, 45). However, not all leukemic cells express HLA-E and other studies have shown relatively low levels of HLA-E expression on primary leukemic blasts (19, 46) and since HLA-E is expressed in complex with a signal peptide from certain MHC class I molecules, its expression is also low in MHC class I-deficient blasts, and in this regard, the HLA-E/NKG2A interaction can be considered to be analogous to that of MHC class I/inhibitory KIRs.

Can allogeneic HSCT break NK tolerance? In the above studies, drawn from both T-cell-replete and T-cell-deplete transplants, with or without cyclosporine A, NK cell expression of at least one inhibitory receptor remained necessary for NK cell functional competency as $NKG2A^{-}$ NK cells that were KIR^{-} or expressed only non-self KIR remained hyporesponsive (17, 29, 44). There is, however, evidence that NK cell tolerance can be broken post-transplant. In one study, unlicensed NK cells that express single non-self inhibitory KIR were found to have increased cytokine secretion and cytotoxicity at 3–6 months post-transplant compared to their respective donors, and this effect was independent of NKG2A expression (47). The mechanism underpinning this is unclear but there are clues from murine models. In mice, NK cell function can be restored *in vitro* with IL-2 or IL-12 + IL-18, or strong stimulation via activating receptors (41, 48, 49). *In vivo*, NK cell tolerance can be broken by infection with *Listeria monocytogenes* and murine cytomegalovirus (MCMV) (50–52). Indeed, NK cell activation early post MCMV infection is dependent on pro-inflammatory cytokines and independent of activating receptor ligation, and licensed and unlicensed NK cells were similarly activated and produced similar levels of IFN-γ and granzyme B (52, 53). Since CMV reactivation is a common complication of allogeneic HSCT, it is possible that a similar mechanism underpinned the clinical observation. Unlicensed NK cells that break tolerance are not merely bystanders but can have specific protective function. In the MCMV model, unlicensed NK cells proliferated more robustly than licensed NK cells and were more effective in controlling MCMV infection because, unlike licensed NK cells, they were not inhibited by MHC class I expression on target cells (52). Similarly, unlicensed NK cells have been shown to be the primary mediators of antibody-dependent cell-mediated cytotoxicity during monoclonal antibody treatment for neuroblastoma (54). In HLA-matched and mismatched allogeneic HSCT, the risk of acute myeloid leukemia relapse is lower in patients who lack one or more HLA ligands to inhibitory KIRs ("missing KIR-ligand" hypothesis) (55, 56), which further supports the importance of unlicensed NK cells as a mediator of GVL effects (**Figure 1**).

Since the first reports on the protective effect of KIR-ligand mismatching on leukemia relapse more than 10 years ago (5, 57), our understanding of NK cell education has evolved and the "missing self" hypothesis is more complex than it initially seemed. It is now known that mature NK cells can undergo "re-education" following transfer into a different MHC environment; thus, mature responsive NK cells from wild-type mice become hyporesponsive when transferred to MHC class I-deficient mice and vice versa (58, 59). Furthermore, the education process requires the MHC class I to be expressed on all or most cells, or hyporesponsiveness is dominantly induced (41); and both non-hematopoietic as well as hematopoietic cells may be involved in NK cell education (58, 60). These new insights suggest that recipient MHC class I-mediated NK cell education may diminish the anti-leukemic effect of "missing self" NK alloreactivity, and are consistent with the observation that alloreactive donor NK cells were detectable mainly in the first 3 months post-transplant (57).

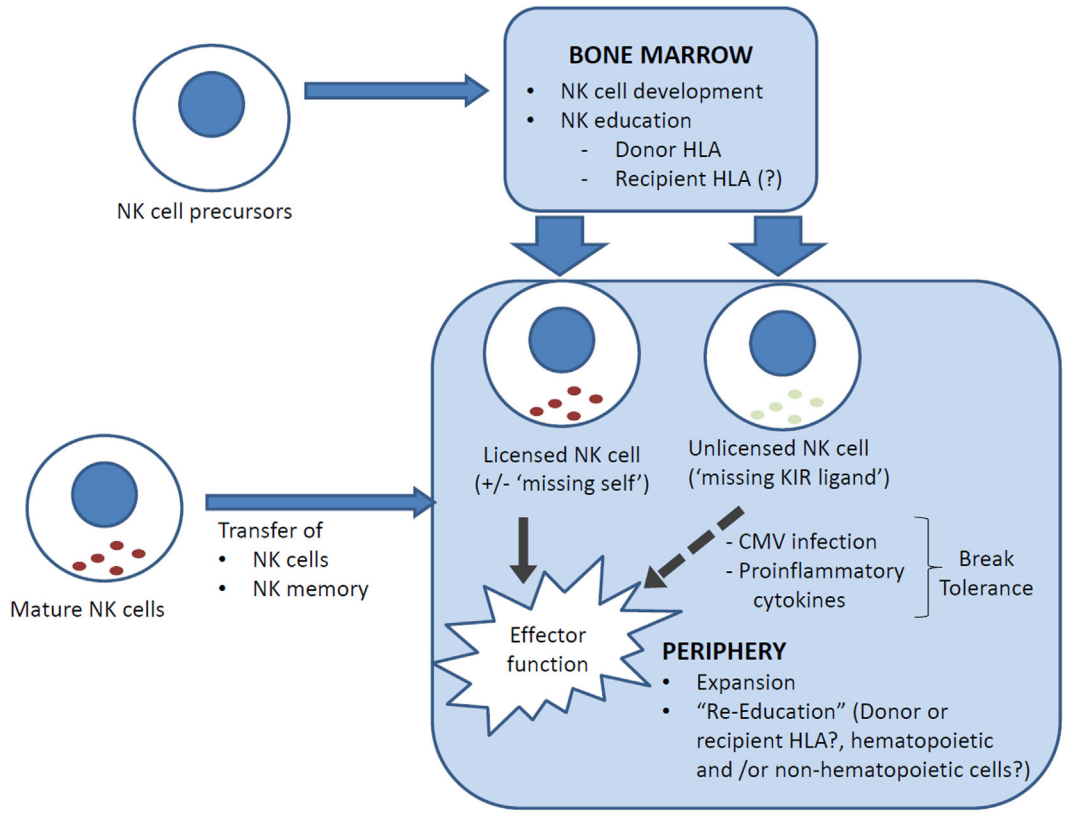

FIGURE 1 | NK cell reconstitution and education following allogeneic HSCT. Reconstituting NK cells can be derived from (i) NK cell precursors, ranging from hematopoietic stem cells through to common lymphoid progenitors, that differentiate into NK cells in the bone marrow, and (ii) transferred mature NK cells, which carry with them a mature NK phenotype and NK memory. NK cells are educated in the bone marrow but can be "re-educated" in the periphery. It is uncertain whether recipient cells (generally non-hematopoietic only post transplant) are involved in NK cell education. In HLA-mismatched transplants, NK cells that are licensed by inhibitory KIRs that recognize a ligand (HLA) that is expressed only by the donor and not the recipient may lyse recipient cells ("missing self") and contribute to the GVL effect. Conversely, an NK cell that has not encountered a cognate MHC class I for its inhibitory KIR(s) is "unlicensed" and hyporesponsive, but can acquire functional competency when stimulated by pro-inflammatory cytokines, for example, in the setting of CMV reactivation.

IMPACT OF CMV ON NK RECONSTITUTION

Cytomegalovirus reactivation is a common and life-threatening complication following allogeneic HSCT. In healthy individuals, CMV serostatus accounts for a significant proportion of the variability in NK cell immunity (61–63). Similarly, in allogeneic HSCT, CMV reactivation influences the frequency, phenotype, function, and/or repertoire of the reconstituting immune system (64–70). Interestingly, CMV reactivation is also associated with lower risks of leukemia relapse (71, 72), and understanding its influence on the immune landscape may provide insight into new therapeutic approaches to enhance the GVL effect.

The concept that NK cells can acquire immunological memory with features that are classically associated with T and B cell responses is largely established by studying CMV infection in mice (73, 74) and humans (61, 62, 75–78). In mice, MCMV infection induces the expansion of NK cells that express the activating receptor, Ly49H, which recognizes the viral protein m157 on the surface of infected cells (74, 79–81). Ly49H$^+$ NK cells undergo marked expansion, followed by contraction, and establishment of a long-lived memory population that mounts a more effective protective response than naive NK cells against MCMV but not heterologous infections (73, 74, 76). In humans, CMV infection induces an expansion of NK cells that express NKG2C (75, 76, 82, 83), an activating killer lectin-like receptor that binds HLA-E, which is upregulated by CMV UL40 protein (84, 85). These NKG2C$^+$ memory NK cells are CD56^{dim}CD57^{bright} and have a highly differentiated phenotype in regard to cytokine secretion and degranulation (75). They are preferentially negative for NKG2A (76, 82, 83) and are biased toward the expression of self-specific "licensing" inhibitory KIRs: KIR2DL3 in HLA-C1$^+$ individuals and KIR2DL1 in HLA-C2$^+$ individuals (76).

Similarly, in allogeneic HSCT, CMV reactivation is followed by an increase in the proportion of NKG2C$^+$ NK cells within 2–4 weeks, which persists for at least a year (86, 87). These NK cells also have a more mature NKG2C$^+$CD57$^+$ phenotype and are predominantly KIR$^+$, especially for inhibitory self-KIRs (KIR2DL2/3), and secrete more IFN-γ than NKG2C$^-$ NK cells (67, 87). CMV-seropositive recipients without overt CMV reactivation have an NK memory phenotype intermediate between patients with CMV reactivation and CMV-seronegative recipients,

but only if they received a sibling allograft and not umbilical cord blood, which is generally considered to be CMV naive (67). This observation would suggest that donor NK memory could be transferrable. In support of this hypothesis, the same group had previously demonstrated that the NKG2C$^+$ NK cells that emerged following CMV reactivation had increased levels of IFN-γ production when the donor was CMV seropositive rather than CMV seronegative (88). Furthermore, NK memory is undoubtedly transplantable in experimental mouse systems (74). The contribution of NK memory transfer in clinical allogeneic HSCT remains to be ascertained as NK reconstitution is primarily attributed to new NK cells generated from hematopoietic precursors rather than the expansion of NK cells from the graft, although this too remains to be conclusively demonstrated. NKG2C is not the only activating receptor relevant to CMV infection. CMV infection in healthy individuals also expands NK cells that express other activating receptors, including the activating KIRs: KIR2DS2 and KIR2DS4 (76). In allogeneic HSCT, recipients of umbilical cord blood transplant from donors with homozygous deletion of *NKG2C,* which represents 4% of the healthy population, have increased numbers of CD56^{dim}NKG2A$^-$ActivatingKIR$^+$ NK cells following CMV reactivation (69).

More recently, a distinct subset of FcRγ (also known as FcεRIγ)-deficient NK cells has been identified in CMV-seropositive individuals (89, 90). They are predominantly, but not exclusively, NKG2C$^+$, and respond poorly to CMV-infected lung fibroblasts, but display enhanced antibody-dependent expansion, degranulation, and cytokine secretion (61, 90). FcRγ-deficient NK cells can be detected in some patients at 6–12 months after umbilical cord blood transplantation, but only if they had prior CMV reactivation (62). This memory-like FcRγ$^-$ NK phenotype is the result of epigenetic modification with hypermethylation of the *FCER1G* promoter (62). Epigenetic silencing also results in a deficiency of the cell signaling proteins SYK and EAT-2, and transcription factors PLZF and IKZF2, within this population (61, 62). The significance of this newly described NK population in allogeneic HSCT remains to be investigated.

IMPACT OF GVHD ON NK RECONSTITUTION

Natural killer cell numbers were found to be lower in patients with acute and chronic GVHD (16, 91), but it is not known if these were casually related given the confounding effects of T cells, immunosuppression, and other clinical variables. On the other hand, acute GVHD is associated with the secretion of pro-inflammatory cytokines, for example, IL-12 and IL-18 (36), which are known to promote NK cell functional maturation. Acute GVHD is also associated with elevated levels of soluble ST2 (92), which serves as a decoy receptor to modulate the IL-33/ST2 axis (93, 94). This raises the possibility of effect on NK cells as IL-33/ST2 axis augments NK cell production of IFN-γ in response to IL-12 (95), and is important in MCMV-specific expansion of naive and memory Ly49H$^+$ NK cells (96). At present, all these concepts remain speculative and require further investigation.

INNATE LYMPHOID CELLS

The lineage marker-negative innate lymphoid cells (ILCs) are a recently identified family of lymphoid cells that are preferentially located at barrier surfaces and can rapidly secrete immunoregulatory cytokines that correspond to the T_H1, T_H2, or T_H17/T_H22 immune response (97, 98). Their role in allogeneic HSCT is gradually being elucidated and it has been recently shown that recipient-derived intestinal ILCs are important in mediating protection from gut GVHD (99, 100). However, the nature and role of donor-derived ILC reconstitution remains largely unknown at present (101).

CONCLUSION

Natural killer cells reconstitute rapidly after HSCT but the delayed acquisition of a mature phenotype and functional competency argues for strategies to enhance functional NK cell reconstitution. These strategies can include adoptive transfer (102–104), with or without *ex vivo* expansion and cytokine activation (105, 106), graft engineering (20), donor selection according to KIR haplotype, and exogenous cytokine administration (107). Key unanswered questions relevant to optimizing NK-mediated anti-leukemic and anti-viral immunity include: what are the desired phenotypic characteristics of NK cells in this regard? What are the relative roles of unlicensed and licensed NK cells? Does NK cell memory contribute to long-term tumor immune surveillance? How are NK cells educated in HLA-mismatched transplantation and does this change over time? Additionally, what is the nature of ILC reconstitution post allogeneic HSCT? The answers to these questions are important in improving transplant outcome and further experimental and clinical studies are needed.

AUTHOR CONTRIBUTIONS

MU wrote the manuscript. GH conceptualized and edited the manuscript. S-KT conceptualized, wrote, and edited the manuscript.

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Intrinsic Contribution of Perforin to NK-Cell Homeostasis during Mouse Cytomegalovirus Infection

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In addition to their role as effector cells in virus control, natural killer (NK) cells have an immunoregulatory function in shaping the antiviral T-cell response. This function is further pronounced in perforin-deficient mice that show the enhanced NK-cell proliferation and cytokine secretion upon mouse cytomegalovirus (MCMV) infection. Here, we confirmed that stronger activation and maturation of NK cells in perforin-deficient mice correlates with higher MCMV load. To further characterize the immunoregulatory potential of perforin, we compared the response of NK cells that express or do not express perforin using bone-marrow chimeras. Our results demonstrated that the enhanced proliferation and maturation of NK cells in MCMV-infected bone-marrow chimeras is an intrinsic property of perforin-deficient NK cells. Thus, in addition to confirming that NK-cell proliferation is virus load dependent, our data extend this notion demonstrating that perforin plays an intrinsic role as a feedback mechanism in the regulation of NK-cell proliferation during viral infections.

Keywords: mouse cytomegalovirus, perforin, NK cells, bone-marrow chimera, Ly49H, m157, proliferation

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INTRODUCTION

Natural killer (NK) cells play a crucial role in the early stage of mouse cytomegalovirus (MCMV) infection; however, the contribution of NK cells varies among different mouse strains (1). In C57BL/6 mice, the activating NK-cell receptor Ly49H mediates resistance to MCMV infection, because of the specific binding of virally encoded m157 protein (2, 3). NK cells exert their function by releasing antiviral cytokines and by cytolytic mechanisms mediated by perforin and granzymes. Mice lacking the Ly49H receptor fail to exert significant virus control by NK cells during the early post-infection (p.i.) days, because of the fact that MCMV expresses immune evasion mechanisms able to avoid or decrease other means of NK-cell engagement (4–6). In addition, MCMV downregulates major histocompatibility complex class I (MHC-I) molecules to avoid detection by $CD8^+$ T cells (7), but at the same time, the virally encoded *m04* protein escorts sufficient MHC-I complexes to the cell surface to ligate inhibitory Ly49 receptors and avoid NK-cell recognition (8). Thus, one can generalize that NK cells play a dominant role in MCMV control only in mice expressing Ly49H receptor and deletion of either the *m157* gene or blocking of the Ly49H receptor abolish the control in most of the organs (9–12). Perhaps the best evidence for the role of Ly49H/m157 interaction in MCMV control

by NK cells is illustrated by strong selection pressure imposed by NK cells, resulting in numerous mutations and deletions in the *m157* gene after passing the virus through the Ly49H$^+$ host (13).

Our research group along with other research groups has previously shown that NK-cell response to MCMV modulates subsequent CD8$^+$ T-cell response and that both specific activation of NK cells and perforin-dependent mechanisms are involved (14–18). In C57BL/6 mice infected with wild-type (WT) MCMV, CD8$^+$ T-cell response was markedly weaker compared to mice infected with the virus lacking *m157* gene, suggesting that specific virus control *via* Ly49H ligation dampens CD8$^+$ T-cell response (19). It was previously shown that Ly49H ligation *via m157* enhances NK-cell proliferation (20). Perforin deficiency in NK cells compromises MCMV control, in spite of the fact that proliferation and production of cytokines were stronger than in WT NK cells expressing perforin (21). The immunoregulatory impact of NK cells on CD8$^+$ T cells was still evident in perforin-deficient C57BL/6 (Prf1$^{-/-}$) mice. Under these conditions, perforin-deficient NK cells regulate CD8$^+$ T-cell response mostly by secreting inhibitory cytokine IL-10 (21). It remains unclear whether the enhanced proliferation of NK cells in Prf1$^{-/-}$ mice is caused by a high virus load or if it represents a homeostatic function of perforin.

Here, we aimed to elucidate the immunoregulatory potential of perforin with the emphasis on NK-cell proliferation and differentiation during infection. For the same, we used a model of bone-marrow chimeras possessing NK cells with or without perforin and tested their response to MCMV. We found that in addition to virus load-dependent Ly49H$^+$ NK-cell proliferation, perforin has an intrinsic role as a feedback mechanism in the regulation of NK-cell homeostasis during viral infections.

RESULTS

Perforin Deficiency Enhances IFN-γ Secretion and Proliferation of NK Cells during Early MCMV Infection

To assess the impact of perforin on NK-cell response to MCMV, C57BL/6 and Prf1$^{-/-}$ mice were infected with either WT MCMV or the virus mutant lacking *m157* (Δ*m157*), and virus titer was determined 3 days p.i. (**Figure 1A**). The proportion of mice in each group was subjected to depletion of NK cells by anti-NK1.1 monoclonal antibodies (mAbs). As shown previously (11, 22), MCMV control in C57BL/6 mice was almost completely dependent on Ly49H/m157 interaction. However, in perforin-deficient mice, the virus control by NK cells was essentially abolished, and no difference between WT virus and Δ*m157* mutant was found in spite of the fact that significantly more NK cells in perforin-deficient mice perforin produced IFN-γ, in comparison with WT control mice (**Figure 1B**). In C57BL/6 mice infected with Δ*m157* virus, we also found higher frequency of IFN-γ-producing NK cells in comparison with WT MCMV-infected mice, which correlates with a higher virus load and higher level of IFN-α and IL-12 in sera of Prf1$^{-/-}$ mice (**Figure 1C**). However, in Prf1$^{-/-}$ mice, higher level of cytokine production was observed irrespective of the virus used. Importantly, our results suggest that enhanced proliferation of NK cells in the absence of perforin is also driven by specific ligation of the NK-cell receptor Ly49H, because Ly49H$^+$ cells proliferate much more strongly in mice infected with WT virus, as compared with virus lacking *m157* [**Figure 1D**; (20, 21)].

It is well established that MCMV infection drives maturation of NK cells toward a terminally differentiated phenotype (23). NK cells derived from MCMV-infected Prf1$^{-/-}$ mice behaved in a similar fashion; however, the maturation was even more enhanced than in WT mice (**Figure 2A**). On day 6 p.i., the vast majority of NK cells in Prf1$^{-/-}$ mice were of terminally differentiated CD27$^-$CD11b$^+$ phenotype. This pattern was observed even in mice infected with Δ*m157* virus, although our data indicate that Ly49H/m157 interaction further enhanced maturation of NK cells (**Figure 2B**). Further evidence for terminal differentiation of NK cells in the absence of perforin was provided by following the expression of KLRG1 at different time points after infection. As shown in **Figure 2C**, almost all NK cells in Prf1$^{-/-}$ mice expressed KLRG1 on day 6 p.i., once again confirming acquisition of a completely mature phenotype (24).

In total, here, we showed that in the absence of perforin, NK cells proliferate far more and differentiate faster. In addition, a higher frequency of NK cells secrets IFN-γ, as compared to NK cells derived from control mice. It remains unclear whether these phenotypes of NK cells reflect the inability of Prf1$^{-/-}$ mice to control the virus *via* NK cells, or if perforin plays an additional, so far uncharacterized, regulatory function.

Enhanced Accumulation of Prf1$^{-/-}$ NK Cells Is Their Intrinsic Function

Although the results described above clearly demonstrated the different functional properties of NK cells in the absence of perforin, we were not able to confirm that this is indeed the intrinsic effect of perforin on NK cells. This is because all these phenotypes (enhanced proliferation, faster maturation, and increased production of cytokines) could simply be a consequence of higher virus load in Prf1$^{-/-}$ mice as compared to control C57BL/6 mice. To compare the proliferation and differentiation of Prf1$^{-/-}$ and WT NK cells in the presence of identical virus loads, we generated bone-marrow chimeric mice (**Figure 3A**). Bone-marrow cells derived from C57BL/6 mice (CD45.1$^+$) and Prf1$^{-/-}$ mice (CD45.2$^+$) were transferred at equal ratio into γ-irradiated C57BL/6 (CD45.1$^+$CD45.2$^+$) recipient mice. Eight weeks later, chimerism was confirmed (**Figure 3B**), and mice were infected with either WT, Δ*m157* MCMV, or left uninfected. Seven days later, mice were euthanized, and splenic and liver lymphocytes were analyzed (**Figures 3C,D**). In control uninfected chimeric mice, the frequency of Prf1$^{-/-}$ NK cells was similar to WT NK cells, indicating no apparent impact of perforin on the generation of NK cells in steady-state conditions. However, in agreement with the results shown in **Figure 1**, a much higher frequency of Prf1$^{-/-}$ NK cells was found both in spleen and liver in WT MCMV-infected chimeric mice, as compared to WT NK cells (**Figure 3C**). Although the absence of Ly49H/*m157* interaction resulted in a dramatic drop of NK-cell frequency, some differences between Prf1$^{-/-}$ and WT NK cells were preserved. Lower frequency of splenic NK cells and decreased differences between

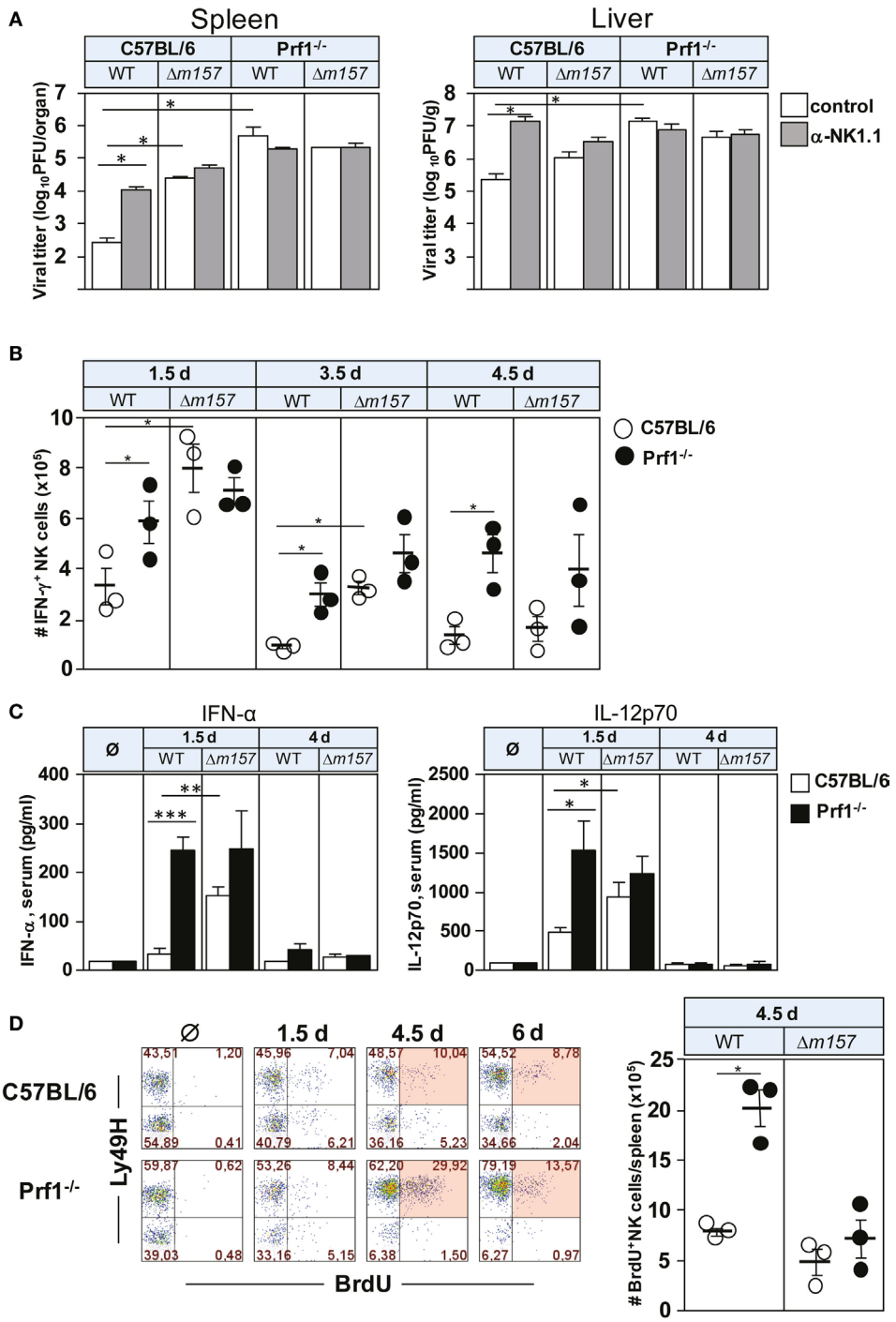

FIGURE 1 | Perforin deficiency enhances IFN-γ secretion and proliferation of NK cells during early MCMV infection. C57BL/6 and Prf1$^{-/-}$ mice were infected intravenously with 2 × 10^5 PFU of indicated viruses. **(A)** Mice, either NK-cell depleted or NK-cell undepleted before infection, were euthanized 3 days p.i. and titers in spleen (per organ) and liver (per gram) were determined. **(B)** On days 1.5, 3.5, and 4.5 p.i., splenocytes were assessed for IFN-γ production by CD3ε$^-$NK1.1$^+$ NK cells. **(C)** On days 1.5 and 4 p.i., serum levels of the indicated cytokines were determined. **(D)** On days 1.5, 4.5, and 6 p.i., mice were i.p. injected with 2 mg of BrdU and euthanized 3 h later. The frequencies of BrdU$^+$ CD3ε$^-$NK1.1$^+$ NK cells of both Ly49H$^+$ and Ly49H$^-$ subsets are depicted for wild-type (WT) MCMV infection (left). The number of BrdU$^+$ CD3ε$^-$NK1.1$^+$ NK cells on day 4.5 p.i. following WT and Δ*m157* MCMV infection is shown (right). Representative data of at least two independent experiments with three to five mice per group are shown. Data are presented as means ± SEM. Asterisks denote significant values: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

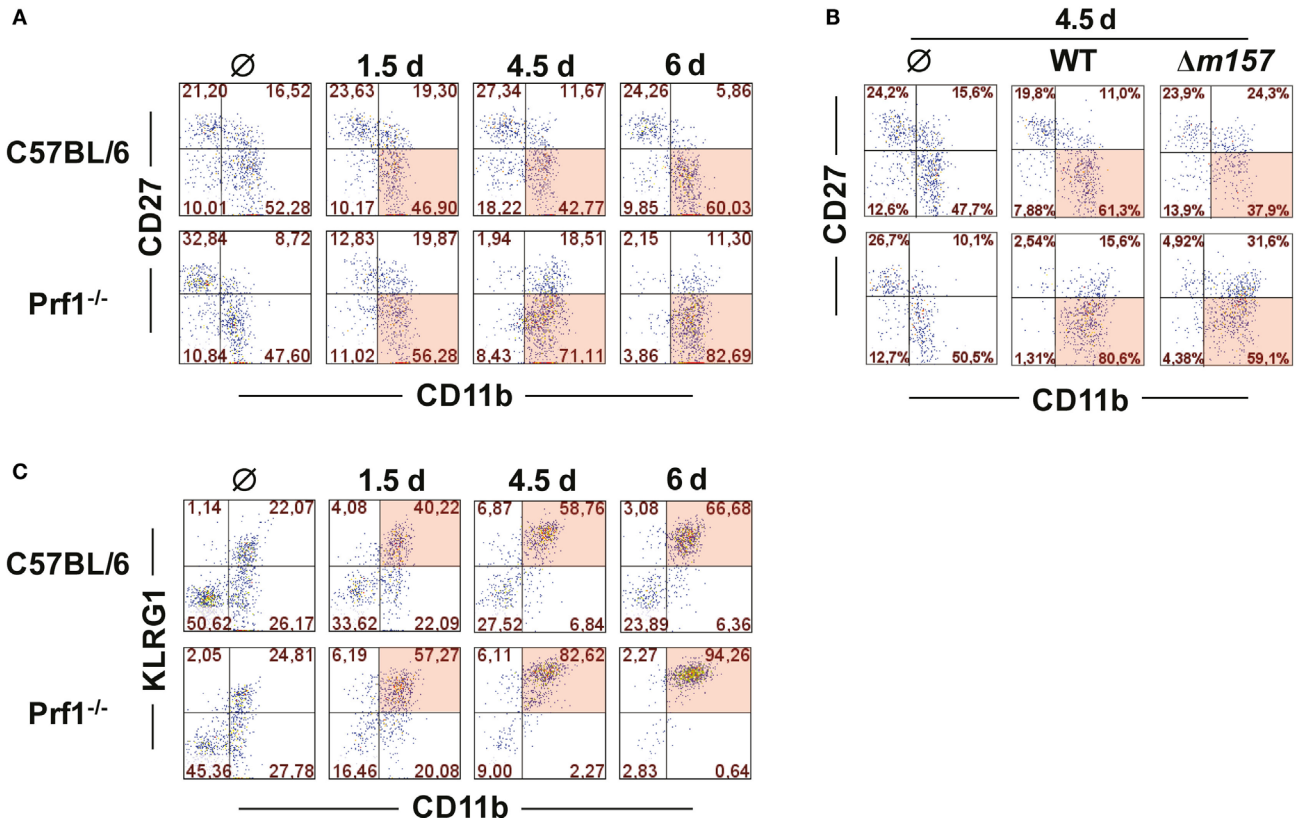

FIGURE 2 | Perforin deficiency enhances maturation of NK cells during early MCMV infection. C57BL/6 and Prf1$^{-/-}$ mice were infected intraperitoneally with 10^6 PFU of MCMV **(A,C)** or intravenously with 2×10^5 PFU of MCMV **(B)**. **(A)** At indicated time points, splenic CD3ε$^-$NK1.1$^+$ NK cells were analyzed for expression of CD27 and CD11b markers. **(B)** On day 4.5, maturation pattern of NK cells was determined for WT and Δ*m157* MCMV infection. **(C)** Frequencies of KLRG1 and CD11b coexpression on CD3ε$^-$NK1.1$^+$ NK cells at indicated time points p.i. are depicted. Representative data of at least two independent experiments with three to five mice per group are shown.

perforin-deficient and perforin-sufficient NK cells in mice infected with Δ*m157* MCMV are likely a consequence of high virus load, as shown previously (19, 21). In addition, the results suggest that enhanced proliferation of perforin-deficient NK cells requires their stimulation *via* a specific receptor. Notably, no significant differences in frequency of Prf1$^{-/-}$ and WT CD8$^+$ T cells were found (**Figure 3D**). A slightly lower frequency of CD8$^+$ T cells in Prf1$^{-/-}$ mice was observed, regardless of infection. Thus, we demonstrated that, in chimeric mice, the enhanced accumulation and differentiation of Prf1$^{-/-}$ NK cells is their intrinsic function, which becomes apparent during virus infection.

Intrinsic Function of Perforin on NK-Cell Proliferation and Maturation Is Ly49H Dependent

In **Figure 1**, we have shown that, during MCMV infection, NK cells of Prf1$^{-/-}$ mice proliferated more strongly, allowing them to reach the terminally differentiated phenotype faster than NK cells from control C57BL/6 mice. To rule out the impact of a different virus load, next we tested maturation pattern of NK cells in bone-marrow chimeras (**Figure 4A**). Here again, we observed that the frequency of terminally differentiated perforin-deficient NK cells was higher than the frequency of terminally differentiated WT NK cells. Moreover, the results also showed that the Ly49H/m157 interaction does play a role, because the differences between Prf1$^{-/-}$ and WT NK cells were not significant in mice infected with Δ*m157* virus. In line with this, the percentage of Ly49H$^+$ Prf1$^{-/-}$ NK cells was higher in comparison with Ly49H$^+$ WT NK cells (**Figure 4B**). Thus, the intrinsic function of perforin on NK-cell proliferation and differentiation is more evident if NK-cell activation is driven through ligation of specific receptor.

What could be the mechanism by which perforin affects proliferation and maturation of NK cells? Higher frequency of Prf1$^{-/-}$ NK cells in MCMV-infected mice could be either the consequence of changes in their transcriptome, enhanced proliferation (as indicated in **Figure 1C**), or prolonged survival. In bone-marrow chimeras, no differences in the expression of two key transcriptional factors regulating NK-cell development and differentiation, Eomes and T-bet, were detected between Prf1$^{-/-}$ and WT NK cells (**Figures 4C,D**). Next, we tested the NK-cell expression of Ki-67, a marker of-cell proliferation, and Bcl-2, an anti-apoptotic protein. As shown in **Figure 4E**, Ki-67

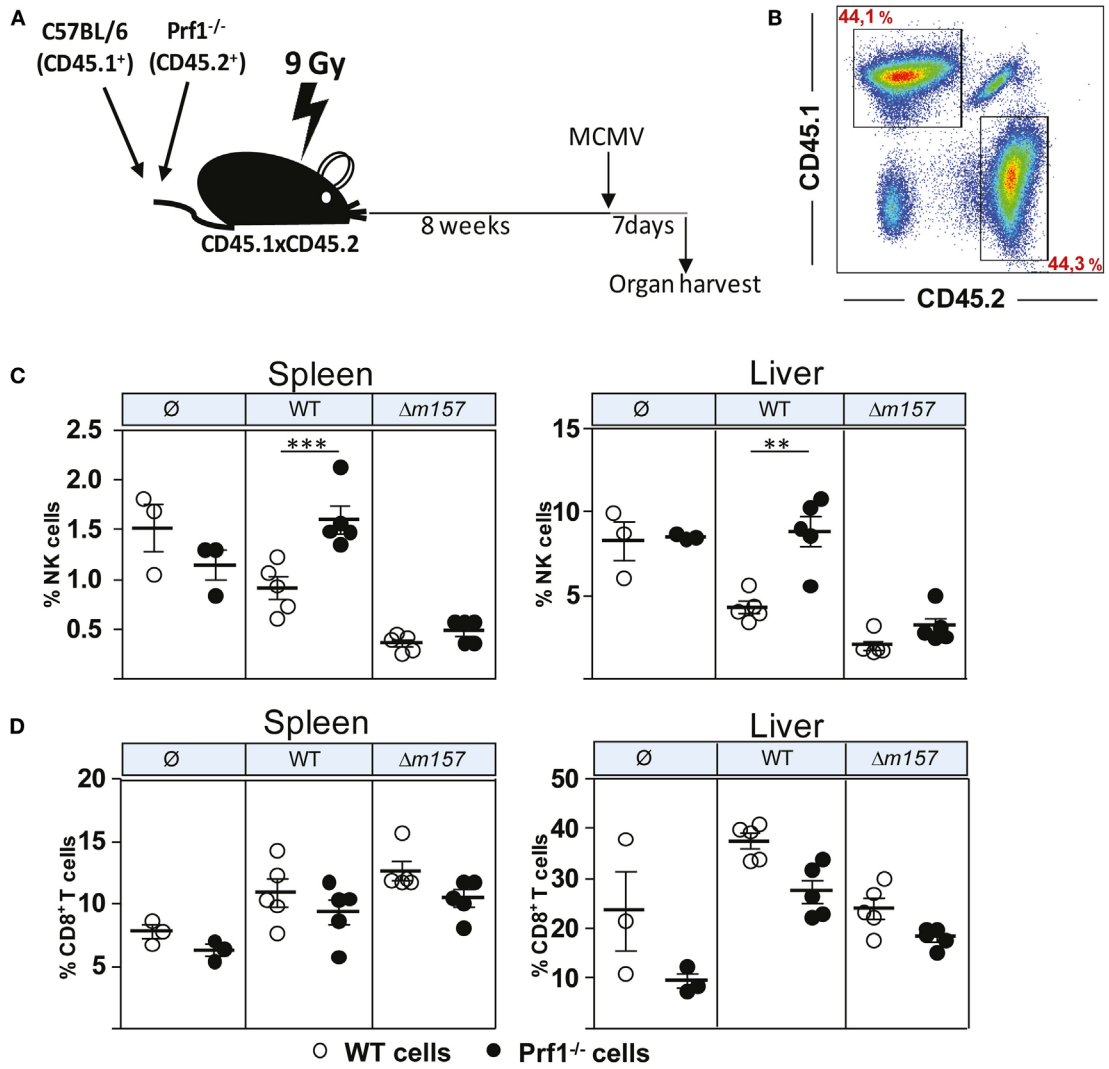

FIGURE 3 | Enhanced accumulation of perforin-deficient NK cells following MCMV infection in Prf1$^{-/-}$/C57BL/6 chimeric mice. **(A)** Mixed bone-marrow chimeric mice were prepared by transferring a 1:1 mixture of C57BL/6 (CD45.1^{+}) and Prf1$^{-/-}$ (CD45.2^{+}) bone-marrow cells to γ-irradiated CD45.1 × CD45.2 mice. Chimeric mice were i.p. injected with 5 × 10^{5} PFU of WT or Δ*m157* MCMV and euthanized 7 days p.i. **(B)** Representative dot plot showing reconstitution efficiency of bone-marrow chimera. **(C)** Frequency of NK cells and **(D)** CD8^{+}T cells in spleen (left) and liver (right) of Prf1$^{-/-}$/C57BL/6 chimeric mice is shown. Representative data of two independent experiments with three to five mice per group are shown. Data are presented as means ± SEM. Asterisks denote significant values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

was expressed in a greater proportion of Prf1$^{-/-}$ NK cells than in WT NK cells. This was the case even in mice infected with the virus lacking *m157* gene. In contrast to Ki-67, expression of Bcl-2 appears to be lower in Prf1$^{-/-}$ NK cells (**Figure 4F**).

Our next goal was to confirm that Prf1$^{-/-}$ NK cells indeed have a stronger capacity to proliferate compared to WT NK cells. To that aim, we used the adoptive transfer model in which we transferred CFSE-labeled Prf1$^{-/-}$ (CD45.2^{+}) and WT (CD45.1^{+}) splenocytes into MCMV-infected CD45.1^{+}CD45.2^{+} recipients (**Figure 5A**). Mice were euthanized on day 5 p.i., and CFSE dilution in NK cells was analyzed. In line with our data obtained in bone-marrow chimeras, we observed higher proportion of donor derived Prf1$^{-/-}$ NK cells compared to Prf1$^{+/+}$ NK cells following adoptive transfer (**Figure 5B**). In agreement with Ki-67 expression, CFSE was more diluted in Prf1$^{-/-}$ NK cells than in Prf1$^{+/+}$ NK cells (**Figure 5C**). Thus, our results confirmed that, in addition to serving as a major cytolytic molecule, perforin also plays an important intrinsic role by influencing proliferation capacity and differentiation of NK cells during virus infection.

DISCUSSION

Here, we describe a novel function of perforin in the regulation of NK-cell proliferation and differentiation during MCMV infection. In agreement with the previously described studies,

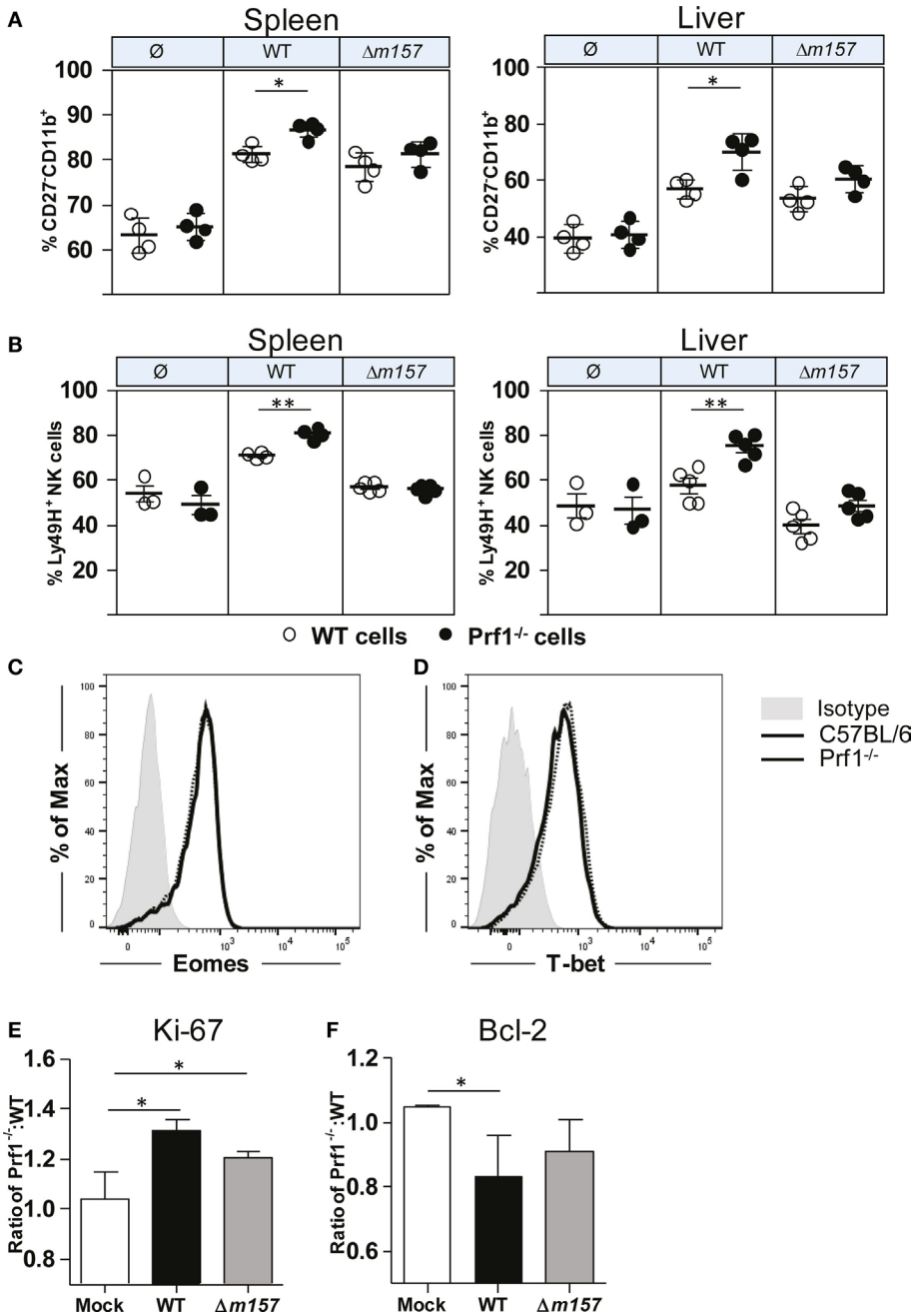

FIGURE 4 | NK-cell phenotype in Prf1$^{-/-}$/C57BL/6 BM chimeric mice. **(A)** Percentage of terminally differentiated CD27^{-}CD11b^{+} and **(B)** Ly49H^{+} NK cells is shown in spleen (left) and liver (right) of WT MCMV, Δ*m157* MCMV, and mock-infected Prf1$^{-/-}$/C57BL/6 BM chimeric mice. Representative histograms of **(C)** Eomes and **(D)** T-bet are shown. Relative expression of **(E)** Ki-67 and **(F)** Bcl-2 is shown calculated as ratio of expression of each marker in Prf1$^{-/-}$ NK cells and C57BL/6 NK cells. Representative data of two independent experiments with three to five mice per group are shown. Data are presented as means ± SEM. Asterisks denote significant values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

we first confirmed that in the absence of perforin, NK cells proliferate more strongly upon infection, compared to NK cells from control mice expressing this cytolytic molecule. Furthermore, this enhanced proliferation is accompanied with increased IFN-γ secretion and NK-cell maturation. The enhanced proliferation is much more evident if activating NK-cell receptor Ly49H is engaged. Yet, it remains unclear whether this is only a consequence of higher virus load, because the virus control is almost completely compromised, and infection is accompanied by high level of proinflammatory cytokines in the absence of perforin. To provide equal conditions for both perforin-deficient and perforin-sufficient

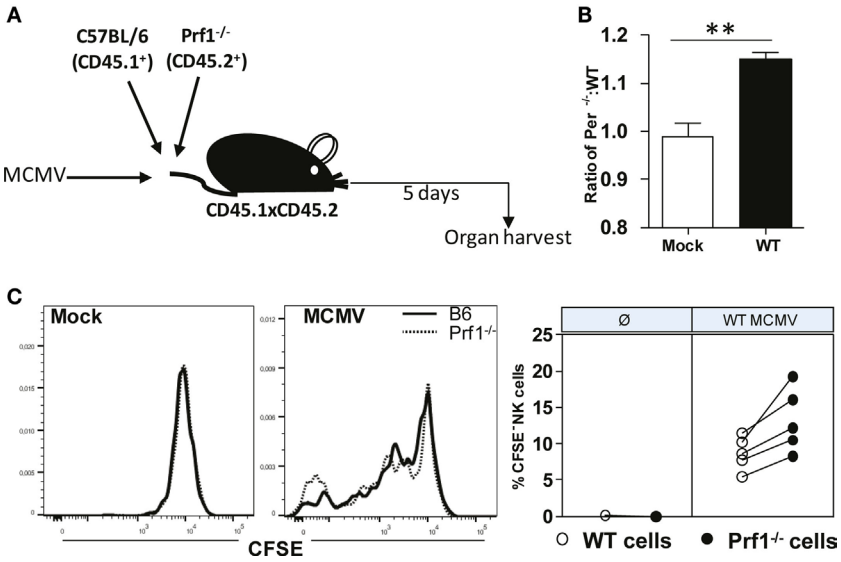

FIGURE 5 | Enhanced dilution of CFSE by perforin-deficient NK cells following MCMV infection in Prf1$^{-/-}$/C57BL/6 chimeric mice. **(A)** A 1:1 mixture of C57BL/6 (CD45.1$^+$) and Prf1$^{-/-}$ (CD45.2$^+$) CSFE-labeled splenocytes were adoptively transferred to CD45.1 × CD45.2 mice that were infected with MCMV 3 h prior to transfer. **(B)** Ratio of number of adoptively transferred Prf1$^{-/-}$ and C57BL/6 NK cells is depicted. **(C)** Proliferation of adoptively transferred Prf1$^{-/-}$ and C57BL/6 NK cells in spleen was determined by analyzing CFSE dilution on day 5 p.i. Representative histograms for mock and MCMV-infected mice (left), and quantification of CFSE$^-$ NK cells (right) are shown. Asterisks denote significant values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

NK cells, we used bone-marrow chimeras and demonstrated that perforin-deficient NK cells still proliferate more and differentiate faster, confirming the intrinsic function of perforin in NK-cell response to viral infections. Note that these differences were absent in uninfected animals, confirming that perforin deficiency does not compromise NK-cell maturation and differentiation under normal conditions.

At first glance, our results are in disagreement with the results published by Lee et al., who showed that coculture of NK cells with cells expressing *m157* gene results in equal proliferation of perforin-deficient and perforin-sufficient NK cells (21). However, these two models are not fully compatible, because *in vivo* infection conditions provide a different microenvironment, which is apparently essential for the phenotype that we observed. Several mechanisms could explain higher levels of perforin-deficient NK cells: (i) higher virus load and stronger cytokine response, (ii) homeostatic elimination by cytolytic cells, and (iii) prolonged interaction and enhanced activation of NK cells *via* the intercellular synapses with the infected cells. Lee et al. showed that an increase in virus load correlates with enhanced proliferation of NK cells (21). However, in bone-marrow chimeras, perforin-deficient and perforin-sufficient NK cells are exposed to the same virus load and cytokine environment. Another way by which perforin deficiency could result in enhanced accumulation of NK cells could be as a consequence of decreased elimination of these cells by perforin-expressing effector cells. It is well established that perforin regulates immune cell homeostasis. During LCMV infection, perforin-mediated killing is involved in deletion of anergic antigen-specific CD8$^+$ T cells (25). Furthermore, regulatory T cells can induce death of tumor infiltrating NK and CD8$^+$ T cells in a perforin-dependent manner (26). NK cells can also eliminate other cells as a part of their homeostatic function. Crouse et al. have previously shown that, in mice lacking type I IFN receptor, CD8$^+$ T cells are highly susceptible to NK-cell killing in a perforin-dependent manner (27). NK cells can also commit fratricide in certain conditions and by doing so participate in NK-cell homeostasis (28, 29). However, the differences in accumulation of NK cells between Prf1$^{-/-}$ and WT mice should be abolished in chimeric mice. Although, in this work, we were unable to characterize the ultimate mechanisms by which perforin regulates NK-cell maturation, it is worth mentioning that the recent study by Jenkins et al. showing that perforin-deficient NK cells form prolonged synapses with target cells (30). This leads to repetitive calcium signaling and enhanced production of cytokines. Interestingly, perforin-deficient NK cells remained in synapse with their targets for a significantly longer period of time than granzyme A- and B-deficient NK cells, but there was no difference in the case of CD8$^+$ T cells lacking these cytolytic molecules. In addition, as proposed by Sad et al., prolonged interaction because of inability of cytotoxic cells to kill target cells may be sufficient for sustained stimulation and activation of effector cells (31). It needs to be tested whether or not this could explain better survival and enhanced differentiation of perforin-deficient NK cells.

Negative feedback mechanisms are essential for any physiological function. Here, we described the novel regulatory function of perforin in homeostasis of NK cells during virus infection, and excluded several mechanisms that have been described to be involved in homeostatic regulation of NK cells and other effector cells. Further studies are required to fully elucidate this novel immunoregulatory role of perforin during viral infection.

MATERIALS AND METHODS

Mice

C57BL/6, Prf1$^{-/-}$ (32), C57BL/6 CD45.1^{+}, and C57BL/6 CD45.1^{+}CD45.2^{+} mice were housed and bred under specific-pathogen-free conditions at the Central Animal Facility of the Medical Faculty, University of Rijeka, in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. The Animal Welfare Committee at the University of Rijeka approved all animal experiments described in this paper. Eight- to 12-week-old mice were used in all experiments.

Viruses

Mice were injected intravenously (i.v.) or i.p. with 2–5 × 10^5 PFU of the tissue culture (TC)-grown virus in a volume of 500 µl of diluent (PBS or DMEM media). Bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 has previously been shown to be biologically equivalent to MCMV strain Smith (VR-1399) and is hereafter referred to as WT MCMV (33). In addition to MW97.01, the mutant virus lacking *m157* gene was used (9).

Depletion of Lymphocyte Subsets and the Determination of Viral Titers *In Vivo*

The depletion of NK cells *in vivo* was performed by intraperitoneal injection of 300 µg of purified MAb PK136 (34) at 1 day before infection and 1 day p.i. For quantifying viral infectivity in organs, virus titers were determined by standard plaque assay, as described previously (35).

Flow Cytometry and Intracellular Staining

Splenic leukocytes were prepared, as previously described, and in order to decrease non-specific staining, Fc receptors were blocked with 2.4G2 mAbs (36). The following *mAbs* were purchased from eBioscience or BD Pharmingen, and cell-surface staining was performed specifically for the following antigens: anti-CD3ε (145–2C11), anti-NK1.1 (PK136), anti-Ly49H (3D10), anti-CD27 (LG.7F9), anti-CD11b (M1/70), anti-CD8α (53–6.7), anti-IFN-γ (XMG1.2), anti-CD19 (1D3), anti-KLRG1 (2F1), anti-Ki-67 (SolA15), anti-Bcl-2 (10C4), and PE-Cyanine7-labeled streptavidin (SA-PE-Cy7). For the *in vivo* cell proliferation assay, mice were i.p. injected with 2 mg of bromodeoxyuridine (BrdU; Sigma) and euthanized 3 h later. To detect incorporated BrdU, splenic leukocytes were first stained for surface antigens and then fixed, permeabilized, refixed, treated with DNase I, and intracellularly stained according to the manufacturer's protocol (BrdU flow kit; BD Pharmingen). For the detection of IFN-γ expression by NK cells, incubation was performed in RPMI medium supplemented with 10% of fetal calf serum (FCS; Gibco) for 5 h in the presence of 500 IU/ml of interleukin-2 (IL-2) at 37°C, with 1 µg/ml of brefeldin A (eBioscience) added for the last 4 h of incubation. After incubation, cells were first-surface stained and then fixed and permeabilized using Cytofix/Cytoperm solutions (BD Pharmingen) followed by intracellular IFN-γ staining, according to the manufacturer's protocol. Staining of Ki-67 was done with the FoxP3 staining buffer set (eBioscience). For Bcl-2 intracellular staining, permeabilization and fixation of cells were done with the Intracellular Fixation and Permeabilization Buffer Set (eBioscience). Flow cytometry was performed on FACSCalibur and FASCAria (BD Bioscience; San Jose, CA, USA), and data were analyzed using the FlowJo software (Tree Star).

Quantification of Serum Cytokine Levels

Serum levels of IFN-α were determined by an enzyme-linked immunosorbent assay (ELISA) kit for IFN-α (PBL Biomedical Laboratories), according to the manufacturer's instructions. Serum levels of IL-12 were determined by Bio-Rad mouse cytokine multiplex assay, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Bone-Marrow Chimeras

Bone-marrow chimeras were prepared using 8-week-old mice as donors and recipients. Briefly, C57BL/6 (CD45.1^{+}CD45.2^{+}) recipient mice were γ-irradiated with 9 Gy. After 24 h, recipient mice were injected i.v. with 10^7 of donor bone-marrow cells containing a 1:1 mixture of C57BL/6 (CD45.1^{+}) and Prf1$^{-/-}$ (CD45.2^{+}) bone-marrow cells. Recipient mice were maintained on antibiotic water containing enrofloxacin for 2 weeks following irradiation. Chimerism was evaluated 7 weeks post-transfer, and chimeras were used in experiments after 8 weeks of reconstitution.

Adoptive Transfer of CFSE-Labeled Splenocytes

Splenic lymphocytes were isolated from C57BL/6 and Prf1$^{-/-}$ mice and mixed at a ratio of 1:1. Splenocyte mixture was labeled with CFSE, as described previously (37). Briefly, cells were washed twice with PBS and suspended in 5-µM CFSE solution, followed by 12 min of incubation at 37°C. The cells were washed three times in complete RPMI media supplemented with 5% FCS and resuspended in complete RPMI. To verify the CFSE labeling of cells, samples were analyzed by flow cytometry. Recipient, non-irradiated C57BL/6 CD45.1^{+}CD45.2^{+} mice were injected i.v. with 5 × 10^7 of CFSE-labeled cells.

Statistical Analysis

Statistical analysis was carried out using Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistically significant differences between two data sets in cytokine assays and phenotype analyses were determined by the unpaired two-tailed Student's *t*-test, and *P* values of <0.05 were considered significant. Differences in viral titers were determined by two-tailed Mann–Whitney *U*-test.

ETHICS STATEMENT

The study was approved by the ethical committee of the Animal Welfare Committee at the University of Rijeka.

AUTHOR CONTRIBUTIONS

MA, IB, BP, AK, Stefan Jordan, and JA performed experiments. Slaven Jurkovic provided technical support. Stipan Jonjić,

AK, MA, IB, BP, and JA designed experiments and wrote the manuscript.

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Microchip Screening Platform for Single Cell Assessment of NK Cell Cytotoxicity

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Here, we report a screening platform for assessment of the cytotoxic potential of individual natural killer (NK) cells within larger populations. Human primary NK cells were distributed across a silicon–glass microchip containing 32,400 individual microwells loaded with target cells. Through fluorescence screening and automated image analysis, the numbers of NK and live or dead target cells in each well could be assessed at different time points after initial mixing. Cytotoxicity was also studied by time-lapse live-cell imaging in microwells quantifying the killing potential of individual NK cells. Although most resting NK cells (≈75%) were non-cytotoxic against the leukemia cell line K562, some NK cells were able to kill several (≥3) target cells within the 12-h long experiment. In addition, the screening approach was adapted to increase the chance to find and evaluate serial killing NK cells. Even if the cytotoxic potential varied between donors, it was evident that a small fraction of highly cytotoxic NK cells were responsible for a substantial portion of the killing. We demonstrate multiple assays where our platform can be used to enumerate and characterize cytotoxic cells, such as NK or T cells. This approach could find use in clinical applications, e.g., in the selection of donors for stem cell transplantation or generation of highly specific and cytotoxic cells for adoptive immunotherapy.

Keywords: NK cells, cytotoxicity, single cell analysis, microchip, screening, microscopy, fluorescence, immune synapse

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INTRODUCTION

Cytotoxic effector lymphocytes, such as natural killer (NK) cells and T cells, are important for immune defense against cancer and viral infections, the traits that have made these cells valuable in adoptive cell therapy. However, their activity is also associated with detrimental conditions, such as autoimmunity or graft-versus-host disease (GVHD), after allogeneic hematopoietic stem cell transplantation (HSCT). Upon activation, both effector cell types are able to kill abnormal cells through release of toxic granules containing perforin and granzymes at the tight intercellular contact formed at the immune synapse (1, 2).

NK cell activation relies on a balance between activating and inhibitory signals from a range of cell surface receptors recognizing ligands on the target cell surface. Inhibitory signals are mediated

by MHC class I proteins that are expressed by most normal cells. However, some infections and transformations lead to down-regulation of MHC class I and/or upregulation of activating NK cell ligands rendering them susceptible to NK cell attack. A functional NK cell repertoire is generated through cellular education, resulting in a heterogeneous NK cell population with varying capacity to respond to stimuli (3–6). Little is known about the functional consequences of education and how this relates to the individual NK cell cytotoxic response observed. However, clinical trials using NK cells from haploidentical donors for cell therapy have shown encouraging results indicating that interindividual differences in NK cell recognition and responsiveness can be used to treat disease (7). Importantly, these studies also established a link between the number of alloreactive NK cells in the graft and patient survival. However, one limitation is that there are few efficient methods to enumerate the fraction of cytotoxic NK cells from a donor sample for a given donor–recipient pair. Thus, new methods to quantify the fraction of alloreactive NK cells and cytolytic potential of individual NK cells could be valuable for the process of selecting donors for therapy.

During the past years, several new tools for single cell analysis have been developed, and some of those have been used to dissect T or NK cell heterogeneity in terms of phenotype, cytotoxicity, or cytokine release (8–22). Here, we use a previously reported microchip platform (23, 24) to screen the cytotoxic response of human peripheral blood NK cells against transformed human cells. This tool complements currently used population- and flow-based techniques as it quantifies the fraction of cytotoxic cells and resolves the cytotoxic potential of individual cells. We find donor-to-donor differences in the fractions of cytotoxic NK cells, a dependence on the choice of target cell and significant heterogeneity in cytotoxic capacity of individual cells.

MATERIALS AND METHODS

Microchip and Holder

Fabrication of microchips was performed as previously described (24). Briefly, microwell layout was defined by lithography followed by deep-reactive ion etching and surface oxidation growth. The microwells were sealed at one end by anodic bonding of a thin (175 μm) glass to the silicon, and the wafer was diced into individual microchips (22 mm × 22 mm × 475 μm). During loading and imaging, the microchip was placed in a custom-made holder with plastic lid held together by four magnets. To reduce evaporation, but maintain oxygen and carbon dioxide exchange, a 35-mm Petri dish lid was placed over the holder lid. Before cell seeding, the chip was covered with cell medium and primed in vacuum to allow liquid to enter the wells.

Cells and Reagents

All experiments with human cells were performed according to local ethics regulations. Human NK cells were isolated from PBMCs of anonymous healthy donors by negative selection using the NK cell Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. Freshly isolated NK cells were maintained in RPMI 1640 cell culture media (Sigma-Aldrich) supplemented with 10% human serum (Blood bank, Karolinska Hospital), 50 U/mL penicillin–streptomycin, 2 mM L-glutamine, 1× non-essential amino acids, 1 mM sodium pyruvate (all from Sigma-Aldrich), and naive NK cells were used within 2 days. For the activation of NK cells, 200 U/mL of IL-2 (Peprotech) was added to the cell culture media, and the NK cells were then used within 6–9 days. The NK cell purity of the isolated cell population was >95% $CD3^-CD56^+$ as confirmed by flow cytometry. The leukemia cell line K562 and human embryonic kidney (HEK)293T cells were cultured in RPMI 1640 media supplemented with 10% FBS, 50 U/mL penicillin–streptomycin, and 2 mM L-glutamine (all from Sigma-Aldrich).

For fluorescent staining before imaging, cells were washed, incubated with the appropriate dye dissolved in RPMI 1640 for 10 min at 37°C, washed, and used for experiments. Final staining concentrations were 0.5–1.0 μM for CellTrace Calcein Green AM, 5 μM FarRed DDAO-SE (target cells), and 0.4–0.6 μM CellTrace Calcein Red-Orange AM (NK cells) (all dyes were from Invitrogen). The family of CellTrace calcein dyes freely diffuses over the cell membrane. Once inside the cell, cytoplasmic enzymes hydrolyze the dye, causing the polarized product to leak out much more slowly than it entered. Calcein dyes give a uniform fluorescent cytoplasmic staining as long as the membrane is intact making them suitable for detection of cell viability. On the other hand, DDAO forms covalent bonds to primary amines present everywhere in the cell and remains detectable after cell death has occurred.

Microscopy and Image Analysis

Images were obtained at 10× magnification using any of four inverted confocal microscopes (Olympus IX81, Zeiss LSM 510 Meta, Zeiss LSM 780, or Zeiss LSM 880) equipped with environmental chambers maintained at 37°C, 5% CO_2, and motorized stages enabling automatic screening. Screening the whole chip required acquisition of 400 separate images, taking ~45 min. Often, analysis was performed only on parts of the chip.

The number of effector cells, as well as live and dead target cells in each well, was quantified either automatically by a software routine developed in Matlab or by a combination of automatic and manual analysis. Briefly, the image analysis software first identified the microwells using the transilluminated channel. Thresholds were applied to the fluorescence channels. Remaining objects were convolved with a Gaussian chosen to be of approximately the same size as the cells to improve circularity and to simplify separation of clustered cells. An algorithm based on the circular Hough transform (25) was applied to search for roughly circular objects. NK cells, due to their irregular non-circular morphology, were instead identified using an algorithm for finding connected components corresponding to single NK cells (25). The classification of live and dead target cells was performed by comparing the green (calcein green) and red (DDAO) fluorescent intensity from each individual target cell. The threshold was set either manually or automatically by selecting the threshold that maximizes the mean of the pairwise Euclidean distances between the intensity values of live and dead targets cells. The

software then returned a numbered map of the detected wells and the number of objects in each well, together with a heuristically chosen figure of goodness describing the chances that the software had made a correct determination of the number and status of cells. This figure was based on several factors, such as large numbers of cells in a single well, the existence of large contiguous objects (likely to be multiple cells), bright objects that could not be identified as cells, and amount of overlap of identified cells. Accuracy of the automatic counting was decreased at screens performed at later times due to decreased fluorescence intensity (mainly affecting the NK cells) and debris from dying target cells in some of the wells. In such cases, manual counting was performed.

Statistical Analysis

Yates Chi-square test was used to evaluate significance. p-Values above 0.05 were considered not significant (n.s.), whereas p-values below 0.05 were considered significant and marked by stars ($0.01 < p < 0.05$ marked as *, $0.001 \leq p \leq 0.01$ marked as **, and $p < 0.001$ marked as ***).

RESULTS AND DISCUSSION

Microchip Cytotoxicity Assay

The microchip platform consisted of a silicon–glass microchip held in place by an aluminum plate, a polydimethylsiloxane (PDMS) gasket, and a plastic lid, and it was designed to fit in a conventional inverted fluorescence or confocal microscope (**Figure 1A**). The microchip contained 32,400 individual wells (≈50 μm × 50 μm) arrayed to facilitate screening with a 10× objective (**Figure 1B**). Deep wells (300 μm) prevented cells from escaping the wells, and thin glass at the bottom (175 μm) allowed high-resolution imaging (18, 24).

To assess single cell cytotoxicity, NK cells were labeled with the fluorescent viability dye calcein orange and target cells with calcein green and DDAO. This allowed detection of live NK cells and distinguishing live from dead target cells (**Figures 1C,D**). Target cells were seeded on the chip and allowed to sediment in to the microwells, then NK cells were added creating an E:T ratio of ≈1:2.5–5, with the higher E:T ratio used to screen for serial killing NK cells. The chip was then screened to assess the number of effector cells and live/dead target cells in each well at the beginning of the experiment. In addition, in experiments requiring more precise enumeration of NK cell-mediated killing events, a prescreen of seeded target cells was acquired before seeding the effector cells. The cells were then left to incubate under physiological conditions (typically for 6 h) to allow effector-mediated lysis to occur before the chip was screened again. The incubation-screening cycle was repeated until the experiment was stopped. The number of effector cells and live/dead target cells was counted with the in-house automatic counting routine, and the cytotoxic potential of effector cells was assessed.

Screening of NK-Mediated Killing of Tumor Cells

To investigate NK-mediated killing in the microchip, human peripheral blood NK cells were isolated from healthy donors and activated in IL-2 for 6–9 days. On the day of experiment, NK cells were labeled and mixed on the chip with labeled target cells. Target cells used were either the cell line K562 originally derived from a human patient chronic myeloid leukemia (CML) in blast crisis (26) or HEK293T cells. Cytolytic and non-cytolytic NK

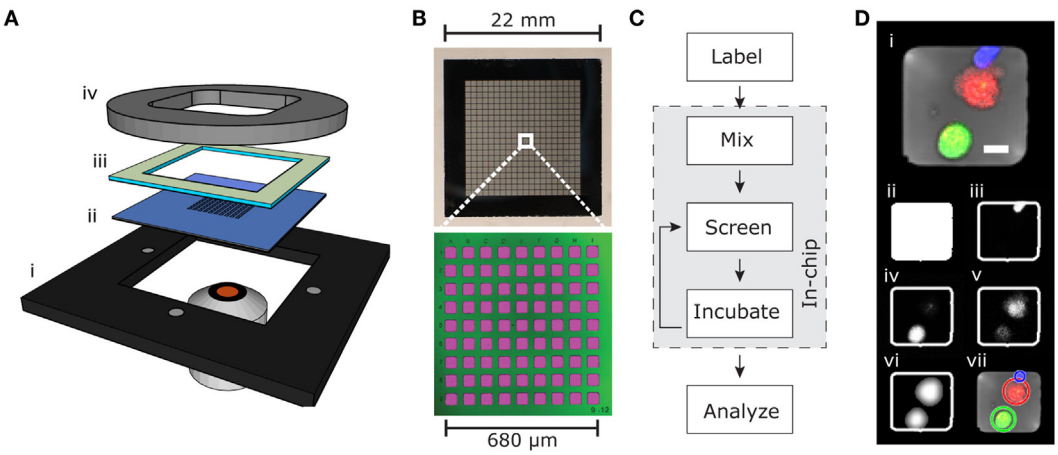

FIGURE 1 | Microchip screening platform. **(A)** Holder (i), microchip (ii), PDMS gasket (iii), and plastic lid (iv). **(B)** Detailed view of the silicon–glass microchip showing photograph of the whole chip (top) containing 32,400 microwells arranged in 20 × 20 subunits each containing 9 × 9 microwells (bottom). The bottom image is produced by confocal fluorescence imaging of microwells filled with fluorescent solution (pink) and reflection from the silicon–glass interface (green). **(C)** Flow chart outlining the screening process. **(D)** Example RGB-transmitted light composite image (i) from an individual well containing one live target cell (green), one dead target cell (red), and one NK cell (blue). Scale bar represent 10 μm. The automated software-detected microwell is shown in (ii). The blue channel (iii) shows the calcein orange-stained NK cell, and the green channel (iv) shows the calcein green-stained target cell. The red channel (v) contains both weak DDAO fluorescence from the live target cell and stronger DDAO fluorescence from the dead target cell. The filtered image of the target cells (vi) is used for the automated software detection of live and dead target cells shown as circles in (vii).

cells were observed against both types of target cells (examples in **Figure 2A**). To assess the level of NK killing, wells containing single NK cells and at least one live target cell at time T_{0h} were analyzed after 6 (T_{6h}) and 12 h (T_{12h}), and the number of wells where target cell death had occurred was counted. NK cells from three donors were tested against both types of target cell. Target cell death was observed in ≈20–70% of wells for HEK293T and in ≈30–70% of wells for K562. Spontaneous target cell death was detected in only a few percent of the wells (**Figure 2B**). We did note a background level of target cell death seen as a fraction (~5–7%) of the target cells being dead already at T_{0h} (Figure S1A in Supplementary Material). This fraction was higher in wells containing NK cells (≈10–15%) (Figure S1B in Supplementary Material), due to NK cell-mediated killing occurring before the first imaging was completed at T_{0h}. As a consequence, the fraction of cytolytic NK cells presented in **Figure 2B** is slightly underestimated. The data showed that the fraction of cytolytic NK cells was somewhat higher for the K562 target cells and that the donor-to-donor differences seemed to be more pronounced for HEK293T cells. The fractions of cytolytic NK cells increased marginally between T_{6h} and T_{12h}, indicating that not all lysis was completed during the first part of the assay (**Figure 2B**). Together, these experiments show that this screening approach allows the fraction of lytic effector cells within populations to be determined, differences between donors or types of target cells to be detected, and the change of NK cell-mediated lysis to be monitored over time.

Time-Lapse Imaging of NK Cell Immune Surveillance

Time-lapse imaging of human NK cells interacting with K562 cells in microwells was also performed. K562 target cells followed by resting (non-activated) NK cells were seeded across the microchip at an E:T ratio of ≈1:3 and imaged at 10 parallel positions, each covering 81 microwells, every 3 min for 12 h (examples of time-lapse sequences can be found in Movies S1–S3 in Supplementary Material). From three independent experiments, 1295 NK cells and 3323 target cells distributed in 775 microwells were analyzed based on the criteria that only microwells containing at least 1 NK and 1 target cell were studied. We scored 425 killing events committed by 326 NK cells, indicating that ≈25% of the NK cells exhibited cytotoxicity. As expected, this number is lower than what was found for IL-2-activated NK cells (**Figure 2**). In wells with single NK cells and ≥3 live target cells, 72% of the NK cells did not kill any target cells, whereas the remaining cells were cytotoxic (**Figure 3A**). A small fraction of NK cells (≈1%) was scored as "serial killers," as they killed three or more target cells during the assay. One NK cell was observed to kill as many as eight target cells (**Figure 3B**; Movie S3 in Supplementary Material). Although serial killers have been described before among IL-2-activated human peripheral blood NK cells (16, 17, 27), the phenomenon has been less studied among resting NK cells. We currently know very little about these NK cells with extraordinary lytic potential, partly because they are "invisible" in population-based methods or in flow cytometry where high cell surface expression of $CD107a^+$ is used as a sign for degranulation and cytotoxicity after stimulation with target cells. Given that the fraction of serial killers under steady-state conditions is low, efficient methods for studying the response of single NK cells are needed to gain better understanding of these cells.

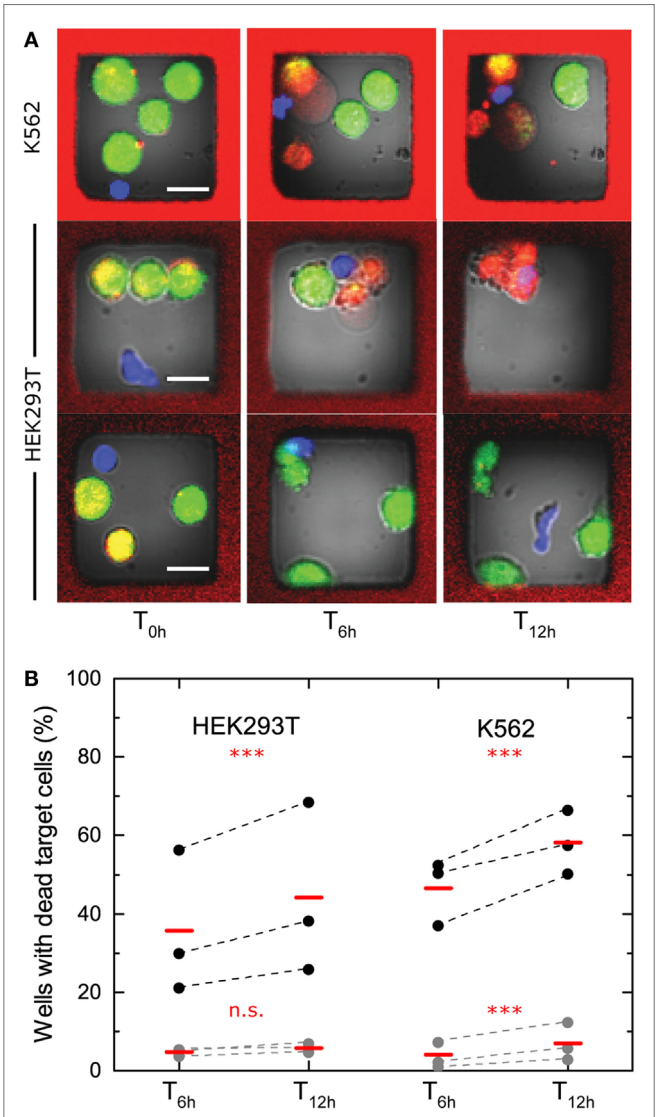

FIGURE 2 | Cytotoxicity screening of IL-2-activated human peripheral blood NK cells. (A) Examples of images acquired at times 0, 6, and 12 h of wells containing single NK cell and three to four K562 (top) or HEK293T (bottom) target cells. Target cell death was observed by loss of green fluorescence from calcein and increased red fluorescence from DDAO. The NK cells shown in the top two rows both kill three target cells during the assay, whereas the NK cell shown at the bottom row is non-cytolytic. Scale bars represent 15 μm. Images have been resampled and adjusted for brightness and contrast. **(B)** Fraction of cytotoxic NK cells assessed after 6 and 12 h with HEK293T target cells (left, three separate NK cell donors, $n_{total} = 997$) or K562 target cells (right, three separate NK cell donors, $n_{total} = 1808$). Spontaneous target cell death is shown in gray ($n_{total, HEK293T} = 2423$, $n_{total, K562} = 3467$). Data points from individual donors are connected by dashed lines and mean values are shown with red bars. The presented data are based on target cells dying during the assay, i.e., death events scored between the screens at T_{0h}, T_{6h}, and T_{12h}. Statistical analysis was performed on total number of observations for each cell type and significance is indicated by red stars or letters.

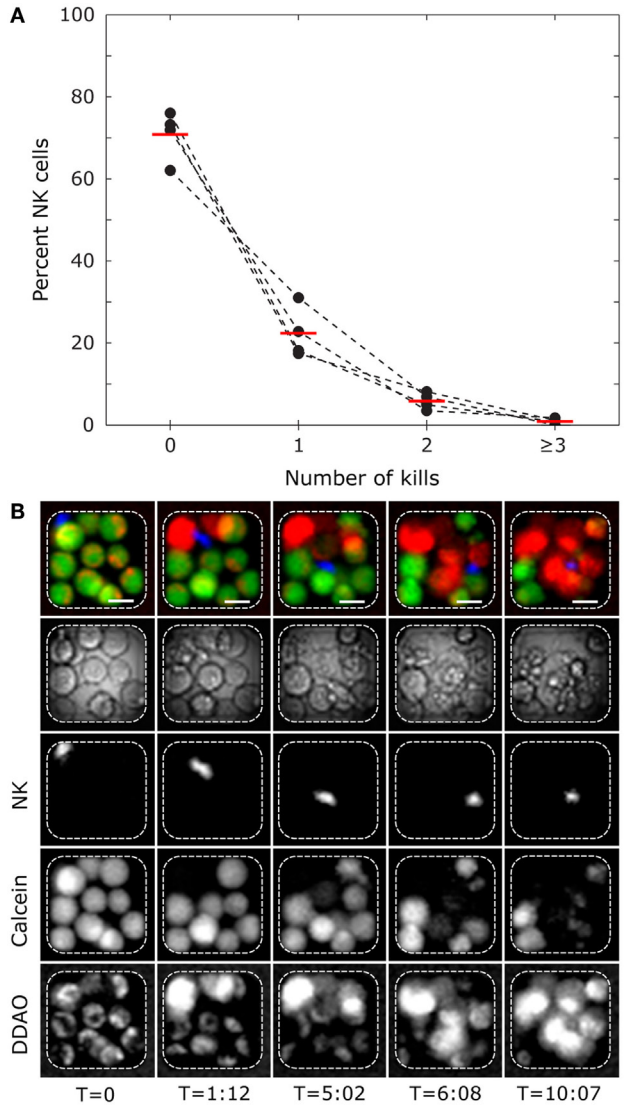

FIGURE 3 | Single-cell level cytotoxicity of resting human polyclonal NK cells revealed by time-lapse imaging in microwells. (A) Frequency of NK cells killing between 0 and ≥3 target cells. Wells containing single NK cells and at least three live K562 target cells were selected for analysis. Presented data are from four donors (black circles connected by dashed lines) with mean values shown by red bars (n_{total} = 322). **(B)** Time-lapse sequence showing a serial killer that eliminates 8 target cells in the 12-h experiment. The rows show from top to bottom: RGB composite of fluorescent channels for NK (blue) and target cells (green and red), transmitted light, calcein orange NK channel, target cell calcein green, and target cell DDAO channels. Each column represents different time points (hours:minutes). Scale bars represent 15 μm. Images have been resampled and adjusted for brightness and contrast.

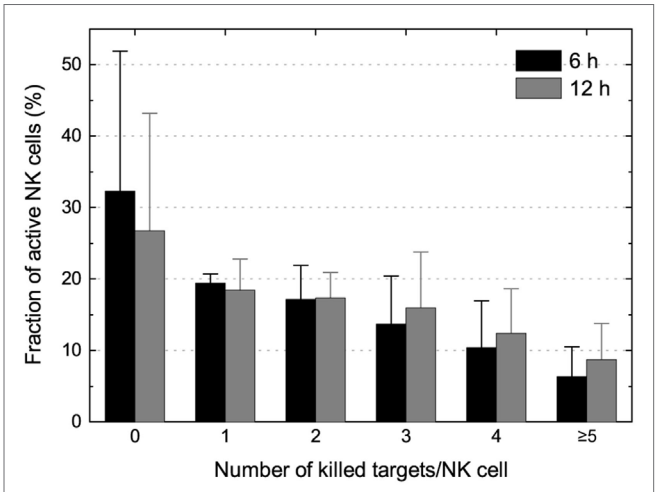

FIGURE 4 | Enumeration of killing potential and assessment of NK serial killing. Distribution of NK cells killing 0, 1, 2, 3, 4, or ≥5 K562 target cells at T_{6h} (black bars) and T_{12h} (gray bars). Wells were selected for analysis based on having a single live NK cell at T_{0h} (and ≤1 live NK cell at later time points) and ≥5 live target cells before NK cell seeding (T_{-NK}).

Assessing Serial Killing by NK Cells

Finally, we decided to further evaluate the cytolytic potential of individual NK cells by adapting the assay toward accurate enumeration of killing events and detection of NK serial killers. The screening assay was adapted in three ways. (1) In the first cytotoxicity screening, it was observed that some NK cells killed targets before imaging at T_{0h} (Figure S1B in Supplementary Material); therefore, a prescreen of the seeded K562 cells were acquired prior to addition of the NK cells (T_{-NK}). (2) The seeding density of target cells was doubled in order to give most NK cells a chance to kill multiple target cells. (3) Only wells that contained ≥5 live target cells at T_{-NK} and a single live NK cell were selected for further analysis.

Pooling data from three individual experiments of IL-2-activated NK cells and K562 target cells showed that the fraction of NK cells gradually decreased with the number of killing events scored (**Figure 4**). Comparing the screens performed at T_{6h} and at T_{12h} showed that the majority of killing took place during the first 6 h of the assay. However, as evident from the decreased population of NK cells killing zero target cells when comparing T_{6h} and T_{12h}, a small fraction of the NK cells did not perform their first kill until after 6 h. This could be caused by the requirement of initial NK–target cell interactions before activation and killing (28), but a contribution from spontaneous target cell death cannot be excluded. Interestingly, higher fractions of cytotoxic NK cells were observed in the experiments where the target cell density was higher. This is consistent with our previous report that high target cell density can increase the likelihood of NK cell cytotoxicity but could also be coupled to heterogeneity in target cells susceptibility or motility of the NK cell (16, 29).

Taken together, NK cells that killed three or more target cells were responsible for 70% of the killing, and from this cohort, the most potent NKs (killing ≥5 targets) were responsible for 20–25% of the total target cell death. Thus, despite representing a minority of the total population, the serial killing NK cells play an important role in target cell elimination (17).

CONCLUSION

We report functional measurements allowing enumeration of cytotoxic cells and assessment of cytotoxic potential of individual cells in polyclonal populations of NK cells. The ability to monitor a single cell's cytolytic capacity over time in a high throughput

fashion is an advancement on standard techniques, such as isotope release assays (chromium/europium) or some flow-based methods, which perform bulk measurement of target cell death. While population measurements give a general overview, they fail to dissect how the sum of individual cellular responses contributes to the overall response. Flow-based CD107a release assays can yield single cell information on cellular degranulation but provides no information on, e.g., neither the number of target cells killed nor the temporal single cell history. On the flip side, the method presented here does require some technical know-how, is more laborious, and time consuming than population-based assays. These could be seen as limiting factors, e.g., clinical utility. However, this method offers a precise single cell tool, allowing small cell numbers to be observed over extended periods of time and seems especially useful when screening for rare events. We see these advantages as strong indicators of utility within hematopoietic stem cell transplantation where small, to date difficult to detect, populations of cells drive clinically beneficial graft-versus-leukemia effects or severe illness, such as GVHD. With a proof-of-concept, the clinical utility will outweigh the technical disadvantages and drive innovation and advancement toward an easier streamlined approach. Thus, we believe that the platform presented here, together with previously reported lab-on-a-chip tools, represent strong complements to flow- and population-based assays for evaluating NK cytotoxicity.

AUTHOR CONTRIBUTIONS

KG conducted a major part of the experiments, analyzed data, and wrote the article. LB performed some of the experiments, analyzed data, and developed image analysis software. K.Gust performed experiments. EF performed experiments and analyzed data. KO developed image analysis software. TF manufactured microchips and designed microchip holders. PO analyzed data. OM developed image analysis software. BV performed initial experiments. HB developed image analysis software. KK designed experiments. MU designed experiments. BÖ conceptualized experimental principle, designed the study, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00119

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REVIEW
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Underground Adaptation to a Hostile Environment: Acute Myeloid Leukemia vs. Natural Killer Cells

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Acute myeloid leukemia (AML) is a heterogeneous group of malignancies which incidence increases with age. The disease affects the differentiation of hematopoietic stem or precursor cells in the bone marrow and can be related to abnormal cytogenetic and/or specific mutational patterns. AML blasts can be sensitive to natural killer (NK) cell antitumor response. However, NK cells are frequently defective in AML patients leading to tumor escape. NK cell defects affect not only the expression of the activating NK receptors, including the natural cytotoxicity receptors, the NK group 2, member D, and the DNAX accessory molecule-1, but also cytotoxicity and IFN-γ release. Such perturbations in NK cell physiology could be related to the adaptation of the AML to the immune pressure and more generally to patient's clinical features. Various mechanisms are potentially involved in the inhibition of NK-cell functions in AML, including defects in the normal lymphopoiesis, reduced expression of activating receptors through cell-to-cell contacts, and production of immunosuppressive soluble agents by leukemic blasts. Therefore, the continuous cross-talk between AML and NK cells participates to the leukemia immune escape and eventually to patient's relapse. Methods to restore or stimulate NK cells seem to be attractive strategies to treat patients once the complete remission is achieved. Moreover, our capacity in stimulating the NK cell functions could lead to the development of preemptive strategies to eliminate leukemia-initiating cells before the emergence of the disease in elderly individuals presenting preleukemic mutations in hematopoietic stem cells.

Keywords: natural killer cells, acute myeloid leukemia, immunoediting, natural killer receptors, immune escape of cancer, aging and cancer

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INTRODUCTION

In recent years, the field of cancer immunology has known a growing interest due to development of innovative therapeutic strategies in various malignant pathologies. Since the first hypothesis by P. Ehrlich at the beginning of the twentieth century suggesting that the organism could defend itself against tumor cells (1), through the "Immunosurveillance" theory developed by Burnett (2) and Thomas (3) in the late 1950s, and into the more recent "three Es of the immunoediting" suggested

by Schreiber et al. (4); scientists and clinicians learnt that not only cancers were capable of inhibiting the tumor-specific immune response but also host immune cells could potentially be restored or manipulated to eliminate tumors cells. Therefore, therapeutic strategies combining conventional chemotherapy treatments and reinforcement of the self anticancer immunity appear as very promising. Recent successes in the use of immune checkpoint inhibitors to restore the T-cell response against solid tumors are in favor of such approaches (5). Interestingly, the observation that immune cells need external interventions to recover an activity against the autologous tumors demonstrates, as a negative, the adaptation process engaged by tumor cells in order to expand despite the patient's immune system. Leukemic diseases are particularly suitable to study the dialog with the immune system, as they develop in the same bone marrow (BM) environment as normal hematopoiesis, are well molecularly characterized and also because they invade the organism through the circulation network, so directly in contact with the circulating immune cells.

ACUTE MYELOID LEUKEMIA: A LONG-TERM MALIGNANT PROCESS, SIDE EFFECT OF AGING

Acute myeloid leukemia (AML) is a heterogeneous group of diseases characterized by the proliferation of a hematopoietic progenitor clone blocked in its differentiation (6, 7). The blockage can concern each maturation step of the myeloid precursors, including granulocytic, monocytic, megakaryocytic, and erythroid precursors. In general, the disease develops in the BM, and the presence of malignant clones inhibits the normal hematopoiesis not only by reducing space available for healthy hematopoietic stem cells (HSCs) but also by direct inhibition (8). This inhibition leads to marrow failure associated with cytopenia. The annual overall incidence of the disease is 3.8 cases per 100,000 adults in western countries, but it increases to 15 cases per 100,000 for elderly over 60 years (9). Many advances have been made in the molecular characterization of the disease and the evaluation of molecular markers in specific cytogenetic AML subsets is now a standard procedure for patient's diagnosis and risk stratification (9, 10). Moreover, attempts were also developed to categorize AML based on mutation profiling (11–13) or on gene expression profiling, associated or not with recurrent acquired mutations identified in routine diagnosis (14–17). Finally, a new paradigm is taking form in our understanding of the connection between aging and leukemia with the identification of recurrent mutations in genes involved in the epigenetic regulation of the HSCs genome (*DNMT3A*, *TET2*, and *ASXL1*), acquired with age in healthy HSC, and leading to clonal hematopoiesis associated with increases in the risk of hematological cancer, including AML (18–20). The demonstration that healthy HSC could integrate mutations originally identified in AML is coherent with the identification of leukemia stem cell (21), with the potential to initiate a malignant clone at the origin of the disease. Indeed, preleukemic HSCs, defined as a pool of HSC with recurrent *DNMT3A* mutations but without the additional mutations observed in AML blasts, were found in AML patients (22).

Altogether, these observations are in favor of the hypothesis that HSCs accumulate somatic mutations and give rise to AML-initiating cells following a clonal selection process (23) at diagnosis and also after relapse (24). This long duration of the malignant development process, in parallel with patient's aging, questions the nature of the stimuli leading to this evolution, why particular successive mutations are required to ensure AML survival and proliferation, and how the organism's environment, including the immune system, can deal with the emerging preleukemic and leukemic cells.

THE NATURAL KILLER CELL: A MAJOR ANTITUMOR EFFECTOR CELL

Among the different immune partners, natural killer (NK) cells were defined, at the time of their discovery, as being capable to directly eliminate tumor cells (25–28). NK cells are lymphocytes from the innate immunity, therefore characterized by the absence of rearranged antigen-specific receptors, such as T-cell or B-cell receptors. This population was recently assigned to a newly described family of innate lymphocytes, comprising various innate lymphoid cells (ILCs) (29). Innate lymphocyte populations show some analogies with the subdivision observed for the T-lymphocytes family with the $CD8^+$ cytotoxic T-cells, and the Th1, Th2, and Th17 $CD4^+$ T-cells. Similarly, conventional NK cells constitute the cytotoxic innate lymphocytes with capacities to eliminate infected or transformed target cells, whereas ILC subsets are capable to support the development of the local immune response through the production of cytokines, such as IFN-γ (ILC1 subset), IL-5 and IL-13 (ILC2 subset), or IL-17 and/or IL-22 (ILC3 subset). NK cells were first categorized as type 1 cells such as Th1 cells because of their capacity to produce IFN-γ, but the expression of perforin and granzymes authorized to distinguish the cytotoxic ILC, i.e., the NK cell subsets, and the helper ILC1 (30). This role sharing could suggest that innate and adaptive lymphocyte populations can interact and support each other to initiate and sustain the immune response (31).

Natural killer cells represent 5–10% of the blood lymphocytes. Two major NK cell subsets are present in blood and secondary lymphoid organs (32). The $CD56^{dim}CD16^+$ NK cells constitute the vast majority of NK cells in blood (90–95%). They are highly cytotoxic but can also produce significant amounts of cytokines, such as IFN-γ and TNF-α, after stimulation by a sensitive target (33). The expression of the FcγRIII CD16 ensures the capacity for NK cells in mediating the antibody-dependent cellular cytotoxicity (ADCC). By contrast, the $CD56^{bright}CD16^{low/-}$ NK cell subpopulation is mainly found in lymph nodes whereas they represent about 10% of blood NK cells (32, 34). The $CD56^{bright}$ NK cells store less intracellular cytolytic vesicles containing perforin and granzymes than their counterpart, but they can secrete large amounts of cytokines in response to an inflammatory environment (32). In addition to the cytokine-mediated triggering, NK cell functions are regulated by a balance between inhibitory and activating signals provided through regulatory receptors on the cell surface (35).

NK Cell Functions Are Tightly Regulated

Natural killer cells are tightly regulated by numerous receptors that either trigger or inhibit the cell's functions. To allow the distinction between healthy and abnormal cells (i.e., infected or tumor "stressed" cells) is the ultimate goal of this balance. Indeed, NK cells detect modified target cells that display perturbations in the expression of surface ligands (35).

Through the recognition of some HLA class-I molecules on the target cell, receptors, such as some of the killer immunoglobulin-like receptors (KIRs) or the lectin heterodimer CD94/natural killer group 2, member A (NKG2A), inhibit NK cell functions. Originally described as the "Missing self" theory (36), the physiological function of these receptors is to detect loss or reduction of the class-I antigen-presenting molecules on the surface of tumor cells, a frequent alteration observed in cancer cells (37) and viral infections (38) at the origin of the escape from T-cell-mediated immunity. The absence of HLA class-I molecules on the target cell surface will therefore lead to an absence of inhibition of the NK cell functions. However, chronic exposure of NK cells to HLA class-I loss tumor variants can also lead to NK cell anergy as an escape mechanism. Such anergy can be reversed in presence of IL-12 and IL-18 (39).

Optimal NK cell triggering will also require activation signals provided by activating receptors that detect ligands on the target. A majority of cancers of all cell types express, at variable levels, stress-induced molecules, including the MHC class-I-related chains A and B (MICA/B) and the UL16-binding proteins (ULBPs) (40). These proteins are recognized by the activating lectin-homodimer NK group 2, member D (NKG2D) receptor on NK cells, resulting in the elimination of the tumor (41). Importantly, ataxia telangiectasia, mutated (ATM), and ATM- and Rad3-related (ATR) protein kinases activation as a response to DNA damage can stimulate NKG2D-ligands (NKG2D-L) surface expression (42). Other pathways regulate the expression of certain NKG2D-L [reviewed in Ref. (43)] and participate in leukemic physiology, including alterations in the microRNAs repertoire (44), the heat shock stress pathway (45), which can induce MICA and MICB gene expression, or the activation of the PI3K pathway that is often constitutively activated in leukemia (46) and can stimulate the expression of the mouse NKG2D-L RAE-1. Stress signals associated with DNA damage response, including reactive oxygen species (ROS), can also promote the expression of the poliovirus receptor (PVR or CD155) recognized by the activating receptor DNAX accessory molecule-1 (DNAM-1) (CD226) (47, 48). Interestingly, the DNAM-1 ligands PVR and Nectin-2 were observed on many cancers and DNAM-1 can collaborate with other activating NK cell receptors to mediate killing of tumor cells (49, 50). The natural cytotoxicity receptors (NCRs), such as NKp30, NKp44, and NKp46, were also implicated in the recognition of tumors and notably AML (51, 52) even if ligands are expressed at low levels (53). B7-H6, a ligand for NKp30, and MLL5, from which a short isoform is recognized by NKp44, can be expressed on AML blasts (54, 55). AML cells are also recognized by NKp46 (56), but the ligands involved have not yet been identified. Additional receptors or coreceptors can induce NK cell activation in a cell-to-cell interaction with a target, including the adhesion molecule lymphocyte function-associated antigen-1 (LFA-1), and the signaling lymphocytic activation molecule (SLAM) family receptors, such as 2B4 (CD244), CRACC (CD319), or NTB-A (57). LFA-1 will bind to the intercellular adhesion molecules-1 (ICAM-1 or CD54) expressed on most AML cells (56). The SLAM receptors will be involved in homotypic interactions, except 2B4 (CD244), which will recognize CD48. To date, CD48 was the only SLAM family receptor frequently observed on AML cells (52).

A Direct Role for NK Cells in the Antitumor Immune Response

The original identification of NK cells as tumor killers has been abundantly confirmed in a wide variety of cancers. The higher susceptibility of murine models lacking NK cells to spontaneous or carcinogen [methylcholanthrene (MCA)]-induced tumors was in favor of a direct role of these lymphocytes in the elimination of malignant cells (4). Numerous *in vitro* and *in vivo* models demonstrated the direct implication of perforin together with IFN-γ produced by cytotoxic cells, including NK cells (58, 59), or of the activating NK receptors (41, 50, 60). In addition, the death receptor pathways involving the Fas-ligand (FasL) receptor and the TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, both induced on NK cells by either IFN-γ or IFN-α/β, were also involved in the antitumor function of NK cells (61, 62). In human, an indirect evidence for a role for NK cells *in vivo* came from the observation of the association between the natural cytotoxicity quantified in blood and the risk of cancer (63). The positive correlation between solid tumor infiltrating NK cells and good prognosis also suggests that NK cells could directly eliminate tumor cells *in vivo* (4). Importantly, in addition to a direct cytotoxicity, NK cells promote the antitumor response through the production of IFN-γ, which is required for the early phase of Th1 priming and polarization in the lymph node (64) and also for the stimulation and polarization of macrophages (65).

AML ESCAPE FROM NK CELLS: IMMEDIATE AND LONG-TERM PROCESSES

In vivo AML sensitivity to NK cell-mediated cytotoxicity has been shown by Ruggeri et al. in patients treated by haploidentical HSC transplantation (66). This team reported a lower incidence of relapse in patients transplanted with graft containing alloreactive NK-cell clones against recipient cells. By contrast, the absence of such NK-cell incompatibility was associated with a high relapse rate (66, 67). This observation was based on the existence of KIR/KIR-ligand (i.e., HLA class-I molecules) mismatches in the responsive donor/recipient pairs. The absence of cognate ligands for the inhibitory KIRs allowed NK cell activation by AML cells and elimination of the leukemic target. This illustration of the "missing-self" theory (36) found its counterpart in the observation that the activating KIR2DS1 can also provide a significant reduction of AML relapse in donor/recipient pairs where recipient expresses specific HLA-C ligands (68). According to these results, the selection of the donor may be of importance in order to optimize the graft-vs.-leukemia effect expected from the HSC transplantation. Therefore, haploidentical and umbilical cord

blood transplantations would be the most suitable transplantation settings to find KIR/KIR-ligands mismatches. A few studies analyzed the role of KIR/KIR-ligand mismatch in cord blood transplantation setting with contradictory results either demonstrating the advantage of such treatment in myeloid leukemia (69) or, by contrast, showing a higher risk of acute graft-vs.-host disease without curative advantages after reduced intensity conditioning (70). Cytomegalovirus (CMV) infection or reactivation in transplanted patients could explain these discrepancies, as CMV-driven NK cell expansion and maturation could participate in the reduction of the relapse risk (71). Clinical trials using infusions of IL-2-activated haploidentical NK cells in AML patients showed encouraging results with *in vivo* expansions of donor NK cells and complete remissions (CR) in certain patients, suggesting an antitumor NK-cell-mediated immune response (72, 73). However, patient's autologous NK cells often show defects at diagnosis. Activating receptors, such as DNAM-1, and the NCR, such as NKp30 and NKp46, present reduced expression levels on NK cell surfaces in a large proportion of patients (74–76). The inhibitory receptor CD94/NKG2A can also be upregulated on patient NK cells (77). In parallel to the phenotypic perturbations, cytotoxic activity and IFN-γ release are also decreased (76–78). These defects were associated with pejorative outcomes, including increased relapse risk and/or reduced overall survival. Interestingly, NK cell phenotype and function are normalized after chemotherapy treatment, underlying the role for AML blasts in decreasing NK cell's abilities (75, 76). Altogether, these observations imply that AML inhibits the autologous NK cell response through several mechanisms (**Figure 1**).

Leukemogenesis and Immune Escape

Tumor microenvironment plays a critical role in the inhibition of the antitumor immune response (79). Leukemogenesis occurs mainly in the BM, the primary site for the healthy hematopoiesis.

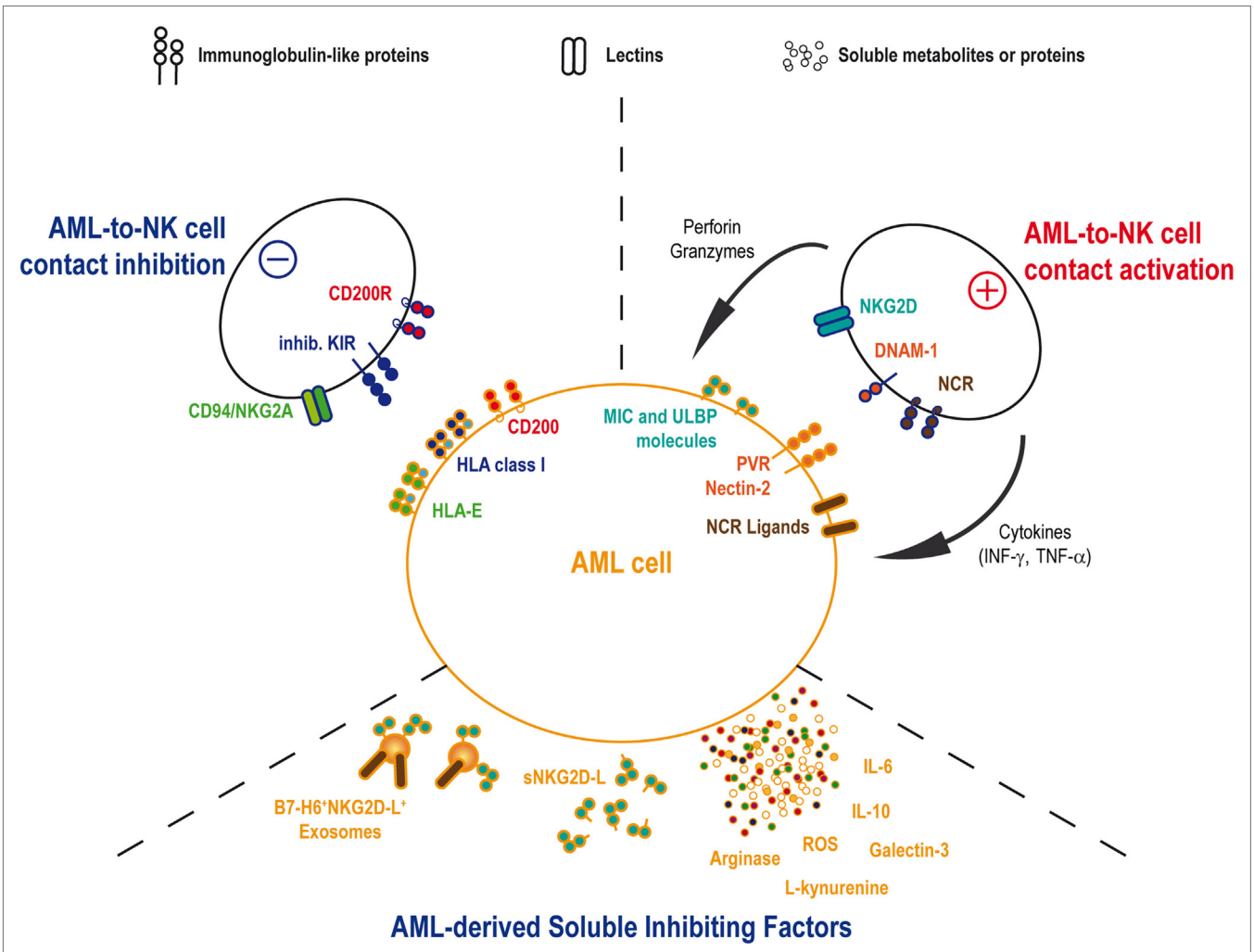

FIGURE 1 | AML and NK cells interactions. AML and NK cells will interact by cell-to-cell contact as well as soluble factors. NK cell functions, including cytotoxicity and cytokines release, will be activated through the binding of activating receptors onto the cognate ligands on AML blast. Conversely, AML cell will attempt to inhibit NK cell functions by mobilizing ligands for inhibitory NK receptors. In the same time, AML will secrete some inhibitory soluble agents in order to reduce NK cell efficacy to detect and eliminate leukemia.

This suggests that leukemia BM environment could modify the immune cell differentiation process and also that healthy immune populations may influence leukemogenesis. Such interference was demonstrated in myelodysplastic syndromes (MDS), a heterogeneous group of myeloid disorders displaying low to high risk to give rise to secondary AML (80). As observed in AML patients, blood NK cells from MDS patients show severe defects with downregulation of activating receptors, including NKG2D and DNAM-1 (81), reduced cytotoxic activity (81, 82), and increased apoptosis rate in response to IL-2 stimulation associated with reduced proliferation *in vitro* (82). Importantly, increased NK cell defects were also associated with high-risk MDS, characterized by higher International Prognostic Scoring System (IPSS) Score, presence of excess blasts, abnormal karyotype, and hypercellularity (83). BM NK cells in MDS patients are also deeply affected by the disease, suggesting that MDS BM environment could play a role in those defects (81–83). In AML, BM environment influences healthy hematopoiesis by affecting BM cell populations. Notably, BM stromal cells show low proliferative rate as well as genetic and epigenetic alterations (84). Similar numbers of healthy $CD34^+CD38^-$ HSC were found in the BM of AML patients and healthy individuals. However, normal $CD34^+CD38^+$ progenitors were found reduced in BM of AML patients likely resulting from a differentiation block at the HSC-progenitor progression (8). Consequently, similar to MDS, NK cell differentiation in the BM seems to be affected by AML. A recent work by Vasold et al. suggested a role for mesenchymal stromal cells and hypoxia in the reduction of NK cell cytotoxic activity against autologous AML blasts highlighting the importance of the BM stroma in the emergence of abnormal mature immune cells in the peripheral blood (85). Connected to this observation, we recently described that AML cell transcriptional program is intimately associated with the deepness of the NK cell defects (76). NK cell deficiencies were associated with an increased risk of relapse. AML blast transcripts coding for proteins involved in cytokine/cytokine receptor and chemokine pathways were found severely diminished in patients with defective NK cells. In return, IFN-γ production by $CD56^{bright}$ NK cells is almost abrogated at diagnosis in those patients (76). Altogether, these observations would indicate that AML blasts can modify BM environment, including stromal, precursor, and mature immune cell populations. In addition, the immune pressure, notably provided by NK cells, could influence the AML transcription program leading to AML escape and a more pejorative outcome.

Cell-to-Cell Contact and Defective Immunological Synapse

Interactions between immune cells and targets constitute a multistep process where first immune cells build conjugates with the target partner and then initiate a reorganization of proteins localized at the membrane at the cell-to-cell contact point [reviewed in Ref. (86)]. This includes actin mesh polymerization and centrosomes polarization (87). The supramolecular activation cluster (SMAC) recruits regulating NK receptors, in parallel with ligands on the target cell, together with costimulatory and adhesion molecules in order to integrate and amplify intracellular signaling. NK cells will therefore polarize cytolytic vesicles toward the immunological synapse (IS) (i.e., the target cell) and secrete perforin and granzymes within the intercellular space (86). At the resolution of the synapse, NK cells express low levels of activating receptors and need a period of time to regain their full function (88).

Even if leukemic cells are sensitive to allogeneic NK-cell-mediated lysis, alterations in the expression of some activating receptors, including NCR and DNAM-1, on the autologous NK cells suggest that NK-to-AML interactions can be defective in patients. Such observation has been made for T cells with a reduction in actin polarization and phosphotyrosine signaling in T-cell/AML blast conjugates (89). Reduction of NKR expression by autologous NK cells was partially associated with the presence of cognate ligands on AML cells as *in vitro* coculture of NK cells with AML cell lines or primary blasts decreases the NKR levels (56, 90). However, this phenomenon is not proportional to the ligand expression levels on the target, suggesting additional mechanisms controlling the profound NKR downregulation observed *in vivo*. When studying the IS between NK and AML cells, NK cells showed defects in the polarization of their cytolytic granules toward the IS against AML blasts (56). Consequently, defects in NKR expression may also be the result of a continuous exposure to ligands in incomplete cytotoxic synapses against AML blasts, leading to an exhaustion of the NK-cell cytotoxic activity. Therefore, NK cell cytotoxic activity is progressively switched off, whereas AML cells survive and proliferate. Whether the decrease in IFN-γ production observed in patients is also the result of perturbations in the IS or in downstream NKR signaling pathways requires further studies. The molecular mechanisms responsible of this cytotoxic defect need also to be identified. Inhibitory KIRs can reduce the autologous anti-AML response as demonstrated in autologous HSC transplantations (91). Nevertheless, inhibitory KIRs likely play a very early role in the building of the IS during polymerization of the actin network and would be only marginally implicated at later stages to inhibit the lytic granules' polarization (86). Other receptors could be good candidates in the negative regulation of NK functions, such as CD96, as demonstrated by the higher resistance of $Cd96^{-/-}$ mice to solid tumors (92). On the other side, CD200 (also named OX2) expressed at high levels on some AML has been recently identified as a suppressor of patient's NK cell cytotoxic and cytokine secretion functions in the antitumor response (93).

Soluble Molecules

In addition to cell-to-cell contact-based inhibition, NK cells' functions are inhibited by various soluble molecules, including soluble ligands of regulatory NK receptors, cytokines, such as TGF-β or IL-10, metabolic compounds, such as ROS, and tryptophan or arginine catabolites.

Some AML clones have adopted strategies to inhibit NK cells with specific soluble compounds. To date, NKG2D and NKp30 were the main targets described for such inhibition. Soluble ligands can be released by tumor cells into the extracellular environment. NKG2D-ligands (NKG2D-L) and the NKp30 ligands B7-H6 are cleaved by metalloproteinases, either matrix metalloproteinases (MMP) or A disintegrin and metalloproteinases

(ADAMs) (94–99). Soluble NKG2D-L was found in the serum of patients with solid tumors or hematological cancers. Moreover, soluble MICA release is regulated by chaperones, such as ERp5, modulating MICA structure with the support of the heat shock protein GRP78 to induce conformational changes allowing its cleavage (100, 101). Despite a high heterogeneity in the surface expression of NKG2D-L by AML, the majority of patients shows soluble forms of MICA, MICB, or ULBP2, alone or more often in combination with up to five soluble NKG2D-L (102). As observed in solid tumors (103), the presence of soluble NKG2D-L in AML patients' serum is associated with a reduction of the surface NKG2D expression leading to a decrease in NKG2D-mediated NK-cell's activity (102). Importantly, a recent work by Deng et al. showed that the soluble form of MULT-1, a mouse high-affinity NKG2D-L, released by tumors, can stimulate NK-cell cytolytic function, and induce tumor rejection in mice (104). Activating soluble NKG2D-L was not identified in human yet, but such molecule, if it exists, could be of interest for inducing antitumor NK-cell function.

B7-H6 shedding by solid tumors seems to induce a reduction of the NKp30-mediated tumor cell recognition by NK-cells (96). In contrast to soluble NKG2D-L, this process would be due to a reduction of the B7-H6 expression on the tumor cell surface rather than to a direct inhibition of the NKp30 expression on the NK cell. No observation of B7-H6 shedding by AML has been made yet but the expression of B7-H6 on AML cells together with the reduction of expression of NKp30 on patient's NK cells would justify studying this pathway in AML patients.

Soluble NKG2D and NKp30 ligands can be also released in the serum bound to tumor-derived exosomes (TEX) (105, 106). Exosomes from solid tumors or leukemia/lymphoma cells can present MICA, MICB, and ULBP molecules leading to an inhibition of the NKG2D-mediated NK cell activation (105, 107). In the same way, a reduction of NKp30 can be observed when NK-cells are incubated with B7-H6 positive exosomes produced by myeloid subsets in inflammatory conditions (108). By contrast, exosomes carrying BAG6, another NKp30 ligand, are necessary to activate NK cells in order to eliminate chronic lymphocytic leukemia (CLL) cells, whereas soluble BAG6 lead to tumor evasion (106). In addition, soluble galectin-3 produced by solid tumor cells works as an inhibitory ligand of NKp30 (109). In line with this observation, higher levels of galectin-3 gene expression in BM are an independent unfavorable prognostic factor for overall survival in patients with AML (110). Altogether, these observations would suggest that soluble or exosomes bound NKp30 ligands could also interfere, in parallel with other soluble ligands, with NK cell functions in AML patients.

Imbalance in serum cytokines can be responsible for perturbations in the regulation of the antitumor response. In contrast to reduced TGF-β levels in plasma of AML patients as compared to healthy individuals (75, 111), IL-10 was found significantly higher together with the proinflammatory cytokines IL-6 and TNF-α. Curiously, high levels of IL-6 and low levels of IL-10 are associated with poor outcome (111). Even if such cytokine environment is probably more related to the AML physiology, it still can influence immune cell properties. Indeed, high levels of IL-6 were shown to impair perforin and granzyme B expression and reduce NK cell cytotoxic activity in individuals with autoimmune diseases (112), with heart failure (113), and cancer patients treated with recombinant IL-6 (114).

In parallel, high levels of small immunosuppressive molecules, side products of the leukemic cell metabolism, can be released by AML blasts. ROS participate to NK-cell defects in the expression of activating receptors, such as NKp46 and NKG2D (115). Interestingly, they are highly produced by AML with specific mutation patterns, such as activating mutations in RAS family members or FLT3/ITD mutations (116, 117). Arginine metabolism is also enhanced in AML blasts leading to an immunosuppressive environment. High levels of production of active arginase II by AML blasts can induce an accumulation of this enzyme in the plasma of patients, resulting in significant inhibition of lymphocyte proliferation (118). In addition, the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) is also expressed by leukemic blasts, whereas it is absent from normal hematopoietic $CD34^+$ stem cells (119). IDO catalyzes tryptophan degradation by producing L-kynurenine, which can directly affect NK cell phenotype and cytolytic function through the inhibition of the cytokine-induced upregulation of NKp46 and NKG2D (120). In addition, IDO activity can stimulate the emergence of $CD4^+CD25^{high}$ regulatory T-cells (Tregs) (121) capable of inhibiting NK cell functions by TGF-β release (122) or IL-2 starvation (123).

AML AND CONVENTIONAL TREATMENTS: RESISTANCE TO CHEMOTHERAPIES

Acute myeloid leukemia treatment by conventional chemotherapy eliminates tumor blasts and leads to the achievement of CR in 70–80% of patients younger than 65 years (6). Elimination of circulating AML blasts allows the recovery of NK cell phenotype and functions (75, 76), and a sustained autologous NK cell activity can support a continued CR (124). However, at least half of patients will eventually relapse (6). A major limitation for success in chemotherapy of AML is dominance of drug-resistant subpopulations of cells. AML cells also can achieve the resistance phenotype through modification of multiple and diverse pathways, such as inactivation of the mitochondrial apoptotic machinery, decreased expression of proapoptotic agents, upregulation of antiapoptotic molecules, and promotion of drug efflux. Although daunorubicin (DNR) is the most efficient and widely used anthracycline to treat AML, resistance to this drug remains a critical problem (125–127). In this regard, the intrinsic and acquired resistance of AML to drug treatment remains a fundamental challenge for improving patient outcome. One of the consequences of acquisition of drug resistance by leukemic cells is the appearance of cross-resistance against immune effector cells. We have recently demonstrated that the acquisition of resistance to DNR resulted in the acquisition of cross-resistance to NK cell-mediated cytotoxicity. MiR microarray analysis revealed that this cross-resistance was associated with miR-181a downregulation and the subsequent upregulation of MAP3K10 and MAP2K1 tyrosine kinases and the BCL-2 (BCL-2 and MCL-1) family. These studies point to a determinant role of miR-181a in the

sensitization of leukemic resistant cells to DNR and NK cells and suggest that miR-181a may provide a promising option for the treatment of immuno- and chemoresistant blasts (128).

In contrast, previous studies showed that NKG2D-L can be upregulated on the AML cell surface after treatment with various molecules, including the histone deacetylase inhibitor valproic acid (VPA) (129, 130). In the same way, the administration of all-*trans*-retinoic acid (ATRA) can also increase the NKG2D-L expression levels on acute promyelocytic leukemia, a particular subtype of AML with a *PML-RARA* gene fusion (130). Importantly, such increased expression of NKG2D-L cannot be observed in patients treated with chemotherapy in absence of ATRA or VPA (130). These observations suggest that chemotherapy can stimulate an anti-AML NK-cell–mediated response. Recently, we showed that cytarabine-resistant cells become more susceptible to NK-mediated cell lysis as compared to parental cytarabine-sensitive cells. The increased susceptibility correlates with the induction of ULBP 1/2/3 and NKG2D-ligands on target cells by a mechanism involving c-Myc induction (131). These studies could help to improve the efficacy of NK-cell-based therapy that allows for better designing of NK-based immunotherapy.

IMMUNOTHERAPEUTIC STRATEGIES

Given the sensitivity of AML to NK-cell-mediated lysis, strategies to enhance or restore NK cell functions in patients could be of interest besides conventional chemotherapy. Numerous methods have been developed during the last few years in order to either modulate immunity against tumors using immunomodulatory drugs (IMiDs) or cytokines or to specifically target or activate NK cells against leukemia cells. Such treatments, used alone or in combination with chemotherapy, aim to eliminate chemoresistant tumor cells.

Immunomodulatory Drugs

Immunomodulatory drugs are structural and functional analogs of thalidomide (132). To date, two molecules have been approved in MDS, multiple myeloma (MM), or mantle cell lymphoma (MCL): lenalidomide and pomalidomide. Alone (for MDS or MCL) or in combination with dexamethasone (MM), as a second or third line of treatment, IMiDs improve both time-to-progression and overall survival of patients. Several studies have also explored the synergistic effect of IMiDs with rituximab for the treatment of CLL (133) or MCL (134). Beyond their direct effect on cancer cell proliferation and angiogenesis, these molecules stimulate antitumor effectors, including B, T, and NK cells (135). Hence, by their broad range of effects, IMiDs represent a novel strategy for immunotherapy as evidenced by numerous ongoing clinical trials, in many cancer settings, including AML (136).

In the case of NK cells, IMiDs increase the expression of activating receptors, notably NCR (137, 138). These molecules induce expansion of NK cells as confirmed by immunomonitoring studies in several clinical trials (137, 139, 140). Enhanced NK cell ADCC or natural cytotoxicity is largely mediated *via* IL-2 produced by T cells (141). In addition, we have recently shown that lenalidomide enhances tumor cell recognition by NK cells by improving the stability of the immune synapse (56). Finally, IMiDs increase tumor infiltration by NK cells in murine models (142).

Cytokines

Several cytokines of the IL-2 family are essential for NK cell survival, expansion, and activation, but so far, only IL-2 has an approval for clinical use. In the family of IL-2, IL-15 and IL-21 share some characteristics such as activation and proliferation of NK cells, and the common γ-chain for their receptor (143). IL-2 is able to induce expression of NKG2D, NKp44, and NKp46 on NK cells (49, 82, 144). In MDS, however, following *in vitro* IL-2 stimulation, NK cells do not recover a normal cytolytic activity when compared to healthy volunteers (82). Moreover, IL-2 fails to induce NK cell proliferation compared to healthy volunteers, but rather increases the rate of apoptotic NK cells (82). So far, the therapeutic use of IL-2 for the treatment of hematological malignancies has been hampered by a peripheral toxicity (145) and an unwanted expansion of Tregs (146). Conclusions of clinical trials report modest antitumor activity when used as a monotherapy. Therefore, the use of IL-2, especially at high doses, might be restricted to *ex vivo* expansion of NK cells given problems of *in vivo* toxicity (145).

In contrast to IL-2, IL-15 and IL-21 may represent a better alternative because these cytokines do not expand Tregs. Accordingly, many clinical trials currently aim to demonstrate an efficient NK cell-mediated antitumor response with *in vivo* or *ex vivo* IL-15-expanded NK cells in AML. Hence, in the absence of clinical approval for IL-15, several groups are testing the possibility to expand NK cells *in vitro* before reinfusion into patients.

IL-15 plays a major role in the proliferation, differentiation, survival, and functions of T and NK cells (147, 148). *Ex vivo* exposure of NK cells from AML patients to IL-15 enhance NKp30, NKp46, NKG2D, and NKG2C surface expression. Accordingly, this increase of receptor expression correlated with an enhanced natural cytotoxicity against autologous AML cells (147, 149). In addition, in hematological malignancies, low levels of circulating IL-15 after BM transplantation are predictive of risk of relapse (150). In line, NK cell recovery in stem cell transplantation is strongly correlated with plasmatic concentrations of IL-15 (149).

The serum concentration of IL-15 increases dramatically following administration of cytotoxic agents (147, 150). For some authors, this elevation of serum IL-15 could be related to the depletion of lymphoid populations that normally consume circulating IL-15 or to inflammation induced by chemotherapy (149). *In vivo* injections of the IL-15/IL-15Rα heterodimer result in significant expansion of γδ, $CD8^+$ T, and NK cells (148). Recently, this cytokine has become available for use in early phase clinical trials as an alternative to IL-2 (147, 148). IL-15 is currently assessed as a therapy for various solid tumors, including refractory metastatic melanoma, metastatic renal cell cancer.

IL-21 has been proven safe in phase I clinical trials with signs of clinical activity (151). IL-21 stimulation of NK cells mainly results in enhanced NK cell functions. *Ex vivo*, IL-21 is capable of inducing NK cell maturation and stimulates the production of IFN-γ and cytotoxic properties of NK cells (152–154). Several clinical trials have reported the effect of IL-21 therapy on immune system after administration in patients with metastatic

melanoma and renal cell carcinoma (151). Although NK and T cell numbers were temporarily decreased during administration of IL-21, the cells had higher expression of CXCR3, hyaluronan-mediated motility receptor (HMMR), IFN-γ, perforin, and granzyme. In addition, NK cells from patients displayed an enhanced cytotoxicity capacity (151). These results were confirmed in a phase II trial for metastatic melanoma (155). In the absence of clinical approval, IL-15 and IL-21 are also used *ex vivo* to expand and activate NK cells for further infusion in patients. NK cells stimulated *ex vivo* by the leukemic cell line K562 expressing membrane-bound IL-15 or IL-21 showed a strong proliferation and cytolytic activity with a higher proliferation rate and an increase in telomere length for IL-21-activated NK cells (156). NK cells expanded *ex vivo* by membrane-bound IL-15 are currently infused into MDS or AML patients (phase I clinical trial #NCT02123836).

Bi- and Trispecific Killer Cell Engager

Several monoclonal antibodies (mAbs) directed against tumor antigens have been generated and are currently used in the clinics. The most famous therapeutic mAbs remains the anti-CD20 rituximab, which is widely used to B-cell-related diseases and cancers. Several mechanisms of action have been identified and one of these is the recognition of the Fc part of the human or humanized IgG1 or IgG3 isotypes by CD16 expressed by NK cells and myeloid cells. Upon engagement of CD16, the cells are activated and kill the targeted cells. Unfortunately, several studies have shown that the polymorphism of CD16 and the engineering process may alter ADCC efficacy. Bispecific killer cell engagers (BiKEs) are engineered antibodies with dual specificity, for a tumor antigen like CD19 or CD20 for B-cell-related diseases and CD16 targeting NK cells. The anti-CD16 part of the BiKEs bypasses the disadvantages of classical mAbs (157). For instance, AFM13, a BiKE targeting CD19 and CD16, has been recently tested in phase I and II trials (#NCT01221571 and #NCT02321592, respectively) in non-Hodgkin lymphoma.

In vitro studies demonstrated that CD33 × CD16 BiKEs trigger NK cell activation against AML cell lines and primary targets through CD16 signaling, leading to cytokine and chemokine production (158). As a consequence, significant increases in NK cell cytolytic activity led to induction of target cell apoptosis at high and low target to effector ratios. In a study based on NK cell isolation from patients with MDS, authors showed that CD33 × CD16 BiKE potently activates blood and marrow MDS–NK cells at all disease stages to lyse $CD33^+$ MDS and $CD33^+$ myeloid-derived suppressor cells (MDSCs) targets (159). Noteworthy, MGD006, a CD123 × CD3 BiKE is tested in a phase I trial (#NCT02152956), confirming the current explosive attention to BiKEs as potent therapeutic tools for AML and other cancers. In the same way, a CD30 × CD16 bispecific tetravalent chimeric antibody (TandAb) was used in a phase I clinical trial in patients with relapsed or refractory Hodgkin lymphoma showing good tolerance and tumor targeting (160). However, with respect to AML treatment, the use of BiKEs remains limited due to the heterogeneity of tumor antigen in this disease.

Recently, several new reagents were developed in attempt to enhance the targeting of malignant cells. Gleason et al. have generated a trispecific mAb (TriKE) directed against CD19, CD20, and CD16 (159). This TriKE, efficiently engaged NK cells against $CD19^+CD20^+$ leukemic targets, as proven by a strong cytotoxicity and IFN-γ production. To increase NK-cell activating properties, Miller et al. have developed a TriKE targeting CD33 and CD16 that contains IL-15 (161). This reagent not only mediates CD16 directed cytotoxicity against $CD33^+$ leukemic cells but also sustains NK-cell activation and persistence by the IL-15 linker.

In conclusion, targeted cellular immunotherapy with BiKEs and TriKEs are promising approaches in terms of effector cell retargeting and induction of efficient antitumor response and are currently being developed and evaluated for targeting of various malignancies (162, 163).

Antibodies Directed against NK Cell Inhibitory Receptors

Among strategies to improve the recognition of tumor cells by NK cells, blocking the inhibitory interactions is appealing. Inhibitory molecules, such as KIR and NKG2A, are expressed at the surface of NK cells and inhibit NK cell activation *via* their ligands (HLA-C and HLA-E, respectively). In the case of cancer patients, expression of KIR and NKG2A, as well as expression of their ligands at the surface of tumor cells, has been described in several solid cancers and leukemias (164–166). Subsequently, activation of NK cells is likely prevented and leads to NK-mediated immune evasion. Inhibition of these mechanisms by blocking antibodies is currently being assessed.

IPH2101 is a fully human IgG4 that blocks the interaction between the major subset of KIR (KIR2DL1, KIR2DL2, and KIR2DL3) and their cognate ligands (167, 168). A second generation of anti-KIR mAb, lirilumab (IPH2102/BMS-986015) with a stabilized hinge was generated (167, 168).

In vitro studies showed that IPH2101 augments NK cell-mediated lysis of KIR-ligand matched tumor cells and enhances NK cell-mediated ADCC against antibody-bound tumors (168–170). The therapeutic potential of IPH2101 has also been demonstrated in preclinical mouse models (171, 172), which have formed the basis for clinical trials evaluating IPH2101 in patients with cancer (173). Blocking NK inhibition with the anti-KIR IPH2101 antibody has been proven to be safe in early phase clinical trials in patients with AML and MM (174, 175) and enhances *ex vivo* NK cell cytotoxicity against MM cells (175).

However, a phase II study in MM patients did not reveal lasting objective responses (173). IPH2101 has also been assessed *in vitro* in combination with lenalidomide and potentiates NK-cell cytotoxicity toward autologous myeloma cells. This combination is currently being tested in a phase I clinical trial in MM patients (#NCT01217203) (176). The second generation anti-KIR lirilumab was also shown to synergize with Lenalidomide to increase NK cytotoxicity of myeloma patients treated with Daratumumab (anti-CD38) (177). *In vitro* and *in vivo* lirilumab enhances NK activity against $CD20^+$ lymphoma cells (167). With respect to AML, lirilumab is currently tested in patients in CR for long-term maintenance (#NCT01687387), and for the treatment of patients with refractory/relapsed AML (#NCT02399917). Although safe,

this therapeutic mAb did not induce impressive clinical improvement so far. First of all, cytotoxic effectors expressing KIRs (NK cells, αβ $CD8^+$, and γδ T cells) use other inhibitory KIRs that are not targeted by lirilumab (NKG2A, KIR3DL, and CD85j/ILT2). In an autologous setting, it is likely that these cells may still remain tolerant to leukemic cells. In addition, lirilumab also recognizes KIR2DS1 and KIR2DS2; blocking these receptors may, in contrast, unfavor tumor cell clearance (168).

Noteworthy, a newly engineered mAb directed against NKG2A has been generated by Innate Pharma (IPH2201) and is currently tested in clinical trials (phase I/II) in ovarian cancer, squamous cell carcinoma, and refractory CLL (#NCT02459301, #NCT02331875, and #NCT02557516, respectively). It is tempting to speculate on the efficacy of this new reagent in AML treatment, as these cells are expected to express HLA-E.

Other inhibitory receptors, including PD-1, LAG-3, or TIM-3, usually classified as "inhibitory checkpoint receptors" may influence NK cell activity. The increasing interest for the PD-1/PD-1-ligands axis on T-cells in cancer therapy legitimated the analysis of PD-1 expression on NK cells in various pathological situations. In a mouse model of glioblastoma, NK cell functions against mouse glioma stem cells and the survival of the mice can be ameliorated by blocking either PD-1 or the PD-1 ligand B7-H1 (also named PD-L1) (178). In human, data describing a role for PD-1 in the regulation of NK cells are relatively scarce. Wiesmayr et al. observed the expression of PD-1 on NK cells in pediatric patients with post-transplantation lymphoproliferative disorders caused by EBV infection. The presence of PD-1 was associated with a reduced expression of NKp46 and NKG2D and NK cell function impairment, and blocking the PD-1 inhibitory pathway could restore IFN-γ secretion (179). The PD-1/PD-L1 axis was also involved in the modulation of NK-cell functions against MM (180). Interestingly, IFN-γ can induce the expression of PD-L1 on AML cells leading to the inhibition of the antileukemic response mediated by T-lymphocytes (181) and NK cells (182). Therefore, the anti-PD-1 mAb Nivolumab is tested in a phase II clinical trial in AML patients in remission with high risk of relapse (#NCT02532231).

Chimeric Antigen Receptor–NK Cells

Another strategy to improve antitumor immunity has arisen from recent advances in cell genetic modification that have allowed the specific targeting of tumor cells by cytotoxic effectors. Most of the tools generated are chimeric antigen receptor (CAR)-T cells, i.e., T cells engineered to express a receptor for tumor antigen (for instance, CD19 in the case of B-cell leukemia) coupled to activate signaling adaptors. The few clinical trials with CAR-T cells have obtained somewhat promising results that should be strengthened by other studies. Genetic modification of NK cells has been more recently performed, but not yet with myeloid tumor specificity. For instance, Töpfer et al. generated NK cell lines or primary NK cells targeting PSCA, a prostate cancer antigen (183). As expected, these cells react against PSCA positive tumor cell lines by secreting IFN-γ and killing these target cells. In line with this observation, several others have been designed, based on NK-92 or other NK cell lines (184–187). This new strategy is promising although the costs may remain a serious limitation.

CONCLUSION

Acute myeloid leukemia is the most common myeloid leukemia, usually treated with a combination of anthracyclines and cytarabine in a first attempt to achieve CR. The consolidation phase of the treatment aims to prolong CR and eventually to cure the disease. However, disease heterogeneity (cytogenetic and mutation profile, deepness of BM failure, resistance to treatment) and patient's general condition (age, secondary AML) led to an unfavorable prognosis for many patients. Over time, AML develops various mechanisms to protect itself from the patient's immune system and more precisely from NK cells. The long-term coexistence of leukemia-initiating cells, and then tumor blasts, with NK cells, first in the BM and later in the periphery, can explain the emergence of NK cell defects together with immunoresistant AML cells. The antitumor function of NK cells, demonstrated after allogeneic HSC transplantation, justifies developing methods in order to restore, stimulate, or induce NK cell activity in AML patients. Treatments combining the elimination of the peripheral leukemic blasts using conventional chemotherapy, together with the chemoresistant leukemic-initiating cells, targeted by immune mediators, including NK cells, appear very attractive. However, we could consider the opportunity to stimulate NK cell antileukemic functions before the emergence of the disease. Indeed, the recent observation of preleukemic mutations in healthy elderly individuals' HSC questions the capacity of the immune system to eliminate or tolerate the presence of leukemia-initiating cells in the BM at advanced age. In that context, stimulating the immunosurveillance mediated by NK cells could be a promising preemptive strategy against AML.

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The Residual Innate Lymphoid Cells in NFIL3-Deficient Mice Support Suboptimal Maternal Adaptations to Pregnancy

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Uterine NK cells are innate lymphoid cells (ILC) that populate the uterus and expand during pregnancy, regulating placental development and fetal growth in humans and mice. We have recently characterized the composition of uterine ILCs (uILCs), some of which require the transcription factor NFIL3, but the extent to which NFIL3-dependent cells support successful reproduction in mice is unknown. By mating *Nfil3*$^{-/-}$ females with wild-type males, here we show the effects of NFIL3 deficiency in maternal cells on both the changes in uILCs during pregnancy and the downstream consequences on reproduction. Despite the presence of CD49a^{+}Eomes^{-} uILC1s and the considerable expansion of residual CD49a^{+}Eomes^{+} tissue-resident NK cells and uILC3s in pregnant *Nfil3*$^{-/-}$ mice, we found incomplete remodeling of uterine arteries and decidua, placental defects, and fetal growth restriction in litters of normal size. These results show that maternal NFIL3 mediates non-redundant functions in mouse reproduction.

Keywords: mouse models, uterine NK cells, placenta, pregnancy, lymphocyte subpopulations

INTRODUCTION

Human and murine uterine NK cells (uNK) are innate lymphoid cells (ILCs) that produce factors active on both uterine arteries and fetal placental cells (1–3), contributing to successful reproduction (4–6). The role of uNK in pregnancy may have coevolved with that of variable immune system genes in reproduction (7, 8). We and others have recently shown that various ILC groups are present in both human and mouse uterus and, similar to ILCs in other tissues, uterine ILCs (uILCs) may also play roles in pregnancy (9, 10). For example, IFN-γ produced by uNK cells is the key factor required for uterine vascular adaptation during murine pregnancy (11), and various other ILC populations produce IFN-γ, including conventional (cNK) and tissue-resident NK cells (trNK), uterus and liver ILC1s, and gut NCR^{+} ILC3s (12). On the other hand, other cytokines produced by uILCs may affect

pregnancy and reproduction, e.g., IL-5 produced by uILC2s and IL-17 and IL-22 produced by uILC3s (9, 10). Indeed, mice lacking lymphocytes, including uILCs, show impaired uterine arterial modifications (11) and fetal growth restriction (13). Defective uterine vascular remodeling and shallow trophoblast invasion are associated with great obstetrical syndromes, including preeclampsia and fetal growth restriction (14), and unbalanced cytokine production may lead to preterm labor (15).

Mice lacking the basic leucine zipper transcription factor NFIL3/E4BP4 display immune abnormalities (16), including severely reduced numbers of cNK cells (17) as well as reduced ILCs in several tissues (18–21). NFIL3 also regulates the emergence of common ILC precursors (22). However, the requirement for NFIL3 during development, homeostasis, and function of different ILCs is lineage and tissue specific. Indeed, *Nfil3*$^{-/-}$ mice retain trNK cells in uterus, liver, salivary glands, and skin, as well as thymically derived NK cells (23–25), and we have shown that they also have uILC3s (9). Moreover, the few residual cNK cells in *Nfil3*$^{-/-}$ mice exhibit normal effector functions in response to mouse cytomegalovirus infection (26). How the absence of NFIL3 impacts maternal responses to the endocrine and immunological changes occurring during gestation is unknown. We have begun to answer these questions by describing the changes in uILCs during pregnancy in *Nfil3*$^{-/-}$ mice mated with wild-type (WT) males. Using these mice, here we show that absence of maternal NFIL3 lead to a dramatic reduction of cNK cells and uILC2s during pregnancy. Despite the expansion of residual CD49^{+}Eomes^{+} trNK cells and uILC3s and the presence of abundant CD49^{+}Eomes^{-} uILC1s at midgestation, the uterine vasculature in *Nfil3*$^{-/-}$ females failed to complete the vascular remodeling process necessary to ensure ample and steady blood supply to the fetoplacental unit. This was associated with placental abnormalities and reduced fetal growth, although litter sizes were normal. The results demonstrate that NFIL3 regulates important functions for reproduction in mice, including key aspects of uILCs.

RESULTS AND DISCUSSION

Curtailed Expansion of Nfil3$^{-/-}$ uNK Cells in Response to Pregnancy

We asked if the residual uNK in virgin *Nfil3*$^{-/-}$ females require NFIL3 to expand at midgestation. To do this, we quantified CD3^{-}NK1.1^{+}NKp46^{+} uNK numbers in both virgin and pregnant *Nfil3*$^{-/-}$ females and compared them with numbers in WT females. Both *Nfil3*$^{-/-}$ and WT females were mated with WT males to restrict NFIL3 deficiency to maternal tissues. Pregnant uteri were dissected into decidua – which is the transformed mucosa that forms in response to blastocyst implantation in mice – and the muscular outer layer, the myometrium, which includes a transient lymphoid structure that forms in rodents during pregnancy, the mesometrial lymphoid aggregate of pregnancy (MLAp). CD3^{-}NK1.1^{+}NKp46^{+} uNK expanded from 2.3×10^4 cells in virgin to 13.7×10^4 cells in pregnant WT mice (5.9-fold increase). The expansion in *Nfil3*$^{-/-}$ mice was less pronounced, from 0.9×10^4 cells in virgin to 2.4×10^4 cells in pregnant mice (2.7-fold increase). Most of this expansion in both WT and *Nfil3*$^{-/-}$ dams was due to accumulation of uNK cells in the myometrium (**Table 1**).

Based on the expression of the two integrins CD49a (ITGA1, VLA-1) and DX5 (CD49b, ITGA-2, VLA-2), uterine CD3^{-}NK1.1^{+}NKp46^{+} cells can be divided in two subsets: CD49a^{+}DX5^{-} and CD49a^{-}DX5^{+} cells, which resemble similar subsets in liver trNK and cNK cells, respectively (25). **Figure 1A** shows a representative flow cytometry analysis, **Figure 1B** a diagrammatic representation, and **Table 1** summarizes the quantification of the expansion of the two subsets during pregnancy. The expression of CD49a and DX5 has been described as mutually exclusive on the two subsets both in liver and uterus (25); however, we found that DX5 was expressed on most uterine CD49a^{+} cells. In *Nfil3*$^{-/-}$ mice, the expression of DX5 on many CD49a^{+} cells increased at midgestation, albeit less than on cNK cells in WT mice (**Figure 1A**). NFIL3 may regulate expression of integrins (27), and we found that in *Nfil3*$^{-/-}$ dams, expression of DX5 on some of the CD49a^{+} cells became even higher than that on cNK cells (**Figure 1A**).

Ex vivo, both CD49a^{+} cells and cNK cells stained positive for Ki-67, a marker strictly associated with cell proliferation (**Figure 1C**). Given the considerable expansion and the lower rate of proliferation in decidual cNK cells in WT mice (**Figures 1A,C**), it is conceivable that some of decidual cNK cells are cNK cells that came from the periphery. Another possible explanation is the *in situ* generation from progenitors, and both scenarios remain to be tested. Like the hepatic CD49a^{+} cells, also uterine CD49a^{+} cells were CD69^{+} and CD62L^{-} (**Figure 1C**).

TABLE 1 | Numbers of uterine CD49a^{+} and CD49a^{-} NK cells in WT and *Nfil3*$^{-/-}$ mice.

	Spleen (×10^6)	V. uterus (×10^4)	Myometrium (×10^4)	Decidua (×10^4)
WT mice				
NK1.1^{+} NKp46^{+}	1.613 ± 0.139	2.345 ± 0.358	11.9 ± 0.07	1.84 ± 0.031
cNK	1.515 ± 0.131	0.809 ± 0.206	2.215 ± 0.127	0.768 ± 0.070
CD49a^{+}	0.098 ± 0.027	1.536 ± 0.293	9.581 ± 0.582	1.071 ± 0.229
***Nfil3*$^{-/-}$ mice**				
NK1.1^{+} NKp46^{+}	0.035 ± 0.023*	0.879 ± 0.044	1.932 ± 0.01***	0.459 ± 0.007**
cNK	0.019 ± 0.009*	0.057 ± 0.026*	0.030 ± 0.014***	0.012 ± 0.004***
CD49a^{+}	0.008 ± 0.029*	0.816 ± 0.005	1.932 ± 0.763***	0.447 ± 0.053*

*Leukocytes were enriched from virgin (v. uterus) and pregnant (gd9–10.5) uteri (myometrium separated from decidua) and stained for CD45, NK1.1, NKp46, CD3, DX5, and CD49a. Conventional NK cells (cNK) are identified as CD49a^{-}DX5^{+}. Based on percentages of positive cells, absolute cell numbers were calculated out of total live singlet CD45^{+} lymphocytes. Statistically significant differences between WT and Nfil3$^{-/-}$ for each subset are indicated (*P < 0.05; **P < 0.01; ***P < 0.001, unpaired t-test, n = 2–5 independent experiments).*

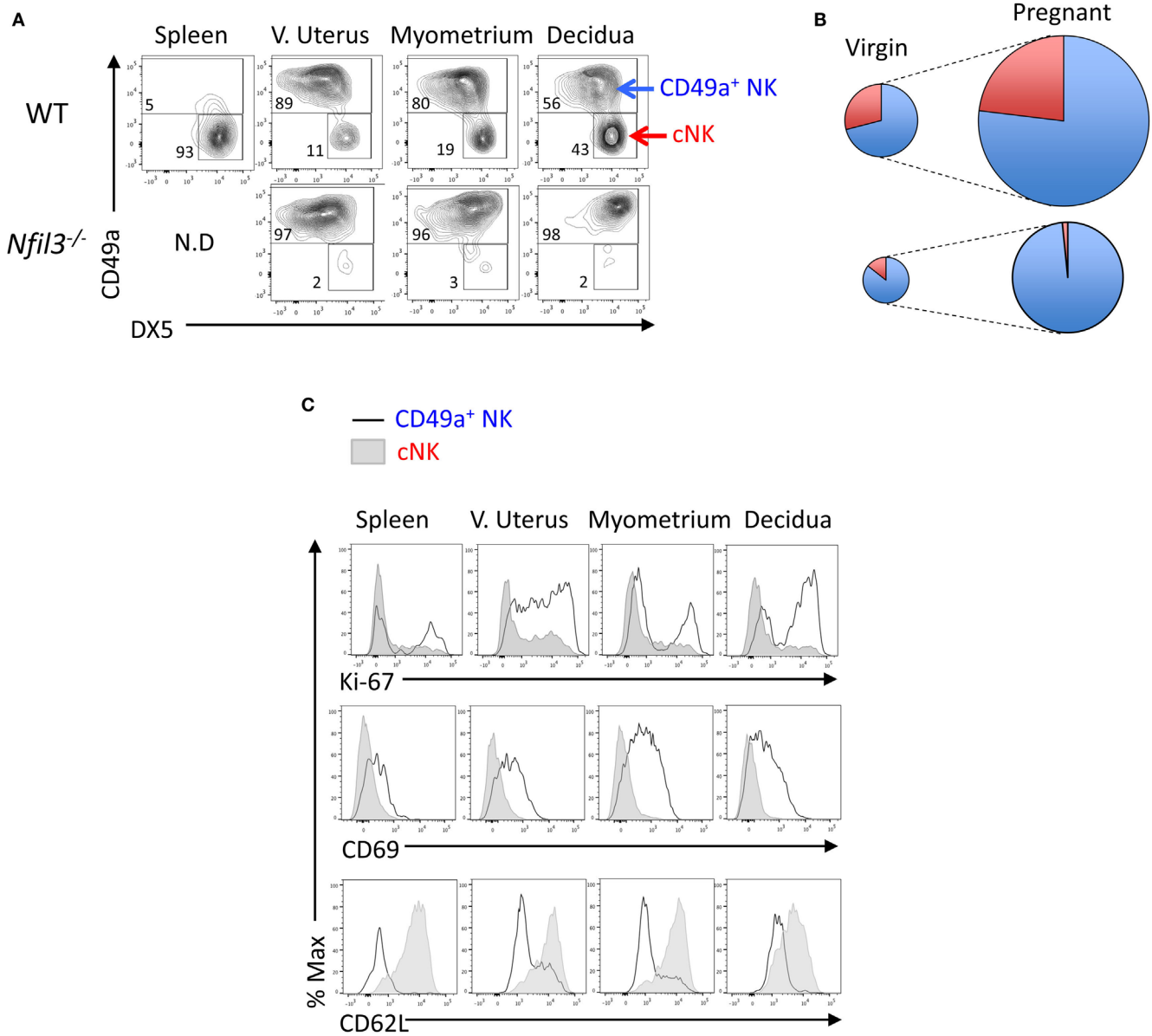

FIGURE 1 | Reduced expansion of uNK cells in *Nfil3*$^{-/-}$ dams. **(A)** Representative flow cytometry plots showing separation of CD45^{+}CD3^{-}NK1.1^{+}NKp46^{+} NK cells in CD49a^{+} uNK and CD49a^{-}DX5^{+} (cNK) cells in the indicated tissues in WT and *Nfil3*$^{-/-}$ females. **(B)** Visual representation of composition and expansion of CD49a^{+} uNK (blue) and CD49a^{-}DX5^{+} cNK (red) in the uterus of virgin and pregnant WT and *Nfil3*$^{-/-}$ mice. **(C)** Representative flow cytometry histograms of intracellular Ki-67, surface CD69 and CD62L in *ex vivo* CD49a^{+} uNK, and CD49a^{-}DX5^{+} cNK in the indicated tissues of WT females. Data representative of two to three independent experiments, n = 4–20 mice per group.

Phenotyping Uterine CD49a^{+} and CD49a^{-} Cells

Expression of Eomes, among ILCs, defines NK lineages, whereas high granularity and staining with the lectin *Dolichos biflorus* agglutinin (DBA) in mice are features associated with uNK cells. We analyzed Eomes expression, granularity, and DBA reactivity on the two subsets of CD49a^{+} and CD49a^{+} uterine cells gated on CD45^{+}CD3^{-}CD19^{-}NK1.1^{+}NKp46^{+} cells. All CD49a^{-} cNK and most CD49a^{+} cells expressed Eomes in WT mice (**Figure 2A**), thus marking them as "bona fide" NK cells. The minority of the CD49a^{+} cells that did not express Eomes are Eomes^{-} uILC1s (9). uNK have also been known as "granulated" cells in several species (28). The granularity of CD49a^{+} cells was more pronounced than that of cNK cells (**Figure 2A**). Perhaps the most distinctive feature of mouse uNK cells is the positivity for DBA staining (29), although more recently, it has become apparent that some uNK cells are not reactive for DBA (30, 31), may be the largest producer of IFN-γ (32) and, if inhibited, contribute to insufficient uterine

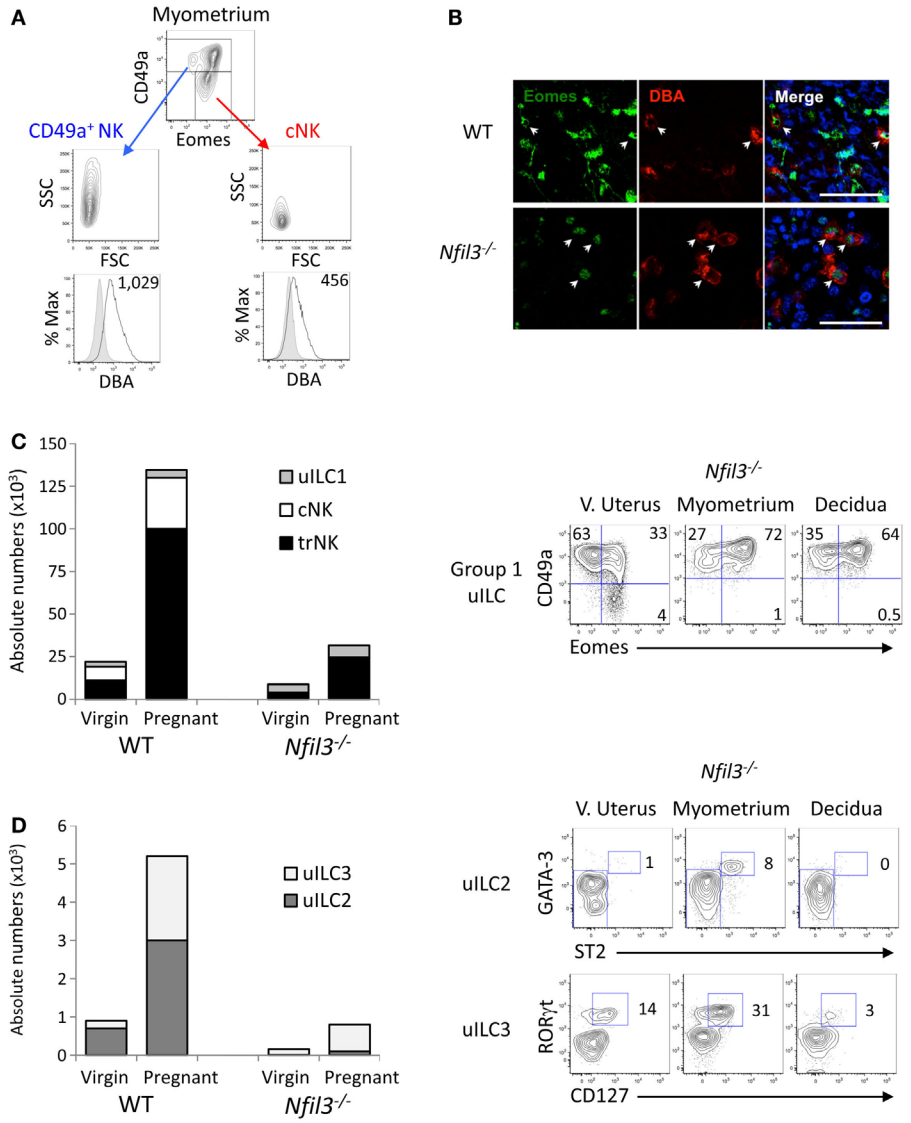

FIGURE 2 | Uterine ILCs in virgin and pregnant *Nfil3*$^{-/-}$ mice. **(A)** Representative flow cytometry histograms of intracellular Eomes, granularity (SSC), size (FSC), and reactivity for *Dolichos biflorus* agglutinin (DBA) lectin (GMFI is indicated) in *ex vivo* CD49a^{+} NK and CD49a^{-} cNK in the indicated tissues of WT females. The negative control cell population for DBA reactivity is made up of CD3^{+} and CD19^{+} cells. **(B)** Representative immunofluorescence stainings of gd9.5 implantation sites (decidua) from WT and *Nfil3*$^{-/-}$ pregnant females mated with WT males. Almost all DBA^{+} uNK (red) express intranuclear Eomes (green), as indicated by arrowheads. Scale bar = 50 μm. **(C)** Absolute numbers and composition in group 1 ILCs in the uterus from WT and *Nfil3*$^{-/-}$ virgin and pregnant females (left graph). Representative flow cytometry plots of the changes in group 1 ILC composition from virgin to pregnant *Nfil3*$^{-/-}$ mice (right panel). Gated on CD45^{+}CD3^{-}CD19^{-}NK1.1^{+}NKp46^{+} cells, uILC1 are defined as CD49a^{+}Eomes^{-}, uterine trNK cells as CD49a^{+}Eomes^{+}, and cNK cells as CD49a^{-}Eomes^{+}. Data representative of two to five independent experiments. **(D)** Absolute numbers and composition in uILC2s and uILC3s in the uterus from WT and *Nfil3*$^{-/-}$ virgin and pregnant females (left graph). Representative flow cytometry plots of the changes in uILC2 and uILC3 composition from virgin to pregnant *Nfil3*$^{-/-}$ mice (right panel). GATA-3^{hi}ST2^{+} uILC2s are gated on CD45^{+}CD3^{-}CD19^{-}CD11b^{-}NK1.1^{-}NKp46^{-}CD90.2^{+} cells. RORγt^{+}CD127^{+} uILC3s are gated on CD45^{+}CD3^{-}CD19^{-}CD11b^{-}NK1.1^{-}CD90.2^{+} cells. Data representative of two to three independent experiments.

adaptations to pregnancy (13). DBA staining mostly colocalized with Eomes staining in tissue sections of both WT and *Nfil3*$^{-/-}$ dams (**Figure 2B**). Although both CD49a^{+} cells and cNK cells stained positive for DBA, CD49a^{+} cells showed a brighter staining than cNK cells (**Figure 2A**), suggesting that these cells may relate more closely to what traditionally has been referred to as a major subset of uNK cells. In line with this, CD49a^{+} cells produced less IFN-γ than cNK cells after stimulation with PMA and ionomycin or with IL-12 and IL-15 (not shown).

Expansion of Group 1 Uterine ILC1 Subsets in Pregnancy

The data here and those in the accompanying manuscript (33) corroborate our recently published data, which show that

Eomes expression defines two populations of group 1 ILCs within the CD49a$^+$ subsets, which, along with cNK cells make up the three subsets of group 1 uILC1, defined collectively as CD45$^+$CD3$^-$CD19$^-$NK1.1$^+$NKp46$^+$ cells (9). The three subsets are CD49a$^+$Eomes$^+$ trNKs, CD49a$^+$Eomes$^-$ ILC1s, and CD49a$^-$DX5$^+$ cNK cells (9). We next set out to define how pregnancy and the lack of NFIL3 affect the changes in these three subsets of group 1 ILC1s. Eomes$^+$ trNK cells expanded about sevenfold in *Nfil3*$^{-/-}$ mice (**Figure 2C**). This expansion was nearly as extensive as in pregnant WT mice; however, trNK cells were still over fivefold less numerous in *Nfil3*$^{-/-}$ dams than in WT dams, presumably because they were already decreased in virgin mice. Eomes$^-$ uILC1s did not seem to expand in *Nfil3*$^{-/-}$ dams and expanded very little in pregnant WT dams too. CD49$^-$DX5$^+$ cNK cells did not expand at all in *Nfil3*$^{-/-}$ dams, whereas they expanded 3.7-fold in WT dams (**Figures 1A** and **2C**; **Table 1**). These results show that the population of uterine group 1 ILC that expand the most during pregnancy in WT mice is trNK cells, with some expansion of cNK cells and little or no expansion of Eomes$^-$ uILC1 cells (**Figure 2C**; **Table 1**). NFIL3 is required for development and expansion of uterine cNK cells and for development of trNK cells, but is dispensable for the expansion of trNK cells and the development of Eomes$^-$ uILC1s.

Expansion of Uterine ILC2 and ILC3 in Pregnancy

NFIL3 is important also for the development of other ILCs. *Nfil3*$^{-/-}$ mice are severely deficient in ILCs in several tissues, such as the intestine or the lungs (19–21). Recently, we have shown that only uILC2s strictly require NFIL3 to develop in the uterus of virgin mice (9). Indeed, uILC2s were not detectable in virgin *Nfil3*$^{-/-}$ mice and barely detectable in pregnant *Nfil3*$^{-/-}$ mice (**Figure 2D**), and uILC3s were present in virgin *Nfil3*$^{-/-}$ mice but they did not expand as in WT mice during pregnancy (**Figure 2C**).

It will be interesting to study the potential role of uILC2s and uILC3s during murine pregnancy, for example, in MLAp formation. Moreover, ILC3s are the most abundant non-NK ILCs in the human uterus (9, 10), and they may play roles in human pregnancy.

Altogether, the data show that NFIL3 may serve different roles in different uILC lineages. Homeostatic development of trNK in virgin mice, but not expansion in response to pregnancy, requires NFIL3, whereas the opposite seems to be true for uILC3s. On the other hand, cNK and uILC2s require NFIL3 for both development and expansion.

Maternal, Placental, and Fetal Abnormalities in Nfil3$^{-/-}$ Dams

Our data show that *Nfil3*$^{-/-}$ mice exhibit defects in the development and/or the expansion of uterine cNK cells and other uILCs during pregnancy. The residual trNK cells, as well as the Eomes$^-$ uILC1s and the uILC3s found in *Nfil3*$^{-/-}$ dams, may however sustain the uterine adaptations necessary for placentation and fetal growth. In order to test this, we compared uterine arterial changes, litter sizes, and placental and fetal growth in WT and *Nfil3*$^{-/-}$ dams mated with syngeneic WT mice. In contrast to what was found in allogeneic pregnancies (34), we found normal litter sizes. Therefore, the lack of NFIL3 does not impact on the number of live births in our crosses. Previous work with mice that do not develop uNK cells or have hypofunctional uNK cells showed that these mice too have normal litter sizes. However, analysis of uterine tissues and fetoplacental growth revealed clinically relevant abnormalities in these mice (11, 13).

To assess the role of residual ILCs in *Nfil3*$^{-/-}$ mice during pregnancy, we used stereology and immunohistochemistry to analyze morphological aspects of implantation sites in *Nfil3*$^{-/-}$ females mated with WT males. DBA$^+$ uNK cells were readily detected in uterine sections of both WT and *Nfil3*$^{-/-}$ mice, including in the decidua and in the MLAp (**Figures 3A,B**). The MLAp is reduced in size in NK-deficient mice (11) or in mice engineered to have hypofunctional uNK cells (13), and we found here that it was also the case in *Nfil3*$^{-/-}$ dams (**Figures 3A,B**). Vascular walls were thicker with incomplete loss of smooth muscle actin (SMA) in *Nfil3*$^{-/-}$ dams (**Figures 3B,C**), a phenotype typical of NK-deficient mice (11, 13). Impaired arterial remodeling in uNK-deficient mice results in placental abnormalities and fetal growth restriction. *Nfil3*$^{-/-}$ dams and their fetuses displayed phenotypes similar to those of NK-deficient mice (**Figure 4A**). Conversely, WT females mated with *Nfil3*$^{-/-}$ males had normal-sized fetuses and placentas (**Figure 4B**). The comparison of *Nfil3*$^{-/+}$ with *Nfil3*$^{+/-}$ conceptuses showed that the reduction in fetal weight and the increase in placental weight is a phenotype of maternal origin (**Figure 4C**).

The labyrinth zone of mouse placenta is analogous to the human placental villi, where nutrient and gaseous exchange take place. The volume of the labyrinth zone was larger in placentas of *Nfil3*$^{-/-}$ dams (**Figures 4D,E**). Trophoblast and maternal blood spaces, as well as the thickness of the barrier separating the fetal and maternal blood spaces, were increased in placentas of *Nfil3*$^{-/-}$ dams (**Figure 4E**). These abnormalities, with the exception of the surface area of placental exchange, mirror those in NK-deficient *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ dams (**Figure 4F**) and are consistent with altered placental hemodynamics secondary to deficient spiral artery remodeling (35, 36). The reason and the significance of the divergent phenotype of the surface area of placental exchange in *Nfil3*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ dams are unclear; however, it may reflect the difference in cellularity in the two strains, with residual ILCs and other lymphocytes in *Nfil3*$^{-/-}$ dams and total lack of all lymphoid cells in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ dams.

In a mouse model of allogeneic pregnancy, Fu et al. found increased Th17 cells in the decidua of *Nfil3*$^{-/-}$ dams, which was associated with fetal demise, and they suggested that the absence of uNK cells in *Nfil3*$^{-/-}$ dams might have caused Th17 expansion and breakdown of tolerance (34). We find here that some NK are instead present in *Nfil3*$^{-/-}$ dams at midgestation, alongside Eomes$^-$ uILC1s, and uILC3s. In conclusion, our results show that the residual uILCs in *Nfil3*$^{-/-}$ dams are not sufficient to mediate normal uterine adaptations to pregnancy. Placentation and fetal growth is compromised in the absence of NFIL3, which emerges as an essential mediator of non-redundant functions for reproduction in mice.

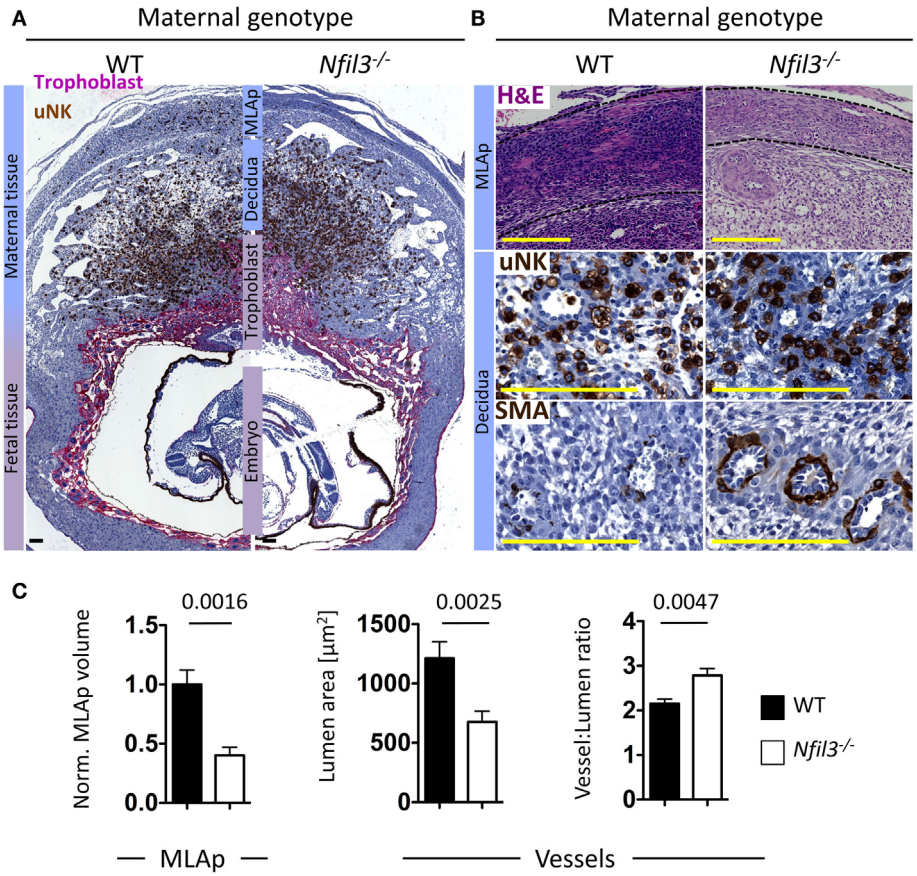

FIGURE 3 | Abnormalities in uterine tissues of *Nfil3*$^{-/-}$ dams. **(A)** Representative immunohistological staining of gd9.5 implantation sites from WT and *Nfil3*$^{-/-}$ pregnant females mated with WT males. DBA^{+} uNK cells (brown) within the mesometrial lymphoid aggregate of pregnancy (MLAp) and the decidua and in proximity of the invading trophoblast, which is stained with cytokeratin (purple). Bar = 500 µm. **(B)** H&E and immunohistological staining of MLAp and decidua at higher magnification showing MLAp area, distribution of uNK around vessels, and presence of residual smooth muscle actin (SMA) within the vascular wall. Bar = 500 µm. Indicated is the maternal geotype. All females were mated with WT males. **(C)** Stereological quantification of MLAp (left), vascular lumen area (middle), and relative wall thickness (right). Means ± SEM of data representative of 4–6 litters per genotype. Statistical differences were calculated with an unpaired Student's *t*-test.

MATERIALS AND METHODS

Mice

C57BL/6J mice were purchased from Charles River UK and were used as WT mice in all experiments. *Nfil3*$^{-/-}$ and *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice were in C57BL/6J background as previously described (17, 37). All mating experiments used virgin females mated either with C57BL/6J males or with *Nfil3*$^{-/-}$ males as indicated. Gestation day (gd) 0.5 was counted at noon of day of the appearance of a copulation plug. Virgin and pregnant females were age matched (7–12 weeks). All procedures were conducted in accordance with the University of Cambridge Animal Welfare and Ethical Review Body and United Kingdom Home Office Regulations.

Cell Preparation

Ovaries, mesometrium, and cervix were removed from all uteri in ice-cold PBS without Ca^{2+}/Mg^{2+}. Virgin uteri were entirely minced with scissors. For gd9.5 and gd10.5 mid-gestated uteri, myometrium tissue (including MLAp) were first dissected from the decidua and pooled. The decidua basalis (containing placenta) tissues were also separately minced and pooled. Embryos, yolk sacs, and decidua parietalis were discarded. Minced tissues were first softened in the predigestion solution, 1× HBSS (PAA) containing 5 mM EDTA (Sigma), 15 mM HEPES solution (Life Technologies), and 10% FCS (Life Technologies) under moderate rotation (220 rpm) for 2 × 15 min at 37°C. Then, the cell suspension was filtrated through a 100-µm cell strainer and the minced tissues were rinsed from remaining EDTA with 1× PBS. This flow-through fraction containing intraepithelial lymphocytes is stored on ice by the time the minced tissue undergoes enzymatic digestion. The latter are dissociated in RPMI 1640 containing 2% FCS, 0.1 WU/ml Liberase DH, and 30 µg/ml DNase (Roche) for 30 min at 37°C under gentle agitation. Digested tissues were fully dissociated with a syringe plunger against a 100-µm cell strainer. Pre-digest and post-digest flow-through solutions were pooled and washed in Ca^{2+}/Mg^{2+}-free PBS containing 5 mM EDTA for enzyme inactivation. The cell suspension was overlayed on a 80/40% Percoll (GE Healthcare Life Sciences) gradient and the

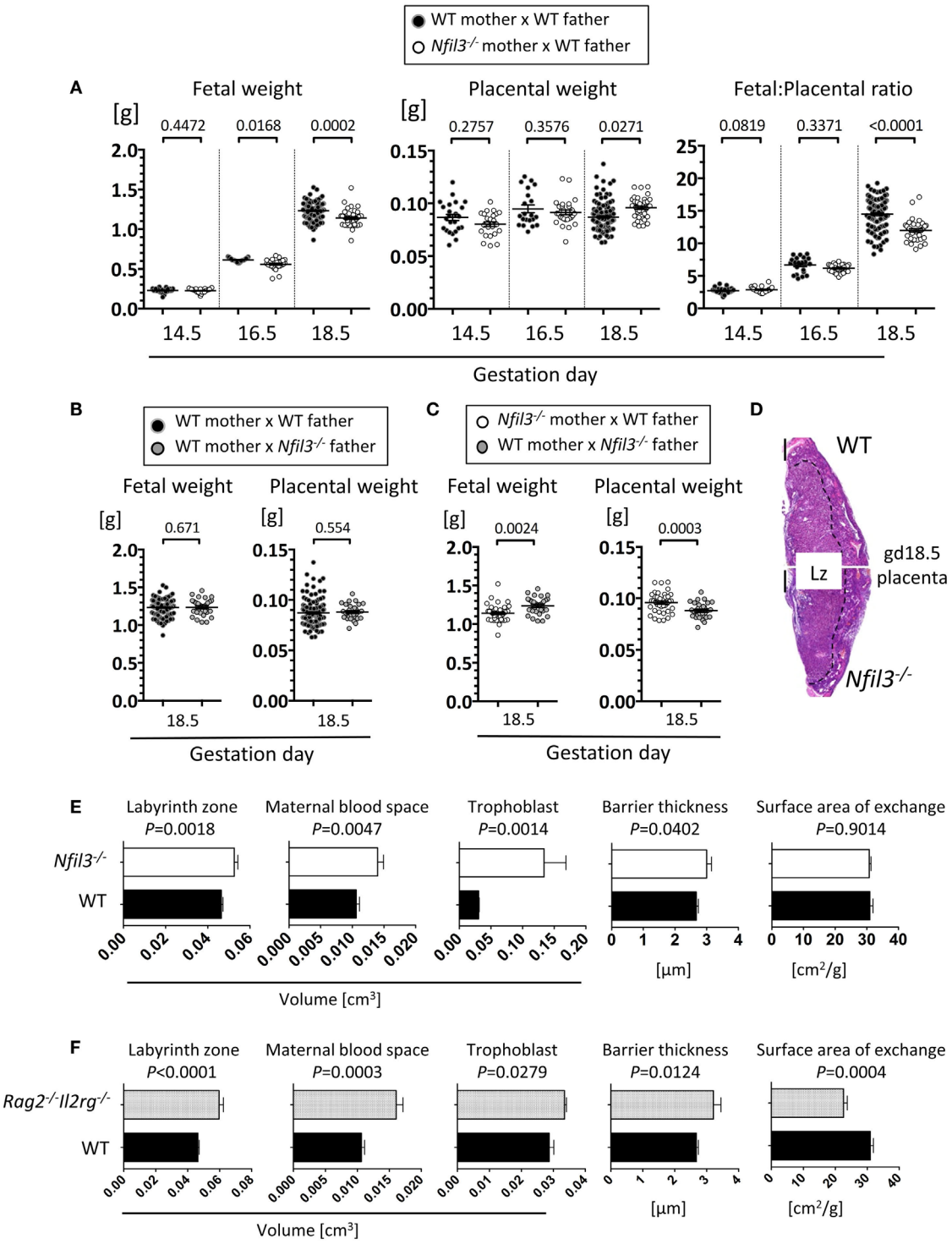

FIGURE 4 | Fetal and placental abnormalities in *Nfil3*$^{-/-}$ dams. **(A)** Fetal weight, placental weight, and ratio of fetal/placental weight of conceptuses from either WT or *Nfil3*$^{-/-}$ females mated with WT males. Means ± SEM of data representative of 3–18 litters per maternal genotype per gestational age. **(B)** Fetal and placental weights of conceptuses from WT females mated either with WT or *Nfil3*$^{-/-}$ males. Means ± SEM of data representative of 4–5 litters per maternal genotype. **(C)** Fetal and placental weight of heterozygous offspring from either *Nfil3*$^{-/-}$ dams **(A)** or *Nfil3*$^{-/-}$ fathers **(B)**. Means ± SEM of data representative of 4–5 litters per cross. **(A–C)** Statistical differences were calculated using mixed effect modeling taking into account the clustering of pups from the same litter. **(D)** Representative H&E staining of gd18.5 placenta from WT and *Nfil3*$^{-/-}$ dams. The discontinuous line demarcates the labyrinth zone (Lz). **(E,F)** Stereological evaluation of the volume of labyrinth zone, maternal blood space, trophoblast, barrier thickness, and surface area of exchange in placentas from *Nfil3*$^{-/-}$ dams **(E)** and in placentas from lymphocyte deficient *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ dams **(F)**, both compared to those from WT dams. Data representative of 5–11 placentas per genotype for Lz volume and 4–9 placentas for Lz structure (MBS, trophoblast, BT, and SA). Statistical differences were calculated with an unpaired Student's *t*-test.

leukocyte fraction was collected at the interface. Cells from spleens and livers (from virgin mice) were similarly prepared except from the EDTA pre-digest step [adapted from Ref. (38, 39)].

Flow Cytometry

Conjugated Abs anti-mouse CD45 (clone 30-F11), CD3ϵ (500A2 or 17A2), CD19 (6D5), NK1.1 (PK136), NKp46 (29A1.4), CD90.2 (30-H12), ST2 (RMST2-2), CD49b (DX5), NK1.1 (PK136), NKp46 (29A1.4), CD62L (MEL-14), CD127 (A7R34), RORγt (Q31-378), anti-human/mouse CD11b (M1/70), Eomes (Dan11mag) and CD16/32-Fc blocking (93), anti-rat/mouse CD49a (Ha31/8), anti-human/mouse GATA-3 (TWAJ and 16E10A23), and Ki-67 (B56) were purchased from Biolegend, eBioscience, BD Biosciences, or R&D Systems. Transcription factors and Ki-67 were stained using the FoxP3 staining buffer set (eBioscience) according to the manufacturer's instructions. Fixable viability dyes eFluor 780 and eFluor 506 (eBioscience) was used to exclude dead cells. Cells were stained with DBA (Vector Labs) after the fixation/permeabilization step. Samples were acquired on a LSR Fortessa (BD Biosciences) using FACS DiVa and analyzed using FlowJo (Tree Star).

Immunohistochemistry

Total pregnant uteri were fixed 6 h at room temperature (RT) in formalin and embedded in paraffin. Sections were serially cut at 7 μm and stained with hematoxylin and eosin (H&E) at 49 μm intervals using standard methods. For chromogenic IHC cytokeratin/DBA double staining, proteinase-K mediated antigen retrieval was performed, and sections were incubated with polyclonal rabbit anti-cytokeratin (1:1000, DAKO, Z0633) at 4°C, followed with biotinylated universal pan-specific antibody (1:500, Vector lab, BA-1300) for 1 h at RT and labeled using VECTASTAIN ABC-AP KIT (Vector lab, AK-5000) and SIGMA *Fast* (Sigma-Aldrich, F4523). After colorimetric detection, sections were blocked using Avidin/Biotin Kit (Vector lab, SP-2001) and incubated with biotinylated DBA-lectin (6.6 μg/ml) for 30 min at RT, labeled using ABC kit (Vector lab, PK6100) and DAB, Diaminobenzidine (Sigma-Aldrich, D4168). For SMA, SMA single staining, paraffin sections were subjected to citrate buffer heat-induced epitope retrieval (HIER) for 3 min, blocked using Mouse On Mouse (MOM) kit (Vector lab, MKB-2213), and incubated with mouse anti-human SMA (1:100, DAKO, M0851) for 30 min at RT, followed by MOM biotinylated anti-mouse IgG reagent (Vector lab, MKB-2213), labeled using ABC kit (Vector lab, PK6100) and DAB (Sigma-Aldrich, D4168) and counterstained with hematoxylin. All immunohistochemical stainings included isotype controls. For dual immunofluorescence staining of DBA and Eomes, 7 μm thick cryosections of uterine tissue at gd9.5 were fixed in 100% acetone at 4°C for 20 min. Sections were incubated with AF488-conjugated rat anti-mouse Eomes (eBioscience, Dan11mag, 1:50) for 60 min at RT and sequentially with FITC-conjugated donkey anti-rat IgG (Jackson Immunoresearch, 712-095-153, 1:150), AF488-conjugated rabbit anti-fluorescein/Oregon Green (Life Technologies, A-11090, 1:200), and AF488-conjugated donkey anti-rabbit IgG (Life Technologies, A-21206, 1:100) for 30 min each at RT. Sections were incubated with 6.6 μg/ml biotinylated DBA and labeled using AF555-conjugated streptavidin (Life Technologies, S-32355, 1:500), each for 30 min at RT. Sections were counterstained with DAPI. Images were acquired using a Leica SP5 confocal microscope and analyzed using ImageJ software to adjust brightness and contrast.

Stereology Analysis of MLAp Volume and Decidual Arteries

Tissue volumes were quantified from serial sections using the Cavalieri method (40). Briefly, MLAp cross-sectional areas were calculated on serial sections and volumes were calculated by accounting for the spacing between the sections as previously described (41). Arterial remodeling was evaluated in the central decidua basalis on sagittal sections and veins localized at the periphery were excluded. Three to five implantation sites from randomly chosen uterine horn were analyzed per litter. In every implantation site, three to five largest vessels were measured in triplicate (three sections with distance of 49 μm apart). Lumen area and vessel-to-lumen ratios were quantitated by using outlines tools from NDP viewer (Hamamatsu) and Aperio ePathology (Leica Biosystems) softwares. SMA IHC staining was used for further qualitative assessment of arterial remodeling (41).

Stereology Analysis of Placentas

Midsagittal cross-sections of placental tissue (gd18.5) were stained with H&E using standard protocols. From each of nine WT and five *Nfil3*$^{-/-}$ mice, the placenta closest in weight to the litter mean was selected for stereology analysis. The fractional volumes of the labyrinth zone (Lz) were assessed by point counting (10× objective lens, the newCAST Computer Assisted Stereological Toolbox system Visopharm, Hoersholm, Denmark) with systematic random sampling (42). Assuming a density of 1 mg mm^{-3}, fractional volumes were converted to absolute volumes in cubic millimeter by multiplying by total placental volume (43). The other placental halves were fixed in glutaraldehyde and embedded in Spurr's epoxy resin and a single mid-line section taken (1 μm thickness) then stained with toluidine blue. Volumes of maternal blood space and trophoblast in the Lz, surface area, and thickness of the barrier were calculated at 100× magnification using stereological methods that were described previously (42).

Statistics

Data were analyzed using paired or unpaired parametric Student's *t*-test or non-parametric Mann–Whitney test. Fetal and placental weights were analyzed using a mixed model approach to test the effect of maternal genotype and account for both gestational age and clustering of observations by litter (44). $P < 0.05$ was taken as statistically significant for all tests. Analyses were performed using GraphPad Prism and IBM SPSS.

AUTHOR CONTRIBUTIONS

HB, AM, and FC conceived the project. SB, J-MD, AS-P, LG, JK, EB, HY, SJ, LV, DH, and KP performed experiments. SB, J-MD, AS-P, LG, JK, AF, GB, and FC designed experiments and analyzed data. SB, J-MD, and FC wrote the manuscript.

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Cytokines Induce Faster Membrane Diffusion of MHC Class I and the Ly49A Receptor in a Subpopulation of Natural Killer Cells

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Cytokines have the potential to drastically augment immune cell activity. Apart from altering the expression of a multitude of proteins, cytokines also affect immune cell dynamics. However, how cytokines affect the molecular dynamics within the cell membrane of immune cells has not been addressed previously. Molecular movement is a vital component of all biological processes, and the rate of motion is, thus, an inherent determining factor for the pace of such processes. Natural killer (NK) cells are cytotoxic lymphocytes, which belong to the innate immune system. By fluorescence correlation spectroscopy, we investigated the influence of cytokine stimulation on the membrane density and molecular dynamics of the inhibitory receptor Ly49A and its ligand, the major histocompatibility complex class I allele H-2D^d, in freshly isolated murine NK cells. H-2D^d was densely expressed and diffused slowly in resting NK cells. Ly49A was expressed at a lower density and diffused faster. The diffusion rate in resting cells was not altered by disrupting the actin cytoskeleton. A short-term stimulation with interleukin-2 or interferon-α + β did not change the surface density of moving H-2D^d or Ly49A, despite a slight upregulation at the cellular level of H-2D^d by interferon-α + β, and of Ly49A by IL-2. However, the molecular diffusion rates of both H-2D^d and Ly49A increased significantly. A multivariate analysis revealed that the increased diffusion was especially marked in a subpopulation of NK cells, where the diffusion rate was increased around fourfold compared to resting NK cells. After IL-2 stimulation, this subpopulation of NK cells also displayed lower density of Ly49A and higher brightness per entity, indicating that Ly49A may homo-cluster to a larger extent in these cells. A faster diffusion of inhibitory receptors could enable a faster accumulation of these molecules at the immune synapse with a target cell, eventually leading to a more efficient NK cell response. It has previously been assumed that cytokines regulate immune cells primarily via alterations of protein expression levels or posttranslational modifications. These findings suggest that cytokines may also modulate immune cell efficiency by increasing the molecular dynamics early on in the response.

Keywords: major histocompatibility complex, natural killer cells, molecular dynamics, cytokine, fluorescence correlation spectroscopy, plasma membrane, Ly49 receptors, molecular diffusion

INTRODUCTION

Natural killer cells play an essential role in innate immunity and protect the host against viral infection and tumors (1). Murine NK cells express a family of inhibitory receptors called Ly49 receptors, which have major histocompatibility complex (MHC) class I molecules as ligands. MHC class I is expressed by virtually all healthy cells, but can be downmodulated upon infection or in tumorigenesis. In the absence of sufficient cognate MHC class I expression, NK cells proceed to kill the target cells due to lack of signaling through their inhibitory receptors, a process called missing-self recognition (2).

Cytokines are important modulators of NK cell activity. Whereas IL-15 is important for NK cell survival, maturation, and priming, interleukin-2 (IL-2) and type I interferons are prototypical cytokines of NK cell activation (3, 4). IL-2 is currently used for clinical NK cell activation in cancer treatment (5). The increased cytotoxicity *in vitro* and *in vivo* induced by cytokines is dependent on the upregulation of numerous proteins, including several adhesion molecules, as well as effector molecules (4). Just a brief stimulation with IL-2 augments adhesion and cytotoxicity, primarily against missing-self targets (6). IL-2 also augments the NK cell dynamics at a cellular level. After several days in IL-2 culture, NK cells display a more migratory phenotype and a more dynamic migratory pattern (7). However, IL-2 stimulation may not affect all NK cells equally, since a minority of IL-2 stimulated NK cells were observed to perform the majority of kills (8). Type I interferons, such as interferon alpha and beta (IFN-α + β), are also strong inducers of NK cell cytotoxicity, primarily during viral infections (9, 10). Type I interferons, in addition, strongly upregulate MHC class I on many cell types, including lymphocytes (11, 12).

When IL-2 binds to its receptor, an association with the cytoskeleton is induced, and the diffusion rate of the receptor complex is slowed down (13). However, although much is known about the cellular dynamics in response to cytokines, very little is known about how cytokines affect molecular dynamics beyond its own receptor. This is despite the vital role of lateral diffusion of molecules within membranes for all diffusion-limited bimolecular interactions. Examples of such reactions are ample, and also involve reactions crucial for immune cell regulation and activation. For instance, lateral diffusion of receptors is responsible for the formation of micro-clusters and the subsequent immune synapse in T cells (14). The diffusion rate of ligands impacts the degree of T cell activation (15), and the activation of CD4 T cells is regulated by the diffusion rate of lck between the CD3 and CD28 receptors (16).

Apart from interacting with its ligands in *trans*, on target cells, Ly49 receptors also interact with their MHC class I ligands in *cis,* in the membrane of the NK cell itself. Such *cis* interactions prohibit Ly49 from interacting with MHC class I in *trans* (17). Thus, the total number of receptors that are "free" and, therefore, available to interact with MHC class I in *trans* is decreased by *cis* interactions. Since Ly49 receptors bound in *cis* do not signal negatively, the sequestration of receptors in *cis* limits the total inhibitory input that the NK cell can receive, consequently lowering the threshold for NK cell activation. *Cis* interactions are also suggested to be important for NK cell education, the process where NK cells are enabled to react on the lack of expression of self-specific MHC class I on target cells (18). The surface expression of MHC class I can affect the proportion of Ly49A that is bound in *cis*. In a transfected cell line, cells with a high surface expression of MHC class I displayed high proportions of Ly49A bound in *cis*, and vice versa (19). This is typical of a diffusion-limited bimolecular interaction in steady state. The molecular density within the cell membrane, as well as the diffusion rates of these proteins, are thus important factors in determining the rate of receptor–ligand interactions between Ly49A and H-2D^d in *cis*. This could ultimately be important to determine the biological outcome of cell–cell interactions.

In this study, we investigated the influence of cytokine stimulation on the MHC class I and Ly49 membrane dynamics and density using fluorescence correlation spectroscopy (FCS) (20). It was previously shown that activating receptors diffuse in different patterns on educated versus uneducated NK cells, thus, coupling the diffusive pattern of receptors to NK cell functionality (21). In this paper, we found that despite the well-known upregulation of MHC class I by type I interferons (11, 12), the membrane density of mobile H-2D^d did not increase after cytokine stimulation. In line with this, there was no indication that the proportion of Ly49A receptors bound in *cis* increased after cytokine stimulation. Instead, we identified a subpopulation of NK cells that exhibited a particularly fast diffusion rate of both the inhibitory receptor Ly49A and of the MHC class I molecule H-2D^d upon cytokine stimulation. This is to our knowledge the first report on the effect of cytokines on molecular dynamics within the membrane of immune cells. The rapid diffusion may enable a faster decision process within the NK cell to kill or not to kill a target cell.

MATERIALS AND METHODS

Experimental Animals

Mice expressing only the H-2D^d allele of MHC class I on C57BL/6 background were used. The generation of the mouse strain has been described previously (22). The mice were bred and maintained at the animal facility at the Department of Microbiology, Tumor and Cell biology, Karolinska Institutet, Sweden. The study was carried out in accordance to Swedish laws and regulations from Swedish board of agriculture (Jordbruksverket), approved by the northern Stockholm commission for ethics in experiments on animals, ethical permit N418-12.

Cell isolation and Cytokine Stimulation

Natural killer cells were enriched from freshly isolated murine splenocytes by negative selection of all other cells, using MACS^{TM} NK cell isolation kit mouse II (Miltenyi Biotec Norden^{AB}, Sweden)

Abbreviations: FCS, fluorescence correlation spectroscopy; OPLS-DA, orthogonal projections to latent structures, with discriminant analysis; PCA, principal component analysis.

according to the manufacturer's recommendations. After isolation, 2.5×10^6 cells/ml were resuspended in alpha-MEM medium supplemented with 10 mM HEPES, 10% fetal bovine serum, 2 μM beta-mercaptoethanol, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C, 5% CO_2 for 4 h, with or without cytokines. Cytokine concentrations were 1000 U/ml IL-2 (ImmunoTools, Germany) or 200 U/ml of each for IFN-α + β (Nordic BioSite, USA). In some experiments, cells were treated with 2 μg/ml Latrunculin B (Invitrogen, ThermoFisher Scientific, Sweden). Latrunculin B was added 15 min before measurement start, after cells were added to chambered glass slides to sediment (see below).

Immunofluorescent Labeling and Flow Cytometry

Fc receptors were blocked using Innovex Fc blocker solution (Innovex Biosciences, Richmond, VA, USA). The cells were then surface stained with antibodies. For spectroscopy and microscopy, anti-H-2D^d (clone 34-5-8S) and anti-Ly49A (clone JR9.318) antibodies were used. The anti-H-2D^d antibody was conjugated with MFP488 (MoBiTec GmbH, Germany) or Abberior Star-635 (Abberior GmbH, Germany) for spectroscopy, or Alexa647® dye (Life technologies, Sweden) for flow cytometry, according to the manufacturers' protocols. The anti-Ly49A antibody was purified from hybridoma supernatants in our lab and conjugated to Abberior Star-635. Antibody conjugates were purified using PD minitrap G-25 kit column (GE healthcare Bioscience, Sweden). The 34-5-8S and JR9.318 antibodies do not affect the diffusion behavior of the investigated surface proteins (19). However, the 34-5-8S antibody breaks the *cis* interaction between H-2D^d and Ly49A (23). In flow cytometry measurements, anti-H-2D^d and anti-Ly49A antibodies were employed in different samples, so as to avoid artificially high levels of "free" Ly49A caused by the presence of the anti-H-2D^d antibody. Antibodies for flow cytometry were Ly49A (clone YE1/48.10.6)-PE, NK1.1-PerCP-Cy5.5, and CD3-FITC (Biolegend and BD Biosciences). Cells for flow cytometry were subsequently stained with Live/dead ® fixable aqua dead cell stain kit 405 nm (Life Technologies, USA).

Flow cytometry data were acquired in a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed in FlowJo (version 9.7). NK cells were gated on the CD3 negative and NK1.1 positive lymphocyte population, as determined by forward and side scatter. The percentage difference between controls incubated at 37°C without cytokines and cytokine-treated cells was calculated using mean fluorescence intensity (MFI).

Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy measurements were performed using a Zeiss 510 microscope equipped with a Confocor 3 system (Carl Zeiss Microimaging GmbH), a 30 mW Argon-ion (488 nm), a 5-mW Helium–Neon (633 nm) laser, and a C-Apochromat 40x/1.2 NA water objective (24). The room temperature was 19°C. Calibration measurements of aqueous solutions of Alexa-647 (2 nM) and Rhodamine-110 (20 nM) with the laser excitation attenuated to 10, 5, 3, 1, and 0.5% of the maximal laser powers were first acquired. Cells were suspended in a 1:1 mixture of phosphate buffered saline and transparent RPMI medium, containing 0.5% fetal bovine serum. They were allowed to sediment in chambered cover glass slides (Fisher Scientific, Sweden) coated with Poly-L-Lysine (Sigma-Aldrich) for 20 min before experiment start. The sample identity (control, IL-2, and IFN-α + β stimulated) was blinded for the operator. The focus was positioned on the upper cell membrane. Only live (as judged by a distinct, rounded nucleus in the differential interference contrast image) Ly49A positive NK cells were selected for FCS measurements. Laser excitations for membrane measurements were 25–45 μW (representing 0.15–0.3% of maximal power for the 488 nm laser and 0.5–1% for the 633 nm laser). Five to seven 10-s repeats were measured per cell. Free antibodies in solution were measured with the same concentration and power as used for the cells, in non-stick-treated wells (PLL-PEG, Surface Solutions) to avoid depletion of antibodies from the solution.

FCS Analysis

Autocorrelation curves were generated by the Confocor 3 software and further analyzed using a MATLAB-based algorithm with a graphical user interface for fitting (25, 26). Free dyes and antibodies were fitted with 3D diffusion with one diffusion component and one triplet state (27):

$$G_{3D}(\tau) = \frac{1}{N} * \left(1 + \frac{\tau}{\tau_D}\right)^{-1} * \sqrt{\left(1 + S^{-2} * \frac{\tau}{\tau_D}\right)} * \left(1 + T1 * (1 - T1)^{-1} * exp\left(\frac{-\tau}{\tau_T}\right)\right) + 1 \quad (1)$$

Parameter definitions: N is the mean number of fluorescent entities within the focal volume, τ is the correlation time, τ_D is the average residence time in the focal detection volume (translational diffusion time), T1 is the average probability for the fluorophores to be in the triplet state, τ_T is the relaxation time for the singlet–triplet state transitions, and S denotes the ratio of height to waist diameter for the focal volume (axial to radial radii). The focal waist radius (ω) for the green and the red detection channels were calculated for each experimental day from the known diffusion coefficients of the free fluorophores, D; for Rhodamine-110, 3.3×10^{-10}, and for Alexa-647, 4.4×10^{-10} m^2/s (28, 29).

$$\omega = \sqrt{\frac{\text{observed}\,\tau_D}{4 * D}} \quad (2)$$

For cell measurements, individual repeats displaying bleaching, large clusters of bright entities, or cell movements, were removed. Remaining repeats were averaged and autocorrelation curves fitted. Due to the lack of *a priori* knowledge about any subpopulations of H-2D^d or Ly49A with different diffusion rates, the average diffusion rate per cell for each surface antigen was defined. Likewise, the underlying data did not contain enough information to fit models with different modes of movement (e.g., free diffusion versus constrained diffusion or active transport).

Thus, all lateral molecular movement was considered to be diffusion. The molecular transit time within cell membranes was, thus, fitted by a 2D diffusion model with one diffusion component and one triplet state (28):

$$G_{2D}(\tau) = \frac{1}{N} * \left(1 + \frac{\tau}{\tau_D}\right)^{-1} * \left(1 + T1 * (1 - T1)^{-1} * exp\left(\frac{-\tau}{\tau_T}\right)\right) + 1 \quad (3)$$

The diffusion coefficients of membrane-bound H-2D^d and Ly49A were obtained from the experimentally determined τ_D values (Eqs. 2 and 3). Densities of diffusing entities of H-2D^d and Ly49A were calculated from *N* from Eq. 3 and the focal waist radius ω for the individual color channels, using Eq. 4.

$$\text{Density} = \frac{N}{(\omega^2 * \pi)} \quad (4)$$

The molecular brightness, i.e., the detected fluorescence intensity per moving entity, was calculated from the overall detected photon count rate/*N*. To compensate for technical day-to-day variations, all cell and free antibody brightness values were adjusted based on the brightness of free fluorophores in the same experiment. To avoid a putative influence from background signal in cells where the antigens were expressed at a too low level, cells with a brightness of <33% of free antibodies were excluded from further analysis. For cells where one color channel was excluded, the other color channel was still used. Cells having a residence time τ_D >500 ms were also excluded from analysis. Such long τ_D could indicate movement of the whole cell, or membrane protrusions rather than movement of the individual molecules in the membrane.

Antibody Binding Efficiency

To define the binding efficiencies of H-2D^d (clone 34-5-8S) and Ly49A (clone JR9.318) antibodies, transfected cell lines with GFP-coupled variants of the respective proteins were employed. For H-2D^d, a previously described EL4-D^d-GFP cell line was used (30). The Ly49a cDNA was amplified by polymerase chain reaction from primary mouse NK cells and cloned with a GTC GAC GGC AGC CAA AAA ACC linker downstream of GFP into the *Sal*I site of the MigR retroviral construct. For Ly49A binding efficiency, AA8-CHO cells were transfected with the EGFP–Ly49a construct according to the manufacturer's protocol (Lipofectamine®2000, Invitrogen, Sweden). Both antibodies were labeled with Abberior star-635. Autocorrelation curves were measured and further analyzed as described above. Relative antibody binding efficiencies were determined by dividing the density of antibodies at the cell membrane with the density of labeled construct within the same measurement.

Multivariate Analysis

The FCS dataset was investigated using the software SIMCA, version 14 (Umetrics AB, Umeå, Sweden). Only cells containing values for all H-2D^d and Ly49A variables (brightness, density, and diffusion) after application of threshold were used. Underlying trends in the data were investigated by principal component analysis (PCA) and orthogonal projections to latent structures and discriminant analysis (OPLS-DA) (31–33).

Principal component analysis is a projection method where data are analyzed without bias. Systematic variation in the data is summarized into latent variables, called scores (T). T1 is the direction in *N*-dimensional variable space summarizing the most of the systematic variation and T2 visualizes the second most variation. One individual PCA model was built per cell treatment group (data not shown). Outliers were detected using the Hotellings T2 ellipse, a two-dimensional representation of the 95% confidence interval. Cells outside of the Hotellings T2 ellipse were classified as outliers. Two outliers were identified for control, one for IL-2, and one for IFN-α + β and excluded from the subsequent OPLS-DA analysis.

Orthogonal projections to latent structures and discriminant analysis is similar to PCA in that it is a projection method, but it analyzes the data in relation to one selected Y variable (in our case, the classification of cells as control, IL-2 or IFN-α + β-stimulated) (33). All Y-related variation is visualized in the first "predictive" principal component, while subsequent "orthogonal" principal component(s) display variation unrelated to Y. In our model, only the first predictive component was significant.

Cell Size Analysis

Overview confocal images were captured during the same sessions as FCS measurements. At least 11 z-sections of cells were captured, with the middle section of the z-stacks centered on the widest cellular diameter for the majority of cells. The image z-stacks were projected in ImageJ and analyzed in Cell Profiler using application-specific modules (34). Circular cell edges were identified and the cell area was calculated.

Statistical Analysis

Statistical tests were performed in the software Prism (version 6.0, GraphPad, La Jolla, CA, USA). Outliers in the FCS data were identified using the Grubb's test (35). If a cell was identified as an outlier in any variable for Ly49A or H-2D^d, the entire cell was removed from further analysis for that color channel. D'Agostino–Pearson omnibus test for normal distribution was performed. The data were not normally distributed; however, transformation by the natural logarithm resulted in normally distributed populations for density and diffusion (36). The transformed data were, thus, further analyzed by one-way analysis of variance, ANOVA, and Holm–Sidak's multiple comparison test. For brightness, H-2D^d in IL-2 stimulated cells, and Ly49A in IFN-α + β stimulated cells, natural logarithm transformation did not lead to normality. Brightness of cytokine-treated cells was, therefore, compared to the control by Kruskal–Wallis one-way ANOVA and Dunn's multiple comparisons test. The same test was also applied for the cell size. The geometric mean and 95% confidence intervals were calculated (36). For flow cytometry data, upregulation of Ly49A and H-2D^d expression after cytokine stimulation was assessed by one-way ANOVA, Dunn's multiple comparisons test on selected pairs of variables. *P*-values of $0.05 < p < 0.01$, $0.01 < p < 0.001$, and $p < 0.001$ were considered statistically significant (noted as *, **, and *** in figures).

RESULTS

Cytokine-Stimulated NK Cells Exhibit Faster Diffusion Dynamics of H-2D^d and Ly49A at the Plasma Membrane

The molecular density and diffusion coefficients are important parameters for the rate of molecular interactions, and thus for subsequent biological outcomes. FCS measures molecular diffusion rates with high accuracy, and give an estimate of molecular densities, also within the plasma membrane of cells (28). We, therefore, investigated the impact of short-term cytokine stimulation on the molecular dynamics within the plasma membrane of the inhibitory receptor Ly49A, and its ligand, H-2D^d, using FCS. Freshly isolated NK cells from mice expressing only the H-2D^d allele of MHC class I were used. All NK cells were thus educated. NK cells were enriched by negative selection to a percentage of 85 ± 5% (data not shown). The cells were thereafter cultured for 4 h, either with IL-2 or IFN-α + β, or without cytokines. H-2D^d and Ly49A were each labeled with antibodies that have been used previously for FCS measurements, and do not induce dimerization or affect the diffusion behavior in any other detectable fashion (19). FCS measurements were performed on the top of the cell membrane (**Figure 1A**). A series of autocorrelation curves were generated and averaged for each NK cell, and then fitted to Eq. 3. Representative curves from each stimulation group are shown in **Figures 1B,C**.

We first investigated the impact of cytokine stimulation on the diffusion rate. H-2D^d diffused rather slowly in resting cells (**Figure 2A**; **Table 1**). Interestingly, H-2D^d diffused significantly faster on both IL-2 and IFN-α + β stimulated NK cells, compared to control cells (**Figure 2A**; **Table 1**). The diffusion of Ly49A was significantly faster than that of H-2D^d in resting cells and, similarly to H-2D^d, increased significantly on IFN-α + β stimulated NK cells (**Figure 2B**; **Table 1**). Notably, in addition to a slight shift of the whole population toward faster diffusion, several cells in both stimulated NK cell populations exhibited a markedly faster diffusion, compared to any of the control cells (**Figures 2A,B**). The diffusion rate of H-2D^d co-varied significantly with that of Ly49A in the same cell, in both control and cytokine-stimulated cells (**Figures 2C–E**). Thus, some, but not all, NK cells exhibited a markedly faster diffusion of both H-2D^d and Ly49A after stimulation with either IL-2 or IFN-α + β.

The reason for the relatively slow molecular motion of H-2D^d in resting NK cells could be either that the molecules are specifically interacting with other proteins, or that the diffusion is physically restricted. This restriction could in turn be due either to crowding, if the membrane is densely populated with proteins, or confinement by the cytoskeleton. To investigate the role of the cytoskeleton, we treated NK cells with Latrunculin B, which disrupts the actin cytoskeleton. Latrunculin B had no effect on the diffusion rate of neither H-2D^d nor Ly49A on resting NK cells. After stimulation with either IFN-α + β or IL-2, there was a tendency of faster movement of Ly49A, albeit not statistically significant (Figure S1 in Supplementary Material). This suggested that the slow movement of H-2D^d is due either to crowding or to interaction with other molecules.

Negligible Changes in Density of Diffusing H-2D^d and Ly49A upon Cytokine Stimulation Despite Increased Total Cell Surface Expression

Type I interferons are known to upregulate MHC class I on most cell types. We investigated the overall surface expression of Ly49A and H-2D^d per NK cell after cytokine stimulation, using flow cytometry. NK cells were isolated and stimulated as for the FCS experiments. H-2D^d was modestly but significantly upregulated on Ly49A$^+$ NK cells by IFN-α + β stimulation, but not by IL-2 (**Figure 3A**, dark gray bars). Ly49A was upregulated by IL-2, while there was no statistically significant difference after IFN-α + β stimulation (**Figure 3A**, light gray bars). Ly49C/I, which has the MHC class I allele H-2K^b as ligand, was upregulated to approximately the same extent (**Figure 3A**, checkered bars). Thus, both a Ly49 receptor with and one without a specific MHC

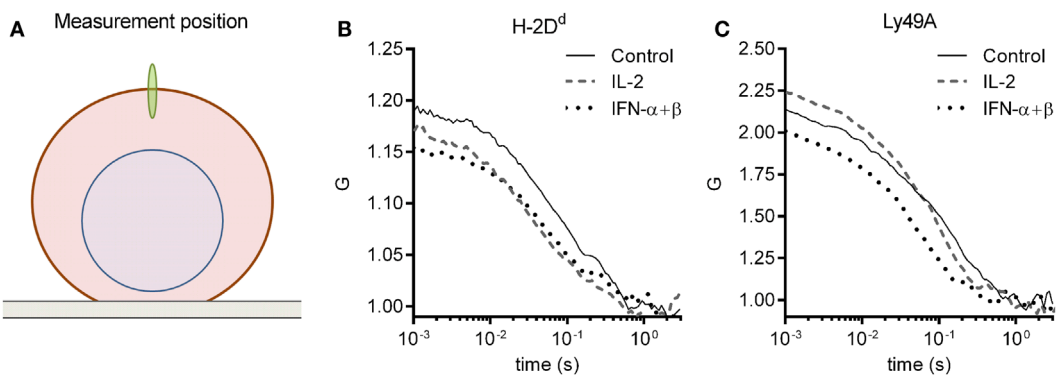

FIGURE 1 | FCS measurement and representative autocorrelation curves. The autocorrelation curves of H-2D^d and Ly49A were measured in freshly isolated murine NK cells after 4 h of cytokine stimulation. **(A)** A schematic illustration of the positioning of the focus (not to scale). FCS measurements were performed on the upper cell membrane. **(B,C)** Representative FCS autocorrelation curves for a control, an IL-2, and an IFN-α + β stimulated cell for **(B)** H-2D^d entities, and **(C)** Ly49A entities. The cells were selected based on a location close to the center in the multivariate PCA analysis for each group (see Materials and Methods for description of the PCA analysis).

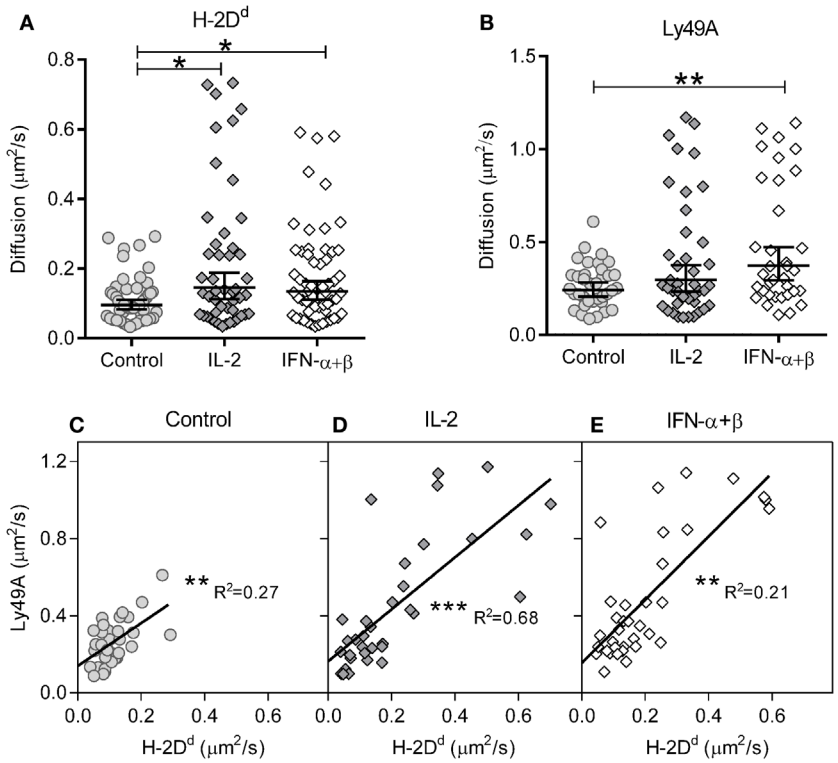

FIGURE 2 | Diffusion rates of H-2D^d and Ly49A are increased after 4 h of cytokine stimulation. The diffusion rates were measured in freshly isolated murine NK cells after 4 h of cytokine stimulation. **(A)** Diffusion rates of H-2D^d in individual cells. **(B)** Diffusion rates of Ly49A. Cells in A and B are the same as in **Table 1**. The lines show Geometric mean, error bars show 95% confidence interval. **(C–E)** Linear regression of Ly49A and H-2D^d diffusion coefficients in the same cells, 35, 32, and 33 cells for control, IL-2, and IFN-α + β, respectively. **(C)** Diffusion rate in control cells. **(D)** Diffusion rate in IL-2 stimulated cells. **(E)** Diffusion rate in IFN-α + β stimulated cells. * = $0.05 < p < 0.01$, ** = $0.01 < p < 0.001$, *** = $p < 0.001$ in A, B compared to control, in C–E for slope difference compared to 0. R^2, least squares goodness of fit.

class I ligand in the host were upregulated at the cellular level. The mRNA levels of H-2D^d in NK cells increased after both types of cytokine stimulation already after 2 h: 1.4 times increase for IL-2 and 1.8 times increase for IFN-α + β (data not shown).

The molecular density is, however, a more relevant parameter for the rate of molecular interactions than the total expression level per cell. Stimulation with these cytokines did not result in increased densities of neither H-2D^d nor Ly49A at the cell surface (**Figures 3B,C**; **Table 1**).

A potential reason for overall increase in protein expression while maintaining density would be a simultaneous increase of the overall cell membrane area. We, thus, determined the average cross-sectional area of stimulated and unstimulated NK cells. By acquiring Z-stacked 2D overview confocal images of several cells per image, the widest point of each cell was identified. The cross-sectional area increased significantly after 4 h of IFN-α + β stimulation, but not by IL-2 stimulation (**Figure 3D**). Thus, the increased cell size, rather than an increased cell surface density, could account for the increased expression level per cell of H-2D^d after IFN-α + β stimulation.

Fluorescence correlation spectroscopy is not capable of detecting single molecules if they are diffusing as a complex. An analysis of the brightness of moving entities can instead give an indication of whether more than one molecule diffuse together. H-2D^d displayed a slightly but significantly higher brightness after IL-2 stimulation (**Figure 3E**; **Table 1**). This could potentially indicate a higher degree of homo-clustering. No significant differences in the brightness were observed for Ly49A (**Figure 3F**). The average brightness of freely diffusing antibodies in solution was 0.82 ± 0.12 kHz for the H-2D^d and 8.2 ± 0.53 kHz for the Ly49A antibody. These values were somewhat lower than for the entities diffusing in the membrane (**Table 1**). However, only H-2D^d in IL-2 stimulated cells were significantly brighter than the free antibody ($p < 0.05$), strengthening the conclusion of a clustering in this situation. An increased clustering of H-2D^d goes in line with the slightly higher fluorescence intensity per cell detected by flow cytometry (**Figure 3A**, not statistically significant).

In conclusion, the early H-2D^d and Ly49A upregulation in NK cells by IFN-α + β correlate with an increased cell size, rather than a higher cell surface density. An increased clustering of H-2D^d was indicated after IL-2 stimulation. The increased Ly49A expression after IL-2 stimulation at the cellular level was, however, not explained, neither by an increased density of diffusing Ly49A, nor an increased cross-sectional area of the cell.

To estimate the actual density of diffusing H-2D^d and Ly49A entities, we next determined the binding efficiency of the H-2D^d and Ly49A antibodies using transfected cell lines expressing GFP-coupled versions of the respective antigens. Fluorescent antibodies labeled 18.3 ± 5.6% of GFP-coupled H-2D^d and 10 ± 5.9% of GFP-coupled Ly49A expressed on the cell membrane (data not shown). Thus, both with and without correcting for binding efficiency, H-2D^d was more densely expressed compared to the Ly49A receptor on the membrane of resting NK cells, which goes well in line with previous reports that most Ly49A receptors are bound in *cis* on H-2D^d expressing NK cells (37, 38).

TABLE 1 | Density, brightness, and diffusion rate obtained from FCS analysis.

Values from FCS		Control L95, GM, U95	IL-2 L95, GM, U95	IFN-α + β L95, GM, U95
Density (N/μm^2)	H-2D^d	43.3, **50.5**, 58.9	35.6, **42.4**, 50.4	45.6, **54.5**, 65.0
	Ly49A	3.46, **4.21**, 5.11	3.04, **3.79**, 4.72	2.78, **3.47**, 4.35
Brightness (kHz)	H-2D^d	0.84, **0.99**, 1.18	1.17, **1.34**, 1.54	0.92, **1.10**, 1.32
	Ly49A	7.0, **8.7**, 10.7	8.4, **10.5**, 13.0	7.9, **10.2**, 13.2
Diffusion coefficient (μm^2/s)	H-2D^d	0.082, **0.095**, 0.110	0.112, **0.145**, 0.188	0.110, **0.135**, 0.164
	Ly49A	0.207, **0.242**, 0.283	0.233, **0.296**, 0.376	0.294, **0.373**, 0.472

The bold is to highlight the geometrical mean, in contrast to the confidence interval limits. L95, lower 95% confidence interval limit. GM, geometric mean. U95, upper 95% confidence interval limit. Geometric mean and 95% confidence interval limits calculated from data transformed by the natural logarithm, and thereafter back-calculated to raw data format. Values are not corrected for antibody brightness and binding efficiency. H-2D^d = 55, 48, and 58 cells, Ly49A = 36, 41, and 35 cells, for control, IL-2, and IFN-α + β, respectively. Data pooled from seven independent experiments.

The Total Amount of Ly49A Receptors Able to Interact in *trans* Increase by IL-2 Stimulation

For detection of Ly49A in the FCS experiments, we used an antibody clone (JR9.318), which detects both free and *cis*-bound Ly49A (37, 39). The total levels of Ly49A per cell could, therefore, be measured. However, if the fraction of *cis*-bound receptors changed, that would not be detected. The relative amount of free Ly49A at the cellular level was, thus, estimated by another antibody clone (YE1/48). This antibody binds less efficiently to Ly49A that is bound to H-2D^d in *cis* (37). Also when using this antibody, the amount of detected Ly49A was significantly increased after IL-2 stimulation. There was also a smaller, albeit

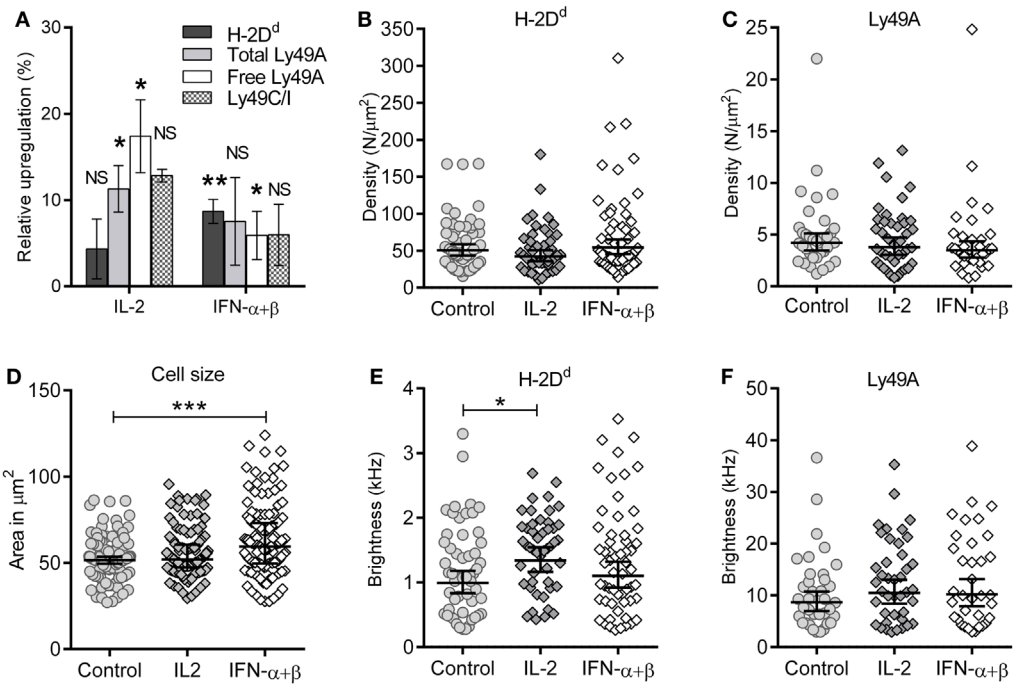

FIGURE 3 | Maintained membrane density despite upregulation of H-2D^d and Ly49A at the cellular level. (A) Expression of cell markers: H-2D^d, "total" Ly49A, Ly49C/I, and "free" Ly49A (not *cis*-bound), as measured by flow cytometry after 4 h of cytokine stimulation. Percentage upregulation calculated with respect to a 37°C incubated control without any cytokines. The difference in upregulation of "free" Ly49A between IL-2 and IFN-α + β stimulation was statistically significant ($p = 0.02$), other differences between the two cytokines were non-significant. Significances were tested by one-way ANOVA, Dunn's multiple comparisons test on selected pairs of variables. Bar graph represents mean ± standard error of the mean ($n = 8$ for H-2D^d, $n = 7$ for Ly49A total and free, $n = 2$ for Ly49AC/I). **(B,C)** Density of diffusing entities measured by FCS; **(B)** H-2D^d entities and **(C)** Ly49A entities. The lines show Geometric mean, error bars = 95% confidence interval. **(D)** Cell cross-sectional area of individual cells at their widest point. The geometric mean of the cell area at the widest point determined for control was 52.8 μm^2 (95% confidence interval limit (CI) 40.7–54.9 μm^2), for IL-2 55.2 μm^2 (CI 52.9–57.5 μm^2, and for IFN-α + β 62.5 μm^2 (CI 59.0–66.0 μm^2). 127, 132, and 130 cells, for control, IL-2, and IFN-α + β, respectively. **(E)** Brightness per H-2D^d entity. **(F)** Brightness per Ly49A entity. The cells in B, C, E, and F are the same as in **Table 1**. **(C–F)** Bar and error = Geometric mean with 95% confidence interval. NS, not significant. * = $0.05 < p < 0.01$, ** = $0.01 < p < 0.001$, *** = $p < 0.001$.

statistically significant upregulation in the IFN-α + β stimulated cells (**Figure 3A**). Thus, IFN-α + β stimulation did not lead to a decrease of free Ly49A receptors, despite the previously well-established upregulation of MHC class I by interferons, and after IL-2 stimulation, there was a substantial increase in the amount of Ly49A receptors free to interact with H-2D^d in *trans*. For the IFN-α + β stimulation, this was in line with the negligible changes in cell surface density of both molecules, as observed by FCS.

Cytokine-Stimulated NK Cells Contain a Subpopulation of Cells with Rapid Diffusion Dynamics of H-2D^d and Ly49A on the Plasma Membrane

To further characterize differences between control, IL-2, and IFN-α + β stimulated cells, a multivariate analysis was performed on the FCS dataset. All six available variables were employed: the diffusion rate, density, and brightness of Ly49A and H-2D^d, respectively. Only cells with no missing data were used (see Material and Methods for exclusion criteria).

Control, IL-2, and IFN-α + β stimulated cells were assigned to individual classes. Differences between these classes were investigated by discriminant analysis (OPLS-DA). The OPLS-DA model could explain 41.3% of the variation between classes, 6.5% of the variation unrelated to class separation, and predicted 3.6% of the variation in the data. Control cells were significantly separated from cytokine-stimulated cells (**Figure 4A**). IL-2 and IFN-α + β-stimulated cells could not be separated from each other with statistical significance. This is visualized by their class-characterizing variables being close in variable space (**Figure 4A**). In the multivariate analysis, it became apparent that a subpopulation among the cytokine-stimulated cells was distinctly separated from the control cells (**Figure 4B**). In the global data set, 29% of the IL-2 and 31% of the IFN-α + β stimulated cells belonged to this subpopulation. However, for the IFN-α + β-stimulated cells, five out of the seven experiments contributed to the subpopulation, whereas the IL-2 subpopulation only came from three out of the seven experiments. IFN-α + β stimulation, thus, led to a more robust appearance of the discrete subpopulation.

We investigated which variables characterized the subpopulation among the IL-2 and IFN-α + β-stimulated cells. The two main defining variables were fast diffusion of H-2D^d and Ly49A for both IL-2 and IFN-α + β-stimulated cells (**Figures 4C,D**). The IL-2-stimulated subpopulation was further characterized by a high brightness and low density of Ly49A (**Figure 4C**). A higher degree of clustering of Ly49A was, thus, indicated in the fast subpopulation after IL-2 stimulation. The IFN-α + β fast subpopulation displayed a low density of both H-2D^d and Ly49A, without statistically significant changes in the brightness (**Figure 4D**). This could be reflecting a dilution of molecules due to an increased cell size (**Figure 3D**).

Conclusively, both types of cytokine stimulations resulted in the emergence of a large subpopulation of NK cells separated from the other cells by a significantly faster diffusion rate of both H-2D^d and Ly49A within the cell membrane. After IL-2 stimulation, there was also an indication of clustering of Ly49 receptors in the subpopulation.

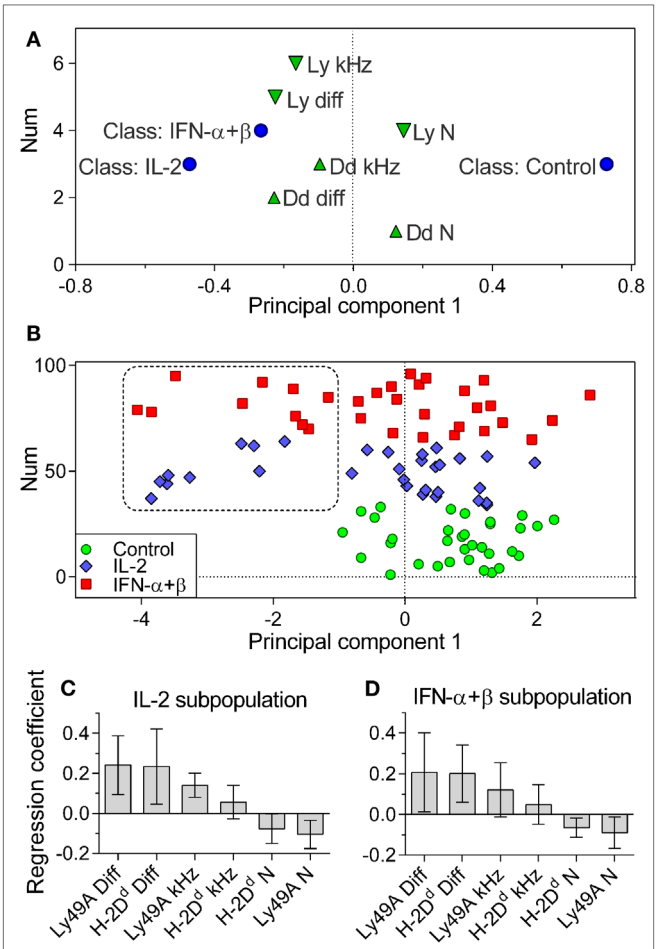

FIGURE 4 | Cytokine-stimulated cells contain a subpopulation of cells with rapidly diffusing entities. Multivariate OPLS-DA analysis of the FCS data. **(A)** Classes: Control, IL-2, and IFN-α + β stimulated cells (circles). Variables contributing to the separation: downward pointing triangles = Ly49A variables, upward pointing triangles = H-2D^d variables. Variables close in space to the class are positively correlated to that class. **(B)** The positioning of individual cells and the identification of a subpopulation of cytokine-treated cells, not overlapping with control cells. Circles, control cells; diamonds, IL-2 stimulated cells; squares, IFN-α + β-stimulated cells. Vertical axis label "Num" refers to the order of the values in the dataset and has no significance for the model. **(C)** Variables characterizing cells in the IL-2 subpopulation. **(D)** Variables characterizing cells in the IFN-α + β subpopulation. Diff, diffusion rate; kHz, brightness; N, density. Error bars in C and D represent the 95% confidence interval. The model was built only on cells where all variables were present for both Ly49A and H-2D^d: 33, 31, and 32 cells for Control, IL-2, and IFN-α + β, respectively. Cells pooled from seven independent experiments.

DISCUSSION

The most marked early impact of cytokine stimulation in this study was an increase in the diffusion rates of both investigated molecules. To our knowledge, this is the first time that an effect of cytokine stimulation on the molecular motion within the membrane of any naïve immune cell has been observed. Cytokines are major controllers of immune reactions, and diffusion rates are vital regulators of the molecular interactions leading to such reactions.

Yet, little is known about how molecular dynamics are affected by cytokine stimulation. The concept of spatiotemporal dimensions at the molecular level in immune cell regulation is relatively new and has become available to study by the development of new fluorescence-based techniques. The principle of induction of faster diffusion of receptors and other immunologically relevant proteins by cytokines may expand to other receptors, immune cells, and cytokines. The full scope of how cytokines affect molecular dynamics, and the biological relevance, is not known at this point. Given the large importance of cytokine stimulation in the immune system, this aspect of cytokine regulation should be further investigated. One example of a situation where cytokine stimulation is important is in NK cell activation to produce more potent killers as immunotherapy for malignant diseases (40).

Ly49A interactions with MHC class I on target cells are essential for the early part of synapse formation and receptor signal integration in the interface between NK cells and target cells (41). The amount of Ly49 receptors bound in *cis* was previously shown to be a diffusion-limited reaction, in similarity to most other protein–protein interactions (19). Ly49 binding of MHC class I in *cis* and *trans* uses the same binding site and the affinity of the two interactions, thus, ought to be in the same range (17). It is, thus, likely that the diffusion rate also affects how fast the Ly49A receptor find a steady state in the binding to MHC class I in *trans*. The higher mobility of H-2D^d and Ly49A could, thus, potentially enable a more rapid formation of an (inhibitory) immunological synapse, and thus a faster decision process whether a target cell is abnormal or not.

It has been observed that a minority of the IL-2-stimulated NK cells perform the majority of kills after several days of IL-2 stimulation (8). Our observation of increased molecular dynamics within a subpopulation could, thus, tentatively be an early sign of this functional heterogeneity within the NK cell population. The subpopulation did not correspond directly to NK cells upregulating CD69 or ICAM-1, since they did not exhibit a similar upregulation pattern on the NK cell population level (data not shown). A difference in molecular dynamics between educated and uneducated NK cells has previously been shown (21). However, since in our case only Ly49A$^+$ NK cells from H-2D^{d+} mice were investigated, all NK cells were educated. Therefore, the heterogenic response could not be explained by a difference in educational status. The fact that the size of the subpopulation differed between experiments suggests that environmental factors in the individual animal and/or the experimental conditions affects how many of the NK cells will respond to cytokine stimulation with a faster molecular movement. It also suggests that a relatively large fraction of the NK cells have the potential to respond with faster molecular dynamics, if stimulated appropriately.

The fact that both H-2D^d and Ly49A moved faster in the same cells after cytokine stimulation could potentially indicate a general change in the cell membrane dynamics. This could at least partly be due to an increased cell surface area, making the proteins in the cell membrane overall less crowded. A crowding effect is not unlikely, given the high density at which H-2D^d was expressed in this study. H-2D^d alone was expressed at around 50 molecules/μm^2, and crowding occurs starting from around 200 proteins/μm^2 expressed in total (19, 42). Crowding in resting NK cells was further supported by the lack of effect of actin cytoskeleton disruption, despite the fact that diffusion of MHC class I has previously been reported to be restricted by the cytoskeleton (43–47). By contrast, there was a tendency of confinement by the cytoskeleton in cytokine-stimulated cells, which would go well in line with a release from crowding by dilution of other surface proteins as a consequence of increased cell size, making the restriction by the cytoskeleton more apparent. Resting murine NK cells, thus, seem to have a membrane densely populated with protein, but ready to within a few hours expand in size and simultaneously also alter their membrane dynamics.

Potential other underlying mechanisms for an increased diffusion rate include alterations of *cis* interactions with other binding partners, such as other Ly49 receptors or ICAM-1, or alterations in the actin cytoskeleton structure, both of which have been shown to be affected by cytokine stimulation (47–50). A faster general diffusion could also tentatively be a reflection of a higher cellular metabolism (51) or a change in the mode of molecular movement, from passive diffusion to active transport.

The diffusion of MHC class I has been studied in a variety of murine and human cell lines. Recorded diffusion coefficients by FCS and fluorescence recovery after photobleaching (FRAP) ranged from 0.01 to 0.9 μm^2/s (19, 43, 45, 46, 52, 53). Single particle tracking gave rise to diffusion coefficients down to 10 times slower than those detected from FRAP and FCS (45). While all previous studies on MHC class I diffusion were carried out in cell lines, we studied the diffusion in resting primary cells. Considering that most cell lines are replicating and, thus, more active than a resting cell, the fact that the H-2D^d diffusion coefficient of 0.095 μm^2/s fits within the range of previously published data is worth to note.

The diffusion of Ly49A was always at least twice as fast as that of H-2D^d. This was surprising; especially since many of the Ly49A receptors interact in *cis* with H-2D^d. The Ly49A diffusion rate was similar to the human inhibitory receptor KIR2DL1 in a cell line system (0.23 ± 0.06 μm^2/s) (54). It is possible that the relatively small fraction of H-2D^d and Ly49A that were bound in *cis* in this study did not make an impact on the average diffusion rate observed. In the FCS part of this study, no detected H-2D^d was bound in *cis* to Ly49A, since the antibody used to detect H-2D^d in the FCS experiments prevents *cis* interaction (23). The detected Ly49A molecules would be a mixture of free and *cis*-bound to H-2D^d, since the H-2D^d antibody binding efficiency was only around 19%. The difference in diffusion rate could, thus, be a result of hindered diffusion of H-2D^d molecules, in line with what has been reported in previous studies (43–47). The diffusion of H-2D^d could putatively also be hindered by clustering to itself or other partners, as discussed above. Different localization with regard to nanodomains, or a greater potential for active transport for Ly49A are other possible explanations for the observed differences.

The amount of "free" inhibitory receptors available to interact in *trans* increased substantially upon IL-2 stimulation. A much smaller increase of free inhibitory receptors was observed after IFN-$\alpha + \beta$ stimulation. The difference between the two cytokines in inducing upregulation of free Ly49A was statistically significant (**Figure 3A**). The more substantial upregulation of MHC class I

induced by IFN-α + β at the cellular level could be one reason for the relatively smaller upregulation of free Ly49A receptors since more of the receptors could be bound in *cis* to H-2D^d. The increased clustering of H-2D^d molecules after IL-2 stimulation (**Figure 3E**) could be another explanation contributing to the lower fraction of Ly49A receptors bound in *cis* observed by flow cytometry, if homo-clustering of MHC class I excludes binding of Ly49A in *cis*. For the IL-2 stimulation, the FCS and flow cytometry results are not completely concordant, as flow cytometry indicated an increase of total Ly49A expression that is still unexplained. A less rounded cell shape, giving a higher total cell surface area without increasing the cross-sectional area, or increased membrane ruffling, could potentially be explanations for this discrepancy. Another reason could be a higher fraction of immobile Ly49A, which would not be detected by FCS.

Even though FCS is a quantitative technique, exact concentrations obtained from FCS data should be regarded with some caution. Since FCS is based on intensity fluctuations, molecules have to be reasonably mobile to be detected. Immobile fractions, as well as bleaching, can contribute to under-estimation of the number of molecules (55). Putative error sources have been listed previously, and the total maximal error in the density determination was estimated to be around 40% (19). Given that the measurement conditions were very similar, this estimation is likely relevant also for this study. Since the error sources are in most cases stable throughout the experiments, the error in relative density between samples should, however, be minor, provided that the immobile fractions do not change. The determination of diffusion rates is also more robust than the density determination, even though also the diffusion rate can be over-estimated in case of bleaching.

Taken together, the heterogenic response to cytokine stimulation within the NK cell population shows that more than one type of response is induced in cytokine-treated cells, indicating subgroups within the NK cell population with different molecular dynamics. These differences are apparent already after 4 h of stimulation. The faster diffusion of inhibitory receptors could influence the rate of accessibility of receptors to target cell interactions, finally making cytokine-stimulated NK cells more efficient in screening of putative target cells. Studies further characterizing these subpopulations, identifying how they functionally differ from other stimulated NK cells, will tell what role this heterogeneity plays in the immune response against tumorigenic cells and infection.

AUTHOR CONTRIBUTIONS

SB-S and ES designed, planned and performed the FCS experiments in **Figures 1**–**3**, prepared figures, and wrote the manuscript. SB-S planned, performed and analyzed the FACS experiments in **Figure 3A** and analyzed the cell size data in **Figure 3D**. ES analyzed the FCS data and performed the multivariate analysis in **Figure 4**. AJS and TK performed the antibody labeling efficiency experiment, and commented on the manuscript. TS wrote the software to analyze the FCS experiments. JW provided feedback on the experimental design, analysis, and the manuscript. SJ designed experiments, analyzed data, provided mice and reagents, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00016

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Natural Killer Cells and Liver Fibrosis

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In the 40 years since the discovery of natural killer (NK) cells, it has been well established that these innate lymphocytes are important for early and effective immune responses against transformed cells and infections with different pathogens. In addition to these classical functions of NK cells, we now know that they are part of a larger family of innate lymphoid cells and that they can even mediate memory-like responses. Additionally, tissue-resident NK cells with distinct phenotypical and functional characteristics have been identified. Here, we focus on the phenotype of different NK cell subpopulations that can be found in the liver and summarize the current knowledge about the functional role of these cells with a special emphasis on liver fibrosis. NK cell cytotoxicity can contribute to liver damage in different forms of liver disease. However, NK cells can limit liver fibrosis by killing hepatic stellate cell-derived myofibroblasts, which play a key role in this pathogenic process. Therefore, liver NK cells need to be tightly regulated in order to balance these beneficial and pathological effects.

Keywords: natural killer cells, liver disease, fibrosis, stellate cells

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INTRODUCTION

Natural killer (NK) cells are innate lymphoid cells (ILC) that can kill virus-infected or transformed cells. Additionally, they regulate adaptive immune responses *via* contact-dependent signals and the secretion of cytokines (1). NK cell cytotoxicity is regulated by activating and inhibitory surface receptors and is additionally modulated by cytokines (2). Inhibitory NK cell receptors include killer cell Ig-like receptors (KIR) in humans and Ly49 family members in mice, both of which interact with MHC I to ensure the self-tolerance against healthy cells. NK cell activation can be mediated by a variety of different surface receptors, such as NKG2D, NKp46, and NKp30 (3). Initially, human NK cells have been divided into two functionally distinct subpopulations based on the expression level of CD56. In recent years, more subpopulations of NK cells have been identified, and we now know that in addition to conventional circulating NK cells, there are also tissue-resident NK cells with distinct phenotypical and functional characteristics (4). Here, we summarize the current knowledge about NK cells in the liver and focus on the role of these immune cells in liver fibrosis.

NK CELLS IN THE LIVER

The liver mainly consists of hepatocytes, which make up approximately 80% of liver cells. Non-hepatocytes include about 20% lymphocytes, 20% Kupffer cells, 40% endothelial cells, 20% stellate cells, and biliary cells (5). NK cells in the liver were first described by electron microscopy of rat liver and initially named "pit cells" (6). They reside in liver sinusoids and can make up to 50% of the liver lymphocyte population in humans (7, 8). This is in contrast to the frequency of NK

cells in peripheral blood, where they only account for 5–15% of lymphocytes. It remains unclear what regulates this enrichment of NK cells in the liver. It is believed that cell-to-cell and cell-to-matrix interactions play an important role in this process (9). For example, NK cell infiltration in the liver can be blocked by neutralizing antibodies against CD2, CD11a, CD18, and ICAM-1 (CD54) (10), suggesting that adhesion to sinusoidal endothelial cells is an important step in their recruitment. Endothelial cells also express vascular adhesion protein-1 (VAP-1) (11), which can be recognized by Siglec-9 and could represent another mechanism of liver NK cell enrichment (12).

Liver NK cells have been extensively compared to peripheral blood NK cells and differ in activation level, cytotoxicity, and maturation (13). In general, liver NK cells are more activated as they express high levels of the activation marker CD69, more perforin, and granzyme B (8, 14–17). As a consequence, they show higher cytotoxicity compared to peripheral blood NK cells. However, they are also less mature compared to peripheral blood NK cells (15, 16, 18, 19).

In humans, NK cells are grouped into $CD56^{dim}$ and $CD56^{bright}$ cells with $CD56^{dim}$ NK cells accounting for up to 90% of all NK cells in peripheral blood and spleen. In contrast, equal numbers of $CD56^{dim}$ and $CD56^{bright}$ NK cells are found in the liver (16, 20). The $CD56^{dim}$ NK cell population in the liver seems to resemble circulating conventional NK cells (cNKs). However, recent evidence suggests that liver $CD56^{bright}$ NK cells differ from cNK and represent a distinct, liver-resident NK cell (lrNK) population dependent on the chemokine receptor CXCR6 (**Figure 1**) (20). lrNKs show increased expression of CD69 and the homing markers CXCR6 and CCR5. Engagement of these receptors by CXCL16 from hepatic sinusoidal endothelial cells (21) and CCL3 from Kupffer cells as well as CCL5 from T and NK cells, respectively, retains lrNK cells in a unique chemokine environment. The development and differentiation of lrNK cells is incompletely understood. Cells corresponding to all described developmental intermediates of NK cells have been identified in the adult human liver (16), indicating that NK cell precursors are recruited from peripheral blood and that lrNK cells may differentiate in the liver.

Conventional NK and lrNK cells have also been identified in mice (**Figure 1**), where NK cells make up only 5–10% of the liver lymphocytes. About half of these murine liver NK cells resemble cNKs, but they are $DX5^-$ and express high levels of TRAIL (22–24). In mice, there is clear evidence that cNK and lrNK originate from different developmental programs. lrNK rely on the transcription factors T-bet and PLZF for their development (25, 26), but they are independent of Eomes (27), which is critical for the development of cNKs (28–30). Mouse lrNK are $CD49a^+$, $DX5^-$ and show expression of homing markers (13). They are similar in their phenotype and development to mucosal group 1 innate lymphoid cells (ILC1) (19).

Interestingly, a hepatic NK cell population has been reported that can display adaptive-like immune memory against haptens or viral antigens (31). This antigen-specific type of NK cell memory is confined to CXCR6-positive hepatic NK cells, which were identified as the $CD49a^+$, $DX5^-$ lrNK population (32). Recently, a human intrahepatic $CD49a^+$ NK cell population was identified that was not detectable in afferent or efferent hepatic venous or peripheral blood (33). These NK cells express KIR and NKG2C, indicative of having undergone clonal-like expansion. They are $CD56^{bright}$ and express low levels of CD16, CD57, and perforin. Because this population was only detected at low frequencies (2.3% of hepatic NK cells) and not in every donor, it might represent a subpopulation of lrNKs. It is interesting to speculate that these cells can also mediate certain kinds of adaptive memory (3). In support of this, antigen-specific memory of hepatic NK cells has recently been shown in macaques following infection and vaccination (34).

The fact that NK cells represent the major lymphocyte population in human liver suggests relevant functions. Indeed, liver NK cells have been shown to influence many physiological and pathophysiological processes, such as viral infections, liver tumorigenesis, liver injury, and inflammation (13). In the following section, we will focus on their role in liver fibrosis.

LIVER FIBROSIS AND NK CELLS

An outstanding feature of the liver is its enormous regeneration capacity that has evolved to protect animals from liver loss by hepatotoxic plants (35, 36). Acute destruction of more than 50% of the liver tissue can be regenerated within a relatively short period of time leading to the perfect restoration of tissue architecture and function (37–39). However, repeated destruction of hepatocytes leads to scar formation and fibrosis (40). Fibrosis is characterized by excess extracellular matrix, which initially compromises liver function only to a minor degree (41). However, fibrosis may progress to cirrhosis, where normal liver architecture is replaced by nodules of hepatocytes surrounded by wide streets of fibrotic tissues, which massively constrict blood flow and reduce liver function. Fibrosis and cirrhosis can, in principle, be caused by any condition that repeatedly kills a critical fraction of hepatocytes, such as alcohol abuse, repeated administration of hepatotoxic drugs, viral hepatitis, cholestatic disorders, or hereditary metabolic liver diseases (42, 43).

Hepatic stellate cells (HSCs) play a key role in pathogenesis of liver fibrosis (44–46). They were described by Karl Wilhelm von Kupffer in 1876, but their role in liver disease was only identified in the 1980s (47). HSCs are located between hepatocytes and the endothelial cells of the sinusoids in the 0.2–1-μm wide extracellular matrix-filled Disse space (**Figure 2**) (48). Activation of HSCs and transdifferentiation to myofibroblasts, the major extracellular matrix-producing cell in fibrotic liver, represents a critical step on the path to fibrosis. Typically, cell death of hepatocytes creates an inflammatory microenvironment, which activates HSCs. Key factors driving HSC activation are transforming growth factor beta1 (TGFbeta1) and platelet-derived growth factor (PDGF) family members. Moreover, numerous cytokines and chemokines released by infiltrating immune cells modify this process (42, 44, 49). Finally, this leads to a situation where extracellular matrix formation by activated HSCs outbalances the mechanisms of collagen degradation by matrix metalloproteases.

The molecular mechanisms of liver fibrosis and HSC activation have been reviewed comprehensively (40, 42, 43). The present article focuses on a specific mechanism, which antagonizes fibrosis formation, namely NK cell-mediated cytotoxic

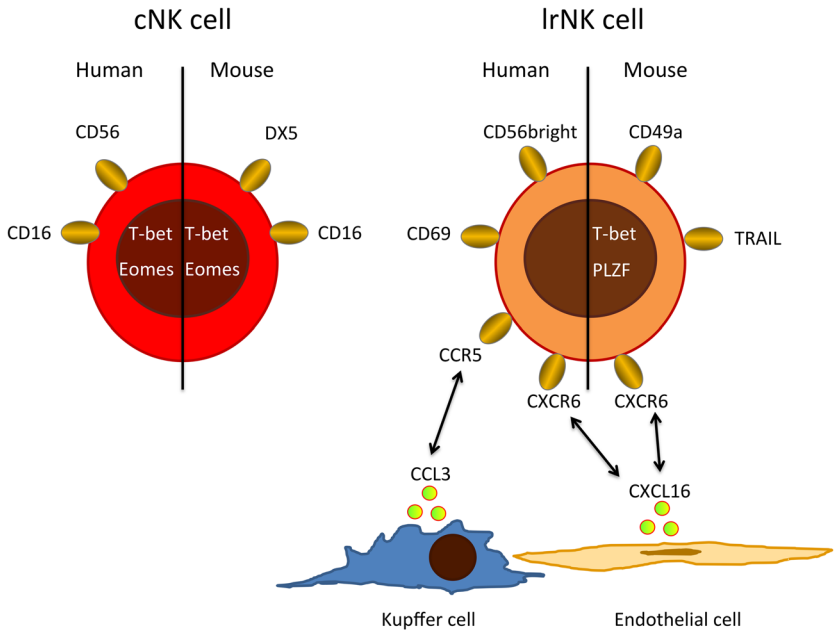

FIGURE 1 | Major phenotypic differences between cNKs and lrNKs. Human cNKs are mostly CD56dim and express CD16, whereas lrNKs show a CD56bright phenotype and are negative for CD16, but express homing markers, such as CXCR6 and CCR5. Possible ligands for these homing-associated receptors are expressed by endothelial cells, Kupffer cells, and circulating cNKs. In mice, lrNKs are $CD49a^{+}DX5^{-}$ and depend on the transcription factors T-bet and PLZF, while cNK are $CD49^{-}DX5^{+}$ and need the transcription factor Eomes for their development.

activity against HSC-derived myofibroblasts, which has been described in numerous articles since its first descriptions in 2006 (50, 51).

To study interactions between NK cells and HSCs as well as their role for liver fibrosis, the most frequently applied experimental tools are the mouse models of liver damage and cultivated human and rodent HSC. In mice, liver fibrosis can be induced by repeated administration of hepatotoxic compounds, such as CCl_4. In this experimental scenario, additional interventions can be performed, such as the elimination of NK cells by antibodies and the genetic deletion of specific receptors and ligands. Alternatively, HSCs can be isolated from liver tissue and brought into culture, where they spontaneously differentiate to alpha-SMA-positive myofibroblasts, which can be tested in killing assays with NK cells. Based on such experimental models, we now have a detailed picture of the interaction between NK cells and HSCs.

In general, the activation of HSCs in response to hepatocyte damage results in changes that increase NK cell stimulation and decrease NK cell inhibition. A key mechanism is that early-activated HSCs produce increased amounts of retinoic acid, which leads to elevated expression of RAE-1 (52). RAE-1 is a ligand for the activating NK cell receptor NKG2D and together with MICA (53) triggers killing of activated HSCs by NK cells. Human and mouse HSCs additionally express a ligand for the activating NKp46 receptor. This also causes HSC killing by NK cells, which ameliorates liver fibrosis (54). Recent data suggest a role of the activating receptor NKp30 in this process (55). In addition to the increased activation of NK cells, reduced inhibition also plays a role in the cytotoxic attack of NK cells against HSCs. Upon activation of HSCs, MHC class I is downregulated, resulting in the reduced engagement of inhibitory NK cell receptors and enhanced killing (50, 56). Experimentally, reducing inhibitory Ly49 receptor expression on mouse NK cells by siRNA-mediated silencing, therefore, increases HSC killing by NK cells and ameliorates liver fibrosis. Inflammatory cytokines can further influence this process. NK cell-derived IFN-γ has antifibrotic effects by inducing HSC apoptosis and cell cycle arrest (57, 58). However, clinical trials with IFN-γ led to disappointing results, and it has been reported that HSC-specific delivery is critical for its antifibrotic effect (59). IFNα has been shown to increase expression of TRAIL on the surface of NK cells (60, 61). Simultaneously, activation of HSCs leads to increased expression of the TRAIL receptor on the HSC surface, resulting in enhanced NK cell-mediated HSC killing. Recombinant expression of human TRAIL on HSCs has been shown to induce HSC apoptosis and blocking TRAIL by antibodies antagonized this effect (62). In contrast, TGF-β levels are elevated during chronic liver injury and suppress the antifibrotic function of NK cells through downregulation of NKG2D and 2B4 surface expression (63–65).

While the mechanisms mentioned above give some insight into the molecular mechanisms about how NK cells antagonize fibrosis, open questions remain. It is unclear which population of liver NK cells is responsible for limiting fibrosis. lrNK may already be in place to directly interact with HSCs. However, the role of lrNK cells in liver fibrosis has not been addressed in detail. *In vitro* experiments mostly use NK cells from human peripheral blood or mouse spleen to study the killing of HSCs. For *in vivo* depletion experiments using antibodies, it is unclear if the tissue-resident

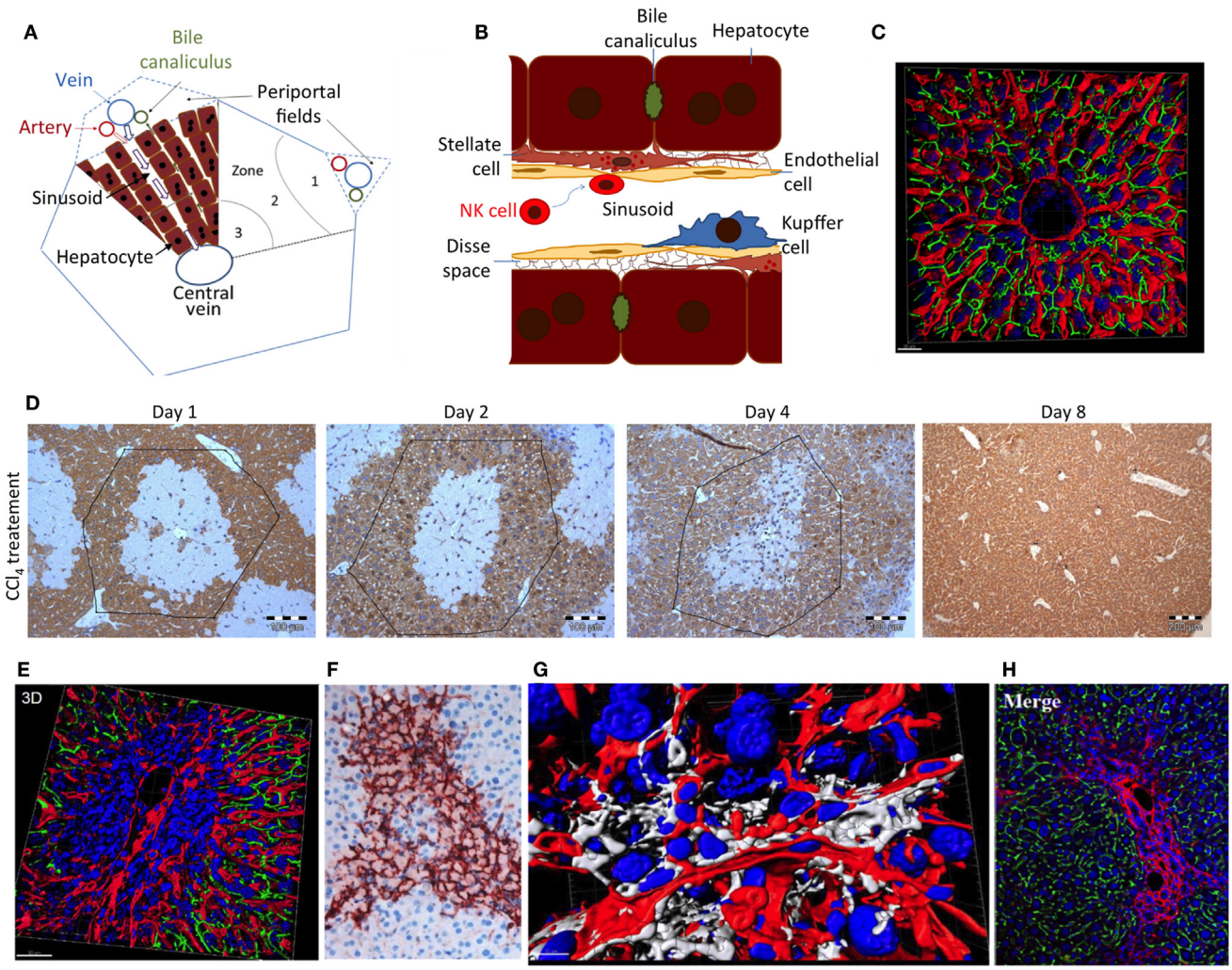

FIGURE 2 | (A) Schedule of a liver lobule and **(B)** sinusoid; **(C)** reconstructed liver lobule showing the sinusoidal endothelial cells (LSEC) in red, bile canaliculi in green, and nuclei in blue. **(D)** Damage and regeneration process after administration of a hepatotoxic dose of CCl_4 to mice. The initial pericentral damage (day 1) is completely regenerated until day 8 after intoxication. **(E)** Necrotic area 2 days after CCl_4 administration, illustrating a pericentral nuclear dense region with compromised microvessels. **(F)** Alpha-smooth muscle actin staining of activated HSC in a necrotic region 2 days after CCl_4 administration. **(G)** Reconstruction of a necrotic region 2 days after CCl_4 administration, visualizing stellate cells in white, LSEC in red, and nuclei in blue. **(H)** Collagen staining to visualize a fibrotic street after repeated CCl_4 doses. Imaging was performed with livers of male C57BL6/N mice as described in Ref. (48); **(D)** from Ref. (37).

lrNK cells can be efficiently depleted. Therefore, it remains elusive what role lrNK cells play during fibrosis.

Hepatic stellate cells play an important role during the regeneration of liver damage, when they infiltrate the wound and by secretion of cytokines, such as HGF, help to orchestrate the regeneration process (66). However, as soon as hepatocytes have repopulated the dead cell area of the wound, the further presence of activated HSCs is deleterious and acts profibrotic. It is therefore important that NK cells remove activated HSCs after they have accomplished their mission in regeneration. Indeed, *in vivo* and *in vitro* experiments show that NK cells kill early activated, but not quiescent or fully activated, HSCs (52, 67). This may be due to an increase of the ratio of activating versus inhibiting mechanisms in early activated HSCs (62). However, so far little attention has been paid to the massive architectural changes during liver damage and regeneration, which may impact NK cell/HSC interactions. In the healthy liver, NK cells float in the sinusoidal blood or roll along sinusoids (**Figures 2A,B**). In this situation, HSCs are shielded from NK cells by endothelial cells (LSEC) (**Figure 2B**). Cytotoxic T cells may overcome the endothelial barrier and probe antigens on subsinusoidal cells by extending cytoplasmic protrusions through sinusoidal endothelial fenestrae (68). However, any shielding function of LSEC is transiently lost during liver damage induction and regeneration (**Figures 2D–G**). Most hepatotoxic compounds that require activation by cytochrome P450 enzymes induce necrosis in the center of liver lobules, visible as a pale region in **Figure 2D**. Numerous immune cells infiltrate the necrotic area leading to high nuclear density (**Figure 2E**). Additionally,

activated alpha-smooth muscle actin-positive HSCs form an alveolar scaffold in the necrotic area between days 2 and 4 after damage induction (**Figure 2F**). In this period, activated HSCs are no longer shielded from immune cells by endothelial cells. With their long delicate protrusions, they get into direct contact with several types of immune cells (**Figure 2G**). Only 8 days after damage induction, all activated HSCs disappear from the tissue. It has not yet been studied whether the direct accessibility of HSCs in damaged regions of the liver to NK and other immune cells is the reason for their abrupt disappearance. Additionally, in liver fibrosis, activated HSCs persist for longer periods. In this situation, they are immured by collagen fibers (**Figure 2H**), which may prevent access of NK cells to their target.

The interaction between NK cells and HSCs is additionally regulated by other cells and processes. Kupffer cells and dendritic cells can enhance NK cell activation under immune stimulatory conditions, such as Toll-like receptor stimulation or viral liver disease (69–71). Regulatory T cells can inhibit NK cell activity and thereby limit their antifibrotic function during viral hepatitis (72, 73).

While NK cell activity may be beneficial for the regulation of liver fibrosis, it can also have negative effects. Indeed, very similar molecular mechanisms by which activated HSCs are removed have also been described for NK cell-mediated killing of hepatocytes. Importantly, RAE-1, MICA/B, B7-H6, TRAIL-receptor, and Fas on hepatocytes, as well as NKG2D, NKp30, and TRAIL ligand on NK cells have been reported to play a role in NK cell-induced hepatocyte death (14, 23, 55, 74–77). This illustrates that NK cell-activating therapeutic strategies (78, 79) should be considered with care, since the tightly controlled mechanisms of selectively killing-activated HSC may easily switch to a situation where also hepatocytes become targeted, which would promote liver damage and aggravate the profibrotic pressure.

When studying the function of NK cells in liver, it is important to mention a potential experimental pitfall. A well-studied example is the misinterpretation of experimental data that NK and NKT cells enhance acetaminophen (APAP)-induced liver damage in mice (80). Since others could not reproduce the result, the experiments have been carefully revisited (81). It was shown that the DMSO used in the original study to dissolve the APAP has adjuvant-like functions and stimulates the activity of NK and NKT cells to enhance APAP-induced liver damage. Interestingly, a solvent control would not be sufficient to avoid this misinterpretation as the adjuvant function of DMSO does not result in liver damage alone, but only in combination with APAP. This has to be taken into account when investigating the role of NK cells in drug-induced liver damage.

OUTLOOK

Natural killer cell cytotoxicity limits HSC-mediated liver fibrosis, and in recent years, we have learned much about the molecular details of this interaction. However, careful *in vivo* analysis will still need to address several important questions, such as the spatiotemporal details of NK–HSC interaction and the role of the different subpopulations of liver NK cells in this process. Additionally, NK cells are part of a larger group of ILC. ILCs are mostly tissue-resident cells with important functions in tissue homeostasis and immunity against pathogens. There are first indications that these novel immune cells can also influence the process of liver fibrosis (82, 83), which may lead to an exciting research field in the future.

AUTHOR CONTRIBUTIONS

All authors participated in writing the manuscript.

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Unique Eomes+ NK Cell Subsets Are Present in Uterus and Decidua During Early Pregnancy

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Decidual and uterine natural killer (NK) cells have been shown to contribute to the successful pregnancy both in humans and mice. NK cells represent "cytotoxic" group 1 innate lymphoid cells (ILCs) and are distinct from the recently described "helper" ILC1. Here, we show that both in humans and mice the majority of group 1 ILC in endometrium/uterus and decidua express Eomesodermin (Eomes), thus suggesting that they are developmentally related to conventional NK cells. However, they differ from peripheral NK cells. In humans, Eomes+ decidual NK (dNK) cells express CD49a and other markers of tissue residency, including CD103, integrin β7, CD9, and CD69. The expression of CD103 allows the identification of different subsets of IFNγ-producing Eomes+ NK cells. We show that TGFβ can sustain/induce CD103 and CD9 expression in dNK cells and decidual CD34-derived NK cells, indicating that the decidual microenvironment can instruct the phenotype of Eomes+ NK cells. In murine decidua and uterus, Eomes+ cells include CD49a−CD49b+ conventional NK cells and CD49a+ cells. Notably, Eomes+CD49a+ cells are absent in spleen and liver. Decidual and uterine Eomes+CD49a+ cells can be dissected in two peculiar cell subsets according to CD49b expression. CD49a+CD49b− and CD49a+CD49b+ cells are enriched in immature CD11b^{low}CD27^{high} cells, while CD49a−CD49b+ cells contain higher percentages of mature CD11b^{high}CD27^{low} cells, both in uterus and decidua. Moreover, Eomes+CD49a+CD49b− cells decrease during gestation, thus suggesting that this peculiar subset may be required in early pregnancy rather than on later phases. Conversely, a minor Eomes−CD49a+ ILC1 population present in decidua and uterus increases during pregnancy. CD49b−Eomes± cells produce mainly TNF, while CD49a−CD49b+ conventional NK cells and CD49a+CD49b+ cells produce both IFNγ and TNF. Thus, human and murine decidua contains unique subsets of group 1 ILCs, including Eomes+ and Eomes− cells, with peculiar phenotypic and functional features. Our study contributes to re-examination of the complexity of uterine and decidual ILC subsets in humans and mice and highlights the role of the decidual microenvironment in shaping the features of these cells.

Keywords: ILC, NK cells, ILC1, Eomes, pregnancy, tissue-resident NK cells

INTRODUCTION

Innate lymphoid cells (ILCs) represent a family of lymphocytes that differ from B and T cells since they lack recombination activating gene (RAG)-dependent rearranged antigen receptors. ILCs share the dependence on Id2 transcriptional repressor and on the common γ chain cytokine receptor for their development. ILCs have been classified into three main groups according to their transcription factor and cytokine profile. Group 1 ILCs express the T-box transcription factor T-bet (*Tbx21*) and mainly express IFNγ and TNF. Group 2 ILCs (ILC2) depend on GATA binding protein-3 transcription factor and produce type-2 cytokines. Finally, group 3 ILCs (ILC3) express the retinoic acid receptor (RAR)-related orphan receptor (ROR)γt, and produce IL-17 and IL-22 (1, 2).

Group 1 ILCs include "helper-ILC1" (hereinafter referred to as ILC1) and "cytotoxic-ILCs," i.e., natural killer (NK) cells (2). ILC1 mainly express IFNγ and TNF and provide defenses against intracellular bacteria and protozoa. Conversely, NK cells, beside IFNγ production, also display cytolytic activity against virus-infected or tumor cells. Unlike ILC1, NK cells also express the transcription factor Eomesodermin (Eomes) (3). ILC1 appear to be resident populations in intestine, liver, and uterus, while NK cells are present in tissues and recirculate in the blood (4–9). A peculiar subset of NK cells residing in murine liver has been recently described and termed tissue-resident NK (trNK) cells (10). These cells express T-bet, but not Eomes. Liver trNK cells display striking phenotypical similarities with ILC1 described in mucosal tissues (11, 12), suggesting a partial overlap between these two cell subsets. Indeed, studies regarding ILC lineage specifications suggested that liver trNK cells are more related to ILC1 than to "conventional" splenic NK (cNK) cells (5). Moreover, Daussy et al. demonstrated that liver NK1.1$^+$T-bet$^+$Eomes$^+$ and NK1.1$^+$T-bet$^+$Eomes$^-$ cells represent two distinct lineages of differentiation, which derive from precursors of medullary and peripheral origin, respectively (6, 9). Of note, peculiar Eomes$^+$ NK cells, differing from cNK and ILC1, have been identified in murine salivary glands and uterus (7, 13).

Innate immune cells are important components of decidual microenvironment during pregnancy (14–16). Among ILCs, we recently identified ILC3 in human decidua during early pregnancy (17). However, the best-characterized and more abundant ILC population is that of NK cells that, during the first trimester of pregnancy, represents up to 70% of decidual infiltrating lymphocytes (DILs). Human decidual NK (dNK) cells are characterized by CD56^{bright}CD16$^-$KIR$^+$CD9$^+$CD49a$^+$ phenotype, are poorly cytolytic, and produce low amounts of IFNγ, as compared to peripheral blood (PB) NK cells (15, 18–20). On the other hand, dNK cells secrete cytokines and chemokines that promote neo-angiogenesis, tissue remodeling and placentation (16). Similar to humans, also murine dNK cells are abundant during the early phase of pregnancy and display unique phenotypic and functional features (21). Since we have previously shown that uterine (u)NK and dNK cells may originate, at least in part, from *in situ* precursors (21) and in light of recent evidences about ILC complexity and differentiation (6, 9, 10), here we re-evaluated the nature of uterine and dNK cells in humans and mice, in order to clarify whether they may be ascribed to ILCs previously identified in other tissues or rather represent unique subsets only present in uterus and decidua.

MATERIALS AND METHODS

Isolation of Human Cells

Endometrial biopsies were obtained from normally cycling women undergoing surgery for ovarian cyst removal at IRCCS AOU San Martino-IST (Genova, Italy). Decidua (d) samples were obtained at 9–12 weeks of gestation from singleton pregnancies of mothers requesting termination of pregnancy for social reasons at IRCCS AOU San Martino-IST (Genova, Italy). The relevant institutional review boards approved the study and all patients gave their written informed consent according to the Declaration of Helsinki. We isolated cell suspensions from decidual and endometrial tissue with GentleMacs (Miltenyi Biotec, Bergisch Gladbach, Germany) and cells were then filtered as previously described (22). Decidua and endometrial infiltrating lymphocytes were isolated by Fycoll (Cedarlane, Burlington, ON, Canada) gradient centrifugation. Lymphocytes obtained were subsequently analyzed by flow cytometry, stimulated for cytokine production, or sorted for subsequent stimulation and culture. In order to isolate ILC subsets and CD34$^+$ cells, DILs were sorted as (CD45$^+$CD3$^-$CD14$^-$CD34$^-$CD56$^+$CD127$^-$CD117$^-$)-NKp44$^+$CD103$^+$, -NKp44$^-$CD103$^+$, and -NKp44$^-$CD103$^-$ cells and CD45$^+$CD14$^-$CD19$^-$CD3$^-$CD56$^-$CD34$^+$ cells at FACSAria (BD Bioscience, San Jose, CA, USA), purity was routinely >95%. Decidual stromal cells (dSC) were isolated as previously described (23). PB NK cells were isolated with Human NK cell enrichment cocktail-RosetteSep (StemCell technologies).

ILC Culture, Analysis of Cytokine Production, and Degranulation

Innate lymphoid cell subsets were cultured in U-bottom 96-well plates (Corning, Tewksbury, MA, USA) in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (vol/vol) FCS (Lonza), 1% (vol/vol) glutamine, and 1% (vol/vol) penicillin, neomycin, and streptomycin antibiotic mixture (Lonza and Cambrex, Charles City, IA, USA). When indicated we added 10 ng/ml IL-15 (Miltenyi) and 5 ng/ml recombinant TGFβ (Peprotech). To perform co-culture experiments, dSC and NK cells were plated at the ratio of 1:5 ± α-TGFβ neutralizing antibody (R&D). CD34$^+$ cells were cultured in the presence of RPMI-1640 medium supplemented with 10% (vol/vol) human AB serum (Lonza), 1% (vol/vol) glutamine, and 1% (vol/vol) penicillin, neomycin, and streptomycin antibiotic mixture in the presence of 10 ng/ml Flt3-L, 20 ng/ml SCF, IL-7, IL-15, and IL-21 (Milteny) ±5 ng/ml TGFβ. For the analysis of cytokine production, cells were stimulated as indicated in figures with 25 ng/ml PMA, 1 μg/ml Ionomycin (Sigma-Aldrich), 50 ng/ml IL-23, 50 ng/ml IL-15, 10 ng/ml IL-12 (Miltenyi), and 100 ng/ml IL-18

(MBL). To perform intracellular cytokine analysis, cells were stimulated 18 h in the presence of Brefeldin A (BD Bioscience). After stimulation, cells were stained for surface markers, fixed with Cytofix/Cytoperm, and permeabilized with Perm/Wash (BD Bioscience) according to the manufacturer's instructions. To perform supernatants (spt) cell analysis, ILCs were stimulated for 72 h, the spt were collected, and cytokine concentration was evaluated by ELISA multiplex assay (Merck Millipore) and analyzed with Magpix system (Luminex). TGFβ produced by dSC was measured by ELISA multiplex assay in spt collected after 1 week of culture in serum-supplemented RPMI-1640 medium. To perform degranulation assays coupled with analysis of IFNγ production, 72-h-cytokine-activated DILs were co-cultured with K562 cells at an effector:target (E:T) ratio of 1:1, in the presence of anti-CD107a and Monensin (BD Bioscience). After 4 h, cells were washed and stained for surface and intracellular markers.

Mice, Collection of Decidual and Uterine Tissues, and Cell Isolation

C57BL/6 mice were purchased from Charles River (Como, Italy). Eomes-GFP reporter mice (6) were maintained and mated at the Animal Facility of the IRCCS-AOU San Martino-IST. All mice were used between 6 and 12 weeks of age. Housing and treatments of animals were in accordance with the Italian and European Community guidelines (D.L. 2711/92 No.116; 86/609/EEC Directive) and approved by the internal Ethic Committee. To time pregnant females, superovulation was induced by intraperitoneal injection of 5 IU of Pregnant Mares Serum (Folligon; Intervet, Italy) followed, 48 h later, by 5 additional IU of hCG (Corulon; Intervet, Italy). Immediately following injection, each female was mated with a syngeneic male overnight. Females with copulation plug were separated and identified as gestation day (gd) 0.5. Mice were killed at different gd by cervical dislocation and uterus was processed as previously described (21). Lymphoid cells present in the implant before gd 9 are of maternal origin since fetal hematopoiesis starts at gd 9. Thus, at gd 5.5 uterus was open and the implants were isolated and processed as a source of decidual tissue; while at gd 10.5 and 14.5, the decidua was separated from the implant by cutting away the mesometrial pole and all decidua derived from the same uterus were pooled. Uterine wall, once cleared out of the implants, was further processed. The uteri of virgin females were isolated and processed entirely. Decidual and uterine tissues were mechanically disrupted. Spleen and liver were also collected from virgin and pregnant mice and single-cell suspensions were prepared as previously described (24).

Flow Cytometry Analyses and Monoclonal Antibodies

Human and mouse cells were stained with the monoclonal antibodies listed in **Table 1**. Before staining with mAbs, murine cells were incubated with FcR blocking reagent (Miltenyi). For intranuclear staining of transcription factor, cells were stained for surface markers, fixed with Fixation/Permeabilization buffer and permeabilized with permeabilization buffer (eBioscience), respectively, according to the manufacturer's instructions. All

TABLE 1 | List of reagents used for flow cytometry analysis.

Marker	Fluorocrome	Supplier	Reactivity
β7	APC	BD Bioscience	h/m
CD3	ECD	Beckman Coulter	h
CD3	PE	Miltenyi	h
CD3	PE/Dazzle594	BioLegend	m
CD3	PacificBlue	BioLegend	m
CD9	PE	Miltenyi	h
CD9	PE	eBioscience	m
CD14	APC-eFluor480	eBioscience	h
CD14	ECD	Beckman Coulter	h
CD34	FITC	Miltenyi	h
CD45	APC-H7	BD Bioscience	h
CD45	biotin	BD Bioscience	m
CD49a	APC-Vio770	Miltenyi	h
CD49a	APC	BioLegend	m
CD49b	PacificBlue	BioLegend	m
CD49b	PE	Miltenyi	m
CD56	PC7	Beckman Coulter	h
CD69	PE	Miltenyi	h
CD69	biotin	eBioscience	m
CD94	FITC	BioLegend	h
CD103	PE	BioLegend	h
CD103	FITC	BioLegend	h
CD107a	FITC	BD Bioscience	h
CD117	APC	Miltenyi	h
CD117	PerCP-Cy5.5	BioLegend	h
CD122	PE	BD Bioscience	m
CD122	biotin	BD Bioscience	m
CD127	BrilliantViolet421	BioLegend	h
CD127	PerCP-Cy5.5	BioLegend	h
CD158a,h	PE	Beckman Coulter	h
CD158b1/b2,j	PE	Beckman Coulter	h
CD158e1,e2	PE	Beckman Coulter	h
CD160	eFluor660	eBioscience	m
CD314 (NKG2D)	PE	Miltenyi	h
CD335 (NKp46)	APC	Miltenyi	h
CD336 (NKp44)	APC	BioLegend	h
CD337 (NKp30)	PE	Miltenyi	h
NKp46	PE	eBioscience	m
NKp46	eFluor660	eBioscience	m
Eomes	AlexaFluor647	eBioscience	h
GranzymeA	PE	BD Bioscience	h
GranzymeB	PE	Life Technologies	h
IFNγ	Alexa647	BD Bioscience	h
IFNγ	PerCP-Cy5.5	eBioscience	h
IFNγ	PECy7	BD Bioscience	m
IL-22	PE	eBioscience	h
NK1.1	APC	BioLegend	m
NK1.1	PerCP-Cy5.5	eBioscience	m
Perforin	PE	Ancell	h
RORγt	PE	eBioscience	h/m
TNFα	eFluor450	eBioscience	h
TNF	PE	Miltenyi	m
TRAIL	VioBlue	Miltenyi	m
Live/dead fixable	Aqua Dead	Life Technologies	
Streptavidin	Alexa-fluor700	Life Technologies	
Streptavidin	PE	Life Technologies	
Streptavidin	eFluor710	eBioscience	

samples were analyzed on Gallios Flow Cytometer (Beckman Coulter) or MACSQuant Analyzer (Miltenyi). Data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA). Unstained cells were used as negative controls and markers set accordingly.

Statistical Analysis

Prism6 GraphPad software was used for statistical analysis. **Figures 2C,D**, **4A,C** and **5B** show one-way ANOVA. **Figures 4E–G** and **5H** show two-way ANOVA. **Figure 5F** show one-way ANOVA plus post test for linear trend. We considered significant p-values ≤ 0.05.

RESULTS

Distinct Eomes$^+$ NK Subsets Are Present in Human Endometrium and Decidua

It has been shown that in tonsil and gastrointestinal epithelium NKp44 molecule is expressed by CD56$^+$CD127$^+$RORγt$^+$ ILC3 and CD56$^+$CD127$^-$CD103$^+$ ILCs (8). Lymphoid cells isolated from human endometrium and decidua contained similar percentages of Lin$^-$CD56$^+$CD127$^+$CD117$^+$ RORγt$^+$ ILC3 (**Figures 1A–C**) that homogeneously expressed NKp44 (not shown) (7, 17). Among Lin$^-$CD56$^+$CD127$^-$CD117$^-$RORγt$^-$ cells, we identified three subsets according to NKp44 and CD103 surface expression (**Figures 1A,D**). In particular, NKp44$^+$CD103$^+$ subset represented a minor fraction of CD56$^+$ cells as compared to NKp44$^-$CD103$^+$ and NKp44$^-$CD103$^-$ cells (**Figures 1A,D**). The frequency of these three cell subsets did not significantly differ between endometrium and decidua. Thus, the presence of these ILC subsets seems not to depend on pregnancy status. NKp44$^+$CD103$^+$, NKp44$^-$CD103$^+$, and NKp44$^-$CD103$^-$ cells expressed T-bet and Eomes (**Figure 1E** and not shown), thus strongly suggesting that they belong to the NK cell lineage. The analysis of markers commonly used to identify endometrial and dNK cells (15) revealed that all three subsets were CD49a$^+$, while CD9 and CD69 were expressed at higher levels by NKp44$^+$CD103$^+$ and NKp44$^-$CD103$^+$ cells than by NKp44$^-$CD103$^-$ cells. In addition, CD103$^+$ subsets expressed integrin β_7 that, together with CD103, can forms the $\alpha_E\beta_7$ heterodimer. Moreover, the main activating NK cell receptors, including NKp46, NKp30, NKG2D, and DNAM-1 were homogenously expressed by all three cell populations (**Figure 1F**). All subsets were CD16$^-$ CD57$^-$and CD94/NKG2A$^+$ (**Figure 1F**), while expressing variable amounts of KIRs (**Figure 1E**).

Decidual Stromal Cells-Derived TGFβ Influences dNK Cell Phenotype

The typical features of dNK cells depend, at least in part, from the influence of decidual microenvironment. Previous reports indicated CD103 and CD9 as markers of exposure to TGFβ (8, 25, 26). Endometrial and dSC were shown to produce molecules of the TGFβ family (27). Accordingly, we found that dSC isolated from different donors produced TGFβ (**Figure 2A**). dSC-derived spt or recombinant (r) TGFβ induced *de novo* expression of CD103 and CD9 on PB NK cells. In addition, in the presence of anti-TGFβ (α-TGFβ) neutralizing antibody, the expression of both markers was inhibited (**Figure 2B**). Next, we evaluated the effect of rTGFβ on the three dNK subsets identified. Only the NKp44$^+$CD103$^+$ subset underwent *in vitro* cell proliferation upon 7 days culture (**Figure 2C**). This result was in accordance with higher *ex vivo* expression of Ki67 (**Figure 2D**). Although TGFβ did not influence cell proliferation (**Figure 2C**), it did affect the phenotypic features of the three subsets. In particular, NKp44$^+$CD103$^+$ retained CD103 expression only when cultured in the presence of rTGFβ (**Figure 2E**). In agreement with previous studies, NKp44$^-$CD103$^-$ cells cultured with rTGFβ acquired CD103 (8, 26). CD9 expression was partially downregulated when cells were cultured in the absence of TGFβ (**Figure 2E**). In addition, rTGFβ reduced the expression of NKp30, particularly in NKp44$^-$CD103$^+$ cells (**Figure 2E**) (28).

We also investigated whether rTGFβ could influence dNK cell differentiation from dCD34$^+$ hematopoietic precursors. In the presence of rTGFβ, dCD34$^+$ cell number fold expansion was affected in two out of three experiments (**Figure 3A**). Differentiation toward Lin-CD56$^+$CD161$^\pm$ cells was inhibited (**Figure 3B**). However, CD56$^+$ cells expressed higher levels of CD103 and CD9 than cells cultured in the absence of rTGFβ (**Figures 3C,D**). Thus, it is conceivable that uterine/decidual microenvironment, enriched in TGFβ, may play a relevant role in the induction of unique features in recruited or *in situ* differentiated NK cells and in the maintenance of dNK cell phenotype.

CD103$^+$ Cells Represent the Major Source of IFNγ in dNK Cells

During the early phases of pregnancy, the balance between inflammation and tolerance is critical (29). A successful pregnancy needs a "regulatory phase" that inhibits immuno-mediated fetal rejection. However, an early "inflammatory phase" favors embryo implantation thanks to the production of cytokines and chemokines that contribute to tissue remodeling and neo-angiogenesis (30). Analysis of cytokines produced by the three dNK subsets revealed that CD103$^+$ cells expressed higher amounts of IFNγ and TNF than CD103$^-$ cells upon stimulation (**Figure 4A**). IL-22 was exclusively produced by ILC3 (**Figure 4A**). dNK cells are classically considered as poorly cytotoxic, in spite of their content of cytolytic granules (15). All dNK subsets expressed similar levels of perforin, granzymes A and B (**Figure 4B**). After 18 h of stimulation with different cytokine combinations, dNK cell subsets were co-cultured with K562 target cells and analyzed for IFNγ production and CD107a expression. Stimulation of dNK subsets with IL-12 or IL-15, or IL-18 did not induce significant IFNγ production (**Figures 4C,D**). Conversely, cells stimulated with IL-12 + IL-15 expressed higher amounts of IFNγ than unstimulated cells. Moreover, cells cultured with IL-12 + IL-15 + IL-18 produced the highest amounts of IFNγ (**Figures 4C–E**), highlighting the known synergy between these cytokines (31). CD107a expression was enhanced upon cell stimulation with IL-15, either alone or in combination with other cytokines (**Figures 4C,D**). Remarkably, although NKp44$^+$CD103$^+$ cells produced the highest amounts of IFNγ and TNF on a per cell basis (**Figure 4E**), this cell subset represented only 2% of

CD56+CD127−CD117− cells (see **Figure 1D**). Indeed, when the amount of cytokines produced was normalized to the relative frequency of the cell subsets, NKp44−CD103+ cells resulted as the most important source of IFNγ (**Figure 4F**). In any case, all three dNK subsets displayed both lower IFNγ production and CD107a expression than PB NK cells (**Figure 4G**), in line with previous reports (23, 26, 32). Thus, decidual microenvironment is likely to affect both the phenotypical and the functional features typical of NK cells.

Murine Uterus and Decidua Contain Eomes+CD49a+CD49b+ and Eomes+CD49a+CD49b− NK Cell Subsets

Previous studies in mice indicated that during midgestation the majority of uterine CD3−NK1.1+ cells express high levels of Eomes (7, 33). Taking advantage of Eomes-GFP mice, we analyzed the expression of Eomes in dNK and uNK cells (identified as CD3−NK1.1+ cells) starting from the early phase

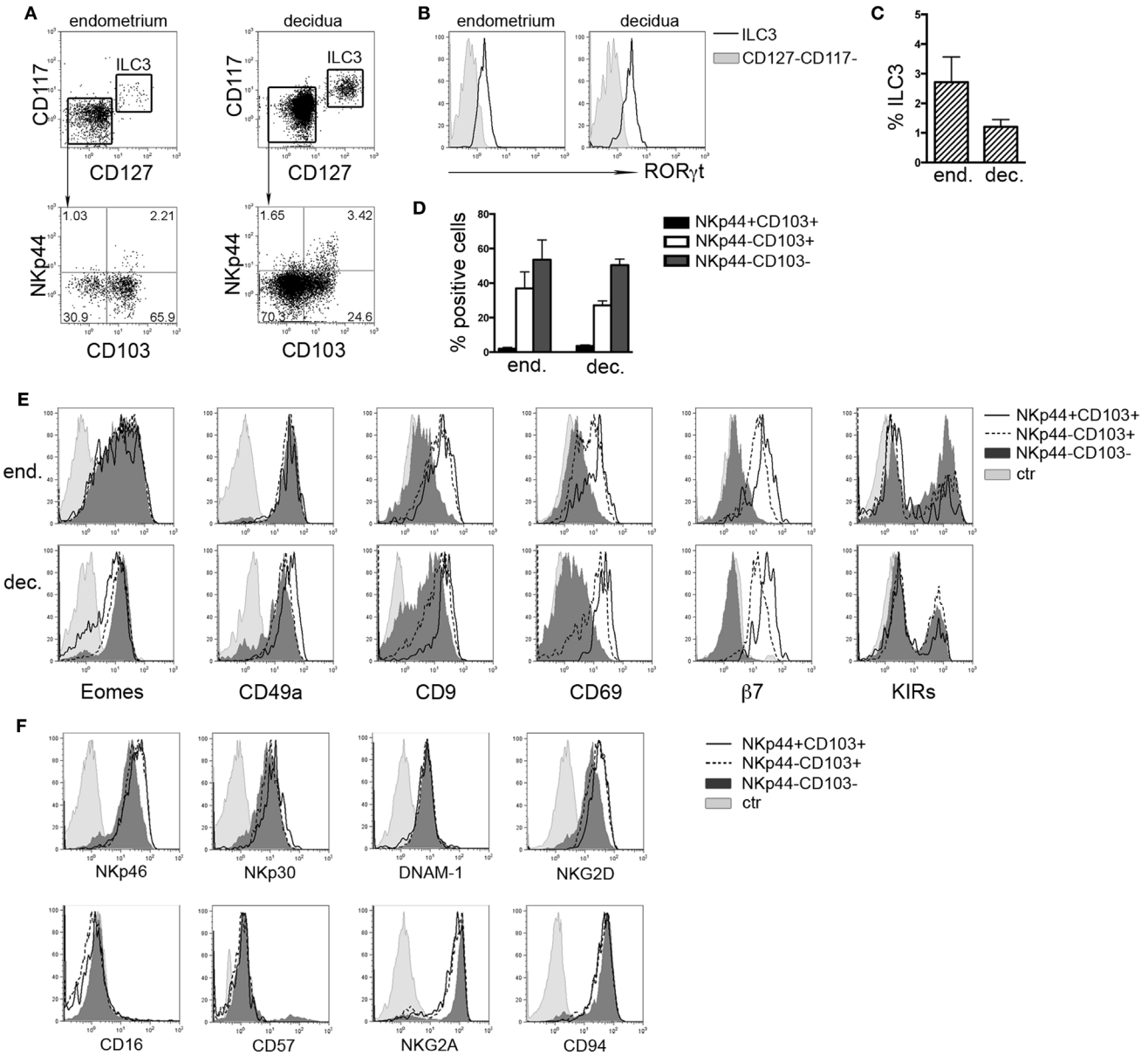

FIGURE 1 | *Ex vivo* characterization of human endometrial and decidual ILC subsets. **(A)** Identification of different ILC subsets by surface marker analysis, after gating on Lin−CD56+ cells. **(B)** Intranuclear expression of RORγt (n = 4–8). **(C)** Mean ± SEM of Lin−CD127+CD117+ ILC3 (n = 7). **(D)** Percentage of different NK cell subsets after gating on endometrial and decidual Lin−CD56+CD127−CD117− cells (mean ± SEM; n = 6 and 28, respectively). **(E,F)** Phenotypic analysis of endometrial **(E)** and decidual **(E,F)** NKp44+CD103+, NKp44−CD103+, and NKp44−CD103− cells. Control (ctr) corresponds to unstained cells. One representative experiment out of 8 performed.

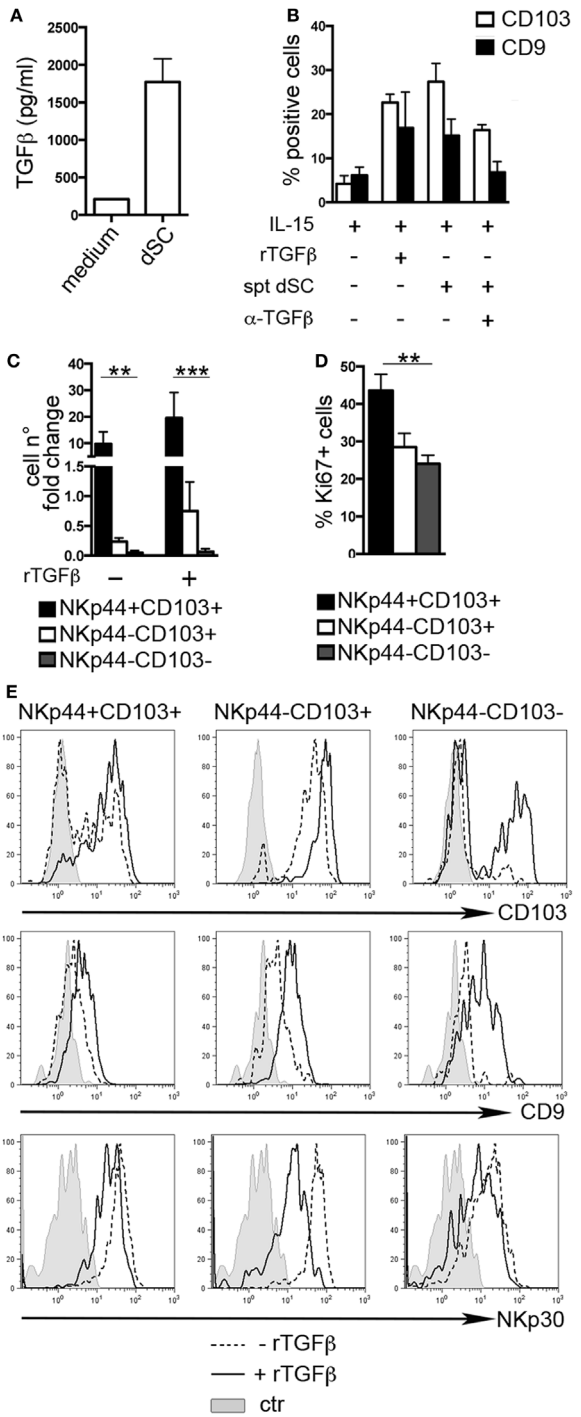

FIGURE 2 | TGFβ modulates human dNK cell phenotype. **(A)** TGFβ concentration (picogram/milliliter) in dSC spt derived from 8 different donors. **(B)** PB NK cells were cultured as indicated for 15 days and analyzed by flow cytometry for the expression of CD103 and CD9; mean ± SEM of positive cells ($n = 5$). **(C)** NKp44$^+$CD103$^+$, NKp44$^-$CD103$^+$, and NKp44$^-$CD103$^-$ dNK cell subsets were sorted and cultured with IL-15 ± rTGFβ. Cell number fold change is depicted, calculated as ratio of cell number at day 20 to that at day 0. **(D)** Ki67 expression on DILs after gating on dNK cell subsets ($n = 6$). **(E)** Sorted dNK cell subsets were cultured for 20 days in IL-15 ± TGFβ and analyzed for the expression of the indicated markers, one representative experiment out of 3 performed. Control (ctr) corresponds to unstained cells.

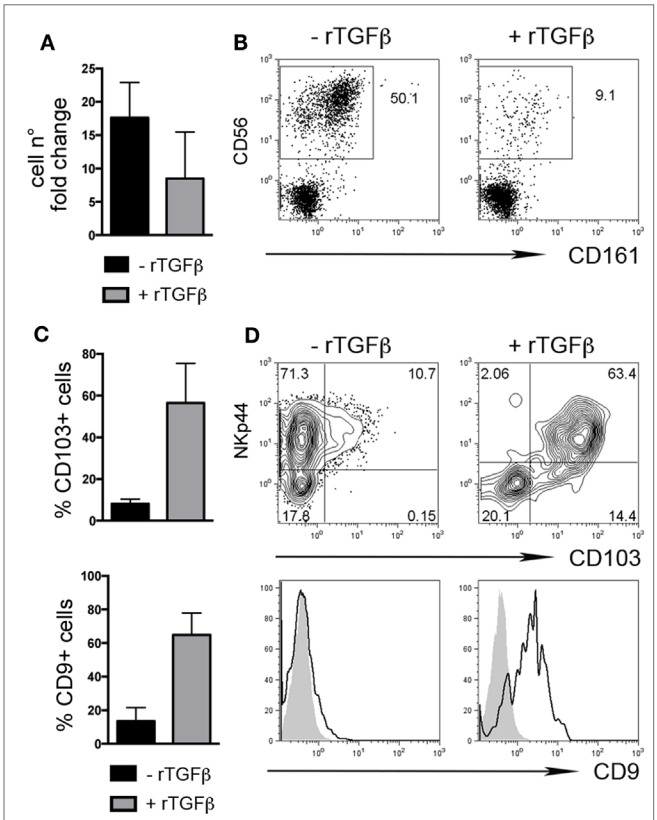

FIGURE 3 | TGFβ modulates human dCD34$^+$ cell differentiation toward CD56$^+$CD103$^+$CD9$^+$ cells. Purified dCD34$^+$ cells were cultured 14 days in the presence of Flt3-L, SCF, IL-7, IL-15, and IL-21 ± rTGFβ **(A)** Cell number fold change calculated as ratio of cell number at day 14 to that at day 0. **(B)** Flow cytometry analysis of *in vitro* differentiated cells after gating on Lin$^-$ cells. **(C,D)** Flow cytometry analysis of *in vitro* differentiated cells after gating on Lin$^-$CD56$^+$ cells, **(C)** mean ± SEM of positive cells ($n = 3$), and **(D)** one representative experiment, gray histogram corresponds to unstained cells, black line gated on Lin$^-$CD56$^+$ cells.

of pregnancy (gd 5.5). The majority of dNK and uNK cells at gd 5.5 were Eomes$^+$ and no differences were detected between pregnant and virgin uteri (**Figure 5A**). A small proportion of CD3$^-$NK1.1$^+$Eomes$^-$ ILC1 was present in uterus and decidua at gd 5.5 and increased in percentages during pregnancy (**Figure 5B**). Both Eomes$^+$ and Eomes$^-$ cells were T-bet positive (not shown). Uterine and decidua NK1.1$^+$Eomes$^-$ cells expressed markers of tissue retention (CD49a, CD160, CD9, and CD69) and TRAIL, while they were negative for β_7 integrin (**Figures 5C,D**). Notably, u- and d-Eomes$^+$ cells displayed a bimodal expression of CD49a and β7 integrin, while they homogeneously expressed CD160, CD9, CD69, and TRAIL (**Figures 5C,D**).

Typically, CD49a identifies liver Eomes$^-$ cells, while Eomes$^+$ cNK cells are CD49a$^-$CD49b$^+$. Strikingly, the simultaneous analysis of Eomes, CD49a, and CD49b allowed the identification of three subsets of NK cells in uterus and decidua: Eomes$^+$CD49a$^+$CD49b$^-$ (population, pop. 1), Eomes$^+$CD49a$^+$CD49b$^+$ (pop. 2), and Eomes$^+$CD49a$^-$CD49b$^+$ cells (pop. 3) (**Figures 5E,F**). The latter population (pop. 3) corresponds to cNK cells, while the other

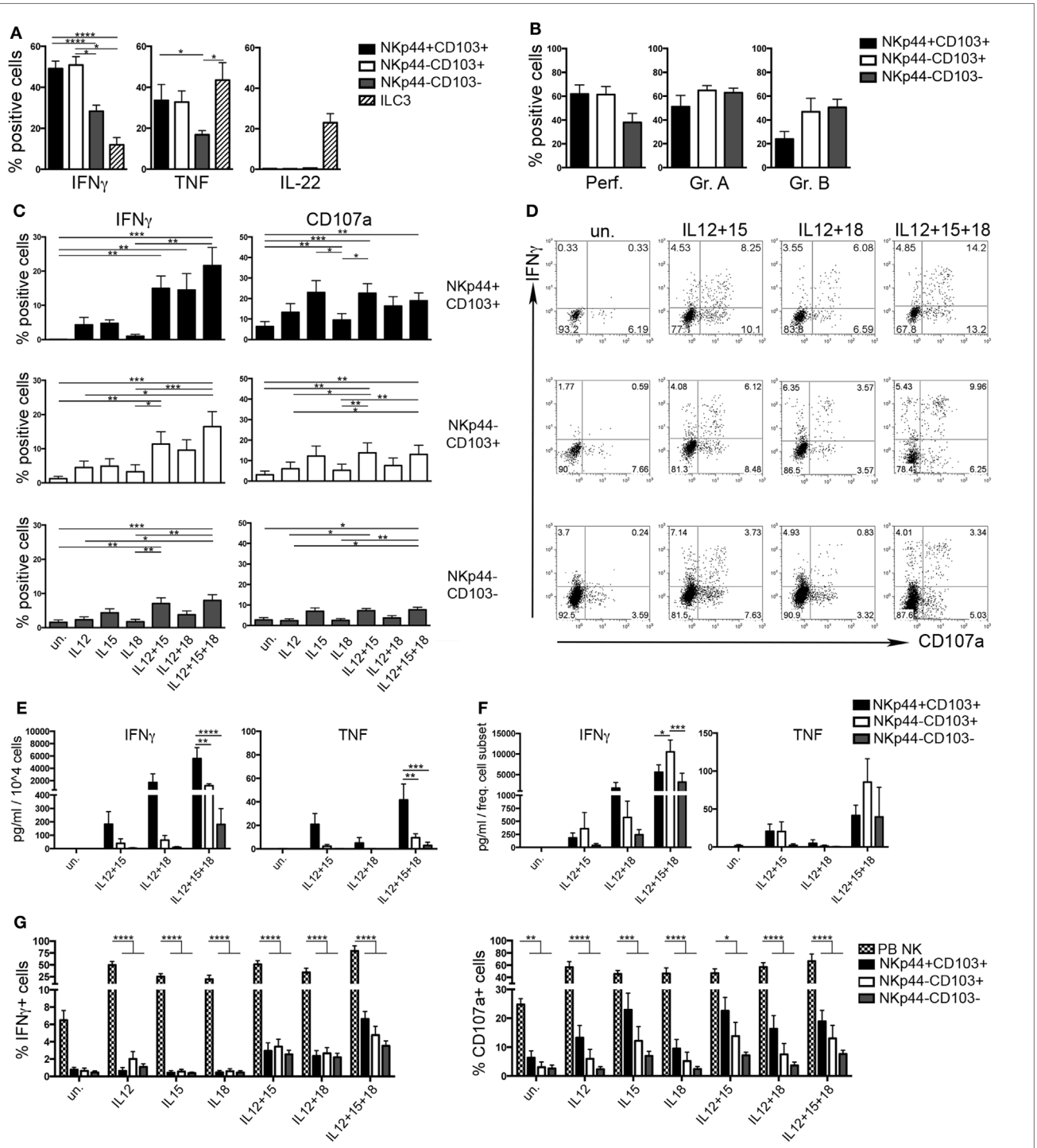

FIGURE 4 | Human decidual NKp44$^+$CD103$^+$, NKp44$^-$CD103$^+$, and NKp44$^-$CD103$^-$ cells produce IFNγ upon cytokine stimulation. **(A)** DILs were stimulated with P + I + IL-23 for 18 h and analyzed for intracellular cytokine expression after gating on the three dNK cell subsets and ILC3. Mean ± SEM of cytokine positive cells (n = 11). **(B)** DILs were analyzed for the intracellular expression of perforin and granzymes. Mean ± SEM of positive cells (n = 6). **(C,D)** DILs were stimulated with IL-12, IL-15, and IL-18 alone or in combination. After 72 h, cells were incubated 4 h with K562 cells and analyzed for the expression of IFNγ and CD107a. **(C)** Mean ± SEM of positive cells (n = 6) and **(D)** one representative experiment. **(E,F)** dNK cell subsets were sorted and stimulated as indicated. Cell spt were collected after 72 h and analyzed by ELISA multiplex assay for IFNγ and TNF. For statistical analysis, within each stimulation condition, data referred to different cell populations were compared with Tukey's multiple comparison. **(E)** Mean ± SEM of cytokine concentration (picogram/milliliter) produced by 10^4 cells (n = 5). **(F)** Mean ± SEM of cytokine concentration (picogram/milliliter) is normalized to the mean frequency (see **Figure 1D**) of each subset (n = 5). **(G)** DILs and PB NK cells were stimulated as indicated. After 72 h, cells were incubated 4 h with K562 cells and analyzed for the expression of IFNγ and CD107a; mean ± SEM of positive cells (n = 6).

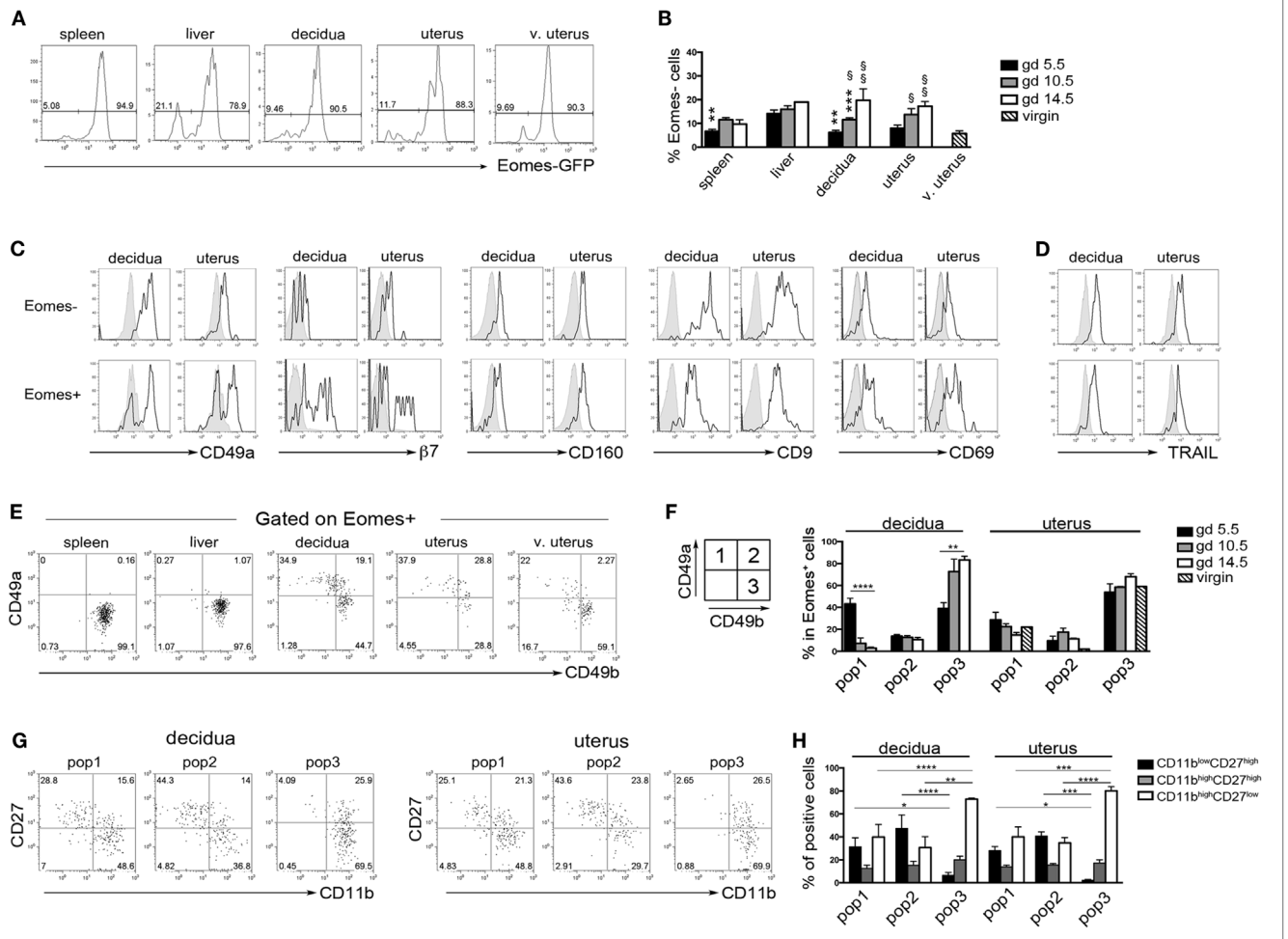

FIGURE 5 | Peculiar subsets of Eomes$^+$ decidual and uterine murine NK cells during pregnancy. **(A)** Analysis of Eomes expression in CD3$^-$NK1.1$^+$ cells in the indicated organs isolated from Eomes-GFP mice at gd 5.5; one representative experiment (n = 25). **(B)** Mean ± SEM of Eomes$^-$ cell percentages among CD3$^-$NK1.1$^+$ cells at different gd ($n \geq 3$). (*) Indicate statistical analysis of data from spleen, decidua, and uterus compared with liver at the same gd. (§) Indicate statistical analysis of data from decidua and pregnant uterus at different gd compared with virgin uterus. **(C,D)** CD3$^-$NK1.1$^+$ Eomes$^+$ and Eomes$^-$ cells isolated from decidua and uterus of Eomes-GFP mice at gd 5.5 were analyzed by flow cytometry for the indicated markers (black line), gray histograms correspond to unstained cells **(C)**, or splenic NK cells **(D)**; one representative experiment ($n \geq 3$). **(E,F)** CD3$^-$NK1.1$^+$Eomes$^+$ cells were analyzed for the expression of CD49a and CD49b. **(E)** One representative experiment (n = 6) at gd 5.5. **(F)** Mean ± SEM of percentage of the different subsets of CD3$^-$NK1.1$^+$Eomes$^+$ cells at different gd in decidua and uterus ($n \geq 3$). **(G,H)** Analysis of CD27 and CD11b expression in the CD3$^-$NK1.1$^+$Eomes$^+$ cells subsets in pregnant uterus and decidua. **(G)** One representative experiment; **(H)** mean ± SEM of percentage of positive cells (n = 4).

two subsets are only present in uterus and decidua. Notably, in decidua the percentages of Eomes$^+$CD49a$^+$CD49b$^-$ (pop. 1) cells decreased during pregnancy, while cNK cells progressively increased (**Figure 5F**). Of note, Eomes$^+$CD49a$^-$CD49b$^+$ (pop.3) cells are enriched in mature CD11b^{high}CD27^{low} cells (24), while Eomes$^+$CD49a$^+$CD49b$^-$ (pop. 1) and Eomes$^+$CD49a$^+$CD49b$^+$ (pop. 2) contain higher percentages of cells displaying an immature phenotype (CD11b$^{low/high}$CD27^{high}), both in decidua and uterus (**Figures 5G,H**). In line with previous results (6), Eomes$^+$ cells produced higher IFNγ and lower TNF than Eomes$^-$ cells (**Figures 6A,B**). In decidua and uterus, Eomes$^+$CD49a$^+$CD49b$^-$ (pop.1) cells mainly produced TNF, while the two subsets of CD49b$^+$ (pop. 2 and 3) cells expressed both TNF and IFNγ **Figure 6C**). Therefore, murine decidua and uterus contain different subsets of group 1 ILCs, including ILC1 (Eomes$^-$CD49a$^+$CD49b$^-$IFNγ^{low} TNF^{high}), cNK (Eomes$^+$CD49a$^-$CD49b$^+$IFNγ^{high}TNF^{low}), and two novel subsets of NK cells (Eomes$^+$CD49a$^+$CD49b$^-$IFNγ$^+$TNF$^+$ and Eomes$^+$CD49a$^+$CD49b$^+$IFNγ$^+$TNF$^+$) characterized by phenotypic and functional features shared by cNK cells and the formerly described trNK cells.

DISCUSSION

In the present study, we show that both human and murine uterine microenvironments are enriched in Eomes$^+$ ILCs, i.e., NK cells. In particular, human endometrium and decidua NK cells include two subsets of CD103$^+$ cells that could be further dissected on the basis of NKp44 expression. We found that decidual CD103$^+$

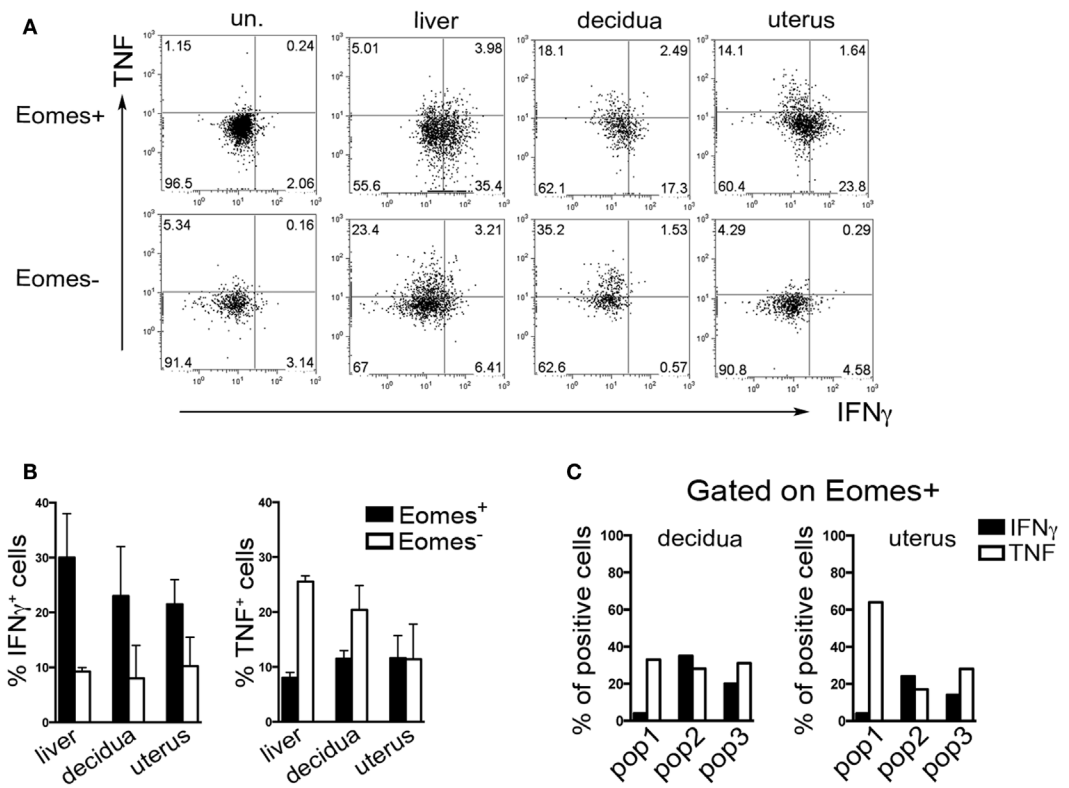

FIGURE 6 | Cytokines production by murine Eomes$^+$ and Eomes$^-$ cells. **(A–C)** Decidua and uterus (CD3$^-$NK1.1$^+$) Eomes$^+$ and Eomes$^-$ cells were sorted from Eomes-GFP mice, stimulated with P + I for 18 h and analyzed for intracellular cytokine expression. **(A)** One representative experiment; **(B)** mean ± SEM of cytokine positive cells (n = 3); **(C)** percentages of IFNγ$^+$ and TNF$^+$ cells in the different Eomes$^+$ cell subsets (see **Figure 5F**). Data derived from a pool of 10 mice.

NK cells produce higher amounts of IFNγ than CD103$^-$ cells and express markers of tissue residency, that were maintained in culture with TGFβ. In murine uterus and decidua, Eomes$^+$ cells are heterogeneous and, besides cNK cells, contain two peculiar subsets dissected by the expression of CD49a and CD49b. In particular, Eomes$^+$CD49a$^+$CD49b$^-$ cells are mainly present during the early phase of pregnancy (gd 5.5) and characterized by TNF production.

Human NK cells represent the most abundant lymphoid population in decidual tissue during the first trimester of pregnancy. This, together with their peculiar phenotypic and functional features, raised many questions regarding their origin. Here, we show that dNK cells express markers suggestive of TGFβ imprinting, such as CD103 and CD9. Eomes$^+$CD103$^+$CD9$^+$ NK cells are already detectable in the endometrium, thus, suggesting that the presence/recruitment of these ILC subsets does not depend on the pregnancy status. Stromal cells present in endometrium and decidua release TGFβ able to induce or maintain expression of these markers on NK cells and on dCD34-derived NK cells. These data suggest that both PB NK cells and dCD34$^+$ precursors may be influenced by decidual microenvironment. Whether a similar TGFβ-dependent mechanism occurs also in mice remains to be determined. A population of CD103$^+$ cells was described also in the human intestinal epithelia (8). Intestinal CD103$^+$ cells homogeneously express NKp44 and are IFNγ producers. Conversely, most endometrial and decidual CD103$^+$ cells do not co-express NKp44 and produce low levels of IFNγ as compared to PB NK cells. Nevertheless, among dNK cells, CD103$^+$ cells represent the major source of IFNγ.

A recent report identified in mice Eomes$^+$CD49a$^+$ cells in the virgin uterus (7). Here, we show that these cells are also present in pregnant uterus and decidua and that CD49b expression allows the further identification of two subsets of Eomes$^+$CD49a$^+$ cells only detectable in these tissues. Eomes$^+$CD49a$^-$CD49b$^+$ cNK cells, which are enriched in IFNγ producing cells, are the predominant Eomes$^+$ subset during midgestation (gd 10.5) when they might contribute to spiral artery modification (34). On the other hand, Eomes$^+$CD49a$^+$CD49b$^-$ cells mainly produce TNF, are abundant at gd 5.5 and subsequently decrease. A minor Eomes$^-$CD49a$^+$ cell population able to produce TNF is also present in decidua and uterus and increases during pregnancy. Thus, a source of TNF is constantly present during early and midgestation.

Studies aiming to characterize ILC subsets in several organs highlighted the complexity of this cell family and suggested that ILCs may display tissue-specific features and developmental requirements. In liver, it has been shown that T-bet$^+$Eomes$^+$ cNK and T-bet$^+$Eomes$^-$ ILC1 differentiate from precursors of medullary and peripheral origin, respectively (6). Our present

data indicate that the majority of uNK and dNK cells express Eomes. They might derive from accumulation of circulating Eomes$^+$ NK cells. However, in a previous study, we showed that only a minor fraction of dNK cells derives from the migration of splenic NK cells (21). Conversely, we showed that Lin$^-$CD122$^+$ NK precursors are present in murine decidua and uterus and give rise to immature NK cells that undergo differentiation during pregnancy (21). Thus, it is likely that Eomes$^+$ NK cells may derive from *in situ* differentiation of precursors of medullary origin. In accordance with this hypothesis, hematopoietic precursors and immature NK cells capable of differentiating toward dNK cells are present also in human decidua and endometrium, supporting an *in situ* development of dNK cells (35, 36).

It is of note that also salivary gland NK cells, although expressing Eomes, display phenotypic features of trNK cells described in liver (13). Salivary gland Eomes$^+$ NK cells are independent of *Nfil3* for their development, while uterine Eomes$^+$ NK cells are reduced in *Nfil3*$^{-/-}$ mice (13). Moreover, Colucci and co-workers (under review) show that *Nfil3* is required for the expansion of Eomes$^+$CD49a$^+$ NK cells during pregnancy. This, together with our present findings, suggests that peripheral tissues different from liver can sustain the differentiation of peculiar Eomes$^+$ NK cells. The developmental relationship among the three subsets of Eomes$^+$ NK cells identified here is unknown. NK cell maturation is a four-stage developmental program that starts at a CD11b^{low}CD27^{low} stage and leads them through the following stages: CD11b^{low}CD27^{high} → CD11b^{high}CD27^{high} → CD11b^{high}CD27^{low} (24). Moreover, CD49b is acquired during NK cell differentiation (37). Here, we show that, among Eomes$^+$ cells, CD49a$^+$CD49b$^-$ cells decrease in percentages during pregnancy, while CD49a$^-$CD49b$^+$ cells increase. Moreover, CD49a$^-$CD49b$^+$ cells are enriched in mature CD11b^{high}CD27^{low} cells, while CD49a$^+$CD49b$^-$ and CD49a$^+$CD49b$^+$ cells contain higher percentages of immature CD11b^{low}CD27^{high} cells, both in uterus and decidua. It may be possible to speculate that the three subsets of Eomes$^+$ NK cells represent developmentally related differentiation stages, although fate-mapping experiments will be needed to test this hypothesis.

Thus, our data indicate that the formerly indicated uNK and dNK actually represent heterogeneous group 1 ILC populations, including Eomes$^-$ ILC1, Eomes$^+$ cNK cells, and two novel subsets of Eomes$^+$ NK cells, phenotypically and functionally distinct from trNK previously described in other tissues. It is conceivable that they may diverge from the Eomes$^+$-developmental pathway under the influence of decidual microenvironment that may contribute to their differentiation. Further studies will be required for better understanding the role of the different ILC subsets in the establishment and maintenance of pregnancy.

AUTHOR CONTRIBUTIONS

EM, PV, and LC designed the study, performed experiments, analyzed data, and wrote the manuscript; DC performed experiments and analyzed data; FL and SM performed experiments; SF selected human samples and interpreted data; TW interpreted data and revised the manuscript; LM and MM supervised the study and wrote the manuscript.

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