NATURAL KILLER CELLS IN TISSUE COMPARTMENTS

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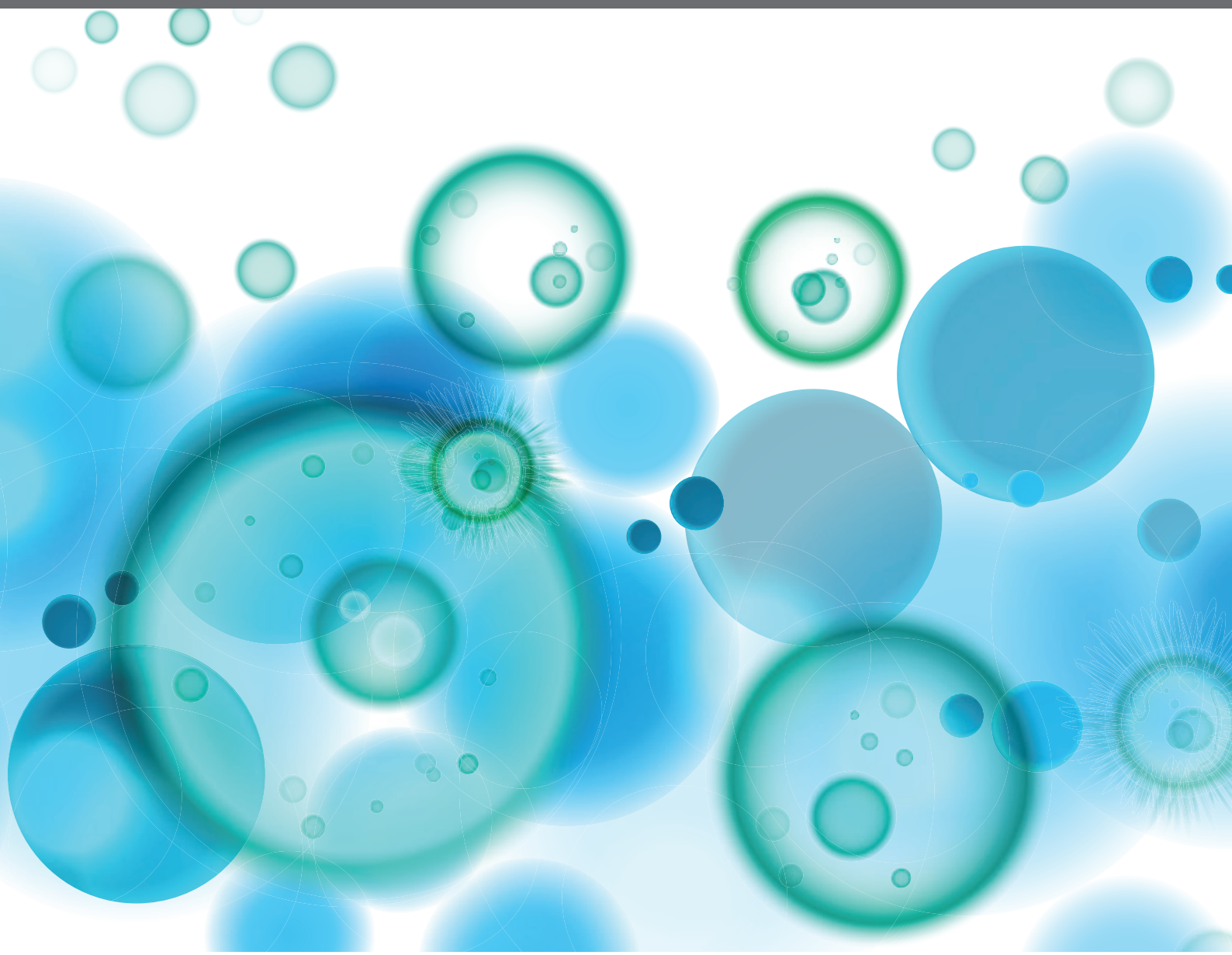
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-612-9
DOI 10.3389/978-2-88963-612-9

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NATURAL KILLER CELLS IN TISSUE COMPARTMENTS

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Citation: Vitale, M., Sivori, S., Caligiuri, M. A., eds. (2020). Natural Killer Cells in Tissue Compartments. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-612-9

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frontiers in Immunology

EDITORIAL
published: 20 February 2020
doi: 10.3389/fimmu.2020.00258

Editorial: Natural Killer Cells in Tissue Compartments

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Keywords: conventional NK cells, tissue resident NK cells, innate lymphoid cells, tissue microenvironment, NK receptors

Editorial on the Research Topic

Natural Killer Cells in Tissue Compartments

Most of our current knowledge about human natural killer (NK) cells comes from studies on cells derived from peripheral blood, also known as "conventional" NK cells (c-NK), but recently, interest in the characterization of NK cells within tissues has increased, and besides recirculating cells, different tissue resident NK cells (tr-NK), each possessing distinct phenotypic profiles, have been described.

This Research Topic gathers the most recent information in the field to consolidate the emerging pictures of NK cells in the different organs, and to explain how the homeostasis of these unique NK cell subsets is normally maintained, or altered in pathologic conditions. The topic has successfully collected articles focused on a number of tissues, covering most of the compartments where NK cells are currently under study.

Two articles focus on the **bone marrow** (BM), where hematopoietic stem cells (HSC), or common lymphoid precursors (CLP) can generate mature NK cells or move to secondary lymphoid organs or peripheral tissues to differentiate under the influence of specific local microenvironments. By reviewing the recent literature and also their own data Bonanni et al. and Bozzano et al. depict a quite complex scenario in which BM, besides supporting NK cell and other innate lymphoid cell (ILC) development, can also orchestrate the NK cell mediated responses to infections. For example, a peculiar $Lin^{-}CD34^{+}DNAM1^{hi}CXCR4^{+}$ CLP subset with the potential of generating fully functional NK cells and reaching peripheral inflamed tissues can exit the BM upon prolonged systemic inflammation. Additionally, mature NK cells can also leave the BM, reach infected peripheral tissues and recirculate from the peripheral blood to the BM. Here, mature NK cells can undergo homeostatic or infection-induced proliferation contributing to their reservoir and also to the generation of "memory-like" long-lived NK cells. In T.Gondii-infected mice, BM NK cells can also induce, via IFNγ, regulatory monocytes to control exaggerated, tissue damaging, inflammatory responses. These NK cells could resemble the human BM-resident NK cell population characterized by low cytotoxicity and high IFNγ production.

The exit from BM of precursors or relatively immature NK cells emphasizes the question on the origins and homeostasis of specialized NK cell populations in specific tissues. This question applies, for example, to **the uterus**. Uterine NK cells (u-NK) represent a heterogeneous population endowed with peculiar functions, spanning from the support of embryo development, to the maternal-fetal tolerance, to the control of infection. Strikingly, this population undergoes important changes upon the transition from the steady state to pregnancy, e.g., u-NK cell frequency dramatically increases in the decidua after embryo implantation. How the dynamics of this population are regulated by the local proliferation of tr-NK cells and/or migration and adaptation of c-NK cells remains an

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *24 December 2019*
Accepted: *31 January 2020*
Published: *20 February 2020*

Citation:
Vitale M, Caligiuri MA and Sivori S (2020) Editorial: Natural Killer Cells in Tissue Compartments. Front. Immunol. 11:258. doi: 10.3389/fimmu.2020.00258

interesting and incompletely addressed question. Based on data from murine virgin or pregnant uteri, Sojka et al. propose a two-wave hypothesis for u-NK cell accumulation during pregnancy. The first wave is due to the local proliferation of tr-NK cells during decidualization, whereas the second, occurring during the placentation, involves the recruitment of peripheral c-NK cells. Importantly, these c-NK cells participate in spiral arteriole remodeling by acting on endothelial and decidual stromal cells in an IFNγ-dependent way.

In healthy pregnancy, the pool of human decidual NKs includes poorly cytotoxic $Tbet^{pos}EOMES^{pos}CD56^{bright}CD16^{-}KIR^{+}$ cells, expressing tissue residency markers (CD69, CD49a, integrin b7, and CD9), and even the inhibitory receptor, 2B4. However, these cells become fully active during viral infections, demonstrating their high plasticity. This issue is discussed by Jabrane-Ferrat, who suggests that the increased NK cell cytotoxicity depends on education via NKG2A- and/or KIR-mediated recognition of HLA molecules on fetal trophoblast cells, and on NKp46 signaling and/or cytokine stimulation during viral infections.

A suppressed u-NK cell phenotype and function may contribute to the progression of endometrial tumors. Degos et al. show that u-NK cells are minimally present in the tumor infiltrate, at least in part secondary to alterations in chemokines (CXCL12, IP-10, and CCL27) and cytokines (IL-1β and IL-6) that are present in the tumor microenvironment. Moreover, tumor resident $CD103^{+}$ u-NK cells are characterized by reduced cytotoxicity and increased expression of inhibitory checkpoint receptors, such as TIGIT, and TIM-3, as compared to recruited $CD103^{-}$ c-NK cells.

Three Research Topic articles focus on human **liver**, an organ in which NK cells represents almost 50% of all intrahepatic lymphocytes. As described in detail by Mikulak et al., human liver contains three NK cell populations showing transcriptional and phenotypic differences: liver tr-NK cells, memory-like NK cells and recirculating c-NK cells. Liver tr-NK cells are enriched in $CD56^{bright}$ NK cells and are characterized by a peculiar transcription factor profile that includes Hobit, Tbet and increased Eomes expression. This profile is in line with the known liver tr-NK cell phenotype $Eomes^{hi}Tbet^{lo}TIGIT^{+}CD69^{+}CXCR6^{+}CD49e^{-}$. Retention of liver tr-NK cells is probably due to their expression of CXCR6, CXCR3, and CCR5. Indeed, these receptors can interact with CXCL16, CCL3, and CCL5 produced by cholangiocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatocytes, thus favoring tr-NK cells homing to liver. According to the authors, these liver tr-$CD56^{bright}$ NK cells also include an interesting small subset expressing CD49a and CD94:NKG2C that could be related to a clonal expansion in response to viral infections. These so–called memory-like tr-NK cells, however, may be different from the adaptive NK cells of the peripheral blood that develop/expand in CMV^{+} or CMV-reactivating donors. The situation is different for $CD16^{+}Siglec9^{+}$ NK cells found in the liver that are $Tbet^{+}Eomes^{+}$ and likely represent c-NK cells recirculating through the liver blood system without being retained in the organ.

Harmon et al. have analyzed the soluble factors produced in the liver microenvironment that can regulate the liver tr-NK cell phenotype in humans. In particular they analyze the role of TGF-β in suppression of Tbet and expression of Eomes in liver tr-NK cells. Notably, blocking TGF-β signaling through pre-treatment with a specific inhibitor reverses the phenotype of liver tr-NK cells toward that of peripheral blood c-NK cells. Interestingly, liver tr-NK cells share many phenotypic characteristics with u-NK cells ($CD56^{bright}$ $Eomes^{hi}$ $CD69^{+}$), probably as a result of TFG-β in both the tissues.

The importance of the local microenvironment in shaping the NK cell compartment has also been demonstrated in non-alcoholic ratty liver diseases, namely in non-alcoholic steatohepatitis (NASH). In this study, Stiglund et al. highlight the phenotypic modification (i.e., an increased expression of NKG2D) of liver tr-NK cells as well as considerable alterations between liver and adipose tr-NK cells, as well as peripheral blood c-NK cells, independent of disease status.

Poggi et al. provide an overview of NK cells in the context of the **gut** lymphoid tissue**.** According to the authors' view, this scarce population, scattered within the lamina propria, can nevertheless play important roles in different pathologies, including inflammatory bowel diseases (IBD), and cancer. Indeed, although it is still uncertain as to the composition of this population in terms of c- or tr-NK cells, different studies referenced in the article suggest peculiar regulatory features of gut NK cells, which may influence TH1/TH17 or TH2 responses in IBD. On the other hand, the authors describe the anti-tumor function of gut NK cells in the context of certain subtypes of colon cancer.

An involvement of NK cells in pathogenic processes is highlighted in the review by Turner et al. on the **kidney**. NK cells represent ∼25% of lymphocytes in healthy human kidney, and contain an important fraction of $CD56^{bright}$ cells (37% of total kidney-NK cells). The expression of CD69, predominant in $CD56^{bright}$ cells, and parabiosis experiments in mice, indicate that kidney harbors both tr- and c-NK cells. tr-NK cells are involved in both acute kidney injury, being attracted, and activated by damaged tubular epithelial cells, and in chronic kidney disease, supporting the progression of interstitial fibrosis. c-NK cells may be of pathogenic relevance during kidney graft rejection, indeed they can induce ADCC against the allograft by the CD16-mediated binding of donor-specific antibodies.

Two Research Topic articles focus on **lung** NK cells. By reviewing different manuscripts in the field, Hervier et al. describe the landscape of NK cells present in the lung. The majority (up to 80%) of these cells displays a mature $CD56^{dim}$CD16+ phenotype and circulate between the organ and peripheral blood. The remaining cells include the lung tr-NK cells that are characterized by the CD16-CD49a+CD69+CD103+ surface phenotype. Also, in this case, the impact of the local microenvironment likely plays a role in shaping the phenotypic and functional features of lung tr-NK cells. Interestingly, Scharenberg et al. describe marked NK cell hyperresponsiveness, particularly in $CD56^{bright}$CD16- NK cells, during Influenza A virus infection, both in human lung and blood.

Bonaccorsi et al. focus on a still poorly investigated issue: the possible role of NK cells in the **carotid atherosclerotic plaques (CAP)** formation. They show that NK cells are present in the plaques, primarily in symptomatic patients, and are enriched for $CD56^{bright}$ $perforin^{low/neg}$ cells and express markers of tissue residency (i.e., CD103, CD69, and CD49a). Such plaque tr-NK cells (CAP-NK) might preferentially be recruited via CCR7 (as high levels of CCL19 and CCL21 could be measured in the plaque) and then induced to up-regulate markers of tissue residency under the influence of local inflammation. According to the authors, CAP-NK cells may participate in the disease process by favoring both the progression of the atherosclerotic process within carotid plaques and promoting plaque instability. Indeed, CAP-NK cells produce the pro-inflammatory cytokine IFNγ, which can also induce matrix metalloproteinases (MMPs) possibly affecting plaque stability. On the other hand, MMPs may also contribute to the abundant shedding of activating NK-receptor ligands found in the plaques.

An additional tissue to be considered when studying NK cells is that of the tumor. Although this issue could be the subject of a dedicated Research Topic, we here present a few contributions highlighting two specific components when considering any NK cell-based immunotherapeutic strategy: NK cell checkpoints and tumor infiltration by NK cells.

Pesce et al. analyze **peritoneal carcinomatosis (PC)** and describe a compromised function of NK cells present in the peritoneal fluid (PF) of high-grade, and even low-grade, PC patients. PF NK cells of low-grade PC patients include a large fraction of immature NKG2A+KIR-CD57-$CD16^{dim}$ cells characterized by a strong downregulation of the main activating NK cell receptors (such as NKp30, DNAM-1, and CD16). By contrast, and perhaps interestingly, PF NK cells of high-grade PC patients have a mature phenotype (KIR+ CD57+ NK cells) but show increased expression of the immune checkpoint PD-1.

Parodi et al. analyze NK cells in setting of tissue **hypoxia**, a condition frequently occurring within the tumor itself. Besides presenting the hypoxia-induced transcriptome of NK cells, the study also highlights how the decreased O_2 tension can influence the pattern of chemokine receptor expression, ultimately favoring the migration of poorly cytotoxic $CD56^{bright}CD16^{dim}$ NK cells. Thus, local conditions, such as hypoxia, can directly act on the expression of specific chemokine receptors and affect migration of specific NK cell subsets, thereby having a significant impact on tumor escape from effective immune surveillance.

In this context, Ingegnere et al. have described a new (virus-free) protocol for the production of NK cells transfected with the CCR7 chemokine receptor. Interestingly, this transfection method could be applied to other chemokine receptors to induce NK cell migration to different tissues and tumor sites. This approach may be combined with the transfection of NK cells with chimeric antigen receptors (CAR) that target tumor-associated antigens (i.e., **CAR-NK cells**).

Although not initially considered in the original framework of this issue on tissue NK cells, we decided to invite some additional contributions focused on **ILCs**, due to the now obvious relationship between NK cell differentiation and other ILC subsets (namely group 1 ILCs), as well as their reciprocal functional interactions. A Review article by Mariotti et al. discusses the topical issue of the checkpoint receptors, their expression on NK cells and other ILCs, and the known and hypothesized implications in cancer, in defense against infectious pathogens, and in pregnancy. Two research articles by Manson et al. and Carvelli et al. focus on trauma and hemorrhagic or septic shock, respectively, and on the related altered immune responses which often result in adverse outcomes. In particular, the changes within ILCs or with innate-like lymphocytes are investigated and the possible consequences on immune homeostasis are discussed.

Finally, a collection of articles on tissue NK cells and ILCs could not be complete without consideration of their common key receptors, the natural cytotoxicity receptors (**NCRs**). Two reviews, by Barrow et al. and Parodi et al., focus on this issue, providing a thorough and updated picture of these receptors in both NK cells and ILCs. Interestingly, both the articles highlight the polyfunctionality of these receptors, consistent with the well-established multifaceted role of NK cells and ILCs in different tissue compartments.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by grants: Fondazione AIRC, grant number IG 2017 id. 20312 (SS); Fondazione AIRC, 5X1000 2018 Project Code 21147 (SS); Ministero dell'Istruzione, dell'Università e della Ricerca, grant number PRIN 2017WC8499_004 (SS); Fondazione AIRC, grant number IG 2014 id. 15428 (MV); Ministero della Salute, grant RF-2018-12366714 (MV); P01 CA163205, R01 CA068458, R01 CA185301, and R35 CA210087, all from the National Cancer Institute (MC).

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

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MINI REVIEW
published: 10 July 2019
doi: 10.3389/fimmu.2019.01569

Bone Marrow NK Cells: Origin, Distinctive Features, and Requirements for Tissue Localization

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NK cell maturation is a continuous process, which initiates in the bone marrow and proceeds in peripheral tissues, where NK cells follow distinct differentiation routes. Drastic phenotypic changes are observed during progression from precursors to mature NK cells, including changes of expression and functionalities of several chemoattractant receptors. Upon differentiation, mature NK cells migrate outside the bone marrow; as well, peculiar subsets of NK cells can also home back to or localize in this anatomic compartment to play specific functions. In humans, NK cells with a tissue resident phenotype have been identified in bone marrow, sharing similarities with tissue resident memory $CD8^+$ T cells; while in mouse, long-lived NK cells undergo homeostatic proliferation in this site during viral infections. The mechanisms underlying NK cell subset localization in the bone marrow have only recently started to be investigated, especially in pathological settings such as tumors or infections. In this review, we discuss the phenotype and function of NK cells as well as their requirements for bone marrow maintenance and/or homing.

Keywords: natural killer cells, bone marrow (bm), infection–immunology, innate lymphoid cell, chemokine receptors

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *07 May 2019*
Accepted: *24 June 2019*
Published: *10 July 2019*

Citation:
Bonanni V, Sciumè G, Santoni A and Bernardini G (2019) Bone Marrow NK Cells: Origin, Distinctive Features, and Requirements for Tissue Localization. Front. Immunol. 10:1569. doi: 10.3389/fimmu.2019.01569

INTRODUCTION

Natural Killer (NK) cells are innate lymphocytes able to recognize and kill cancer or virus-infected cells (1). They account for 5–20% of the mononuclear cells of the peripheral blood and the spleen. They also produce cytokines, among which interferon (IFN)-γ delivers signals to the innate component of the immune system, which activate the inflammatory process in defense of the organism. Activation of NK cell function following interaction with a target cell is the result of the integration of signals generated by inhibitory and activating receptors expressed simultaneously by NK cells and engaged by the ligands present on the target cells (2). By acting early during cell infection or transformation, before and independently of specific immunity, they take part to the first line of the immune response. These characteristics make them fundamental as a defense mechanism.

Recently NK cells have been re-categorized as part of the innate lymphoid cells (ILCs). ILCs have been characterized in three groups. The group 1 comprises cells expressing the transcription factors T-BET and producing the T helper cell type 1 (Th1)-associated cytokine IFN-γ, including NK cells (3).

Conventional NK cells appear to be the only cytotoxic cells, while all the other ILCs follow the pattern of helper CD4 T cells and produce cytokines and other soluble factors that help adaptive immune response development.

The transcription factor EOMES is expressed by NK cells but not ILC1, thus allowing to distinguish this two subsets (4). Mouse and human ILC differentiation process proceeds gradually from hematopoietic stem cells (HSC) to the precursor of the lymphoid line, including the common lymphoid progenitor (CLP) in the mouse and the CLP-like hematopoietic progenitor cells (HPC) in humans (4).

The bone marrow (BM) is considered the main site for ILC differentiation (also termed ILC-poiesis) in the adult, containing a wide spectrum of progenitors and precursors able to give rise to cells having different degrees of multipotency, commitment, and maturation (4). Several multipotent ILC precursors have been defined in mice, including the α-lymphoid progenitor, early innate lymphoid progenitor, common helper innate lymphoid progenitor and innate lymphoid cell progenitor (5). While single-cell RNA-seq approach has been helpful to unravel the complexity of these precursors by improving the definition of markers and transcription factors associated with the ILC fates (6), their pluripotency has been continuously redefined by using different mouse models (7–9).

ILC precursors express several chemotactic receptors and molecules associated with tissue homing, including CXCR5 and CXCR6 (10). In particular, the expression of CXCR6 in these cells has been related to egress from BM, as supported by evidence in *Cxcr6*$^{-/-}$ mice showing accumulation of ILC precursors in this organ (11). Remarkably, these mice have normal numbers of peripheral ILCs, due to *in vivo* mechanisms compensating *Cxcr6* deficiency that include increased proliferation of tissue resident cells (11). Recently, a peculiar ILC precursor has been described in human peripheral blood, which can give rise to mature cytotoxic NK cells and ILC subsets (12). These findings, along with the presence of cells having progenitor phenotypes in several peripheral tissues, imply the existence of homeostatic mechanisms of BM egress for ILC precursors. Thus, one of the paradigms in the ILC field is based on the capacity of ILC precursors to leave the BM and complete their differentiation programs in the tissues. This behavior mainly discriminates helper ILCs, which develop *in situ*, from NK cells, able to recirculate, and has been corroborated by findings obtained from parabiosis experiments, in mice (13, 14).

NK cells develop from a multipotent progenitor, the HSC, in a continuous differentiation process encompassing several stages, characterized by modulation of multiple cell surface markers (15). Developing cells acquire the expression of the IL-15 receptor (IL-15R), including the common β chain of the IL-2 and IL-15 (CD122) (16). The acquisition of the CD122 represents an important step in the NK cell differentiation since IL-15 promotes NK cell differentiation, maturation, and survival and is constitutively produced by BM stromal cells and can be induced in monocytes and dendritic cells *in vivo* (17, 18).

NK cell differentiation and maturation have been traditionally thought to occur exclusively in the bone marrow (BM), but evidence in humans and mice suggests that precursor and immature NK cells can also migrate in secondary lymphoid tissues (SLT) to complete maturation (19). Human NK cells develop from hematopoietic stem cells (HSCs) and during transition from CD56^{high} into CD56^{low}, they undergo a progressive loss of NKG2A and expression of KIRs, CD57, and NKG2C on terminally differentiated NK cells (20–22). Moreover, a new Lin^{-}CD34^{+}DNAM-1^{bright}CXCR4^{+} CLP precursor has been found in the peripheral blood of patients with chronic inflammatory conditions. The phenotype of these cells suggests that they originate from the BM as they still retain the CXCR4 and DNAM-1 receptors, and that they are released from endosteal niches due to bone remodeling occurring during chronic inflammation (23).

Mouse NK cells develop from HSCs encompassing four developmentally related subsets that can be distinguished based on expression levels of the integrin chain CD11b and of a member of the TNF receptor superfamily, CD27.

The bone marrow is not only a place for development and maturation, but BM NK cells perform important functions for defense against infections and tumors linked to their ability to traffick and/or reside in this organ (24–28).

NK CELLS AND OTHER ILC POPULATIONS IN THE BONE MARROW

Several members of the chemokine family influence NK/ILC tissue localization by regulating their release from the BM as well as their tissue homing and retention. Beside this type of conventional NK cells that can be found in circulation, tissue resident NK cells present specific characteristics that involve for example CD69, possibly linked to suppression of sphingosine 1-phosphate receptor-1 expression which retains immune cells in lymph nodes and tissues. Another mechanism is the engagement of chemokine receptors, like for example CXCR6 and CCR5, that are highly expressed on tissue-resident NK cells in human lymphoid tissues and liver, while peripheral blood-derived NK cells can be recognized by expression of CCR7 (29).

NK cell subsets display a differential pattern of chemokine receptor expression. In humans, CD56^{high} NK cells are targeted to lymph nodes *via* CCR7, preferentially express CXCR3 and have higher CXCR4 expression levels as compared with CD56^{low} cells. CXCR1, ChemR23, and CX_3CR1 are expressed only by CD56^{low} NK cells. ILC subsets have differential tissue tropism, reflecting their transcriptional and functional states. Transcriptomic analyses established in the context of the Immgen project have revealed both specific and overlapping expression patterns for chemokine receptors in mouse ILCs (30). CXCR3 is one of the chemokine receptors showing subset specificity. This receptor is typically associated to the type 1 response and in general with T-BET expressing ILCs, including NK cells, ILC1 and a subset of ILC3 expressing NCRs. The chemokine receptors CCR4 and CCR8 are associated, instead, with the type 2 response and are specifically expressed on ILC2. Finally, CCR6 and CXCR5 are found mainly on lymphoid tissue inducer (LTi)-like cells (30–33). Examples of chemokine receptors widely express on ILCs include CXCR4 and CXCR6.

Upon maturation, mouse NK cells start to express sphingosine 1-phosphate receptor (S1P5), and co-expression of KLRG1 and the chemokine receptor CX_3CR1 identifies a late maturation stage with unique functional properties (34). Mature populations

of NK cells accumulate in the BM of CX_3CR1 and S1P5-deficient mice, and display defective translocation from the BM parenchyma to the vasculature indicating that these receptors contribute to the egress of specific NK cell populations from the BM (35, 36). Even though immature $CD11b^{low}$ NK cells represent the predominant population in BM, they are poorly mobilized into circulation due to CXCR4 mediated retention (37).

Besides immature NK cell populations, BM comprises both potentially cytotoxic NK cells, trafficking from the blood, and stably resident cells expressing specific markers of tissue retention (CD69) and chemokine receptors (CCR5, CXCR6). Indeed, a third distinct CXCR6+CD69+ subset of NK cells populating lymphoid tissues, distinct from the conventional $CD56^{high}$ and $CD56^{low}$ NK cells, represents a relevant fraction of human BM NK cells and displays lower functionality, possibly linked to organ-specific immunomodulatory functions (38). Compared to classical NK cells, the BM resident NK (BMrNK) cells display lower proliferative capacity, cytolytic granule content, and expression of KIRs and DNAM1, while they express higher TIGIT levels. Interestingly, this population does not express molecules implicated in localization of resident cells in other tissues such as the adhesion molecules CD49a and CD103 (39, 40).

The role of CXCR6 in promoting BM colonization by these cells was not investigated, but a fraction of immature BM NK cells was found to express CXCR6 also in the mouse, and deletion of *Cxcr6* gene limits the egress of this population into the blood circulation. Similarly to CXCR3 and S1PR5, expression of this chemokine receptor is regulated by the transcription factor TBET since it is suppressed in BM NK cells of TBET knockout mice (41, 42).

Mature ILCs are not usually found in the BM; however, as for NK cells, this site contains a peculiar subset of ILC2 having an immature phenotype. These cells differ from terminally differentiated ILC2 for the lack of the chemokine receptors CCR4 and CCR8, as well as KLRG1 expression (43). Currently, our knowledge on the mechanisms underlying ILC2 egress from BM remains limited. A role for IL-33 in this process has been proposed based on the observation that mice deficient for *Il33* or *St2* show a drastic reduction of ILC2 in the tissues. This is in contrast with the accumulation of these cells observed in the BM. Indeed, when the IL-33/IL-33R axis is disrupted the ILC2 present in the BM shift the expression of chemokine receptors showing increase of CXCR4, which results in increased retention in the BM (44).

Recently, the concept of ILC2 strictly seen as tissue-resident cells has been revised based on their ability to traffick upon activation (45). This paradigm shift, at least for ILC2, is based on the effects of IL-25 administration *in vivo* or helminth infection in mice (45, 46). Indeed, lung localization of inflammatory ILC2 minimally involves the recruitment of ILC2 from the BM. Conversely, the intestine is the reservoir where these cells originate and come from. Remarkably, the lung inflammatory ILC2 generated in the intestine keep a distinct transcriptional profile from the lung-resident ILC2. This interorgan trafficking of inflammatory ILC2 relies on S1P.

BM NK CELLS IN IMMUNE RESPONSES AGAINST INFECTIONS

Beside its role in supporting ILC-poiesis, the BM contains a significant proportion of mature lymphocytes, including NK cells which can participate to the immune response *in situ* or be mobilized into blood to migrate to peripheral tissues during systemic or local microbial infection.

BM NK CELLS AND VIRAL (MCMV, RSV, INFLUENZA) INFECTION

Early studies in mice indicated that systemic type I IFN induction by poly(I:C) treatment, LCMV and MCMV infections elicit NK cell responses (47). These results suggested that one systemic IFN-α/β induction is required to activate blast NK cell precursors located in BM and drive their efflux to peripheral compartments leading to increased NK cell cytotoxic activity and appearance of blast NK cells in the spleen. The mechanisms of BM NK cell mobilization have not been investigated but likely involved regulation of chemokine receptor function since, *Ccl2*- and *Ccr2*-deficient mice showed reduced proportions of NK cells in the liver during MCMV infection (48). The role of CCR2 in BM NK cell response to viral infection is also supported by evidence obtained in a mouse model of respiratory virus infection: NK cells migrated from the BM to the airways of mice and this process was attributed to CCR2-mediated egress from the BM using mixed-BM chimera mice studies (49). The author demonstrated that upon influenza virus infection, a proportion of $Ccr2^{-/-}$ significantly lower than WT NK cells was recovered from the bronchoalveolar lavage of infected mice and corresponded to a mild increase of $Ccr2^{-/-}$ NK cells in the BM (**Figure 1**).

In addition to recruitment in infected tissues, peripheral immature and mature NK cells also home to the BM during influenza infection and can respond to subsequent viral challenge by proliferating there (50). Using an adoptive transfer model, van Helden et al. demonstrated that during a first infection cycle, the BM contained not only immature NK cells but also mature, long-lived NK cells that had migrated back from the periphery to undergo both homeostatic and infection-induced proliferation.

Why do NK cells proliferate in BM upon influenza virus infection? The authors proposed that the BM constitutes a site for maintenance of NK cell immunological memory, a function already documented for plasma cells and memory T cells (51, 52). Homeostatic NK cell proliferation mediated by key cytokines expressed in BM may thus be responsible for the preservation of long-lived NK cells in the absence of viral stimuli; as for NK cells preactivated with cytokines *in vitro*, these NK cells are not pathogen-specific as they responded to the unrelated respiratory syncytial virus similar to influenza virus (50).

Other evidence suggests the existence of a pre-existing pool of BM NK cells with elevated effector capacity: upon systemic viral-like stimulation NK cells display elevated motility in the BM cavities possibly representing a search for accessory cells to interact with and target cells to kill, two processes critical to

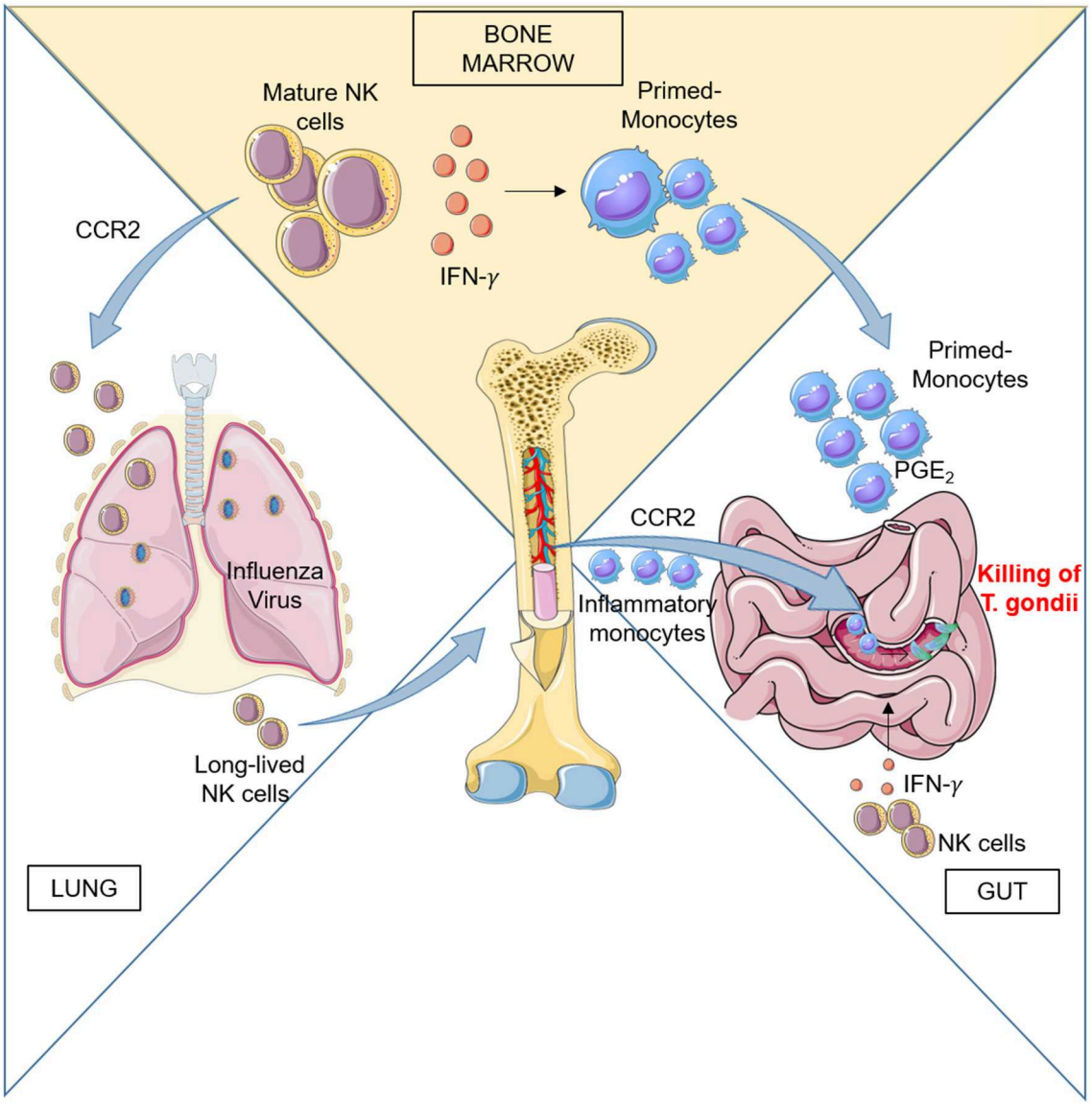

FIGURE 1 | Left: CCR2 regulates BM NK cell recruitment to the lung during influenza virus infection. During infection, activated NK cells home back to BM and persist there to provide a pool of effector cells which proliferate quickly upon subsequent infections. Right: Peripheral NK cells participate to the activation of the immune response during Toxoplasma gondii infection by promoting differentiation of inflammatory macrophages and monocyte-derived DC. Ileitis promoted by inflammation can result in severe tissue damage, but recruitment in the gut of PGE2- and IL-10-producing monocytes generated in BM under the influence of NK cells dampens exaggerated inflammation and ensures epithelial barrier protection.

activate NK cell function and for anti-viral response (53). This response was associated with upregulation of effector molecules, followed within 24 h by induction of genes required for cell cycle and DNA replication. BM NK cell response paralleled that of spleen NK cells, although the formers were faster to proliferate. Using two-photon microscopy, NK cells have been observed leaving the blood sinusoids and displaying distinctive features of strong activation in the BM parenchyma. In this compartment, NK cells increased size and motility and underwent multiple interactions with $CD11c^+$ cells and cell division.

The mechanisms of NK cell migration and/or proliferation in BM in response to virus infections, however, remain unknown. Furthermore, it is not clear if BM NK cells that undergo extensive proliferation during infection in peripheral organs include subpopulations able to perform specific functions *in situ*.

BM NK CELLS IN MOUSE MODELS OF *Toxoplasma gondii* INFECTION

BM NK cell activity during early infection could also impact the myeloid cell compartment *in situ* during *Toxoplasma gondii* (*T. gondii*) infection. *T. gondii* is a protozoan parasite that infects intestinal enterocytes and spreads into the submucosa.

Upon infection, inflammatory monocytes exit the BM and home to the lamina propria where they differentiate into TNF-α/inducible nitric oxide synthase (iNOS)-producing (Tip)-DC that control infection. Inflammatory monocytes failed to exit the BM in *Ccr2*$^{-/-}$ mice, although their number was upregulated following infection indicating that CCR2 is critically involved in the egress of Tip-DC precursors from the BM to the blood, similarly to previous observation in *L. monocytogenes*-infected mice (54–57).

By using depleting antibodies, NK cells were initially shown to be essential for early parasite control (58). Subsequent studies showed that NK cell-produced IFNγ is a dominant, early protective mechanism, possibly acting directly on infected cells or stimulating a cytotoxic T cell response (59, 60). More recent studies performed in a peritoneal infection model, have demonstrated that IFNγ acts both by promoting tissue-recruited monocyte differentiation into IL-12 producing DC at site of infection and the loss of resident mononuclear cell population which are not able to control infection.

Evidence obtained in these and other studies led to the conclusion that inflammatory Ly6C^{hi} monocytes acquire appropriate functions after entry into infected tissues and in response to local signals (57, 61, 62).

Although oral *T. gondii* infection leads to a localized response that resolves without severe pathology, oral infection of certain strains of mice leads to epithelial damage and gut pathology driven by an aggressive type 1 immune response to commensal bacteria. However, despite severe ileitis, *T. gondii*–infected mice can survive the infectious challenge due to acquisition of a PGE2-dependent regulatory function acquired by Ly6C^{hi} inflammatory monocytes (63). Recently Askenase et al. showed that mature BM NK cells are responsible for the acquisition of this regulatory function by interacting with differentiating monocytes before their release into the circulation (26) (**Figure 1**). NK cells in BM were activated by signals derived from infected tissues in the periphery. Acquisition of regulatory function was associated with a MHCII^{+}Sca-1^{+}CX3CR1^{-} phenotype by Ly6C^{hi} circulating monocytes and was a common response to several type of infections, in addition to *T. Gondii*. The authors evidenced that IFNγ was responsible for a dramatic alteration of the transcriptional program of monocyte progenitors in the BM early during infection and before terminal differentiation and egress. BM NK cells were initially the only population producing IFNγ in the BM, with minimal contribution by T cells or type 1 ILCs, while IFNγ production was also observed in T cells at later time. The results demonstrated that IFNγ dependent mechanism of regulation can be designed to prepare monocytes for recruitment to barrier tissues and prevent immunopathology and tissue damage thanks to their capacity to produce PGE2 and IL-10.

Overall, NK cells and probably other leukocytes do not only act as effector cells in the periphery but also regulate myeloid cell differentiation in the BM, thus shaping the immune response during infection.

CONCLUSIONS

Correct localization of NK cells in BM have a fundamental role in several aspects of NK cell-mediated immune response *in vivo*. BM can represent a location suitable for self-renewal and persistence of NK cell population with enhanced functional capacity. This is of clinical importance for the reconstitution of immune compartments during viral infections. It would be of great importance to understand if a similar expansion of memory-like populations could be observed during cancer growth, since experienced BM NK cells of cancer patients may be potentially able to mediating continuous surveillance against the recurrence of cancer. On the other hand, it is not known if the existence of NK cells capable of modifying the function of monocytes in BM applies to cancer growth outside the BM since it may contribute to generation of monocyte with immune suppressive functions.

The origin and phenotype of the BM NK cells promoting this function is not clear. In particular, it has not been investigated whether these NK cells reside stably in the BM, occupying stable niches or whether they migrate there participating to the systemic immune responses similarly to virus infections. In this regard, it would be interesting to clarify if the phenotype of these cells overlaps with that of tissue resident NK cells identified in BM since BrNK cells have enhanced IFNγ production capacity and low killing potential, thus suggesting that they can shape hematopoiesis under specific conditions.

AUTHOR CONTRIBUTIONS

VB wrote the introduction section and contributed to the paragraph concerning NK cells in BM. GS wrote the sections regarding innate lymphoid cells contributing to the introduction section and the paragraph concerning the regulation of ILC distribution in bone marrow. AS contributed to paper writing and revised the paper. GB revised the literature and wrote the paragraphs concerning NK cells in BM and BM NK cells in infection.

ACKNOWLEDGMENTS

The authors were supported by grants from AIRC (5 per mille, Metastatic disease: the key unmet need in oncology, ref: 21147 and MFAG 2018, ref: 21311), Italian Ministry for University and Research (PRIN 2017, Prot. 20177J4E75), and the Sapienza University (Ricerca Universitaria). The authors wish to thank Servier Medical Art templates for the artwork used in the figure of this manuscript, which are licensed under a Creative Common Attribution 3.0 Unported License; https://smart.servier.com.

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

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frontiers in Immunology

MINI REVIEW
published: 28 August 2019
doi: 10.3389/fimmu.2019.02045

NK Cell Precursors in Human Bone Marrow in Health and Inflammation

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NK cells are generated from hematopoietic stem cells (HSC) residing in the bone marrow (BM), similar to other blood cells. Development toward mature NK cells occurs largely outside the BM through travel of CD34+ and other progenitor intermediates toward secondary lymphoid organs. The BM harbors multipotent CD34+ common lymphoid progenitors (CLPs) that generate T, B, NK, and Dendritic Cells and are devoid of erythroid, myeloid, and megakaryocytic potential. Over recent years, there has been a quest for single-lineage progenitors predominantly with the objective of manipulation and intervention in mind, which has led to the identification of unipotent NK cell progenitors devoid of other lymphoid lineage potential. Research efforts for the study of lymphopoiesis have almost exclusively concentrated on healthy donor tissues and on repopulation/transplant models. This has led to the widely accepted assumption that lymphopoiesis during disease states reflects the findings of these models. However, compelling evidences in animal models show that inflammation plays a fundamental role in the regulation of HSC maturation and release in the BM niches through several mechanisms including modulation of the CXCL12-CXCR4 expression. Indeed, recent findings during systemic inflammation in patients provide evidence that a so-far overlooked CLP exists in the BM ($Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$) and that it overwhelmingly exits the BM during systemic inflammation. These "inflammatory" precursors have a developmental trajectory toward surprisingly functional NK and T cells as reviewed here and mirror the steady state maintenance of the NK cell pool by $CD34^{+}DNAM\text{-}1^{-}CXCR4^{-}$ precursors. Our understanding of NK cell precursor development may benefit from including a distinct "inflammatory" progenitor modeling of lymphoid precursors, allowing rapid deployment of specialized $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ -derived resources from the BM.

Keywords: NK cells, CD34+ precursors, inflammation, common lymphoid precursor, DNAM-1, CXCR4

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Emily Mace, Columbia University, United States
Francisco Borrego, BioCruces Health Research Institute, Spain

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *04 June 2019*
Accepted: *13 August 2019*
Published: *28 August 2019*

Citation:
Bozzano F, Perrone C, Moretta L and De Maria A (2019) NK Cell Precursors in Human Bone Marrow in Health and Inflammation. Front. Immunol. 10:2045. doi: 10.3389/fimmu.2019.02045

INTRODUCTION

Natural Killer (NK) cells are innate lymphoid cells (ILC) with potent cytotoxic effector activity, due to their constitutive expression of perforin and granzyme and ready ability to produce high amounts of IFNγ and other proinflammatory cytokines. Their original definition of "born natural killers"(NK) was due to their "perforin-armed" resting condition. Their activity encompasses multiple defense activities including detection and disposal of virus-infected or transformed cells, early detection of pathogens via pathogen-associated

molecular patterns (PAMPs) that are recognized by an array of innate receptors (e.g., TLRs), recruitment other cells involved in immune responses thanks to the early secretion of chemokines (IL-8, RANTES, MIP1a, MIP1b) and cytokines (GM-CSF, IL-6, IL-1) upon PAMP recognition. Subsequently, however, their involvement in the regulation of downstream responses was recognized with continuous coordination of and support to downstream adaptive immune responses through crosstalk with dendritic cells (1, 2) and with T cells (3–5). Over the last 10 years, it became clear that conventional NK cells are part of an extended family of ILC which includes three additional groups of innate cells having remarkable functional parallels with known helper T (Th) cell subsets (6, 7). Within this family, distinctive transcription factor expression and cytokine production characterizes conventional NK cells (Eomes, IFNγ) from group 1 ILC (T-bet, IFNγ), group 2 ILC (Gata3, IL-5/IL-13), and group 3 ILC (Rorc, IL17/IL-22). Several properties of conventional NK cells, and in particular transcription factor expression, clearly set them apart from other ILC subsets (8, 9), and has led to suggest a distinction between "helper" ILCs (ILC1s, ILC2s, and ILC3s) and "cytotoxic" ILCs (NK cells) that parallels the CD4+ Th cells vs. CD8+ CTL duality (10).

The predominant sites of the human body in which NK cells are found include secondary lymphoid organs, bone marrow, liver, lungs, and decidua while an overall minority of body NK cells (<2%) circulates in peripheral blood where they represent 5–15% of blood lymphocytes. Contrary to adaptive T or B cells, their functional specificity does not include somatic rearrangements. Their wide array of activating receptors is germline encoded and delivers potent triggering signals upon recognition of distress ligands expressed by stressed healthy as well as infected or transformed cells (11). NK cell function is tightly regulated by a balance between activating stimuli delivered through activating molecules and inhibitory signals primarily by HLA class I-specific inhibitory receptors (12). Inhibitory signaling recognizes self and, in most instances, overrides routine minor activating distress in order to avoid self-destruction unless NK cell activity is needed to control overt cell infection or transformation (13).

Similar to other blood cells, NK cells are generated from hematopoietic stem cells (HSC) residing in the bone marrow. Following the first experimental evidence of the possibility to rescue mice from lethal irradiation, the bone marrow (BM) has been identified as the main source of HSC in the body with a first estimate of 1 in 10e4 BM spleen-colony forming cells (14, 15). HSC with the characteristics of self-renewal and multipotency, that are able to generate more differentiated precursors along the pathways toward production of erythrocytes, leucocytes and platelets, were indeed thereafter identified in BM (16–18), with a frequency of 1 in 10e5 BM cells (16). According to a strictly hierarchical "stem tree" view where all cells derive from a common ancestor, progressive steps of differentiation of HSC lead to the generation of progressively more oligopotent precursors toward all blood cell lineages. The classical model of hematopoiesis postulates that the earliest fate decision toward NK cells downstream of HSCs is represented by the divergence of lymphoid and myeloid lineages. Erythroid and megakaryocyte lineages branch off before the lymphoid–myeloid split. This step is followed by myeloid–lymphoid divergence in which common lymphoid progenitors (CLPs), and common myeloid progenitors are generated (19). Alternate possibilities of a less stringent stem-root developmental model have been pursued. Thus, there is considerable heterogeneity in reconstituting HSCs, with proof of a less defined hierarchical transition reflecting different propensities for lineage-fate decisions by distinct myeloid-, lymphoid- and platelet-biased HSCs (20, 21). The low level of agreement on some aspects of decisional fate of progeny development in humans is primarily due to different experimental settings. So far, studies have been heterogeneous with regard to different aspects that that include the use of either adult or fetal/newborn materials that may be inadequate for a coherent comparison of results, the different study settings comparing analysis of precursors at steady state vs. repopulation studies with a push toward tissue and body repopulation after transplantation, and finally the exclusive use of healthy donors with a lack of data derived from disease states, in which the developmental push toward differentiation and self-maintenance may more strongly reflect the influence of inflammatory signals and/or of peripheral need caused by accelerated cell turnover.

The purpose of this review is to briefly summarize the findings on classical NK cell precursors in the bone marrow and to recapitulate recent findings on alternate new precursor populations. Only a brief mention to ILC development will be provided, since this is out of the purpose of the present work and may be obtained elsewhere (7, 22).

NK CELL DEVELOPMENT AND INTERMEDIATES IN THE BM

After the first description of multipotent $Lin^{-}CD34+CD45RA+CD10+CD38+$ progenitors in the BM generating *in vitro* T, B, NK, and Dendritic Cells (23), it became clear that the BM was the primary site of where NK cell precursors dwell and may generate NK cells (24). In fact, neither the thymus nor the spleen seemed to be essential for NK cell growth as shown by NK cell persistence and preserved function in their absence (25–27). The role of postnatal as compared to fetal liver in NK cell generation was unclear at the time and still requires further studies in future). Early views on NK cell development considered the BM as the main site for NK precursor growth from HSC and also the site where progressive NK cell development takes place (24).

Early work on BM precursors provided evidence that CD7 expression on $CD34^{+}CD45RA^{+}$ HPCs enriches for NK cell precursors (28). Also co-expression of CD10 on BM $CD34^{+}$ HPCs identified a CLPs generating NK cells (23). These progenitors lacked erythroid, myeloid, and megakaryocytic potential but contained a broad B, T, and NK cell and DC differentiation potential, suggesting that this population might correspond to the human postnatal common lymphocyte precursor (CLP). It was also clear that $CD34^{+}CD7^{-}$ and $CD34^{+}10^{-}$ HPCs also could generate NK cells, albeit with lower efficiency and with more stringent contact requirement

with stromal cells (21, 23, 28, 29). Subsequent studies revealed that CD10 expression on progenitors is associated with a strong bias toward B cell potential with minimal T or natural killer (NK) cell potential (28, 30, 31). Thus, the stepwise process of lymphoid differentiation from multipotent HSC to the earliest lymphoid-primed multipotent progenitor (LMPP) in BM was not characterized by the expression of CD10 (23), but rather of L-selectin (CD62L) expression on CD3-CD14-CD19-(henceforth Lin^-) $CD34^+CD10^-$ progenitors (28). These progenitors were devoid of erythroid or myeloid clonogenic potential corresponding to LMPP and had the ability to seed SLT and thymus through the CD62L homing signal (21, 32, 33). In the same BM setting, CD7 expression alone did not define lymphoid commitment, as a Lin^-CD34+CD38–CD7+ population that had been identified as a LMPP in umbilical cord blood (UCB) (34) was not detected, and low CD7 expression in $CD34^+Lin^-CD38^+CD10^-$ cells was insufficient to define lymphoid restriction as erythroid progenitors could also be detected (28). In UCB, circulating CD34+CD45+CD7+CD10– precursors could generate cells of the three lymphoid lineages, however, with a skewed potential toward the T/natural killer (T/NK) lineages. In contrast, $CD34(^+)CD45RA(^{hi})Lin(^-)CD10(^+)$ HPCs predominantly exhibited a B-cell differentiation potential. Also, a culture of purified CD34+ derived from UCB (without further subset sorting) with SCF, FLT3, IL-7, and IL15 generates *in vitro* $CD3^-CD16^+CD56^+CD244^+CD33^-$ myelomonocytes and highly immature $CD3^-CD16^+CD56^+CD244^+CD33^-$ NK cells that are substantially devoid of cytotoxic activity and of IFNγ production, without growth of T cells or other lieages (35–37).

More recently, Renaux et al. provided evidence that $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ cells purified from BM or UCB represent the unipotent NK cell precursor devoid of potential toward other lymphoid lineages (37, 38). These precursors are also detected in adult tonsils and fetal tissues and are different from Lin^-CD34^+ $CD38^+CD123^-CD45RA^+CD7^+CD10^-CD127^+$ cells, which can undergo different fates including myeloid lineages, and also different from $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$ cells that generated only lymphoid lineages (T, B, ILC, NK cells) (38). Thus, this confirms previous reports on the origin of ILC from CD34+ precursors il SLT or UCB (39, 40). ILC development still bears some areas of uncertainty with need of additional focus (6, 7). Indeed, CD127 expression has been shown to represent a requirement for the fate decision toward ILC development from upstream precursors, which still bear NK, T, and B cell potency (38), and is expressed on ILC but not on NK cells. However, it is transiently not expressed on early innale lymphoid progenitors (EILIP) in the BM (7, 41, 42) and is also lacking on the single-fate NKP, which is supposed to be downstream ILC developmental potency (38).

Overall, therefore, it is clear that the BM harbors, in addition to totipotent HSCs, more committed lymphoid precursors with the ability to generate NK cells, including LMPP, CLP, and single-cell NKP. Also, there are evidences that the developmental fates of NK cells and of "helper" ILCs are intertwined in general up to CD127 retention on CLP. The occurrence of a local NK cell development in BM is not disputed, however, at present is not quantified and poorly defined.

The concept that the BM could not be the predominant site of NK cell development developed after the first reports on secondary lymphoid tissue NK cell composition. Indeed, lymphnodes were harboring predominantly large numbers of $CD56^{bright}$ NK cells adjacent to T-cell-rich areas (43). This led to experiments showing that NK cells with a $CD56^{dim}$ phenotype developed from $CD56^{bright}$ NK cells to take place in SLTs (44). Proof of this concept followed these observations and was substantiated by Freud et al. (45) with the description and characterization in lymphnodes of CD34+ CLP generating $CD56^{bright}$ NK cells *in vitro* (45). Subsequent work confirmed the presence of CD34+ CLP in SLT and thymus, generating $CD56^{bright}$ NK cells. Finally, MMLP and CLP have been recovered from PB and from UCB, and in general are believed to transit from BM through PB toward peripheral tissues for further development. These observations, therefore, supported the concept that the BM is the site where HSC are contained and is the origin of MLP and CSP, and that the vast majority of NK cells may be generated in peripheral tissues (e.g., SLT) as progenies from CLP traveling from the BM.

BM ORGANIZATION OF THE HSC MICROENVIRONMENT

All the so-far described BM NK cell precursor populations have been investigated according to the assumption of an uncharacterized anatomical organization, in which HSC reside in the BM within specialized microenvironments or niches. Animal studies have contributed a wealth of information on hematopoietic organization of the marrow microenvironment. Quiescent HSCs reside in perivascular niches, in which different cell types express or release factors that promote HSC maintenance (46). Quiescent HSCs in mice BM associate specifically with small arterioles that are preferentially found in endosteal BM (47). The production of CXCL12 by cells present in the perivascular region, including stromal cells, sinusoidal endothelial cells, and mesenchymal progenitors, has been shown to support HSC retention. Accordingly, CXCL12 deletion in mice results in constitutive HSC mobilization (48).

Thus, the organization of HSC in BM niches and their retention by CXCL12 raises the fundamental question of whether HSCs and restricted progenitors, including CLP or NKP, reside within distinct, specialized niches or whether they share a common niche. Using CXCL12 knock-in mice and conditional CXCL12 deletion, Ding and Morrison provided evidence that *Cxcl12* was primarily expressed by perivascular stromal cells and at lower levels by endothelial cells, osteoblasts, and some haematopoietic cells (20). Interestingly, deletion of *CXCL12* from endothelial cells depleted HSCs and certain restricted progenitors, but not myeloerythroid or lymphoid-committed progenitors, from perivascular stromal cells, while deletion of *CXCL12* from osteoblasts depleted certain early lymphoid progenitors, but not HSCs (20). Therefore, these findings

provided evidence that different stem/progenitor cells occupy distinct cellular niches in BM. Accordingly, while HSCs reside in a perivascular niche, early lymphoid progenitors are localized in an endosteal niche (**Figure 1**).

This particular organization leaves open the fundamental question of whether the observation of stem/progenitor cells in healthy, steady state conditions actually reflects the whole progenitor potential that is present in the BM and whether this may actually reflect which progenitors/cell types are released from the BM during inflammatory states. Remarkably, support for this question is provided by the observation that chronic inflammation is associated with bone remodeling including endosteal niches, as a result of cytokine-induced modulation of the cells responsible for MMP-9/CXCR4-dependent HSC retention (49, 50). In this regard, proinflammatory cytokines, that include TNFα and IL-1, have been shown to regulate CXCL12 expression, induce lymphocyte mobilization by suppressing CXCL12 retention signals in BM, and to promote the appearance of developing B cells in the spleen (51). Importantly, BM egress could be achieved in the absence of amoeboid migration toward BM exit sites. Accordingly, immature B cell egress from BM has been shown to rely on CXCR4 down-regulation. This passive mode of cell egress from BM also contributes significantly to the export of other hematopoietic cells, including granulocytes, monocytes, and NK cells, and is reminiscent of erythrocyte egress (52).

INFLAMMATION-ASSOCIATED LIN$^-$CD34$^+$DNAM-1^{BRIGHT}CXCR4$^+$ CELL PRECURSORS

Taken together, all the mentioned evidences showing that HSC and CLPs in the BM occupy distinct niches, contribute to generate the concept that BM exit modality in different physiological or pathological conditions differ. Thus, in steady state conditions, mature NK cells and CLP would exit the BM following CXCR4 down-regulation. On the contrary, in the presence of inflammation or inflammatory cytokines, including TNFα, IL-1, and G-CSF, the production of CXCL12 is suppressed and would then allow exit from the BM of lymphocytes lacking CXCR4 down-modulation.

Therefore, the question is whether any "inflammatory" CLP exist, and if so, is the progeny actually superimposable to the one of the so-far characterized CLPs described in BM and other tissues. According to this model and to the interplay between CXCL12 expression and inflammation, CLP and NK cell progenitors released from the BM following inflammatory conditions would be expected to still express CXCR4 (20, 48, 50–52).

Indeed, proof that "inflammatory" or "emergency" progenitors actually exist has been provided by the analysis of patients with chronic inflammatory disorders including patients with HIV-1, chronic HCV infection, TB, as well as COPD or PAPA syndrome (53).

During these inflammatory conditions, Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells can be detected in the PB, where they often represent the majority of CD34+ cells (53). These precursors were found to generate *in vitro* NK cells and T cells but not cells of myelomonocytic lineage under the assayed culture conditions, thus qualifying for a CLP definition. Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ CLP reside in the BM under steady-state conditions, where they represent 10% of CD34+ cells, while they are not or are poorly (<0.5–1% of PBMC) detectable in PB in healthy, uninflamed conditions (53). The circulating pool size of Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells is significantly increased in patients with inflammatory disorders compared to HD. In cART-treated virologically suppressed HIV-1 patients, for instance, they may represent as much as 30% of Lin$^-$ gated PBMC, and over one third of patients had proportions of circulating Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells in excess of 5% of PBMC (53). Notably, the proportion of circulating Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ correlated directly with fibrinogen concentrations and therefore different output from the BM to the PB likely reflect, among other factors, individual differences in systemic chronic inflammation (53).

In addition to high DNAM-1 and CXCR4 expression (which are absent on conventional CD34+ cells in PB or in UCMC), inflammatory or "emergency" CD34+ CLP express HLA-DR, CD38, CD69, while they do not express CD117, CD94, CD123, CD161. A fraction of these mobilized inflammatory CLP variably express no or very low CD7 (0.2–3%) or CD10 (0.2–10%). Therefore, these CLP represent a heterogeneous population that may well contain small proportions of more committed CD34+CD7+CD10+CD127– single-lineage NK precursors similar to those described recently for classical CD34+ cells (38). These inflammation-dependent CLP, however, predominantly contain less committed precursors upstream the NK cell progenitor fate-decision, which have the potential do develop *in vitro* also to CD3+ T cells and to CD3+CD56+ cells. For example, according to the study by Doulatov et al. (54), Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells would surprisingly also fit in the group of megakaryocyte/erythroid precursors, characterized by the CD38+CD10–CD7–Flt3– phenotype. In addition, their lack of CD127 and CD161 expression could rule out their developmental trajectory toward ILCs. This is confirmed by the lack of ILC growth *in vitro* and ILC-compatible transcription (53). However, there is still room for the possibility to find "inflammatory" ILC precursors, in view of the observation that EILIPs transiently lack CD127 (7, 42), and that so-far uncharacterized Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ precursors may be observed in infectious/inflammatory conditions.

Importantly, the chemokine receptor expression of Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells is different from the one of conventional circulating Lin$^-$CD34$^+$DNAM-1$^-$CXCR4$^-$ CLP. Indeed, while the latter predominantly express CD62L or CCR7 (>90%) and therefore appear to be homing predominantly toward SLT, a relevant proportion (35%) of Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells express CXCR3, CXCR1, or CX3CR1 and therefore appear to be poised to a relevant extent to peripheral inflamed tissues, not only to SLT (53).

A peculiar feature of Lin$^-$CD34$^+$DNAM-1^{bright}CXCR4$^+$ cells is represented by the unusual characteristics of their NK and T cell progenies. Under limiting dilution conditions, NK cell progenies grow rapidly and, already 16–20 days after seeding,

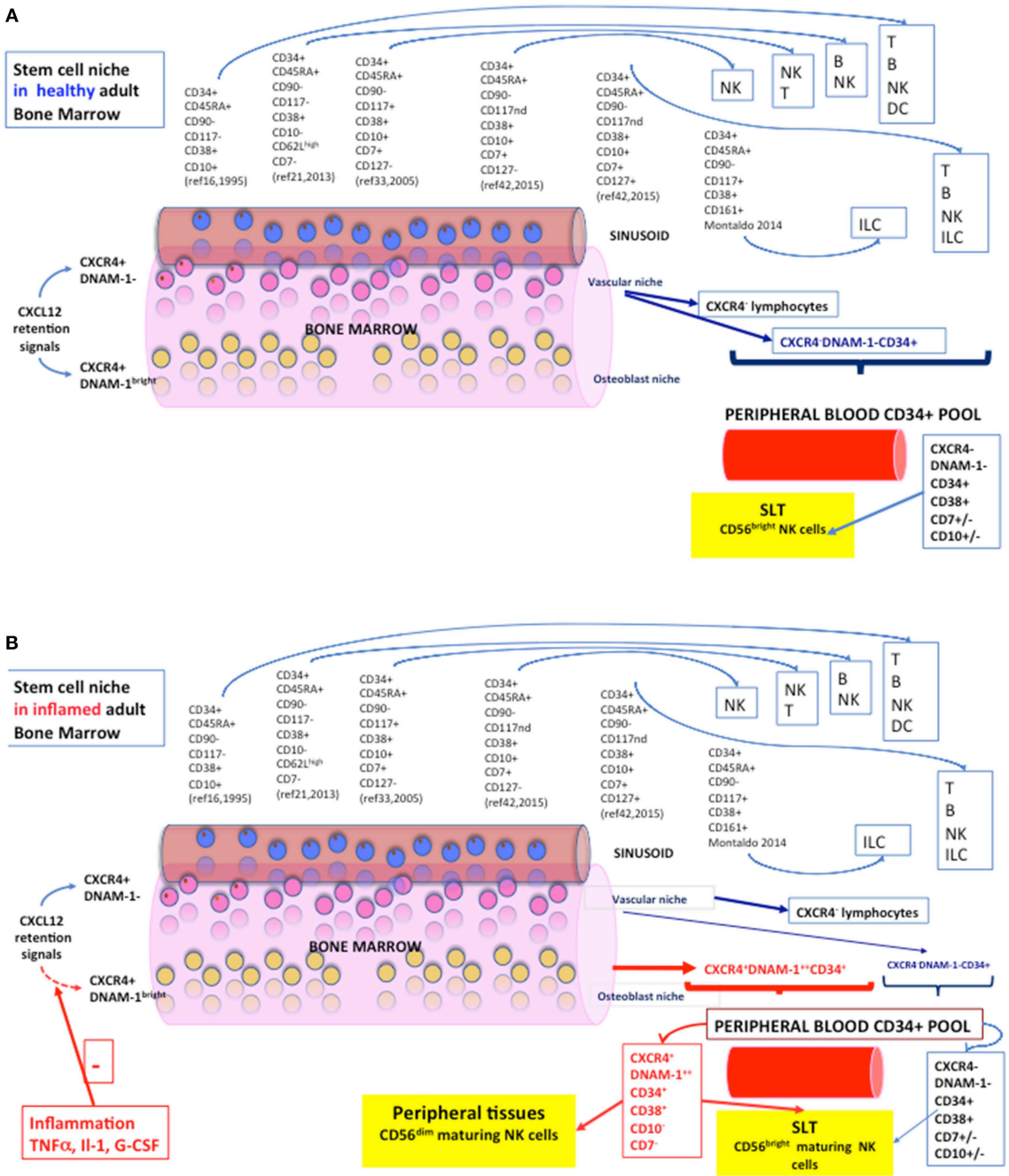

FIGURE 1 | NK cell hematopoiesis in health and during systemic inflammation. A section of Bone Marrow with a sinusoid is represented. Vascular Niche in pink, osteoblast niche representation in yellow. Blue cells represent CXCR4- lymphoid or erythroid cells passively released in the sinusoid. A spectrum of the phenotypes of so far characterized CD34+ NK cell precursors is represented with reported progenies and is indicated by citation numbers. **(A)** Diagram of the Stem Cell Niche and NK cell precursors in Healthy Adult Bone Marrow. Lymphoid cells and precursors exiting passively from sinusoids are indicated by dark blue arrows and constitute the pool of CD34+ cells circulating in peripheral blood. A yellow box defines their trajectory toward SLT (CD62L+CCR7+) and the prevailing phenotype of NK cells grown under standard conditions *in vitro*. **(B)** The Stem Cell Niche and CD34+ NK cell precursors in Adult Bone Marrow during chronic inflammation. During chronic inflammation, inflammatory cytokines and mediators determine a reduction/shutdown of CXCL12 signaling within the BM niches, with decreased retention ability of CXCR4+ HSC and CLP that otherwise populate the BM but do not circulate in PB. Red arrows show "inflammatory" or "emergency" CD34+ cells exting the BM. A different composition of CD34+ peripheral blood pool is accordingly shown during inflammatory conditions (HIV, HCV, COPD, Tuberculosis, PAPA). Travel trajectories of $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ cells toward peripheral tissues are shown as these CD34+ CLPa express CX3CR1+, CXCR1+, CXCR3+, in addition to SLT-homing receptors (CD62L+CCR7+).

have acquired a mature NK cell phenotype with expression of Natural Cytotoxicity Receptors (NKp46, NKp30, NKp44), CD244, HLA-DR, NKG2D, DNAM-1, NKG2A/CD94, Killer-like Immunoglobulin Receptors (KIRs) (53). In addition, they express high levels of Perforin, are cytotoxic toward tumor cell lines, and produce abundant IFNγ. The NK cell progeny of $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ cells, produces IFNγ with an early production pattern (53), which is superimposable to the one observed in mature $CD56^{dim}$ NK cells (34, 53). Since $CD56^{bright}$ NK cells are known to be developmentally upstream of $CD56^{dim}$ NK cells (44, 55) and have a "late" IFNγ production pattern (34), this IFNγ production pattern in the progenies is somehow surprising as one would rather expect a $CD56^{bright}$-like pattern. In addition, this finding is also unusual as classical CD34+DNAM-1-CXCR4- cells generate $CD56^{bright}$-like, poorly functional, maturing NK cells almost exclusively *in vitro*, that have low NCR and very low to absent perforin, NKG2D, DNAM-1 expression and IFNγ production (31, 46). Indeed, NK cells develop from CD34+ precursors following a 4-staged expression of receptors (CD34, CD117, CD94, CD56, CD16), originally described by Freud and coll in CD34+ cells from tonsils and lymph nodes (55), and more recently revised to include six distinct stages (56). In particular, a small fraction of maturing NK cell progenies from "classical" CD34+DNAM-1- precursors may express KIRs only after prolonged culture and IL-21 stimulation (37), while progenies from "inflammatory" $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ cells in the BM and PB readily express KIRs after 16–20 days of progenitor seeding, and may follow a different staged development (53).

CONCLUDING REMARKS

Thus, "inflammatory" $DNAM\text{-}1^{bright}$ $CD34^{+}CXCR4^{+}$ CLP (53), distinct from "classical" $CD34^{+}DNAM\text{-}1^{-}CXCR4^{-}$ progenitors (23, 24, 38, 45, 55–57), stably reside in BM at steady state in a presumed osteoblast niche. They do not circulate in PB in easily detectable amounts, but are ready to be rapidly deployed to the peripheral tissues following stimuli that may include inflammation-induced CXCL12 downmodulation (48, 51, 52) (**Figure 1B**). Recruitment to the periphery includes, for example, G-CSF. Indeed, G-CSF-induced mobilization/harvest protocols for transplantation purposes where $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ cells are released together with other CD34+ HSC/CLPs (53). Importantly, $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ cell frequencies in PB prevail over conventional $CD34^{+}DNAM\text{-}1^{-}CXCR4^{-}$ cells during chronic inflammation. This "emergency" deployment may be mechanistically interpreted to support the model of a CD34+ travel trajectory toward inflamed peripheral tissues—in addition to SLT—when an increased turnover of lymphoid cells occurs at sites of infection/inflammation. This would allow the prompt availability of function-ready NK cells with an unconventional mature CD56dim-like functional activity.

Thus, the current views of NK cell development need to take into account recent evidences. Indeed, our views have been upgraded from a strictly hierarchical stepwise organization of fate decisions for HSCs and CLPs toward progressively more restricted maturing potentials (23, 38, 55, 56) to a comprehensive system where HSC and CLP fate decisions are less strict. In this comprehensive system, they depend on more shared transcriptional programs, additional conditions including local tissue signals (e.g., delivered by stromal cells) and system requirements (steady state vs. recolonization) (58–61). Modeling in the presence of increased peripheral turnover with inflammation has so far been assumed to fall into the "steady state," and has therefore led to the substantial lack of consideration of "inflammatory" CD34+ in studies on NK cell development (24, 54–56). Thus, this explains how these CLPs eluded characterization for a long time. With the demonstration of the regulation of HSC release from distinct niches in the BM (20, 46–48, 50), and the characterization of "inflammatory" CD34+ progenitors in the BM (53), some so-far unanswered questions along the path of NK cell development could be addressed. Indeed, the surprisingly wide spectrum of NK cell phenotypic and functional repertoires (62) still has unanswered aspects, including the origin of tissue-resident NK cells, the origin of such a variety of phenotypic differences, and the exact boundaries for the generation of memory-like NK cells (1, 63–69). Inclusion of $Lin^{-}CD34^{+}DNAM\text{-}1^{bright}CXCR4^{+}$ in the modeling of NK cell development in inflamed BM, SLT and peripheral tissues introduces an additional level of complexity to an already full pattern of developmental steps (56, 59), but will help to address some unanswered questions. In view of the ongoing effort at redirecting NK cells for immunotherapeutic purposes (e.g., anti-KIR, anti-NKG2A mAbs, CAR-NK engineering), the existence of inflammatory $CD34{+}DNAM\text{-}1^{bright}$ precursors with extremely functional NCR+NKG2D+ NK cells could represent a useful tool for immunotherapeutic purposes.

AUTHOR CONTRIBUTIONS

FB, CP, LM, and AD contributed to writing the manuscript.

FUNDING

This work was supported by grants awarded by PRA-MIUR 2019 (AD), Fondazione AIRC per la ricerca sul Cancro: IG 2017 project n. 19920 (LM), and Immunity in Cancer Spreading and Metastasis (ISM) 5 per Mille project n. 21147 (LM).

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

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frontiers in Immunology

REVIEW
published: 01 May 2019
doi: 10.3389/fimmu.2019.00960

Uterine Natural Killer Cells

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Natural killer (NK) cells are members of a rapidly expanding family of innate lymphoid cells (ILCs). While most previously studied NK cells were derived from the mouse spleen and circulate in the blood, recently others and we found tissue-resident NK (trNK) cells in many tissues that resemble group 1 ILCs (ILC1s). During pregnancy, NK cells are the most abundant lymphocytes in the uterus at the maternal-fetal interface and are involved in placental vascular remodeling. Prior studies suggested that these uterine NK (uNK) cells are mostly derived from circulating NK cells. However, the murine virgin uterus contains mostly trNK cells and it has been challenging to determine their contribution to uNK cells in pregnancy as well as other potential function(s) of uNK cells due to the dynamic microenvironment in the pregnant uterus. This review focuses on the origins and functions of the heterogeneous populations of uNK cells during the course of murine pregnancy.

Keywords: uterine natural killer cells, pregnancy, tissue-resident natural killer cells, placenta, maternal-fetal interface, uterine innate lymphoid cells, conventional natural killer cells

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Barbara L. Kee, University of Chicago, United States
Sumati Rajagopalan, National Institute of Allergy and Infectious Diseases (NIAID), United States

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *06 January 2019*
Accepted: *15 April 2019*
Published: *01 May 2019*

Citation:
Sojka DK, Yang L and Yokoyama WM (2019) Uterine Natural Killer Cells. Front. Immunol. 10:960. doi: 10.3389/fimmu.2019.00960

INTRODUCTION

Innate lymphoid cells (ILCs) constitute an expanding heterogeneous family of cells that are found resident in tissues (1–4). Unlike T and B lymphocytes, ILCs do not require RAG-dependent somatic rearrangement for expression of their receptors. ILCs can respond early to eliminate virally infected and transformed cells and provide epithelial barrier immunity. ILCs form complex interactions with tissue-specific cells where they integrate signals and respond appropriately to maintain tissue homeostasis and repair, expanding their functions beyond host immunity.

A recent re-classification categorized ILCs into five subsets based on transcription factors and cytokine production: ILC1s, ILC2s, ILC3s, lymphoid tissue-inducer (LTi) cells and conventional NK (cNK) cells (5, 6). $TBET^+$ ILC1s produce type 1 cytokines IFN-γ, TNF-α and GM-CSF; $GATA3^+$ ILC2s produce type 2 cytokines IL-5 and IL-13; and $ROR\gamma T^+$ ILC3s cells produce IL-17 and IL-22. LTi cells are also $ROR\gamma T^+$ and are important in formation of secondary lymphoid structures but do not produce IL-17 or IL-22. Similar to ILC1s, cNK cells produce IFN-γ but possess a much higher cytotoxic potential, differentiating them from the ILC1s. Because of their tissue occupancy, ILCs are privy to local dysregulation and pathogenic insult and collectively appear to have a diverse toolbox to not only combat infection but also restore tissue homeostasis by initiating tissue repair mechanisms (7–10). Hence, at the tissue site, the multidimensional biology of ILCs allows for a prompt response to meet the needs of the altered tissue.

ILCs are resident in many tissues throughout the body (1). Cells resembling cNK cells and ILC1s are enriched in several organs (11–15). We identified two populations of murine NK cells, tissue-resident (trNK) and circulating cNK cells that occupy non-lymphoid tissues such as the liver, skin, and virgin uterus (13, 14). The virgin uterus contains an abundant number of trNK cells and a few cNK cells, often described as negligible. The highly specialized uterine tissue, with cyclic exposure to sex hormones and invading extravillous trophoblast during pregnancy, contains trNK cells, cNK

cells, and ILC1s, here referred to as uterine NK (uNK) cells to include all subsets (15, 16). In this review we provide an overview of uNK cells, with a focus on mouse.

Conventional NK Cells

Most of our knowledge about the phenotype, function and development of murine NK cells comes from studying NK cells found circulating in the blood and spleen, here termed conventional NK (cNK) cells. The cNK cell population constitutes 2–3% of the lymphocytes in the blood and spleen where they have been extensively studied. Functionally, they are set apart from other ILCs because of their potent cytotoxic capability to potentially kill on contact. ILC developmental studies determined that all ILC lineages are derived from early common lymphoid progenitors (CLPs) that can give rise to NK cells, ILC1s, ILC2s, ILC3s, and LTi cells (17–19). The common progenitor to all helper-like innate lymphoid cell lineages (CHILP) gives rise to PLZF$^+$ ILC precursors that develop into ILC1s, ILC2s, ILC3s, and LTi cells separating them from the NFIL3$^+$ NK precursor that give rise to cNK cells earlier in the developmental pathway (20). Therefore, cNK cells are developmentally distinct members of the ILCs.

Tissue-Resident NK Cells

Circulating cNK cells are widely distributed throughout the body but many tissues, have resident NK cells, termed tissue-resident NK (trNK) cells that are present in the liver, skin, kidney and virgin uterus (13, 14, 21). Although cNK and trNK cells are both absent in IL15Rα-deficient mice demonstrating they both depend on IL-15 signaling in early development, there are several characteristics that distinguish cNK and trNK cells. First, surgical joining of two congenically marked animals in parabiosis studies determined that the cNK cells traffic freely in the circulation while the trNK cells remain in the tissue (13, 14, 16). Second, detailed phenotypic and RNA-seq analyses revealed that cNK and trNK cells differentially express receptors and transcription factors that can be used for their identification. The cNK cells express the integrin DX5 but lack the expression of another integrin, CD49a, and are defined as CD3$^-$NK1.1$^+$CD49a$^-$DX5$^+$. In a mutually exclusive manner for DX5 and CD49a staining, trNK cells lack expression of DX5 but express CD49a and are defined as CD3$^-$NK1.1$^+$CD49a$^+$DX5$^-$. All cNK cells require the transcription factors *Nfil3* and Eomesodermin for development while trNK cells do not. In contrast, *Tbet*, which has a less profound effect on cNK cell development, is required for the development of trNK cells in liver and skin. Interestingly, uNK cells in the virgin uterus are predominantly trNK cells and develop independent of both *Nfil3* and *Tbet* (13), strongly suggesting that they form a lineage distinct from cNK cells and trNK cells in liver or skin. Taken together, these data indicate that cNK and trNK cells represent different lineages of NK cells rather than different differentiation states.

ILC1s

The trNK cells and ILC1s share features but have important differences making it difficult to use the terms interchangeable to define a population. Both trNK cells and ILC1s are resident populations in tissues (1, 13, 14) and both express receptors that have been used to define NK cells such as NK1.1 and NKp46. In the case of the trNK cells in the liver, developmental studies indicate that they use the ILC1 precursor pathway distinguishing them from the cNK developmental pathway (20), making the term ILC1 an appropriate term to define the trNK cells in the liver. However, developmental studies are lacking for ILCs in uterine tissue and trNK cells in the murine virgin uterus develop independent of Tbet, which is required for all ILC1s and liver trNK cells. Therefore, caution needs to be taken when a population is solely defined phenotypically as marker expression may vary among different tissue microenvironments.

UTERINE ADAPTATION THROUGHOUT GESTATION

Uterine adaptation to pregnancy supports fetal growth by the formation of a maternal-fetal interface. Despite structural placental differences between mouse (labyrinth) and human (villous), the uterine tissue response to pregnancy is very similar between the two hemochorial placental species (22), with the fetal chorion directly bathing in maternal blood. These pregnancy-induced responses include uterine receptivity to blastocyst implantation, endometrial decidualization, placental vascular remodeling, and maternal immune cell composition at the maternal-fetal interface. The gestational timeline is well-established during murine pregnancy and continues to be a valuable model to study pregnancy-related physiology and pathology.

The mouse uterus undergoes dynamic changes that accompany the developing conceptus from implantation to the main event, parturition (**Figure 1B**). In C57BL/6J mice, the gestational length is 19.5 days (gd19.5) while in humans it is 40 weeks. When embarking on mouse pregnancy studies, investigators must be aware that specific animal facility characteristics such as food, water, bedding, noise pollution and animal husbandry can all affect gestational length. There are also mouse strain-dependent variations in gestational length so it is important to breed controls of the same genetic background when assessing transgenic models for reproductive fitness (23). One of the most accurate methods for estimating gestational length is a restricted mating period (24). This is recommended and most often done with an overnight breeding strategy in which an estrus-stage dam is placed with a stud male and checked for the presence of a copulation plug before 8:00 am the next day. This method is effective because mice are nocturnal animals and fertilization typically occurs around midnight, the halfway point of a 12 h dark/light cycle (25). If a copulation plug is visualized, the mouse is identified as at gestational day (gd) 0.5, which is important to time accurately because major changes rapidly occur during early stages of mouse pregnancy. For preterm birth studies, a more precise gestational length determination is required and a 2–4 h mating period strategy is critical to follow (24).

The copulation plug, an indication that mating occurred is most often followed by pregnancy, but not always. Following the

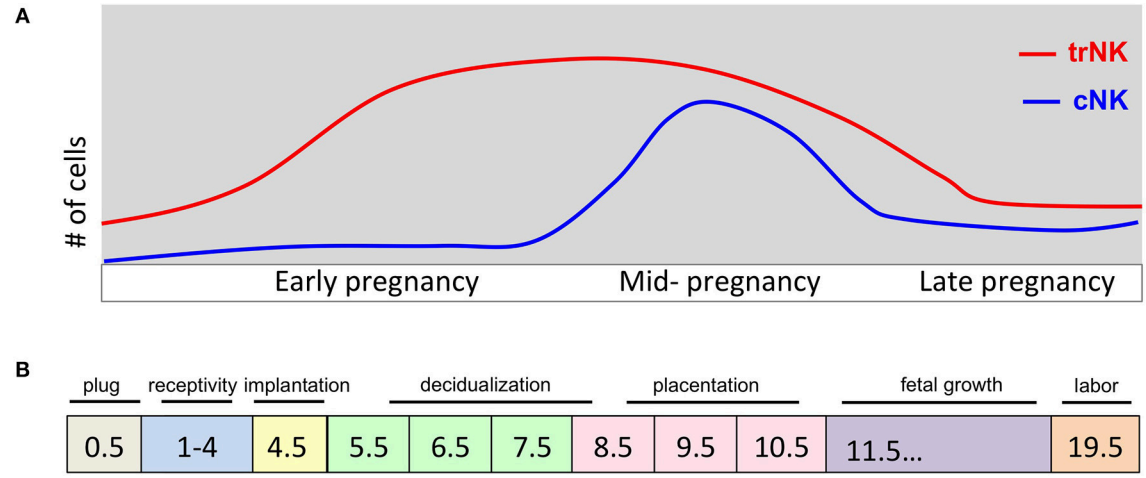

FIGURE 1 | Pregnancy events and uNK cell kinetics during murine pregnancy. **(A)** Schematic diagram of number (y-axis) of trNK and cNK cells during pregnancy (gd on x-axis). During early pregnancy the trNK cells dominate the virgin and decidualized endometrium. By mid-gestation, cNK cells are increased in number and both trNK and cNK cells decline during late pregnancy. **(B)** Schematic diagram of key events during pregnancy at indicated gd's. Uterine adaptation to pregnancy begins shortly after the visualization of a copulation plug and before embryo implantation, identified as window of uterine receptivity. During this time the uterine tissue is prepared for embryo implantation. Embryo implantation triggers the process of decidualization causing extensive proliferation and vascular modification initiating the process of placentation. A fully developed placenta marks mid-gestation.

next couple of days, the uterus needs to experience a necessary estrogen surge at gd 3.5 in order to activate the window of implantation which puts the luminal epithelium in a receptive state to bind the blastocyst at gd4.5 (26). In mice blastocyst implantation initiates the endometrium transformation process called decidualization and the vascular permeability and immune cell accumulation that are associated with the process.

Decidualization begins at gd6.5 and is characterized by extensive cell proliferation and remodeling. Fibroblast cells proliferate and differentiate into decidual cells that assume an epithelial cell-like phenotype. Extracellular matrix remodeling of the endometrial stroma and angiogenesis are initiated during decidualization and continue until the placenta is fully formed. Additionally there is a marked increase in immune cells; specifically uNK cells, beginning with the onset of decidualization (16, 27). The embryo becomes completely surrounded by the decidualized endometrium at which time the primitive placenta, called the choriovitelline placenta, is the main source of nutrition for the developing embryo between gd 6.5–10.5 (28, 29). In human pregnancy, decidualization is triggered during the menstrual cycle, independent of implantation (30). Decidualization is essential for a successful pregnancy to ensue as insufficiency in decidualization can cause infertility and recurrent spontaneous abortion.

The murine definitive placenta, chorioallantoic placenta, is considered fully developed and assumes nourishment of the developing embryo at gd10.5–11.5 when four distinct compartments can be histologically distinguished (**Figure 2**). Farthest away from the fetus is the mesometrial lymphoid aggregate of pregnancy (MLAp) embedded in the myometrium of the uterine wall and specific to murine pregnancy. Underneath the MLAp is the decidua basalis, which contains immune cells, invading trophoblasts and the remodeled vasculature, and which in mouse does not extend into the MLAp. The junctional zone consists of spongio-trophoblast (SpT) and glycogen trophoblast cells (GlyT), and a layer of parietal trophoblast giant cells (P-TGCs) that provides a separation between the maternal decidua basalis and the labyrinth. Closest to the fetus is the labyrinth, the innermost compartment of the placenta. The interhemal membrane unit, also known as the exchange barrier, in the labyrinth is made up of three trophoblast cell types and an endothelial cell layer of the fetal vasculature (**Figure 2** inset). Sinusoidal trophoblast giant cells line the maternal blood sinus, which is separated from the fetal blood capillary by two barrier layers, syncytiotrophoblast I and II. Moreover, the invasive extravillous trophoblast cells are intimate with the maternal immune cells and both are in the same space, the decidua basalis. Together they provide the structure of the remodeled vasculature.

The extent of trophoblast invasion differs between mouse and human, with more extensive invasion into decidua stroma, arteries and myometrium in the latter (31). In human, pregnancy complications linked to inadequate placental perfusion such as preeclampsia and intrauterine growth restriction (IUGR) are associated with inefficient extravillous trophoblast invasion (32). Hence, uterine adaptations to pregnancy establish and maintain the maternal-fetal interface.

MATERNAL-FETAL INTERFACE

The development of the maternal-fetal interface is critical for the successful outcome of pregnancy. The maternal component of the interface is the decidua basalis, which contains the maternal immune cells and the fetal component is the placenta labyrinth, which contains fetal-derived invading trophoblast cells (**Figure 2**). The maternal-fetal interface is common ground for the two allogeneic entities to communicate. At the center are

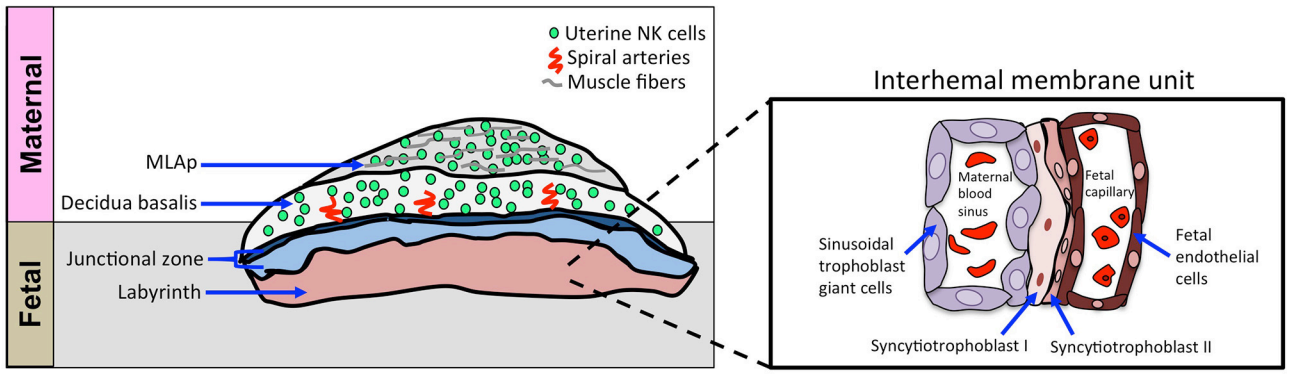

FIGURE 2 | Schematic of cellular structures of mouse definitive chorioallantoic placenta. Schematic diagram of placenta, oriented with maternal tissues above fetal tissues, as indicated. The inset shows a closer view of the interhemal membrane unit in the placental labyrinth. The murine chorioallantoic placenta, at gd11.5, is fully developed. The maternal contributions to the chorioallantoic placenta are the MLAp and the decidua basalis, both regions dominated by uNK cells. The fetal-derived invading trophoblasts can be found in the decidua basalis and with the uNK cells they aid in spiral artery remodeling during placentation. The spongiotrophoblast layer and parietal trophoblast giant cells (P-TGCs) layer make up the junctional zone that separates the placenta labyrinth from the decidua basalis. The labyrinth contains a highly organized cellular barrier called an interhemal membrane unit that separates the maternal blood from the fetal blood. The maternal blood sinus is lined with sinusoidal trophoblast giant cells and separated from the fetal blood compartment by two layers of syncytiotrophoblast cells (SynT-I and SynT-II). The fetal endothelial cells line the fetal capillaries.

the uNK cells that have been implicated in remodeling of the placental vasculature, regulating invading trophoblast cells, and providing immunity.

NK HETEROGENEITY IN THE UTERUS

Granulated metrial gland (GMG) cells were first visualized and characterized by light microscopy over a century ago and more recently by electron microscopy. Identified by morphologists as a prominent cell population containing cytoplasmic granules and occupying the metrial gland during pregnancy, the metrial gland is induced during murine pregnancy and is embedded between the muscle layers of the uterus at the implantation site (33). Since the metrial gland was not of epithelial cells, did not resemble glands histologically, and did not have endocrine or exocrine functions, Croy proposed to rename the structure (34). As a result, two names have been used interchangeably in the literature to replace the term granulated metrial gland: mesometrial triangle and MLAp (34–36). In this review we will refer to this structure as MLAp. Concurrently with the name change, studies revealed that GMG cells belonged to the NK cell lineage and have since been referred to as uterine NK (uNK) cells, as well (37).

During murine pregnancy, uNK cells make up the vast majority of the maternal leukocytes, constituting 70% of the lymphocyte fraction (38–40). Histological analysis revealed uNK cells to occupy both the MLAp and the decidua basalis of the implantation site in early pregnancy, with a decline in both locations at parturition. Heterogeneity among the uNK cells was identified by differences in size and cytoplasmic granule content, which correlated with the maturation status, with the smaller uNK cells mostly residing in the MLAp. Classically identified by histological approaches, uNK cells were detected by the periodic acid-Schiff (PAS) reaction with or without co-reactivity by *Dolichos biflorus* agglutinin (DBA) lectin staining, with DBA reactivity specifically found in the murine pregnant uterus (41). The DBA^+ cells are often referred to as decidual NK cells in the literature. Flow cytometry helped to further characterize uNK cells, which expressed CD45 and NK cell-specific receptors and lacked expression of T cell, B cell and macrophage markers. Analysis of $Rag2^{-/-}\gamma c^{-/-}$ mutant mice, deficient in cNK cells, also indicated absence of uNK cells. When reconstituted with wild-type bone marrow, they showed uNK cell development in the uterus, consistent with bone marrow derivation of uNK cells (42, 43). Taken together, the morphologic, phenotypic and bone marrow reconstitution experiments supported the assignment of uNK cells to the NK cell lineage.

Recently, we used a novel NK reporter mouse to visualize the emergence of uNK cells during pregnancy (**Figure 3**). Since the *Ncr1* gene encodes NKp46, a receptor selectively expressed on all NK cells, $Ncr1^{iCre}$ mice restrict improved Cre (iCre) expression to NK cells (44). $Rosa^{mT/mG}$ mice (45) contain a construct with membrane-bound Tomato constitutively expressed in all tissues. When Cre is expressed, the Tomato cassette and a stop codon are excised, allowing for expression of membrane-bound GFP and fate mapping of essentially all $NKp46^+$ NK cells in $Ncr1^{iCre}$ x $Rosa^{mT/mG}$ mice. We confirmed the GFP^+ cells in the uteri of these reporter mice were indeed NK cells based on flow cytometry phenotypic analyses with NK cell-specific markers (16). An extensive time course revealed that at gd6.5 the decidua basalis contained proliferating GFP^+ uNK cells, prior to the development of MLAp, challenging the proposed idea that the MLAp was a source of immature uNK cells (46). At gd 10.5 (mid-gestation), we found a prominent MLAp structure and a fully developed decidua basalis, both of which contained GFP^+ uNK cells, unlike the placenta labyrinth (16). Shortly after mid-gestation, the GFP^+ uNK cells began to decline in number to essentially non-existent at the implantation site in a laboring dam. Remarkably, at 2.5 days post-partum, GFP^+ uNK cells start to resemble those in the non-pregnant uterus (**Figure 3**). Hence,

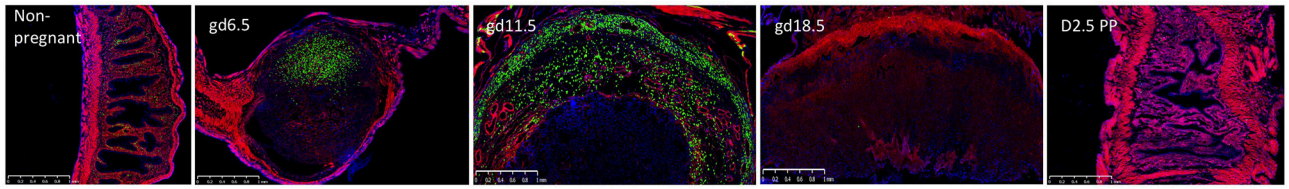

FIGURE 3 | The accumulation and decline of GFP$^+$ uNK cells in the Ncr1^{iCre} X Rosa$^{mT/mG}$ reporter mice during pregnancy. The GFP$^+$ uNK cells accumulate in the decidualized endometrium at gd6.5 and are found segregated to the myometrium and decidua basalis by gd11.5. At gd18.5, (laboring dam) the placenta has remnants of the MLAp and decidua basalis and is essentially devoid of uNK cells. Remarkably by 2.5 days post-partum, the structure of the uterus is remodeled and contains uNK cells. Uterine sections were derived from non-pregnant mouse, different gd's, and 2.5 days post-partum, as indicated.

the *Ncr1*^{iCre} x *Rosa*$^{mT/mG}$ mice allow detection of GFP$^+$ uNK cells with greater sensitivity, particularly with easily detectable GFP$^+$ uNK cells in the MLAp (16) that can be further analyzed by histological analysis and by flow cytometry.

ORIGIN OF UNK CELLS

The origin of uNK cells during pregnancy has been of long standing interest. Whether mouse uNK cells in the pregnant uterus develop *in situ* from progenitor cells in the virgin uterus or home there from the periphery had been addressed using several approaches that include uterine segment transplantation, adoptive transfers and parabiosis. Here we will summarize these studies before describing more recent studies that provide a new hypothesis.

Previously, uNK cell origin was studied by uterine segment transplantation (47, 48). Wild-type (WT) uterine segments from virgin mice were engrafted into the uterine horns of either NK-deficient (*Rag2*$^{-/-}$ $\gamma c^{-/-}$) or NK-sufficient (WT) controls. After the uterine segments established end-to-end anastomosis, the mice were mated and analyzed histologically on gd10. Uterine segments transplanted into WT hosts contained uNK cells but when transplanted into NK-deficient hosts, no uNK cells were found despite having a decidualized uterus originating from WT (NK-sufficient) donors. These data demonstrated that peripheral NK cells homed to the uterus and that the uterus did not contain uNK cells that expanded during pregnancy, with the caveat of possible surgical effects on the host uterine tissue. Regardless, in another approach, adoptive transfer of bone marrow, thymus, lymph node, and spleen or fetal liver cells from SCID mice into alymphoid recipients resulted in detection of donor-derived uNK cells in the pregnant uterus (43), providing further support for NK or progenitor cell homing. This homing to the uterus was independent of chemokine receptors CCR-2 and CCR-5 (49) but specific chemokine receptors have not been identified. However, a recent study disputed these findings as transferred splenic NK cells from *virgin* mice did not home to the pregnant uterus and already present uNK cells appeared to expand (50).

Previously, we reported that murine virgin uteri contain few circulating CD49a$^-$ DX5$^+$ cNK cells and an abundant CD49a$^+$ DX5$^-$ trNK cell population (13). A subset of the CD49a$^+$ DX5$^-$ trNK cell population in the uterus was found to lack the expression of Eomesodermin and identified as ILC1, with trNK cells still dominating the uterus during early pregnancy (15, 16, 27, 51, 52). The accumulation of uNK cells during pregnancy could be due to local proliferation of trNK cells and ILC1s, migration of cNK cells or a combination of both. In the decidua basalis during early pregnancy (gd6.5), trNK cells but not cNK cells were highly proliferative as marked by increased BrdU incorporation and high Ki67 expression (16, 27), with undetectable ILC1s in these studies. Parabiosis experiments with experimentally induced decidualization confirmed that there is minimal contribution from migrating cNK cells to the local proliferating pool of trNK cells in a model of early pregnancy (16). Taken together, these findings indicate that accumulation of uNK cells in *early* pregnancy originates from local proliferating trNK cells.

Our data do not exclude the contribution of cNK cells migrating from the periphery. Although we did not detect any indication of their proliferation, cNK cells increased in number. As previously reported, migration into the pregnant uterus could be one mechanism to account for the increase in cell number in the absence of proliferation. Taken together, we propose a new hypothesis to account for the cNK cell and trNK cell contributions to the pool of uNK cells during murine pregnancy.

We proposed a two-wave hypothesis for uNK cell accumulation in the pregnant uterus that is driven by uterine tissue remodeling events during pregnancy and takes into account uNK cell heterogeneity (**Figure 1A**) (16, 27). The first wave is initiated at the onset of the decidualization process where our parabiosis experiments demonstrated the local proliferation of trNK cells with minimal contribution from the circulating cNK cells (16). The second wave involves the recruitment of cNK cells during the placentation process that includes vascular remodeling. Mice that lack cNK cells but retain trNK cells, such as the *Nfil3*$^{-/-}$ mice, have a major defect in uNK cell accumulation and placentation is suboptimal with aberrant spiral artery remodeling (53, 54). Taken together, these data support the contribution of both trNK cells and peripheral cNK cells to the uNK cell population during pregnancy.

CNK CELL FUNCTION

NK cells can eliminate tumor cells upon contact without prior sensitization, an event known as natural cytotoxicity (55). This is in contrast to T cell-mediated cytotoxicity that requires

major histocompatibility complex (MHC)-dependent antigen recognition. NK cells can also use their cytotoxic machinery and cytokine production to elicit anti-viral immunity early during an infection.

Direct contact with the target cells may engage receptors expressed on NK cells. NK cell receptors are stochastically expressed and an individual NK cell can express several different inhibitory and activation receptors simultaneously, resulting in the potential for many specificities. The NK cell receptor repertoire is dependent on the inherited haplotype of NK cell receptor genes. In the mouse, the genes for Ly49, CD94, NKG2, NKG2D, and NK1.1 (encoded by *Nkrp1*) receptors reside in the NK gene complex (NKC) on mouse chromosome 6 (56).

NK cells recognize their cellular targets via two functional types of surface receptors: activation and inhibitory (55). NK cell inhibitory receptors that engage target MHC-I and deliver negative signals via cytoplasmic immunoreceptor Tyr-based inhibitory motifs (ITIMs) that recruit Tyr phosphatase, SHP1, provide an explanation for missing-self recognition. This mechanism of activation receptor suppression holds true for the inhibitory receptors in mouse and human, lectin-like Ly49s and killer immunoglobulin (Ig)-like receptors (KIRs), respectively, that are functional orthologs. In contrast, ligand binding activation receptor chains couple to immunoreceptor Tyr-based activation motif (ITAM)-containing molecules, CD3ζ, FcεRIγ, or DAP12, that stabilize expression and transmit downstream intracellular signals resembling events found in TCR signaling. Thus, during effector responses, NK cell triggering by its cellular targets is typically dependent on integrating signals from activation and inhibitory receptors.

In the spleens of C57BL/6 mice, cNK cells express NK1.1, NKp46, and Ly49 receptors. Although the Ly49 receptors are also expressed by some trNK cells, their expression differ depending on the tissue from which they are examined. For example, ILC1s in the liver do not express the activation receptors Ly49D and Ly49H and have variable expression of the inhibitory Ly49 receptors (13). The inhibitory receptor Ly49I is differentially expressed on uterine trNK cells and is dependent on the location with no expression in the MLAp while in the decidua basalis expression is similar to that found on cNK cells (16). Hence, it is plausible that during pregnancy uNK cells may respond to their cellular targets using strategies, similar to but distinct, from those used by cNK cells.

Pregnant women with a specific KIR haplotype and fetal HLA-C genotype combination have a significantly higher risk of preeclampsia (57). Similar findings were reported in a cohort of African women, which have more genetically diverse KIR haplotypes and HLA alleles (58), strengthening the interpretation that inhibitory receptors on uNK cells interact with their fetal MHC-I ligands leading to increased susceptibility to preeclampsia. Conversely, genetic association studies indicate that a KIR activation receptor recognizing a fetal HLA ligand protects from preeclampsia (58, 59). Thus, these data suggest uNK cells respond to fetal MHC-I via their inhibitory and activation receptors to control proper placental vascularization and development.

UNK CELL FUNCTION DURING PREGNANCY

Placental Vascular Remodeling

Although uNK cells were thought to belong to the NK cell lineage and contained large cytoplasmic granules, when isolated from the murine pregnant uterus, they possessed essentially no cytotoxic ability to kill prototypic NK cell-sensitive target cells (60–62). This was puzzling because NK cells are defined by their natural ability to kill targets. But their abundance in the pregnant uterus left many to wonder about their function. Since uNK cells have been visualized by microscopy from the very beginning of their discovery, they often were noted to be in close association with trophoblast cells lining the blood vessels. Pioneering work by Croy and colleagues proposed the hypothesis that during murine pregnancy uNK cells modulate placental vascular remodeling.

During pregnancy, spiral arterioles are transformed into high-capacitance, low-resistance, thin-walled vessels with large lumens (63). This vascular adaptation is thought to keep up with the nutritional demands of the growing fetus. Studies of mouse uNK cells support their role in this remodeling. Mice lacking cNK cells have defects in spiral artery remodeling during placentation that were rescued when IFNγ was injected systemically (53, 64–68). In bone marrow (BM) chimeric experiments, the remodeling defects were rescued when BM from NK-sufficient mice, but not BM from IFNγ$^{-/-}$ mice was used. Also, BM from IFNγ receptor-deficient mice was able to rescue, indicating that NK cells did not respond to IFNγ in order to rescue. Thus, IFNγ produced by NK cells contributes to spiral arteriole remodeling by acting on non-NK cells such as endothelial cells and decidual stromal cells but the exact signaling pathway to initiate their cytokine production and other aspects of uNK cell-dependent remodeling have not been elucidated.

Growth Promoting Factors

Recently, uNK cells have been reported to directly stimulate fetal growth by producing growth-promoting factors essential for embryo development prior to the establishment of the placenta (52). The trNK cells specifically produced the growth factors pleiotrophin, osteoglycin, and osteopontin. A decrease in trNK cells secreting these growth factors in the *Nfil3*$^{-/-}$ and aged mice impacted offspring from these dams, which had fetal growth-restricted pups with defects in bone development. The fetal growth deficiency and bone development were restored when the dams were reconstituted with *in vitro* expanded trNK cells that produced sufficient amounts of growth factors. Hence, this study sheds light on additional novel functional roles of trNK cells during early embryo development in pregnancy.

Memory of Pregnancy

In human, first time pregnancies are at a higher risk for miscarriages and preeclampsia, a multifactorial disease characterized by impaired placental perfusion (69). The percentage of preeclampsia is greater among women that are pregnant for the first time when compared to women with repeated pregnancies. Likewise the uterus and placenta differ during a second pregnancy with regard to

placental vascularization and trophoblast invasion (70). The following studies suggest that uNK cells may provide memory to aid in vascular remodeling of the placenta during subsequent pregnancies.

Mandelboim et al. identified a unique subset of human NK cells that only exist in repeated pregnancies (71). They defined these cells as pregnancy-trained decidual NK (PTdNK) cells. The PTdNK cells have a unique transcriptome and epigenetic signature and express NKG2C and LILRB1. When stimulated, the PTdNK cells produced more IFNγ and VEGFα, both important in vascular modification of the placenta in mouse studies (64, 67). In another recent study, single cell RNA-seq analysis of human first trimester decidua identified three uNK cell subsets termed dNK1, dNK2, and dNK3 (72) that all co-expressed CD49a, the receptor used to identify murine trNK cells in the uterus (13). The dNK1 cell subset expressed higher levels of KIRs and *LILRB1* receptors that bind HLA-C and HLA-G molecules, respectively, expressed on extravillous trophoblast. Thus, these studies propose that a previously primed uNK cell subset during the first pregnancy may function to recall subsequent pregnancies and be better equipped to support placental vascular development.

Colucci et al. tracked the emergence and decline of the ILC family of cells during murine pregnancy (51). The trNK cells are most abundant during early pregnancy while the cNK cells peak during placentation. The ILC1 population is dominant before puberty and is essentially not detected again until the second pregnancy, where it is the most abundant population. The ILC1 cells express CXCR6 and phenotypically resemble liver NK memory cells described in the contact hypersensitivity model (73). Taken together, these data provide the intriguing idea where an uNK cell subset provides a protective memory response in subsequent pregnancies and is conserved between mice and humans.

CONCLUSIONS

In both mouse and human, uNK cells are the most prominent immune cells that occupy the maternal-fetal interface. The uNK cells appear to engage and establish complex interactions with the surrounding tissue, which impact their function. As more cell subsets are identified within the heterogeneous uNK cell population, it is anticipated that their functional heterogeneity will extend beyond vascular modification, growth-promotion and memory generation.

AUTHOR CONTRIBUTIONS

DS wrote the manuscript. LY provided the micrographs and WY edited the manuscript.

FUNDING

Work in the Yokoyama lab on uterine NK cells is supported by grant R01-AI140397 from the National Institutes of Health.

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

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frontiers in Immunology

REVIEW
published: 28 June 2019
doi: 10.3389/fimmu.2019.01397

Features of Human Decidual NK Cells in Healthy Pregnancy and During Viral Infection

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The hallmark of human early pregnancy is the accumulation of a unique population of Natural Killer (dNK) cells at the main maternal-fetal interface, the *decidua basalis*. dNK cells play a crucial role in successful placentation probably by orchestrating the invasion of trophoblast cells deep into the *decidua basalis* and remodeling of the maternal spiral arteries. Recent advances in the field emphasize the importance of the local microenvironment in shaping both the phenotype and the effector functions of these innate lymphoid cells. Despite slow progress in the field, *ex vivo* studies revealed that dNK cells sense and destroy infected cells in order to protect the fetus from invading pathogens. In this review, we will discuss key features of dNK cells during healthy pregnancy as well as their functional adaptations in limiting pathogen dissemination to the growing conceptus. The challenge is to better understand the plasticity of dNK cells in the maternal-fetal interface. Such insights would enable greater understanding of the pathogenesis in congenital infections and pregnancy disorders.

Keywords: pregnancy, decidual natural killer, congenital infection, receptor, cytokines

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Paola Vacca, Bambino Gesù Children Hospital (IRCCS), Italy
Ashley Moffett, University of Cambridge, United Kingdom

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *25 February 2019*
Accepted: *03 June 2019*
Published: *28 June 2019*

Citation:
Jabrane-Ferrat N (2019) Features of Human Decidual NK Cells in Healthy Pregnancy and During Viral Infection. Front. Immunol. 10:1397. doi: 10.3389/fimmu.2019.01397

IMMUNOLOGICAL PARADOX OF HUMAN PREGNANCY

Seven decades ago, Sir Peter Medawar wondered: "How does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is an antigenically foreign body?" (1). This interrogation highlighted the immunological paradox of pregnancy. Ever since, the compelling relationship between two mismatched individuals, the mother and her fetus, prompted the development of a novel reproductive immunology research stream. The original theories claiming the antigenic immaturity of the fetus, inertness of the maternal immune system, and the presence of an anatomical barrier between the embryo and its mother have proven wrong. As a result, the modern concept of active immune crosstalk emerged. Recent advances in the field advocate a unique bidirectional immune dialogue involving the fetus and mothers' innate as well as adaptive immune cells; namely innate lymphoid cells, regulatory T cells, macrophages, and dendritic cells (2–7). In this review, we discuss the current understanding of how a unique population of type 1 innate lymphoid cells (ILC-1), the uterine Natural Killer cells found at the maternal *decidua basalis* (called hereafter dNK cells), supports the development of the fetal placenta while maintaining active immune surveillance against invading pathogens.

HUMAN PREGNANCY

Every month, the uterine mucosa or endometrium undergoes singular anatomical changes, the most crucial ones occurring during pregnancy. Implantation of the semi-allogeneic blastocyst is synchronized with massive adaptations of the uterine mucosa which transforms into the *decidua basalis*. The blastocyst produces large amounts of the chorionic gonadotrophin hormone (CGH) to maintain high levels of progesterone. These hormonal changes prevent menstruation, destruction of the decidualized endometrium, regulate immune cell functions, and promote angiogenesis. Other factors, such as the leukemia inhibitory factor (LIF), IL-6, and matrix metalloproteinases, are also highly expressed during the implantation process.

In humans, the embryo is completely embedded within the endometrium and the implantation is termed interstitial hemochorial. The development of the placenta is initiated with the apposition of the trophectoderm layer of the blastocyst to the uterine mucosa. The rapid proliferation of this extra-embryonic cell layer generates a unique type of placental cell, the trophoblast, which will further develop into the floating and anchoring chorionic villi of the placenta. The underlying stromal core of the placental villi originates from the extraembryonic mesoderm. The proliferative cytotrophoblasts (CTBs) follow two differentiation programs (8–12). In the first program, CTBs fuse to form the syncytiotrophoblast (STB), a multinucleated epithelial outer layer of the floating chorionic villi. The STBs, in direct contact with maternal blood, ensure nutrient, and gas exchanges for the conceptus. In the second program, CTBs in the cell column of the anchoring villi differentiate into extravillous trophoblasts (EVTs). CTBs and EVTs exhibit differential expressions of cell adhesion molecules, integrins, growth factors as well as the immune inhibitory molecules Fas Ligand, TRAIL, and Indoleamine 2,3-dioxygenase (IDO) (13). Consistent with the role of some of these factors in dampening T cell response, it is possible that EVTs contribute to fetal tolerance.

Unlike most cells of the body, EVTs express only the less polymorphic HLA-C and non-classical HLA-E and HLA-G molecules (14, 15). It is believed that these HLA molecules mediate recognition of invading EVTs by maternal dNK cells rather than T cells (16, 17). The recognition of HLA-G molecule and HLA-G peptides presented in the context of HLA-E may contribute to NK cell hyporesponsive (18–20). Even if the detailed mechanisms of EVT invasion of the placental bed are still largely unknown, progressive remodeling of maternal spiral arteries by EVTs seems to follow two separate waves (21, 22). In the first 10–12 weeks, endovascular migration and plugging of the maternal arteries prevents blood flow to the intervillous space and creates a hypoxic environment that is necessary for placental and fetal development (23, 24). These original claims were confirmed by *in vivo* monitoring of the oxygen tension at different gestational ages (25, 26). The second wave of EVT invasion, starting around 14 weeks, stops at the inner myometrium. The resulting intramural incorporation of invasive EVTs into the vessel wall and erosion of the trophoblastic plug are needed to establish proper blood flow to the intervillous space of the developing placenta (27–29). These early and late developmental steps result in the establishment of privileged sites, where embryonic trophoblasts intermingle with maternal cells. The best examples are the decidua, hosting a large number of innate immune cells in early pregnancy, and the intervillous space, where maternal blood bathes the chorionic floating villi (**Figure 1**). Flaws in EVT invasion and arteries' remodeling can lead to placental dysfunction and major pregnancy disorders such as preeclampsia, fetal growth restriction (FGR) and recurrent miscarriage (30).

NATURAL KILLER CELLS

Natural killer (NK) cells are cytotoxic innate lymphoid cells known for their active role in immune regulation of leukocyte activation and immune surveillance of microbial infections and malignancies (31–33). Human conventional NK cells in peripheral blood (cNK/pNK/) have been extensively studied in health and disease. NK cells were regarded as innate immune cells, owing to the lack of expression of antigen-specific receptors. NK cell responsiveness is governed by the diversity of their germline encoded activating and inhibitory receptors (NKR). Originally, cNK cells were subdivided into two main subsets; the $CD56^{dim}CD16^{+}$ cytotoxic cells and the $CD56^{bright}CD16^{-}$ cytokine producer cells. However, recent developments in the field regarding NK cell educational programs and the diversification of NKR in response to pathogens, as well as the development of memory-like capacities, suggest the existence of more than two NK cell subsets (34).

Similar to the periphery, distinct subsets of resident NK cells (trNK) have been found in many tissues including the liver, kidney and uterus (35). While trNK share striking similarities with cNK cells, these $CD56^{bright}$ cells exhibit different signatures that are related to their tissue of origin. Similar to tissue-resident T cells, trNK express high levels of CD69, CD103, and CD49a (2, 3, 36–38). Here, we will mainly focus on the aforementioned dNK cells that reside in the decidua.

PREGNANCY AND IMMUNITY: REGULATORS OF THE MATERNAL-FETAL INTERFACE

Decidualization requires coordinated contribution of the uterine glands, stromal cells, and immune cells (4, 39–41). In early pregnancy, the hallmarks of the decidua include the accumulation of immune cells that represent up to 40% of total decidual cells and the histiotrophic nurturing of the developing placenta by the uterine glands. The distinctive $Tbet^{pos}EOMES^{pos}CD56^{bright}$ dNK cell population accounts for almost 70% of total tissue leukocytes (42, 43). Whereas, T cells account for ∼5–10% of total leukocytes, the quasi absence of B or plasma cells suggests it is very unlikely that any antibody response would harm the invading EVTs (44–46). Additional innate immune cells include $CD14^{pos}$ macrophages and dendritic cells, which represent ∼20%. Besides dNK cells, other ILCs are found at the implantation bed including a non-NK ILC1 subset as well as both NCR^{+} and NCR^{-} ILC3 (43, 47, 48). These

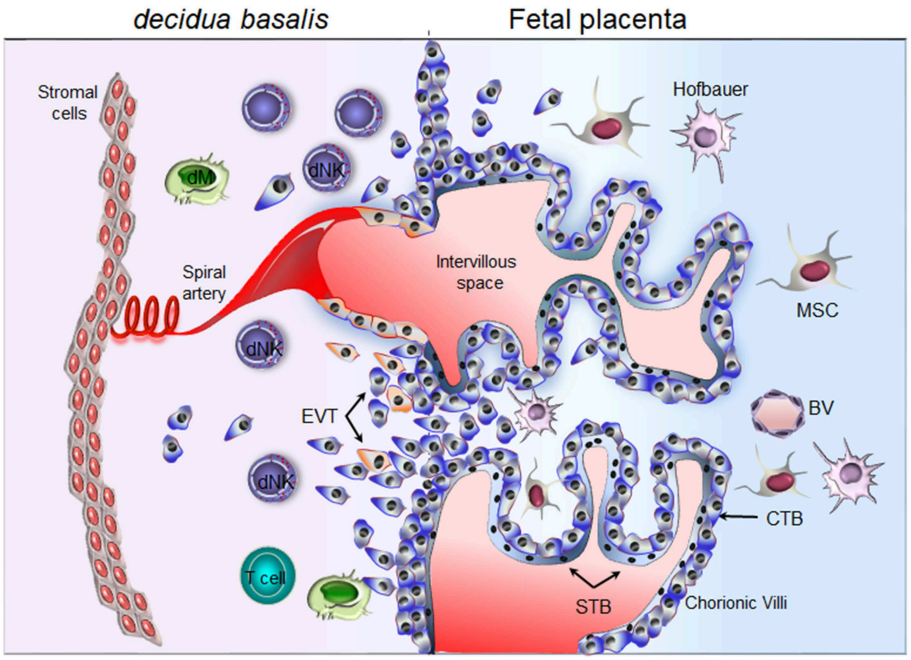

FIGURE 1 | Schematic representation of the maternal-fetal interface. Floating chorionic villi are bathed in maternal blood within the intervillous space. A multinucleated syncytiotrophoblast (STB) outer cell layer covering the chorionic villi. STB layer serves for transport of nutrient and barrier function. A layer of cytotrophoblast cells (CTBs), underlines the STB. CTBs differentiate into extravillous trophoblast (EVTs) and invade the maternal *decidua*. Through the release of soluble factors (cytokines, chemokines, and proangiogenic factors), maternal decidual NK (dNK) cells participate actively in the attraction of invasive EVTs and remodeling of the spiral arteries. Invasive EVTs are also in contact with decidual macrophages (dM) and T cells. Fetal blood vessel (BV), mesenchymal stem cells (MSC), Hofbauer cells (fetal macrophage).

decidual ILCs share similarities with other tissue resident ILCs. Upon *in vitro* stimulation, decidual ILC1 are able to produce IFN-γ while NCR$^+$ILC3 produce IL-22 and IL-8 and NCR$^-$ILC3 produce TNF and IL-17 (43, 47, 48). Finally, in addition to the typical T cell populations (CD8, CD4, γδT cells), the non-pregnant uterine mucosa and first trimester decidua contain a small fraction of mucosal-associated invariant T (MAIT) cells [(49) and unpublished data from our laboratory]. Yet, the exact functional role of decidual ILCs and MAIT cells in pregnancy is not clear.

Decidual NK Cells

The discovery of dNK cells at the implantation site, even before the implantation of the blastocyst, has led to the idea that these cells play a crucial role in normal placentation (50). As a matter of fact, the uterus is undeniably among the peripheral organs that exhibit the highest frequency of NK cells. After ovulation, the surge of IL-15 and prolactin, triggered by the exposure of stromal cells to progesterone, induces a rapid proliferation and differentiation program of uterine NK cells (51). These numbers increase further when implantation is successful and are maintained throughout the second trimester. dNK cell numbers decline from mid-gestation onward to reach a barely detectable level at term. Despite extensive work on dNK cells, we are still lacking essential information about their origin and exact functions. The association of dNK cells with EVTs and their spatiotemporal localization at the vicinity of maternal arteries suggest that these immune cells provide a well-balanced microenvironment to enable proper development and functioning of the placenta yet preclude excessive trophoblast invasion.

Research, performed by several groups has yielded fascinating insights into the phenotype and functional plasticity of dNK cells. In contrast to cNK, dNK cells are poorly cytotoxic and display a unique repertoire of NKR (2–4, 9, 38, 52–54). dNK cells are mainly CD56^{bright}CD16$^-$KIR$^+$ cells but they are distinct from the CD56^{bright} subset found in peripheral blood, both at the functional and phenotypical levels. dNK cells express the tissue residency markers CD69, CD49a, integrin β7, and CD9. Additionally, dNK cells express most of the NKRs including NKp46, NKp80, NKG2D, CD94/NKG2A. Contrary to cNK, the CD94/NKG2C heterodimer and NKp44 receptor are found on a fraction of dNK cells (2–4, 38, 52), although other reports demonstrated no expression of NKp44 only freshly isolated cells (55). Nonetheless, similar to cNK, NKp44 expression can be induced on the large population of dNK cells upon *in vitro* stimulation. 2B4 and LILRB, which is expressed at low frequency, act as inhibitory receptors (54, 55). Likewise, freshly isolated unstimulated dNK cells express inhibitory isoforms of the NKp44 and NKp30, natural cytotoxicity receptors 2 and 3 respectively (3). Furthermore, several chemokine receptors including CXCR3, CXCR4, CCR1, and CCR9 are expressed by these cells (3, 53, 56).

Fine analysis of the killer-cell immunoglobulin-like receptors (KIR) has highlighted a skewed repertoire toward the recognition of the less polymorphic HLA-C, the only classical HLA class I molecule expressed on EVTs (14). However, several of these NKR are expressed only by a fraction of cells, suggesting that dNK cells may come in different flavors. As an example, the majority of dNK cells express the CD49a residency marker and the chemokine receptor CXCR3, whereas a sizeable fraction of cells lacks the expression of CD103 or CXCR4 (3).

The recent discovery of three main pools of dNK cells (dNK1, dNK2, and dNK3) with different immunomodulatory profiles confirmed these findings (57). The dNK1 pool expresses regulatory CD39 ecto-ATPase, which is involved in shifting the balance from a pro-inflammatory to an immunosuppressive environment [reviewed in (58)] and has high levels of the inhibitory as well as activating KIRs (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, and KIR2DS4). Furthermore, the expression of the high affinity receptor for the HLA-G dimer, *LILRB1*, and the active glycolytic metabolism allude to the interaction of the dNK1 subset with fetal EVTs. The dNK2 pool is characterized by the expression *ANXA1* and *ITGB2*. Both dNK1 and dNK2 cell subsets express the activating *NKG2C* and *NKG2E* and inhibitory *NKG2A* receptors. The third subset expresses *CD160*, *CD161*, *TIGIT*, *CD103,* and *ITGB2*. This unbiased reconstruction of the fetomaternal interface further highlights key interactions between dNK cell subsets, invading fetal EVTs and decidual stromal cells (DSC), all needed for the development of embryonic tissues and successful pregnancy. Whether dNK cell subsets with defined characteristics exert common or distinctive functions within the decidual microenvironment is yet to be defined. dNK cell subsets shall either promote or restrain EVT invasion. However, doubt subsists as to whether this would occur through specific ligand–receptor interaction, metabolic adaptations and expression of checkpoint inhibitors or through microenvironment paracrine effect. While additional studies are required to define the exact function of the three dNK cell subsets, it is clear that maternal adaptations, during pregnancy, are designed to restrain harmful dNK cell responses. Thus, a finely tuned dialogue between a given dNK cell pool, decidual cells and invading EVTs, is necessary for the establishment of the fetomaternal interface and for the development of the placenta and fetus.

Origin of dNK Cells

The origin of dNK cells remains subject to controversy. The discovery of $CD34^{pos}$ progenitors in the maternal *decidua* would suggest that dNK cells originate from local self-renewed $CD34^{+}$ progenitors. This perspective is supported by the ability of $CD34^{pos}$ progenitors from the decidua to differentiate into dNK-like precursors, in the presence of DSC and an IL-15-enriched microenvironment (59, 60). The second possible explanation would be that dNK cells arise from NK precursors, as advocated by the presence of $CD34^{neg}CD117^{pos}CD94^{neg}$ NK cell precursors within the uterine mucosa (61). Lastly, dNK cells could originate from cNK cells recruited from the periphery through chemotaxis (59, 60, 62–64). This latter insight is supported by a twofold argumentation; (i) both estrogens and progesterone induce the secretion of CXCL10, CXCL12, CCL2, CXCL8, and CX3CL1 chemokines by DSC and endothelial cells, ensuring the availability of several chemoattractant axes that can promote the recruitment of cNK cells, and (ii) the conversion of cNK cells in the presence of the transforming growth factor-β (TGF-β) or a combination of TGF-β/IL-15 or yet again, TGF-β/5-aza-2′-deoxycytidine into less cytotoxic cells that can promote the invasion of human trophoblast (65–67). In line, we have shown that cytokines enriched within the decidual microenvironment (68–70), namely TGF-β and IL-15, in combination with IL-18 convert the NKp30/NCR3 and NKp44/NCR2 splice variant profile of cNK cells into one similar to that of dNK cells (3). The switch from activating to inhibitory isoforms of NKp44 and NKp30 was associated with decreased cytotoxic function and major adaptations of NK cell secretome, the two hallmarks of the decidual phenotype (**Figure 2**).

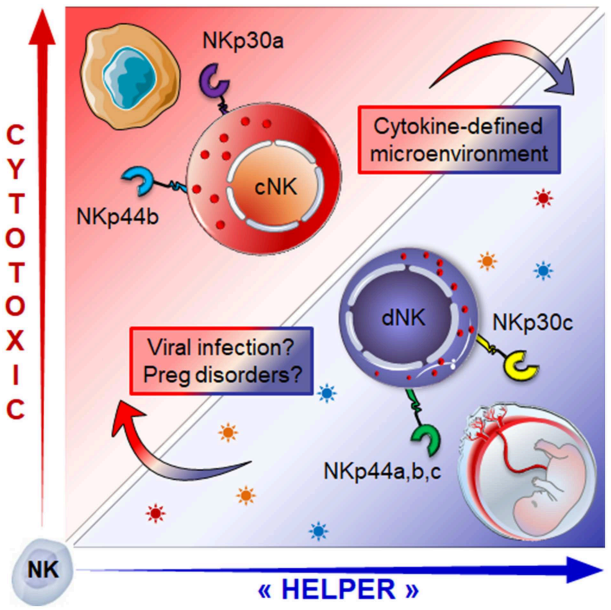

FIGURE 2 | Role of the microenvironment in shaping NK cell phenotype and functions: cNK cells expressing activating isoforms of the NKp30 and NKp44 receptors (NKp30a, NKp44b) which endows them with cytotoxic function, can be converted into dNK like cells. Exposure to a combination of TGF-β, IL-15 and IL-18) drives the isoform expression profile toward regulatory profile (NKp30c, NKp44a,c), a hallmark of dNK cells. This conversion is associated with the switch from cytotoxic to "helper-like" or tolerogenic effector functions associated with major changes in the secretome. How micro-environmental changes during pregnancy disorders or congenital infection might influence the dNK cell plasticity and effector functions is still an open question.

Thus, whether recruited or tissue resident cells, dNK cells are undoubtedly different from other $CD56^{bright}$ NK cell subsets found in the periphery (2). Today, it is undeniably admitted that microenvironment pressure within the *decidua basalis* conditions the education and the generation of dNK cells with unique phenotypic and functional features; a great ability to produce large amounts of soluble factors and a finely tuned cytotoxic function that are both necessary for a successful pregnancy.

dNK CELLS IN HEALTHY PREGNANCY

dNK Cell Effector Functions

Large scale profiling of dNK cell transcriptome and secretome revealed that these cells produce: (i) a large array of cytokines including IFN-γ, TNF-α, GM-CSF, TGF-β, and IL-10; (ii) chemokines including CXCL8 (IL-8), CCL3 (MIP1a), CCL4 (MIP1b), CCL5 (Rantes), CXCL10 (IP-10), and CXCL12 (SDF-1); and (iii) angiogenic factors including Ang-2, PLGF, EGF, VEGF-A, but also VEGF-C that can induce the expression of inhibitory ligands on trophoblasts (4, 56). Yet, most of these *in vitro* studies were conducted under IL-2 or IL-15 stimulation. In our hands and in agreement with single cell transcriptomic analysis, freshly isolated and unstimulated dNK cells barely produce any IFN-γ or VEGF-A (38, 57). With regard to the cytotoxic effector function, dNK cells express functional activating receptors and a payload lytic machinery including granzymes, granulysin, and perforin, but conversely they lack cytolytic activity in healthy pregnancies (2, 3, 52, 71, 72). Defaulting assembly of the immunological synapse and failure of 2B4 receptor to convey activating signals have been proposed as mechanisms that can explain the poor cytotoxic function of dNK cells (54, 71). Nevertheless, these cells are probably educated in the decidua by the binding of their NKG2A and/or KIR to their cognate ligands expressed by the fetal trophoblast cells, they are also highly plastic and can acquire cytotoxic functions upon NKp46 receptor engagement and/or cytokine stimulation [reviewed in (2)].

dNK Cells Control Trophoblast Invasion

The tight regulation of EVT invasion into the maternal decidua is essential for the development of the placenta and the outcome of the pregnancy. Inadequate invasion can have disastrous consequences and result in pathological pregnancy such as preeclampsia, FGR, preterm labor, and recurrent miscarriage. The presence of dNK cells in the vicinity of invasive fetal trophoblasts and spiral arteries is suggestive of their active role in regulating the extent of trophoblast invasion and vascular remodeling. The production of a large panel of soluble factors is the likely mechanism for dNK cells to regulate trophoblast invasion. For instance, secreted CXCL8 and CXCL10 bind to their receptors on invasive trophoblasts and promote trophoblast migration while Ang-2, TNF, and TGF-β inhibit trophoblast invasion (56, 73–76). Whether through specific education programs, direct receptor-ligand engagement or paracrine factors, dNK cells contribute grandly to the appropriate trophoblast invasion (72, 77–79). Further elucidation of how the education program shapes dNK cell functions will probably have upshots in resolving some pregnancy disorders.

dNK Cells Direct Vascular Remodeling

The remodeling of decidual tissue is mandatory to pregnancy success to ensure minimal vessel resistance and high blood flow of nutrients as well as oxygen to the growing conceptus. Even if there are still a lot of controversies regarding different steps of the vascular remodeling process, the invasive EVTs are very like to have an active role in the removal of the smooth muscle media and in the replacement of the endothelium lining deep into the endometrium by mural trophoblast (28, 80, 81). Although lessons from mouse studies highlight the contribution of dNK cells to this process (82), their role is not yet fully elucidated in humans. However, their accumulation along the vascular wall of the changing vessels before endovascular invasion and their production of angiogenic factors is suggestive of an active role in angiogenesis (52, 56). Similar to the extent of trophoblast invasion, specific KIRs express on dNK cells may dictate the fate of vascular remodeling and thus conduct to successful or pathological pregnancies (79, 83).

Do Decidual NK Cells Remember Pregnancy?

While NK cells were considered as short lived cells for many years, accumulating evidence indicate that cNK cells develop long-lasting memory-like phenotype to viruses marked by high cytotoxicity and characterized by the expression CD94/NKG2C and the CD57 terminal differentiation marker (34, 84). Whether dNK cells develop memory-like phenotypes to pregnancy is still a major debate. Nevertheless, efficient development of the fetal placenta in subsequent pregnancies hints to the existence of a "trained" uterine immunity (85). Pioneer work showing the association between maternal activating KIRs expressed on dNK cells and protection against reproductive failure mediated by fetal HLA-C2 (83), suggest that fine-tuning of dNK responsiveness is necessary for successful pregnancy. Later studies provided evidence that dNK cell response is orchestrated by functional education and expression of inhibitory receptors (86). Whether education takes place even before embryo' implantation and how lessons from first pregnancy shape the uterine immune landscape remain to be elucidated.

Recently, the group of Ofer Mandelboim reported the existence of a specific population of NKG2C^{high}LIRB1^{+} dNK cells in the decidua of multigravid women (87). The co-expression of NKG2C and the high affinity receptor for HLA-G dimer suggest that these "trained" dNK cells belong to dNK1 subset (57). The ability of IL15-primed "memory-like" dNK cells to produce high amounts of IFN-γ and VEGF-A upon ligation of NKG2C/E and LIRB1 receptors (87) is a major difference with other "memory" NK cells that produce only IFN-γ. Previous work has clearly established that the physiological pool of dNK cells is governed by the differential expression of NKp30 and NKp44 alternatively spliced isoforms (3). The expression of inhibitory isoforms would act as a secondary innate immune checkpoint that conveys dNK cells with a "support" function contributing to proper placental development and successful pregnancy, while activating isoforms trigger NK cell responsiveness and effector functions. Whether this has physiological relevance to dNK cell training and "memory-like" development is yet to be depicted and warrants further investigations. The development of "memory-like" dNK cells in subsequent pregnancies may explain why deficient placentation are less frequent in subsequent pregnancies. dNK cell hyporesponsiveness in first pregnancies might lead to

deficient placentation and pregnancy disorders. Elucidating the potential role of "trained" dNK cell immunity will constitute a new step toward a better understanding of the pathophysiology of pregnancy disorders and the development of new therapeutic intervention.

dNK CELLS DURING VIRAL INFECTIONS

While many aspects of the immune response are circumvented at the fetomaternal interface to enable an intimate relationship between maternal and fetal cells, many threatening pathogens can jeopardize the harmony of this immune-friendly site. For instance, the growing family of TORCH pathogens which originally includes *Toxoplasma gondii*, other (syphilis, varicella-zoster, parvovirus B19, and others), rubella virus, cytomegalovirus (HCMV) and herpes simplex virus can cause severe maternal and fetal morbidity during pregnancy. Today, the genotype 1 of Hepatitis E virus (HEV-1) and Zika virus (ZIKV) can also be classified as TORCH pathogens (88–92). However, how these viruses reach the developing placenta is still largely unknown and requires active investigations. Lessons from *ex vivo* studies demonstrate that some of these viruses (HCMV, ZIKV, and HEV-1) can use the fetomaternal interface as a replication platform before spreading to the placenta and fetal compartment.

The human cytomegalovirus (HCMV) is a member of the largest virus specie, Betaherpesviridae, with a DNA genome encoding more than 150 proteins (93). HCMV is the most common cause of congenital infections with severe and permanent birth sequelae (94, 95). Even if the transmission rate is much higher in the third trimester, primary infection in the first trimester is associated with high risk of placental pathology and severe congenital syndrome. *Ex vivo* studies demonstrated that replication of HCMV strains in stromal and placental cells results in impaired function and soluble factor secretion (38, 96–99).

The hepatitis E virus (HEV) is a single-stranded RNA virus with five genotypes that can cause acute self-limiting illness in immunocompetent host. During pregnancy, the outcome of infection is quite devastating in some endemic areas where HEV-1 prevales. In fact, HEV-1 infection is associated with a high co-morbidity rate in pregnant women from northern India, due to fulminant hepatic failure associated with severe placental diseases (100, 101). Retrospective studies have estimated vertical transmission in 23–50% of North India cases. However, the regional differences in the course of congenital HEV-1 infection remain unclear. It is highly possible that both environmental and viral factors may contribute to the devastating pregnancy outcome. To provide insights into the genotype-specific pathogenicity of HEV during pregnancy, we *ex vivo* modeled the pathological HEV-1 and less-pathological HEV-3 infection at the maternal-fetal interface using organ cultures of first trimester decidua and fetal placenta. While both HEV genotypes are able to infect the maternal-fetal interface, HEV-1 replicates more efficiently in the decidual and placental tissues as well as in primary isolated stromal cells. The dysregulation of the cytokine microenvironment by HEV-1 caused severe damage to the decidual and fetal placenta tissues (89).

Zika virus (ZIKV) is a mosquito-borne Flavivirus initially isolated in the Zika forest in Uganda in 1947. The recent epidemic wave of ZIKV in the Americas revealed an unprecedented association with a severe congenital syndrome (102, 103). Investigations using decidua and placenta explants have demonstrated that ZIKV replicates in a wide range of cells. In the basal decidua, ZIKV targets EVTs as well maternal macrophages and stromal cells. In the anchoring villi, ZIKV targets the proliferative CTBs and stromal cells of the villous core (90, 91, 104, 105), but not STBs owing to intrinsic antiviral defense mechanisms involving IFN-λ (106). Thus, ZIKV should either overcome the STB restriction mechanisms or exploit alternative strategies to access the fetal compartment.

To date, our understanding of how viruses reach the fetal compartment and whether they exploit common infection routes is still in its infancy. Usually, the vertical transmission rate is quite low in the first trimester of pregnancy, which coincides with high numbers of dNK cells within the placental bed. Whether these immune cells are able to restrain viral spread at the maternal-fetal interface and what is the contribution of placental intrinsic defense mechanisms and restriction factors are yet to be demonstrated *in vivo*.

The first evidence of the involvement of dNK cells in controlling viral infection was described for HCMV. Indeed, we have shown that dNK are able to infiltrate HCMV-infected tissue and to co-localize with infected cells. The exposure of dNK cells to HCMV-infected cells was associated with phenotypic changes and the acquisition of a cytotoxic function involving the NKG2D and CD94/NKG2C-E activating receptors (2, 35, 38). The combination of maternal KIR, namely the expression of KIR2DS1, also increases dNK cell cytotoxic response to HCMV-infected HLA-C2^{+} maternal DSC and prevents viral spread and placental pathology (78). However, even if dNK cells are able to clear HCMV infection from the decidual stroma, placental cells are more resistant to NK cell cytotoxicity.

Beside viruses, the fetomaternal interface can be also threatened by other microbial pathogens such as *Listeria monocytogenes* and *Toxoplasma gondii*. The fact that dNK cells, as well as decidual macrophages and dendritic cells, constitutively express the antimicrobial peptide granulysin (37, 107), would suggest their involvement in controlling these infections. However, it is not clear whether dNK cells can destroy the pathogen while sparing infected maternal DSC and fetal trophoblasts.

Collectively, these findings underscore the importance of early activation of dNK cells in reducing and/or preventing the spreading of pathogens to the fetal placenta. However, we still have to further our understanding on (i) whether dNK cell response can be generalized to other TORCH infections, (ii) whether an exacerbated dNK cell responsiveness and/or changes in the microenvironment would maintain fetal development, (iii) what is the role of "trained" dNK cells in viral confinement and/or spreading, and (iv) how the maternal immune system-dNK cells

as well as others innate and adaptive cells—manages immunity to infections while promoting fetal development.

CONCLUDING REMARKS

Through their wide secretome, dNK cells play a crucial role in the regulation of tissue homeostasis and optimal fetal development. Lessons from *ex vivo* studies demonstrated that these cells are highly plastic and may overcome the negative control of their lytic functions in order to control viral dissemination to the fetal compartment. However, the molecular and functional basis underlying the transition from a poorly cytotoxic status during healthy pregnancy to a fully active one during viral infection are yet to be revealed. It is clear that inhibitory receptors participate actively to the education of cNK cells and to the acquisition of their effector functions.

In addition to being educated and endowed with high functional plasticity, dNK cells can also develop "innate memory-like." As basic knowledge expands, we should envision how to exploit these later developments innate immune memory toward the prevention pregnancy disorders. An example within this notion is the established correlation between the recurrence of miscarriages, FGR or preeclampsia and (i) the KIR repertoire skewing, (ii) KIR/HLA-C match or mismatch, and the (iii) changes in the interval between pregnancies and partners. Another open question is whether dNK cells can expand in response to infected cells and generate a "memory-like" response. Such a memory may generate a natural vaccine against viruses and contribute to the control of viral transmission to the fetus. Beyond pregnancy, understanding mechanisms that regulate the plasticity of dNK cells will be helpful to customize NK cell responsiveness in line with therapeutic requirements.

AUTHOR CONTRIBUTIONS

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

FUNDING

The work was supported by the INSERM, CNRS, and ANRS.

ACKNOWLEDGMENTS

We would like to thank Dr. R. Al-Daccak (UMRS976) and members of NJ-F team for helpful discussions and critical comments on the manuscript.

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

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frontiers in Immunology

ORIGINAL RESEARCH
published: 26 April 2019
doi: 10.3389/fimmu.2019.00877

Endometrial Tumor Microenvironment Alters Human NK Cell Recruitment, and Resident NK Cell Phenotype and Function

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OPEN ACCESS

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: 17 December 2018
Accepted: 05 April 2019
Published: 26 April 2019

Citation:
Degos C, Heinemann M, Barrou J, Boucherit N, Lambaudie E, Savina A, Gorvel L and Olive D (2019) Endometrial Tumor Microenvironment Alters Human NK Cell Recruitment, and Resident NK Cell Phenotype and Function. Front. Immunol. 10:877. doi: 10.3389/fimmu.2019.00877

Endometrial Cancer is the most common cancer in the female genital tract in developed countries, and with its increasing incidence due to risk factors such as aging and obesity tends to become a public health issue. However, its immune environment has been less characterized than in other tumors such as breast cancers. NK cells are cytotoxic innate lymphoid cells that are considered as a major anti-tumoral effector cell type which function is drastically altered in tumors which participates to tumor progression. Here we characterize tumor NK cells both phenotypically and functionally in the tumor microenvironment of endometrial cancer. For that, we gathered endometrial tumors, tumor adjacent healthy tissue, blood from matching patients and healthy donor blood to perform comparative analysis of NK cells. First we found that NK cells were impoverished in the tumor infiltrate. We then compared the phenotype of NK cells in the tumor and found that tumor resident $CD103^+$ NK cells exhibited more co-inhibitory molecules such as Tigit, and TIM-3 compared to recruited $CD103^-$ NK cells and that the expression of these molecules increased with the severity of the disease. We showed that both chemokines (CXCL12, IP-10, and CCL27) and cytokines profiles (IL-1β and IL-6) were altered in the tumor microenvironment and might reduce NK cell function and recruitment to the tumor site. This led to hypothesize that the tumor microenvironment reduces resident NK cells cytotoxicity which we confirmed by measuring cytotoxic effector production and degranulation. Taken together, our results show that the tumor microenvironment reshapes NK cell phenotype and function to promote tumor progression.

Keywords: endometrial cancer, NK cells, Tigit, Tim-3, resident cells, immune checkpoint

INTRODUCTION

Endometrial cancer is the most common cancers of the female genital tract in developed countries. Despite a good survival overall (70% of 5-year survival), prognosis associated with a decreased survival rates (<20% of 5-year survival) are observed in patients with late stages of endometrial cancer. Furthermore, endometrial cancer incidence tends toward increasing as risk

factors risk factor such as obesity and aging are involved. These factors, associated with exogenous estrogen exposure contribute to the spreading of the disease in western countries and might lead to a major public health issue (1, 2).

Histological studies showed that endometrial cancer could be separated in two groups based on histological characteristics. The first group is composed of endometrioid adenocarcinoma, while the second group regroups all the others histology which include carcinosarcoma, clear cell carcinoma, serous adenocarcinoma. Type I endometrial cancers are subdivided following 3 grades, which are associated with the differentiation status of cells ranging from the most differentiated to the less differentiated, which are associated, respectively with a good and a bad prognosis. Type II endometrial cancers are usually associated with a poorer survival than Type I (3, 4).

Endometrial cancers are also separated and classified according to the tumor localization. This anatomical classification designed by the FIGO status is used, along with the histological analysis and the TNM system from the American Joint Committee on Cancer [the extent of the tumor (T), the invasion of lymph nodes (N), the spreading of distant sites (M)] to define the treatment (5). In addition to that, endometrial cancer has been less studied than other tumors such as breast, lung or colorectal cancer, and therefore some aspects of this disease remain poorly understood. This is the case of the Immunology of the endometrial tumor environment. Only few reports show that the immune system is involved in the regulation of endometrial tumor progression. Kondratiev et al. showed that the presence of $CD8^+$ T cells inside the endometrial tumor represents a marker of patient survival (6). Similarly, in another study it was demonstrated that intraepithelial $CD103^+$ $CD8^+$ tumor infiltrating T cells expressed $PD1^+$, were antigen-activated and were associated with improved survival (7).

However, The Cancer Genome Atlas (TCGA) recently sequenced and identified several genomic endometrial cancer patient profiles (8). Indeed, 4 genetic profiles were found and used to stratify the patients with different survival, interestingly the POLE (polymerase ε) mutated profile correlated with the best survival. Some studies showed that this POLE mutated profile is associated with a high $CD8^+$ T cells infiltration, PD-1 expression on TILS, and also $CD4^+$ T cell responses (8–10). This highlights the importance of tumor infiltrating leukocytes in the pathophysiology of endometrial cancer.

Natural Killer cells (NK cells) are cytotoxic innate lymphoid cells that are a major anti-tumoral effector cell type along with $CD8^+$ T cells and γδT cells.

NK cell activation and cytotoxicity relies on a balance between inhibitory and activating signaling. For instance, class I Human Leukocyte Antigens, Ig like transcripts, Tigit (T cell Immunoreceptor with Ig and ITIM domain), inhibitory cytokines (TGF-β, IL-6, IL-32a) provide inhibitory signals for NK cell functions and activation. On the contrary, various activating receptor engagement, such as DNAM1, KIRs, NKp80, NKG2D, and NCRs, along with activating cytokines (IL-15, IL-18, IL-12) lead to an activation of NK cell (11–18). Indeed, activating receptors are triggered by stress-induced, self-molecules, and viral components. In cancers, NK cells are efficient during the elimination phase as they control tumor growth. However, NK cell function is often altered during tumor immune evasion which allows tumor growth and tissue invasion.

Indeed, in colorectal cancer, NK cells infiltration is correlated with a better prognosis and outcome (19–21), in lung cancer NK cells displayed an inhibitory phenotype as there is a downregulation of the expression of the NKp30 and NKG2D, their cytolytic function is also impaired by a reduced production of Granzyme-B (GrzB) (22, 23). Finally, in breast tumors, reports showed that breast cancer cells increase self-tolerance by modifying NK cell phenotype and were unable to repress tumor growth (24).

Therefore, we investigated NK cell profiles in endometrial tumors. For that, we gathered endometrial tumors, tumor adjacent healthy tissue, blood from matching patients, and healthy donor blood to perform comparative analysis of NK cells. First, we found that there were very few NK cells in the tumor infiltrate and that the amount of $CD56^{bright}$ NK cells was increased in the tumor. We then compared the phenotype of NK cells in the tumor and found that tumor resident NK cells (expressing CD103) exhibited more co-inhibitory molecules such as T cell Immunoreceptor with Ig and ITIM domain (Tigit), and T cell immunoglobulin and mucin domain containing 3 (Tim-3) compared to non-resident NK cells. Furthermore, IL-15, a pro-cytotoxic cytokine, was reduced, whereas IL-6, an inhibitor of the STAT-5 pathway and of the NK cell function (25), was increased in the tumor. This led to hypothesize that tumor resident NK cells lost their cytotoxic function. Thus, we tested cytolytic effector production by NK cells and we observed that they were reduced in the tumor, which was confirmed by functional assays. Here we could demonstrate that the endometrial cancer tumor microenvironment is of great influence on resident NK cells as it could reduce their cytotoxic capacity and therefore promotes tumor progression.

MATERIALS AND METHODS

Patients and Ethics

Patients were included in the Paoli Calmettes Institute "GC Bio" clinical trial (NCT01977274) which aims at characterizing gynecological cancers. The GC-Bio protocol inclusion process will last 5 years and the patient's follow up will be done over 10 years. This study has been accepted by the national ethics committee (ANSM, Agence Nationale de Sécurité du Médicament, n°130995B-12 and CPP, Comité de Protection des Personnes, n° CPP 13 62). The registration number of the study is ID-RCB: 2013-A00992-43.

Our cohort includes patients with endometrial cancer prior to any treatment. The lymph nodes invasion assessment was performed using imaging (MRI, CT scan or PET scan) and, when required, pelvic and/or para-aortic lymphadenectomy.

Abbreviations: EC, endometrial cancer; HD, healthy donors; LN, lymph nodes; NK, natural killer; POLE, polymerase-ε; TCGA, The Cancer Genome Atlas; Tigit, T cell Immunoreceptor with Ig and ITIM domain; Tim-3, T cell immunoglobulin and mucin domain containing 3.

Patient blood samples were collected before the initial surgery. A tumoral sample, along with an adjacent non-invaded endometrial tissue sample (assessed by the pathologist macroscopically), were resected during the initial surgery and before any other treatment. The healthy blood was obtained from French Blood Bank (Etablissement Français du Sang-EFS).

Cell Isolation

The tissue samples were weighted, and dissociated manually using scalpels in RPMI 1640 (Gibco). The cells were isolated after filtration on 70 μm then 30 μm filters and centrifuged (300 × *g* for 5 min at 4°C). Tissue supernatants were kept to quantify the cytokines released in the tissue microenvironment. PBMCs were isolated using a Ficoll gradient (Eurobio). Briefly, whole blood was diluted by adding an equal volume of PBS, deposited slowly onto Ficoll media and centrifuged at 800 × *g* for 30 min at room temperature with no break or acceleration. Cells were recovered from the interface with the plasma, washed twice in PBS, then counted and prepared for the experiments. Serum and plasma were also collected and frozen at −80°C before use to allow the quantification of circulating cytokines and chemokines.

Flow Cytometry

Isolated cells were centrifuged, and then stained for 20 min at 4°C in the dark with various mixes of antibodies (listed in **Supplementary Table 1**) in brilliant stain buffer (BD Biosciences), after a wash in PBS, we stained the cells with a viability marker [LIVE/DEAD Aqua (Life Technologies)] for 20 min at 4°C in the dark. For intracellular staining, we used BD Biosciences Cytofix/Cytoperm kit, according to manufacturer's instructions. Briefly, after the extracellular staining, cells were permeabilized in Fixation/Permeabilization solution for 20 min at 4°C, cells were then washed twice in Permwash buffer before intracellular staining during 20 min at 4°C. Appropriate isotype antibodies were used as controls. The entire tube of cells was then acquired on a FACS LSR2 (Becton Dickinson). To assess the absolute cell number we used True-count beads (BD Bioscience). Application settings and sphero rainbow beads (BD Biosciences) were used to ensure reproducible and comparable results between patients and over time. BD DIVA software was used for data acquisition and FlowJo (Treestar) software was used for the analysis.

Functional Assays

Tissue cells (from tumor and non-invaded endometria) were plated in 96 well-plates in RPMI 1640, 10% FCS, 1% of Penicillin/Streptomycin (Gibco), 200 UI/ml of IL-2 (Proleukine) at 37°C with 5% CO_2. After 16 h, we added PMA (Sigma Aldrich, 25 ng/ml), Ionomycin (Sigma Aldrich, 1 μg/ml), GolgiStop (BD Biosciences, 0.4 μl/200 μl), anti-CD107a and anti-CD107b FITC antibodies (BD Biosciences) in the wells and cells were incubated for 6 h at 37°C and 5% of CO_2. Cells were then harvested and stained for both extracellular markers and intracellular cytokines, as described above.

Cytokine and Chemokine Quantification

Human ProcartaPlex Mix & Match assays (eBiosciences/Life Technologies) and the 40 plex Bio-Plex Pro™ Human Chemokine Panel mix were used to test the presence of chemokines and cytokines of interest. The assay was performed according to manufacturer's instructions. Plates were read on the Bio-Plex analyzer (Biorad). We used Bio-Plex Manager (Biorad) to generate standard curve and results, according to manufacturer's instructions. Results were normalized against tissue weight.

Softwares and Statistical Analysis

GraphPad Prism was used to generate graphical results and statistical analyses. Mann-Whitney test (non-parametric *t*-test), or Wilcoxon (paired *t*-test, non-parametric) were used when paired series of tissues were used in the experiment (e.g., same patient adjacent tissue + tumor or tumor from same patient + Ec PBMC). Differences were considered significant with $p \leq 0.05$.

RESULTS

NK Cell Infiltrate Is Altered in the Tumor Microenvironment

The immune infiltrate of tumor and adjacent tissue samples as well as PBMC cell composition was assessed by flow cytometry. After gating alive and singlets cells on the size (FSC) and granularity (SSC), we could identify NK cell subtypes, $CD3^-CD56^{dim}$ cells and $CD3^-CD56^{bright}$ cells (**Supplementary Figure 1**) among the immune cells ($CD45^+$).

To identify the immune infiltrate in the tissue, the number $CD45^+$ cells found per mg of tissue was calculated and we showed that the immune infiltrate in the tumor was higher compared to that of the adjacent tissue (**Figure 1A**). Using the same quantification method, we demonstrated the NK cell infiltration increased along with the $CD45^+$ in the tumor compared to the matching adjacent healthy tissue (**Figure 1B**).

We then described the proportion of NK cells among the $CD45^+$ population. Tumor NK cells represented 6.1 ± 1% of $CD45^+$ cells while in the adjacent tissue they represented 20.4 ± 4% of immune cells (**Figure 1C**). This suggests that the immune infiltrate in the tumor is altered compared to adjacent tissue. Interestingly, blood samples from either patients (EC blood), or healthy donors (HD blood) harbored a similar quantity of NK cells among $CD45^+$ cells (**Figure 1C**). Note worthily, no differences were observed for the profile of NK $CD56^{bright}$ or $CD56^{dim}$ cells between tumor and adjacent tissue, or between blood from patients or healthy donors (**Figure 1D**).

Resident NK Cells Exhibit an Immunoregulatory Phenotype

As NK cells abundance is reduced in tumor compared to healthy adjacent tissue, we wondered if their phenotype might be affected by the tumor microenvironment. For this purpose, we added a CD103 staining to our panel that allowed us to discriminate resident cells and recruited cells. Indeed, $CD56^+CD103^+$ cells represent a population of resident NK cells

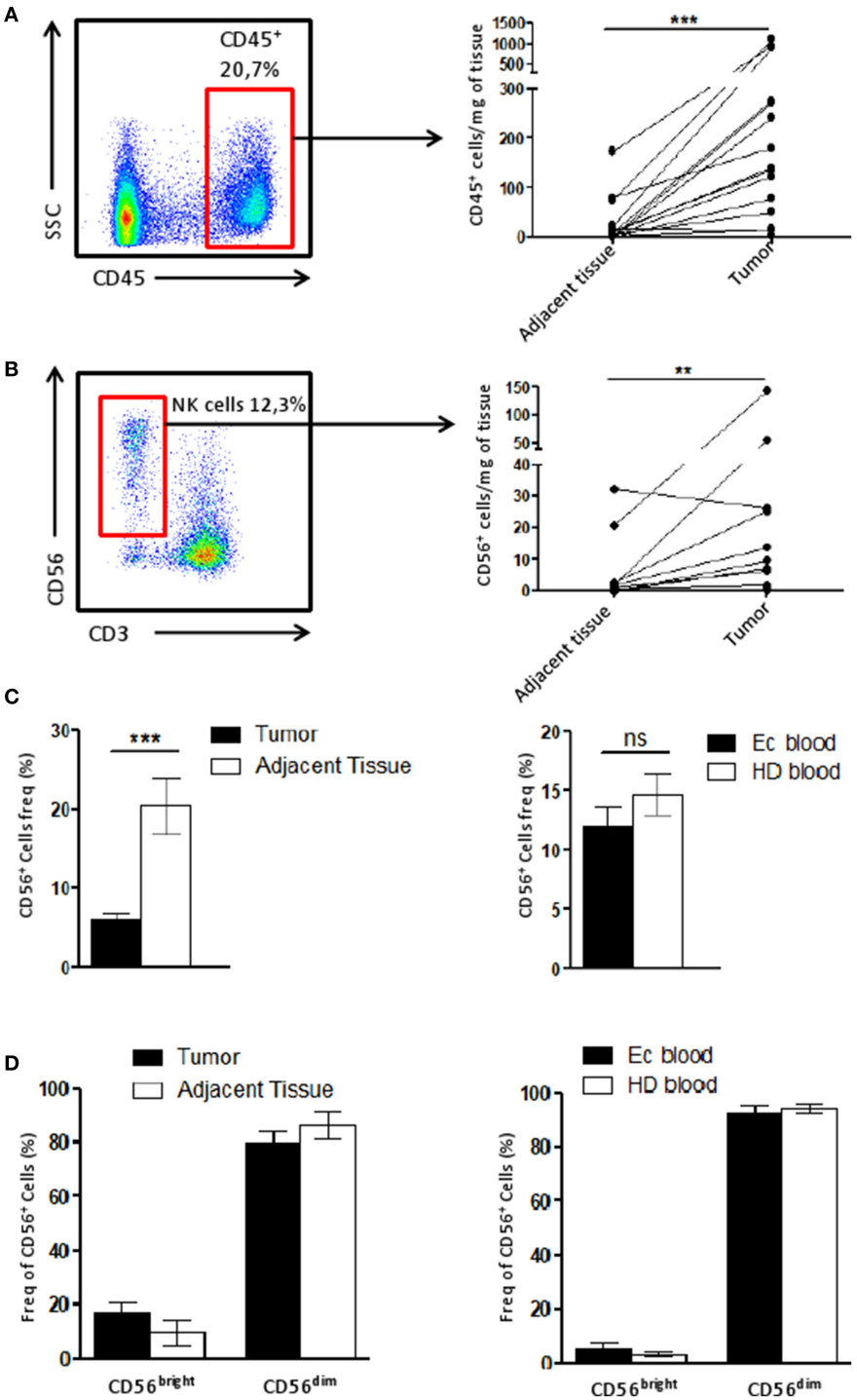

FIGURE 1 | NK cell infiltrate is altered in the tumor microenvironment. **(A)** The number of $CD45^+$ cells recovered per mg of either the tumor or the adjacent tissue ($n = 15$) was plotted here. Briefly, cells were counted by flow cytometry using beads. Each dot-line represents one patient. **(B)** The number of $CD56^+$ $CD3^-$ cells (among $CD45^+$ cells), corresponding to NK cells, recovered per mg of either the tumor or the adjacent tissue ($n = 11$) was plotted here. Each dot-line represents one patient. **(C)** On the left panel, the frequency of NK cells ($CD56^+$ cells) among $CD45^+$ cells in the tumor ($n = 29$), in black, was compared to their frequency in the adjacent tissue ($n = 12$), in white. On the right panel, the proportion of NK cells among PBMCs from either healthy donors patients [HD blood ($n = 13$), in white bar] or endometrial cancer patients [EC blood ($n = 18$), in black bar]. **(D)** $CD56^{bright}$ and $CD56^{dim}$ cells were identified by flow cytometry among the NK cell population in tissues on the left panel (tumor, $n = 13$, in black and adjacent tissue, $n = 6$, in white), and for HD (white bar, $n = 7$) and EC blood (black bar, $n = 4$) on the right panel. Mean ± SEM of different patients' samples, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant ($p > 0.05$).

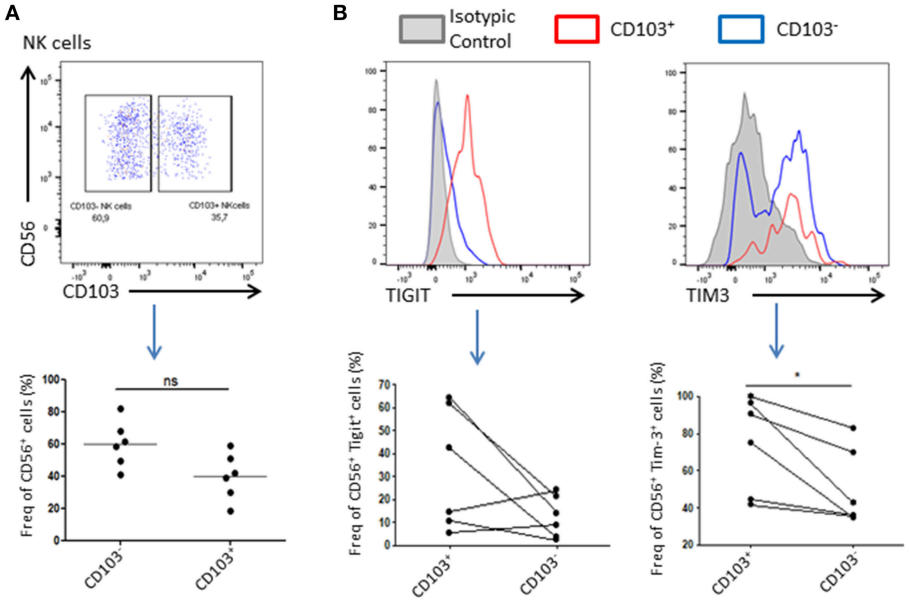

FIGURE 2 | Resident NK cells displayed an inhibitory profile in the tumor. **(A)** Resident NK cells ($CD45^+CD3^-CD56^+$) were identified based on CD103 expression. A representative dot plot of $CD103^+$ and $CD103^-$ NK cells gating is shown. Below, a plot chart of $CD103^+$ and $CD103^-$ NK cells frequency among all $CD56^+$ cells is shown, where each dot represents one patient ($n = 6$). **(B)** Upper panel: Representative graphs of the expression level of Tigit (left panel) or Tim-3 (right panel) in $CD103^+$ NK cells (in red) or $CD103^-$ NK cells (in blue). The isotype control was represented in gray. The positive gate was made using the isotype control (>1% of isotype control staining was considered as positive). Lower panel: The expression of Tigit (left panel) and Tim-3 (right panel) among NK cells $CD103^+$ or $CD103^-$ was assessed and plotted here. Each dot-line represents one patient $n = 6$. $^*p < 0.05$, ns: not significant ($p > 0.05$).

whereas $CD56^+CD103^-$ represent their recruited counterpart (**Supplementary Figure 1**). We found that 40 ± 19% of total NK cells expressed CD103 and were likely to be tumor resident NK cells. Tigit and Tim-3 are usually associated, in cancer, with an exhausted phenotype of immune cells, and weaker immune response as well as anti-inflammatory and anti-cytolytic responses. Indeed, we could observe a trend in Tigit expression, which seemed to increase in $CD103^+$ NK cells, and a significant increase in the expression of Tim-3 in these same cells compared to $CD103^-$ NK cells, suggesting an inhibitory environment prone to abolish the cytotoxic effect of NK cells (**Figure 2B**).

NK Cells Co-inhibitory Molecule Expression Is Higher in Patients With Lymph Node Invasion

Lymph node (LN) invasion in endometrial cancer correlates with advanced tumors stages and therefore, poorer outcome for the patient. As we demonstrated that tumor NK cells showed variability in the expression of the co-inhibitory molecules Tigit and Tim-3 we wondered if their expression was correlated to the severity of the disease. For that, we compared Tigit and Tim-3 expression on tumor NK cells from LN-invaded or uninvaded patients. We showed that NK cells in patient harboring LN invasion had a significant increase in Tigit or Tim-3-expressing NK cells compared to patient with no LN invasion (24.2 ± 6% in LN^+ vs. 4.6 ± 2% in LN^- and 62.8 ± 12% in LN^+ vs. 29.5 ± 5% in LN^-, respectively) (**Figure 3**). Taken together, these results demonstrate that NK cells immunosuppressive phenotype increases with lymph node invasion and therefore, the severity of the disease.

NK Cells Are Shaped by the Chemokines and Cytokines Environment

We then wondered whether the chemical tumoral microenvironment, and more specifically the cytokines and chemokines present in the tissue, could play a role in the NK cell homeostasis. We performed multiplex immuno-assay (Luminex) to assess the presence of inflammatory cytokines involved in the regulation NK cells function, such as TNF-α, IFN-γ, IL-6, IL-15, and IL-1β, in the tumor microenvironment (black bars, **Figure 4A**) and in the healthy adjacent tissue microenvironment (white bars, **Figure 4A**). IL-1β, a pro inflammatory cytokine that participates to both cytotoxic response and tumoral angiogenesis, was the only cytokine that was significantly secreted in higher amount in the tumor (52.3 ± 14.8 pg/ml) compared to the adjacent healthy tissue (4.65 ± 2 pg/ml) (**Figure 4A**). Indeed, nor IL-15 (5.2 ± 1 pg/ml in the tumor vs. 6.2 ± 1.2 pg/ml in the adjacent tissue), TNF-α (62.7 ± 11 pg/ml in the tumor vs. 39.7 ± 9 pg/ml in the adjacent tissue) or IFN-γ (62.7 ± 18 pg/ml in the tumor vs. 93.6 ± 20 pg/ml in the adjacent tissue) were found to be significantly modulated in the tumor compared to the adjacent healthy tissue (**Figure 4A**). The secretion of IL-6, a negative regulator of NK function (25, 26), tended to increase in the tumor rather than in the adjacent tissue as its concentration

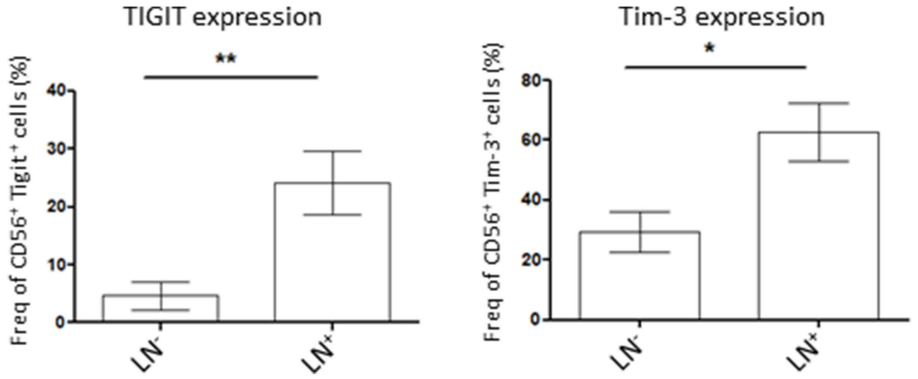

FIGURE 3 | Patient lymph nodes (LN) invasion is associated with higher expression of co-inhibitory molecules in tumor NK cells. Isolated tumor cells were stained for Tigit (left, $n = 7$ for LN^- patients and $n = 5$ for LN^+ patients) or Tim-3 (right, $n = 13$ for LN^- patients and 5 for LN^+ patients) by flow cytometry and their expression was assessed in NK cells ($CD56^+$ cells). Their expression was compared among patient with lymph nodes invasion (LN^+) or not (LN). Mean ± SEM of different patients' samples, $^*p < 0.05$, $^{**}p < 0.01$.

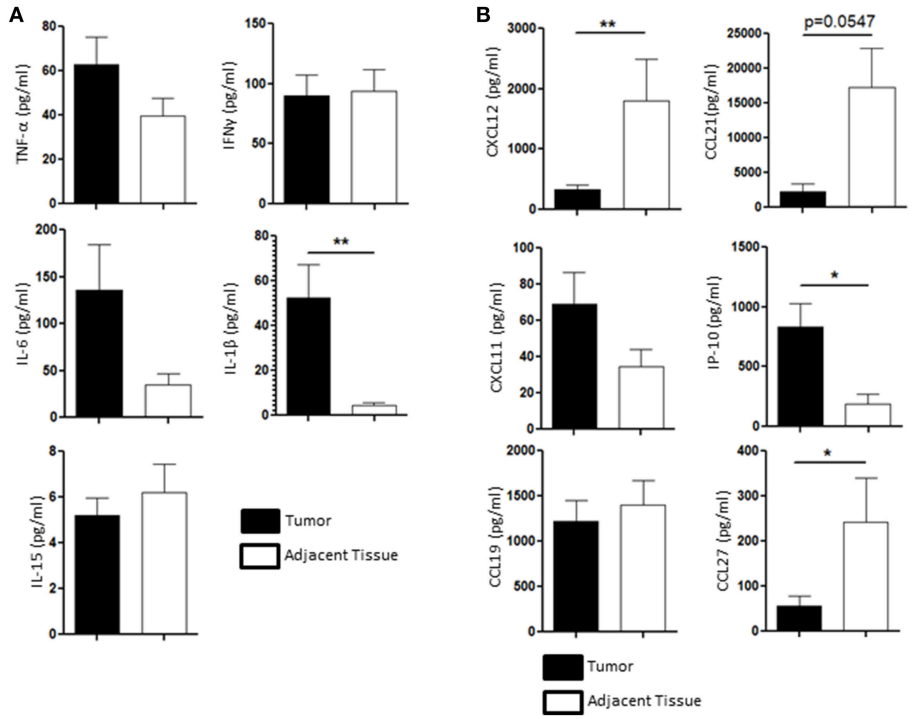

FIGURE 4 | Cytokines and chemokines profile is different between the tumor and the healthy adjacent tissue, regulating NK cells differently. **(A)** The presence of TNF-α, IFN-γ, IL-6, IL-1β, and IL-15 in the supernatant of the tissue dissociation was assessed by Luminex and compared between the tumor (black bars, $n = 11$) and the healthy adjacent tissue (white bars). **(B)** The presence of the following chemokines in the tumor microenvironment (black bars) was compared to the ones found in the healthy adjacent tissue (white bars, $n = 11$): CXCL12, CCL21, CXCL11, IP-10, CCL19, and CCL27. Mean ± SEM of different patients' samples, $^*p < 0.05$, $^{**}p < 0.01$.

reached 135.7 ± 50 pg/ml in the tumor compared to 34.9 ± 11 pg/ml in the adjacent healthy tissue.

We also investigated the presence of CXCL12, CCL21, CXCL11, IP-10 (or CXCL10), CCL19, and CCL27, which participate to the recruitment of specific NK cell populations to the tumor site. CCL19 and CXCL11 were not differentially secreted in the tumor (1221.47 ± 250 pg/ml and 69.1 ± 9 pg/ml, respectively) compared to the adjacent tissue (1399.5 ± 300 pg/ml and 34.6 ± 5 pg/ml, respectively). IP-10 (also known as CXCL10) was found to be enriched in the tumor (835.9 ± 210 pg/ml) compared to the adjacent healthy tissue (188.1 ± 100 pg/ml). We also found that CXCL12, CCL27, and CCL21 were found in higher quantities in the healthy adjacent tissue (1795.6 ± 700 pg/ml, 242.9 ± 95 pg/ml, and 17285.9 ± 6000

pg/ml, respectively) than in the tumor (325.8 ± 72 pg/ml, 56.7 ± 20 pg/ml, and 2313.6 ± 1,000 pg/ml, respectively) (**Figure 4B**). All together, we showed that both cytokine and chemokine production in the endometrial tumors may participate to the alteration of NK cell function, as IL-6 and IL-1β are increased in the tumor, and recruitment, as chemoattractants CCL27, CXCL12, and CCL21 are significantly reduced in the tumor.

NK Cell Function Is Altered in Endometrial Cancer

Finally, we assessed the capacity of tumoral NK cells to mount an efficient anti-tumoral response through the production of known cytolytic mediators such as GrzB (27) and IFN-γ, which can be used as cytolytic function markers (28). For that, we incubated dissociated tumors with IL-2 overnight and then stimulated them with PMA/Ionomycin to induce an immune response by measuring IFN-γ, TNF-α, GrzB production and CD107 expression (a surrogate for degranulation). Here, we could show that tumor NK cells are less responsive than healthy adjacent tissue NK cells. Indeed, tumoral NK cells produced less IFN-γ, TNF-α and GrzB and were less potent at degranulation in 4 out of 5 patients tested (**Figures 5A,B**). Taken together, these results strongly suggest that tumoral NK cells are less efficient than adjacent tissue NK cells to mount an anti-tumoral response in endometrial cancer.

DISCUSSION

Here we showed that NK cells were impoverished in the tumor immune cells population, compared to adjacent healthy tissue. Interestingly, resident NK cells also seemed to harbor inhibitory and exhaustion hallmarks such as high Tigit and Tim-3, which are associated with advanced diseases. The study of the tumoral NK cells function revealed a trend showing that NK cells seemed to be deficient at mounting a cytolytic immune response. We also showed that some of these features could depend on the surrounding microenvironment of NK cells, such as specific chemokine presence in the tumor. Indeed, CCL27 and other NK chemoattractant molecules are less produced by the tumor microenvironment than by the adjacent tissue. We showed that NK recruitment in the tumor is altered compared to the one in the adjacent tissue. Taken together these results showed that NK cells biology is deeply impaired by the tumor.

The NK cell population is one of the main anti-tumor actors of the immune system. These cells are often impaired both phenotypically and functionally in solid tumors such as breast cancer (24), melanoma (29), in colorectal cancer (30), in lung cancer (23), and various other cancers (31–33). Here, we showed that the proportion of NK cells is lower among the immune infiltrate in endometrial tumors than in the adjacent healthy tissue. The reduction of the cytotoxic immune cells infiltration, and more particularly of NK cells, is usually associated with an immunosuppressive effect of the tumor, a poorer outcome and has been widely described in solid tumors (21, 34–36).

The recruitment of immune cells, including NK cells is regulated by tissue produced chemokines, which, in our study, may explain the lack of NK cells in the tumor. Indeed, we could find that the production of various chemokines was modified in the tumor compared to the adjacent healthy tissue. IP-10, also known as CXCL10, is a chemoattractant for NK cells, and can activate NK cells leading to the lysis of tumor cells (37–39). Interestingly, we showed here that IP-10 is strongly produced in the tumor compared to the adjacent tissue, suggesting that an immune response is occurring in the tumor. Though, considering the results we observed from the functional tests, it may not be sufficient to recruit NK cells and to maintain an efficient NK cell-driven cytolytic response. CCL27, is also a NK cell chemoattractant molecule, and is, in endometrial cancer, less produced in the tumor compared to the healthy adjacent tissue. Similarly, CCL21, which has been described to enhance the recruitment of immune cells in the tumor and to participate to the establishment of a potent immune cellular response (40–42), is significantly reduced in the tumor microenvironment compared to the adjacent tissue. The lower levels of NK cell chemoattractants (CCL27, CCL21) in the tumor microenvironment may participate to the immunosuppressive mechanism of the tumor by limiting cytolytic cell recruitment.

The CXCL12-CCR4 axis has been described to promote metastasis in various cancers and should be considered as a potent target to block the disease progression (43, 44). This axis does not seem to be related to disease progression in endometrial tumors as CXCL12 is poorly secreted in the tumor microenvironment. However, CXCL12 is also required for the NK cell recruitment, and its weak production in the tumor may participate to the impoverishment of NK cells in the tumor (41). The recruitment of potent immune cells is one of the first steps of mounting a complete anti tumoral immune response. We showed here that the tumoral microenvironment could suppress the recruitment of immune effector cells by altering the production of chemokines compared to the adjacent healthy tissue.

Another important component of the tumor microenvironment is the production of cytokines. In this study we analyze the production of various pro-inflammatory cytokines such as TNF-α, IL-15, IL-6, IL-1β, and IFN-γ. While we did not see any difference of TNF-α, IL-15, and IFN-γ secretion between the tumor and the adjacent healthy tissue, we showed a trend with a higher production of IL-6 in tumor. IL-6 is known to be a pro-inflammatory cytokine that can act on several immune cells and biological features (45). Its presence in the tissue could be related to an immune response. However, several reports showed that IL-6 is also playing a pro-tumoral role (by promoting the angiogenesis, the tumor progression and metastasis) and can be linked to a bad prognosis in patients (46–48). The IL-6/JAK/STAT3 pathway is also known to negatively regulate NK cell cytolytic response, via the inhibition of the STAT5 pathway, to promote the progression of tumors by inducing a chronic inflammation (46, 49). IL-1β was also found to be highly produced in endometrial tumors. This cytokine is a hallmark of inflammation and, similarly to IL-6, plays a dual role in the tumor immunology. Indeed, it participates to both

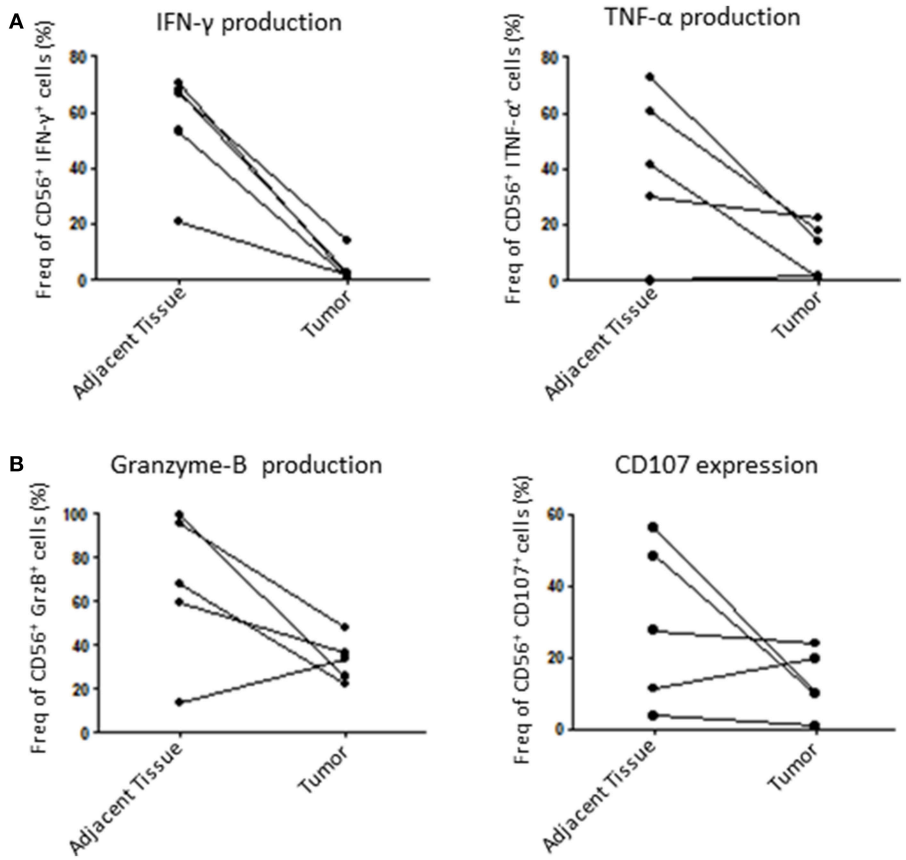

FIGURE 5 | NK cells function is altered in the tumor compared to adjacent tissue. Isolated cells from either the tumor or the matching adjacent tissue were cultured overnight at 37°C. They were then stimulated with PMA/Ionomycin for 6 h and stained for intracellular cytokines. **(A)** We compared the percentage of intracellular expression of IFN-γ and TNF-α, or, **(B),** Granzyme-B (GrzB) and CD107 in NK cells. Each dot/line represents one patient $n = 5$.

anti-tumoral, by contributing to tissue inflammation, and pro-tumoral by enhancing angiogenesis and spreading of the disease (50–53). Therefore, the elevated production of IL-6 and IL-1β in the endometrial tumors might participate to local inflammation as well as cytotoxic effector inhibition and disease progression.

Considering the anti cytolytic effect of the tumor microenvironment, we investigated tumor NK cell phenotype and function and we could find that they were deeply altered. Tim-3 and Tigit are immune inhibitor checkpoints that can suppress the cytolytic activity of NK cells. They were reported to be expressed on immune cell membranes in cancer, thus dampening the anti-tumoral response (54). In this study we showed for the first time, that tumor resident NK cells ($CD103^+$) express Tigit and Tim-3, while recruited NK cells ($CD103^-$) do not. This suggests that resident immune cells were more exhausted, while circulating NK cells could still harbor classical phenotype, therefore, being activated and mount an efficient immune response. Interestingly, we showed that advanced stages of endometrial cancers (which correspond to patient with LN invasion) correlates with a higher exhaustion of NK cells through the expression of Tigit and Tim-3. The increasing expression of such exhaustion markers in advanced stages of the disease makes sense as the immune response is highly inhibited, while, in early stages of the disease, immune effectors are still efficient, and thus, are able to mount an anti-tumoral response. Interestingly, it has been shown in melanoma that NK cells found in the LN metastasis showed a reduced cytotoxicity suggesting an altered function (55). Our data seem to reinforce this idea of altered phenotype or/and function of NK cells along with advance disease.

We also demonstrated that tumor NK cells had reduced production of GrzB suggesting an impaired cytolytic function compared to adjacent healthy tissue NK cells, which could correlate with their exhausted phenotype. Therefore, therapeutic strategies aiming at blocking Tigit and/or Tim-3 signals in advanced stages of cancer seem of interest, and are currently being tested in various clinical trials in association with anti-PD1 or anti-PDL1 (clinical trials: NCT03119428 and NCT03563716) and alone in phase I (clinical trial: NCT03628677). Previous reports already showed that such a strategy would help to restore a potent function of the exhausted NK cells (56, 57). Targeting these molecules in endometrial cancer could therefore, be useful to develop new therapies and restore the immune response.

Altogether we characterized the NK cells found in the tumor microenvironment of endometrial, for the first time and we

showed that resident NK cells harbored an inhibited phenotype, with an impaired function. The inhibition of the NK cells response and the NK cells phenotype also seemed to be related with an aggravation of the disease. The secreted factors such as chemokines and cytokines in the tumor could participate in the establishment or not of NK cells response and we demonstrated here an alteration of their production profile in the tumor compared to the adjacent healthy tissue. The low infiltration of NK cells among the tumor immune cells should be explored and more studies on chemokines and other attracting factors could explain this. Further studies are also required to better characterize the expression of various activating and inhibiting receptors of NK cells which have been reported to be dysregulated in various solid tumors and to identify more clearly which factors are able to shape and inhibit NK cells in the tumor. Here we described and identified potential molecules and functions that were impaired in tumoral NK cells, highlighting new targets to be studied in order to restore the NK cells function in tumor.

ETHICS STATEMENT

Patients were included in the Paoli Calmettes Institute GC Bio clinical trial (NCT01977274) which aims at characterizing gynecological cancers. The GC-Bio protocol inclusion process will last 5 years and the patient's follow up will be done over 10 years. This study has been accepted by the national ethics committee (ANSM Agence Nationale de sécurité du Médicament, n° 130995B-12 and CPP, Comité de Protection des Personnes, n°CPP 13 62). The registration number of the study is ID-RCB: 2013-A00992-43. Written informed consents were obtained from each patients that were included in this study. The healthy blood was obtained from French Blood Bank (Etablissement Français du Sang - EFS).

AUTHOR CONTRIBUTIONS

CD and LG conceived and planned the experiments. CD, NB, and LG carried out the experiments, CD, LG, AS, and DO contributed to the interpretation of the results. MH, JB, and EL provided patient samples. CD and LG wrote the manuscript with support from DO. CD, LG, EL, and DO conceived the original idea. DO and EL supervised the project.

FUNDING

This work was supported by grants from Genentech. CD was funded by INSERM transfert and Roche/Genentech. LG was supported by an ARC grant for young scientist.

ACKNOWLEDGMENTS

The authors thank Philippe Livrati and Sylvaine Just-Landi for excellent technical assistance and help for processing the blood samples; The Departments of Surgical Oncology 2, Pathology and the Clinical Research Department at the Institut Paoli-Calmettes as well as the patients for their contribution to this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00877/full#supplementary-material

Supplemental Figure 1 | NK cell gating strategy within tissue or blood. **(A)** The gating strategy for tumoral NK cells is shown for one representative patient. **(B)** The gating strategy for blood circulating NK cells is shown for one representative patient.

Supplemental Table 1 | Antibodies used in this study.

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Conflict of Interest Statement: The authors declare that this study received funding from Genentech. The funder had the following involvement with the study: Genentech provided a research grant and funded CD's salary. CD was funded by INSERM transfert and Roche/Genentech. DO is a cofounder and stakeholder of Imcheck Therapeutics. DO has licensed patents to Glaxo-Smith Kline, Janssen, and Imcheck Therapeutics. DO has received research grants from Genentech, GSK, Innate Pharma and Imcheck Therapeutics.

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

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frontiers in Immunology

REVIEW
published: 30 April 2019
doi: 10.3389/fimmu.2019.00946

Hepatic Natural Killer Cells: Organ-Specific Sentinels of Liver Immune Homeostasis and Physiopathology

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The liver is considered a preferential tissue for NK cells residency. In humans, almost 50% of all intrahepatic lymphocytes are NK cells that are strongly imprinted in a liver-specific manner and show a broad spectrum of cellular heterogeneity. Hepatic NK (he-NK) cells play key roles in tuning liver immune response in both physiological and pathological conditions. Therefore, there is a pressing need to comprehensively characterize human he-NK cells to better understand the related mechanisms regulating their effector-functions within the dynamic balance between immune-tolerance and immune-surveillance. This is of particular relevance in the liver that is the only solid organ whose parenchyma is constantly challenged on daily basis by millions of foreign antigens drained from the gut. Therefore, the present review summarizes our current knowledge on he-NK cells in the light of the latest discoveries in the field of NK cell biology and clinical relevance.

Keywords: tissue immunity, liver, Natural Kill cell, homeostais, homeostasis

OPEN ACCESS

Edited by:
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Daniela Pende,
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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *01 February 2019*
Accepted: *12 April 2019*
Published: *30 April 2019*

Citation:
Mikulak J, Bruni E, Oriolo F, Di Vito C and Mavilio D (2019) Hepatic Natural Killer Cells: Organ-Specific Sentinels of Liver Immune Homeostasis and Physiopathology. Front. Immunol. 10:946. doi: 10.3389/fimmu.2019.00946

INTRODUCTION

The liver is the largest solid organ in our body receiving every day more than 2,000 liters of blood from dual blood supply. Nearly 80% of blood derive from the gastrointestinal tract via the portal vein, thus being constantly filled of large amounts of foreign antigens. The remaining 20% of blood is supplied from the hepatic artery that together with the portal vein terminates into the capillary system of the liver, sinusoids, and leaves liver parenchyma through the hepatic vein.

This large inflow of antigens makes the liver an important immunological organ in which a unique microenvironment shapes both innate and adaptive immune responses in order to maintain a correct balance between immune tolerance and immune activation (1, 2). Dysregulation of immune cells in the liver is critical in the pathogenesis of several hepatic diseases, including viral hepatitis, autoimmune disorders and tumors. Liver immune compartment consist in diverse innate populations such as Natural Killer (NK) cells, Natural Killer T (NKT) cells, gamma delta (γδ) T cells, and adaptive lymphocytes, such as αβ T cells and B cells (1, 3). On the other hand, liver parenchyma is composed by hepatocytes that represent two-thirds of the total liver cells. Other non-parenchymal cells include liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) (i.e., liver-resident macrophages), cholangiocytes, biliary cells, and hepatic stellate cells (HSCs) (3).

Among the immune compartment, hepatic NK (he-NK) cells that contain both liver resident (lr-NK) or either transient through the adult liver conventional NK (cNK) cells, are particularly

abundant and can account up to 50% of total hepatic lymphocytes (**Figure 1**). These innate immune effectors play key roles in order to retain a certain degree of unresponsiveness to "non-self" antigens, while are ready to attack and eliminate the true dangers to the host (2, 4, 5). Since their discovery in the early 1980s, NK cells have been valued for rapid recognition and clearance of viral-infected, tumor-transformed and stressed cell targets in the absence of antigen specificity (6). Cytotoxicity and interferon(IFN)-γ production represent the main effector-functions of mature NK cells and are controlled by a dynamic balance exerted by an array of inhibitory (iNKRs) and activating (aNKRs) receptors differently expressed on the cell surface (7). The dominant mechanism regulating the priming and activation of resting NK cells is based on the engagement of several iNKRs that bind several alleles of the major histocompatibility complex class I (MHC-I) expressed on the surface of autologous cells. This recognition spares from NK cell killing all "self" targets, thus ensuring a perfect NK cell tolerance toward our own cells. These iNKRs include inhibitory Killer Ig-like receptors (iKIRs) that recognize classical MHC-I alleles and the C-type lectin like receptor NKG2A that forms a heterodimer with the CD94 molecule (CD94/NKG2A), binding HLA-E, a non-classical MHC-I complex (8, 9). Either the decreased expression or the absence of MHC-I on target cells triggers NK cell killing, a phenomenon known as "missing-self hypothesis," via the employment of aNKRs that, in turn, bind their putative ligands expressed on viral-infected, malignant or stressed cells. The Natural Cytotoxicity Receptors (NCRs) NKp30, NKp46 and NKp44, the C-type lectin receptors NKG2D and CD94/NKG2C heterodimer, DNAM-1, SLAM family receptors such as 2B4, and activating KIRs (aKIRs) are the main aNKRs inducing NK cell cytotoxicity (7, 10, 11).

Under homeostatic conditions, human circulating cNK cells represent about 5–15% of circulating lymphocytes and are subdivided into two main subsets defined on the basis of their differential expression of CD56 and CD16, namely $CD56^{bright}/CD16^{neg}$ ($CD56^{bright}$) and $CD56^{dim}/CD16^{pos}$ ($CD56^{dim}$) NK cells (12). It is largely accepted that $CD56^{bright}$ NK cells are the precursors of the more mature $CD56^{dim}$ NK cells, however, the developmental relationship between the different types of human NK subsets has not been finally clarified (13, 14). In this context, a recent study proposes for two $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets distinct ontologies (14). The low amounts of intracellular cytotoxic granules (i.e., Perforin and Granzymes A-B) parallel the poor cytotoxic potential of $CD56^{bright}$ NK cells that are also unable to perform the antibody (Ab)-dependent cellular cytotoxicity (ADCC) in line with their undetectable expression of CD16 (12). On the other hand, this latter subpopulation exerts important regulatory functions through secretion of chemokines and pro-inflammatory cytokines [i.e., IFN-γ, Tumor Necrosis Factor(TNF)-α] in response to different stimuli [i.e., interleukin (IL)-1β, IL-2, IL-12, IL-15, and/or IL-18] delivered by surrounding cells at tissue sites [i.e., macrophages, dendritic cells (DCs), and T lymphocytes] (6, 15, 16). $CD56^{bright}$ NK cells likely give rise to terminally differentiated $CD56^{dim}$ NK cells as they represent the largest population in blood (up to 90%), mostly express lower levels of NKp46 and CD94/NKG2A and higher amounts of iKIRs and CD94/NKG2C heterodimer (17). This population exerts both high cytotoxicity and ADCC given its high constitutive expression of CD16 in response to activation by IL-12, IL-15, and IL-18 (18). Moreover, it has been reported that $CD56^{dim}$ NK cells can also rapidly produce IFN-γ in response to stimulation with IL-2 and/or IL-15 (19). Hence, $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets fulfill distinct roles in immunity, with the first one serving more as immune-modulator and the second population acting mainly as cytotoxic effector. An additional NK cell subset identified on the basis of CD56 and CD16 surface expression is represented by anergic $CD56^{neg}/CD16^{pos}$ ($CD56^{neg}$) cells that are present at very low frequency under physiologic conditions, while pathologically expanded during the course of several disorders, such as viral infections and autoimmune diseases (20, 21). More recently, an unconventional population of $CD56^{dim}/CD16^{neg}$ ($unCD56^{dim}$) NK cells has been described. This latter subset can exert potent cytotoxicity and is extremely rare in healthy donors, while representing the main cNK cell population in the first weeks after haploidentical hematopoietic stem cell transplants (haplo-HSCT). This high frequency of $unCD56^{dim}$ NK cells in aplastic patients affected by hematologic malignancies and receiving haplo-HSCT suggests novel pathways of NK cell ontogenesis and differentiation that are currently being investigated (22, 23).

The distribution of NK cell subsets in human tissues is very peculiar and differs from what we observe in peripheral blood. Notably, $CD56^{dim}$ NK cells are found in high amounts in bone marrow, lung, spleen, subcutaneous adipose tissue and breast tissue. Instead, $CD56^{bright}$ NK cells are present at high frequency in lymph nodes, gut, liver, uterus, visceral adipose tissue, adrenal gland and kidney (24, 25). Hence, other than the phenotypic and functional diversities of these latter two subsets in peripheral blood, the spectrum of human NK cell populations in tissues is much broader and likely depends on specific imprinting given by local microenvironments and by the chronic exposure to foreign antigens/inflammatory stimuli.

Here, we review our current knowledge in regard to he-NK cells with a particular focus on the breadth and generation of he-NK cell heterogeneity, under both homeostatic conditions and during the course of liver diseases.

HETEROGENEITY OF LIVER RESIDENT NK CELLS

The liver is populated by both transient cNK cell subsets and lr-NK cells that are phenotypically and functionally distinguished (**Figure 1**). The first identification of tissue resident NK cells occurred in murine livers and rapidly expanded in other tissues (26). Lr-NK cells soon displayed heterogenous phenotypic profiles in different species with unique anatomical identities that reflect the impact of this peculiar tissue-niche in generating either cytotoxic or tolerogenic lymphocytes.

Murine lr-NK cells carry a $CD49a^{pos}/DX5^{neg}$ phenotype that differs from $CD49a^{neg}/DX5^{pos}$ cNK cells in mouse (27, 28). It

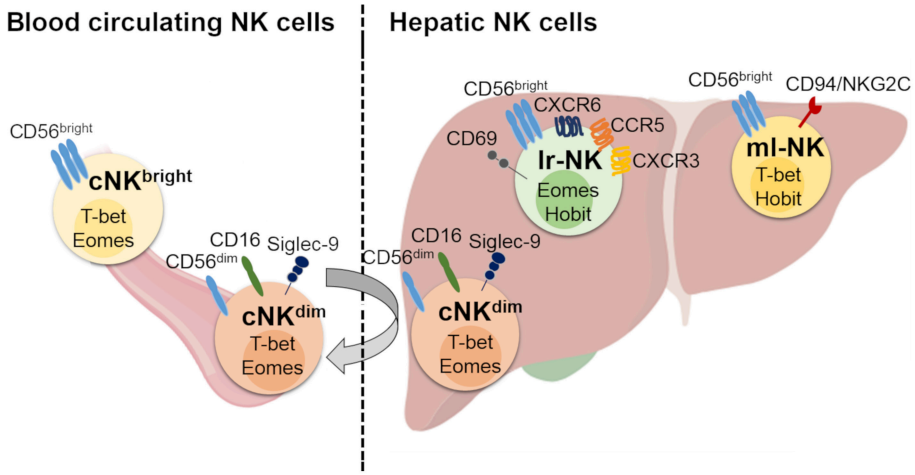

FIGURE 1 | Human hepatic and conventional NK cell subsets, distribution, and phenotype. Human blood circulating conventional NK (cNK) cells contain two main subsets: CD56^{dim} (cNK^{dim}) and CD56^{bright} (cNK^{bright}) cells. Human hepatic NK (he-NK) cell compartment contains: liver resident NK (lr-NK) cells, memory-like NK (ml-NK) cells and transient conventional NK (cNK) cells mainly represented by recirculating cNK^{dim} cells through the liver blood system (gray arrow). Lr-NK and ml-NK cell subsets show transcriptional and phenotypic differences compared to the conventional cNK cell subsets.

is still unclear how the development and differentiation of lr-NK cells is regulated, but this latter subset seems to be more terminally differentiated as it lacks or has decreased expression of CD11b, Ly49, CD43, and KLRG1 (i.e., surface markers present on mature cells) compared to murine cNK cells (27). Several experimental findings indicated that lr-NK and cNK cells likely develop from separated innate lymphoid cell (ILC) lineages (29). Moreover, a common ILC progenitor subset able to differentiate in lr-NK cells but not in cNK cells has been described (30). Indeed, lr-NK and cNK cells rely on different transcriptional factors for their development, since the presence of T-bet deficiency in mice is associated with the depletion of lr-NK cells, while Eomes is critical for the maintenance of cNK cell homeostasis (**Table 1**) (31, 32). More recently, the new transcription factors Hobit and the aryl hydrocarbon receptor (AhR) have been reported to induce the development of different tissue-resident NK cells, including lr-NK (33, 34).

Human lr-NK cells were first described in 1976 and were originally called "pit cells." Only later, they were defined as highly cytotoxic NK cells resident in the hepatic sinusoids (35, 43, 44). Differently from murine and their human counterparts in peripheral blood, CD56^{dim} and CD56^{bright} NK cells are present at similar frequencies in liver and the latter subset likely corresponds to the murine CD49a^{pos}/DX5^{neg} lr-NK cells, as they both share the same transcriptional factor T-bet and are negative for Eomes (**Table 1**) (28). However, CD49a^{pos}/CD56^{bright} lymphocytes account for only 3% of all humans he-NK cells and do account for all CD56^{bright} lr-NK cells. In this regard, it is well known that several murine NK cell markers are not phylogenetically conserved in their human counterparts and this largely explains the absence of a phenotypic match between murine and human lr-NK cells. Only recently, the phenotype of human CD56^{bright} lr-NK cells has been better characterized by disclosing their constitutive expression of the chemokine receptors CXCR6 and CCR5 and of the tissue-residency marker CD69 (28, 45, 46). As a matter of fact, these 3 surface markers are absent on CD56^{dim} he-NK cells. Hence, the CD56^{bright}/CCR5^{pos}/CXCR6^{pos}/CD69^{pos} phenotype identifies human lr-NK cells that also appear to be more heterogeneous in their development pathways compared to murine counterparts as they express high levels of Eomes transcripts rather than T-bet (36, 45, 46). Indeed, only those 3% of human CD49a^{pos}/CD56^{bright} lr-NK cells resulted positive for T-bet, while the transcription factor Hobit was found positive on all CD56^{bright} lr-NK cells (28, 37).

Very little is known about the mechanism(s) regulating both recruitment and retention of NK cells in the liver. Within the hepatic microenvironment, NK cell interactions with LSECs certainly play a key role, as the masking of CD2, CD11a, CD18, and CD54 (ICAM-1) with neutralizing monoclonal Abs (mAbs) block their recruitment to the liver (38). Moreover, the constitutive high surface levels of CXCR3, CXCR6, and CCR5 on lr-NK cells are important in the retention of these hepatic lymphocytes. Indeed, the engagement of these chemokine receptors following the binding with their cognate ligands (i.e., CCL3, CCL5 and CXCL16, respectively) expressed by cholangiocytes, LSECs, hepatocytes, and KCs, is associated with liver homing (45, 47). Sinusoidal endothelial cells also express VAP-1 that, in turn, binds Siglec-9 expressed on cNK cells, thus mediating their migration to the liver (47, 48). This latter pathway seems a mechanism restricted to hepatic trafficking, since VAP-1^{pos} cNK cells do not express L-selectin (CD62L) and CCR7 receptor required for homing in secondary lymphoid tissues (27).

Another important question is whether he-NK cells stably reside in the liver or recirculate through liver sinusoids. Experimental evidence obtained from human transplanted liver revealed that Eomes^{high} lr-NK cells can persist for decades, thus further supporting the idea that these cells represent a long-lived

TABLE 1 | Comparison of NK cell subsets in humans and mice.

Species	Subset	Phenotype	Precursors	Transcription factors	References
Mouse	cNK	$CD49a^{neg}DX5^{pos}$	$Lin^{neg}CD27^{pos}CD107^{neg}CD244^{pos}CD122^{pos}IL7Ra^{neg}$	Eomes	(28–33)
Mouse	lr-NK/ml-lr-NK	$CD49a^{pos}DX5^{neg}$	$PLZF^{pos}Lin^{neg}IL7Ra^{pos}cKit^{pos}a4b7^{pos}$	T-bet, PLZF, Hobit, AhR	(28–35)
Human	cNK^{dim}	$CD56^{dim}CD16^{pos}$	$CD56^{bright}CD94^{pos}NKp80^{pos}CD16^{neg}CD57^{neg}$	T-bet, Eomes	(13, 14)
Human	cNK^{bright}	$CD56^{bright}CD16^{neg}$	$Lin^{neg}CD34^{neg}CD117^{pos}CD94^{neg}CD16^{neg}$	T-bet, Eomes	(13, 14)
Human	lr-NK^{bright} ml-NK^{bright}	$CCR5^{pos}CXCR6^{pos}CD69^{pos}$ $CXCR6^{pos}CD94/NKG2C^{pos}$	$CD56^{bright}Eomes^{low}cNK^{bright}$ lr-$CD49a^{pos}CD56^{bright}$	$Eomes^{high}$, Hobit T-bet, Hobit	(28, 29, 36–42)

tissue-resident subset (49). In addition, $CD56^{bright}$/$Eomes^{low}$ cNK cells recruited to the liver have the potential to become $CD56^{bright}$/$Eomes^{high}$ NK cells. This last piece of data suggests that cNK cells can also represent precursors of their liver-resident counterparts, although the associated mechanisms involved in this process have not yet been disclosed (49). Interestingly, the administration of an anti-α4β1 and -α4β7 integrins mAb (i.e., natalizumab) in patients with multiple sclerosis is associated with an remarkable increased frequency of NK cells in peripheral blood, thus indicating that they can migrate across the tissue endothelial barriers including the hepatic ones (39, 50). However, it is important to highlight that he-$CD56^{dim}$ NK cells are transcriptionally and phenotypically similar to their circuiting counterparts and this evidence indicates that they likely recirculate through the liver blood system without being retained in the organ. This is not the case for lr-$CD56^{bright}$ NK cells that are also transcriptionally different from their homologs in the peripheral blood (45).

The liver is also home of peculiar and newly identified lr-NK cells endowed with unique adaptive traits and showing hapten-specificity (51, 52). The phenotype of these so-called "memory like" NK (ml-NK) cells in mice is $CD49a^{pos}$/$DX5^{neg}$ and matches with murine lr-NK cells (51, 52). It has been also shown that $CXCR6^{pos}$ he-NK cells can retain an unconventional immunologic memory versus viral antigens including inactivated vesicular stomatitis virus (VSV), human immunodeficiency virus (HIV) and influenza (53, 54). Most of the studies characterizing human ml-NK cells focused their investigation on cytomegalovirus (HCMV) infection, that induces the expansion of "specific" $CD94/NKG2C^{pos}$ NK cells able to produce a higher amount of IFN-γ when these "adaptive" NK cells are re-challenged with the same virus (40, 41, 55). Interestingly, it has been reported that the small subset of $CD49a^{pos}$/$CD56^{bright}$ lr-NK cells is characterized by a clonal-expansion of NK cells expressing CD94/NKG2C heterodimer (28). However, the existence of a specific viral-antigen recognized by a given NKRs expressed on human ml-NK cells is still being debated and never formally demonstrated. This is indeed a very hot research topic in the field of NK cell homeostasis that requires further experimental evidence and investigations.

Lr-NK cells are characterized by different features and can kill different targets as well as secrete cytokines. They have higher intracellular amounts of lytic granules (i.e., Granzymes and Perforin) and stronger cytotoxic potentials compared to their circulating counterparts (2, 42, 56). In particular, lr-NK cells are characterized by higher constitutive expression of TRAIL and FasL compared to cNK cells, thus suggesting that the tissue resident subset employs different mechanisms to eliminate targets (i.e., apoptosis) (57). Moreover, both cNK cells and lr-NK cells are able to secrete large amount of IFN-γ, but the latter population is much more efficient in the production of TNF-α and Granulocyte-macrophage colony-stimulating factor (GM-CSF), and in case of murine lr-NK cells IL-2, all key players in inflammatory responses at tissue sites (31, 32, 57).

The need of keeping an optimal degree of immune-tolerance vs. foreign antigens while ensuring a correct immune-surveillance against potential threats (i.e., infections, tumors, aberrant inflammation, and autoimmunity) certainly explains the particularly high level of heterogeneity and complexity of he-NK cells. Indeed, these features are peculiar of the liver microenvironment that is able to induce the non-conventional "long-lived" and "memory like" innate immune effectors also within NK cell compartment.

NK CELLS IN LIVER TOLERANCE AND HOMEOSTASIS

Human liver developed a high degree of immune tolerance as demonstrated by the clinical evidence indicating that liver allografts are less likely to be rejected than other transplanted organs (58). Several actors play different and fundamental roles in the maintenance of liver tolerogenic NK cells (**Figure 2**). KCs produce high doses of IL-10 which was observed to be critical in the control of mice intrahepatic NK cell-mediated alloreactivity (59). Indeed, an impaired ability of liver macrophages to produce this anti-inflammatory cytokine boost the IFN-γ-dependent priming of he-NK cells in response to double strand RNA exposure (60, 61). Moreover, the interplay between cNK cells co-cultured with human hepatic cells, and DCs induces the expansion of tolerogenic T cells (Tregs) via the engagement of CD94/NKG2A, which is a mechanism able to trigger the production of both transforming growth factor-β (TGF-β) and IL-10 (62, 63). Interestingly, *in vitro* stimulation of human cNK cells with apoptotic cells develops tolerance in these innate

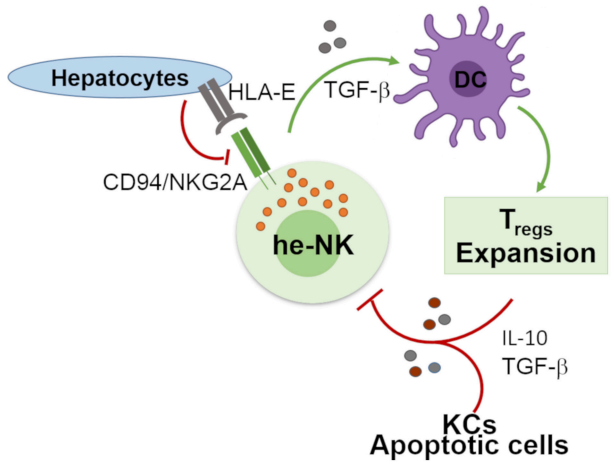

FIGURE 2 | Involvement of he-NK cells in the maintenance of hepatic tolerance and homeostasis. NK cells promote hepatic tolerance by interplaying with hepatocytes via CD94/NKG2A that in a TGF-β-mediated manner modulate DCs that further prompt expansion of tolerogenic $CD4^{pos}CD25^{pos}$ Treg cells. On the other hand, Treg cells along with hepatic KCs and apoptotic cells contribute to the production of immunosuppressive factors IL-10 and TGF-β that can induce tolerogenic he-NK cells. Green arrows show stimulatory connection and red lines inhibition.

effector cells via the secretion of TGF-β that, in turn, suppresses their autocrine IFN-γ production (64).

Different studies demonstrated that he-NK cells are also important in regulating the unique capacity of liver to regenerate itself after tissue damage (65, 66). In this regard, in the *in vivo* model interaction of cNK cells with surrounding different liver-resident cells (i.e., KCs, fibroblast, and stem cells) induces the secretion of growth factors, hormones, cytokines, and chemokines able to induce the proliferation/regeneration of hepatic tissue (67). In particular, the activation of he-NK cells is associated with a *de novo* production of CXCL7, CXCL2, CCL5 and IL-8 that, in turn, can recruit and differentiate mesenchymal stem cells substantially contributing to the so-called *"restitutio ad integrum"* of this organ (65). This is a process that needs to be finely tuned and regulated since paradoxically over-stimulation of mouse he-NK cells can inhibit, rather than promoting, liver regeneration through the aberrant signaling pathway exerted by IFN-γ on those factors (i.e., STAT1, IRF-1, and p21cip1/waf1) regulating hepatocyte proliferation (68, 69). This is the case of *in vivo* activation with high doses of the immuno-stimulant Polyinosinic:polycytidylic acid (Poly I:C) (70).

NK CELLS IN THE PATHOGENESIS OF AUTOIMMUNE LIVER DISEASES

Those mechanisms that make it possible for the liver to develop immunologic tolerance also expose this organ to the onset of immunological diseases. In this context, the presence of dysfunctional he-NK cells can actively contribute to the breach of immunological tolerance and in the appearance of autoimmune-liver diseases including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC) (2, 71).

Although T cells have been reported to play a prominent role in the pathogenesis of AIH, several lines of evidence showed that also autoreactive he-NK cells are expanded in this autoimmune liver disorder (72). Indeed, the *in vivo* administration of Poly I:C in mice induces the onset of AIH in which activated intrahepatic NK cells actively contribute to liver damage (73). Additionally, the low frequency of the inhibitory KIR/KIR-ligand combinations KIR3DL1/HLA-Bw4 and KIR2DL3/HLA-C1 coupled to the high frequency of the HLA-C2 high affinity ligands for KIR2DS1 may contribute to unwanted NK cell autoreactivity in AIH (74). The expansion of aberrant NK cells able to kill autologous cholangiocytes represents also one of the pathogenic mechanisms present during the course of PBC (75, 76). Indeed, the frequency of he-$CD56^{dim}$ NK cells in PBC is higher compared to that of healthy livers. However, it is still unclear whether the expansion of autoreactive he-NK cells targeting autologous biliary epithelial cells is directly associated with breach of liver immune tolerance or if this is a secondary event linked to the high degrees of immune activation and inflammation present in PBC (77). Another mechanism employed by cNK cells to lyse "self" cholangiocytes relies on the engagement of TRAIL pathway. As a matter of fact, the downstream death signal delivered by TRAIL receptor 5 is higher in PBC patients and induces cholestatic liver injury (78, 79). Another study also reported a protective role of intrahepatic NK cells in PBC patients, as the presence of low NK cell/cholangiocytes ratio is associated with higher IFN-γ production. This can induce or increases the expression of MHC-I and -II on cholangiocytes that are, in turn, spared from the lysis exerted by autoreactive NK cells. This latter protective mechanism is particularly relevant in the initial stages of PBC, since it can slow its progression to liver failure (80).

Among the three main liver autoimmune diseases, PSC represents the one whose pathogenesis is still largely unknown. However, the presence of certain HLA alleles or genetic variants of the NKG2D ligand MIC-A had been associated with higher risks of developing PBC. Indeed, both these molecular patterns regulate NK cell recognition of cholangiocytes (81). Similar to AIH and PBC, an increase of he-NK cell frequency was detected in PSC patients (82, 83). The most prominent current working hypothesis postulates that, similar to PBC, the engagement of TRAIL could induce the he-NK-mediated destruction of cholangiocytes in PSC patients (42, 79). Finally, another study reported that lr-NK cells from PSC patients are impaired in their cytotoxicity due to the high levels of local TNF-α production (84). Taken together, these contradictory data and speculations in regard to PSC pathogenesis reflect our general lack of knowledge in regard to the mechanistic roles and clinical impact of he-NK cells in liver autoimmune diseases.

NK CELLS IN LIVER CANCER

Hepatocellular carcinoma (HCC) is the most common leading cause of liver-cancer related death worldwide (85). Among

the main predisposing risk factors of HCC, there are chronic viral infections by hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol related cirrhosis and non-alcoholic steato-hepatitis (86). The liver is also the first site of colorectal cancer (CRC) metastatic dissemination (87). NK cells had been first discovered due to their ability to kill tumor-transformed cells (i.e., immune surveillance) and are able to provide protection in hematological malignancies, solid primary cancers and metastatic lesions (88, 89). This important feature is also valid for HCC as human cNK cells were shown to be highly cytotoxic against HepG2 hepatocellular carcinoma cells (2, 42, 56). Moreover, it has been reported that higher numbers of total tumor infiltrating $CD56^{pos}$ he-NK cells predict a better outcome for HCC in terms of patient overall survival (OS) (90–92). Other retrospective studies showed that high frequencies of the specific intra-tumor NK cell subsets slow the progression of liver cancer, as demonstrated for human $NKp46^{pos}$ he-NK cells in CRC metastatic disease and for $CD11b^{neg}/CD27^{neg}$ he-NK cells in HCC (93, 94). In addition, the selective engagement of NKG2D in both mice and human enhances NK cell anti-tumor activity against HCC since the transcriptional modulation or the interferon-induced expression of this aNKR boosts he-NK cell cytotoxicity and blocks tumor growth (95, 96). This potent anti-tumor NK cell effector-function against HCC seems to be more effective in the early stages of the tumor and decreases as soon as the disease progresses (**Figure 3**). Indeed, lower frequencies of anergic/dysfunctional $CD56^{dim}$ and $CD56^{bright}$ NK cells, characterizes end-stages HCC patients both in peripheral blood and at a tumor site, a phenomenon that is also associated with a parallel expansion of $CD4^{pos}/CD25^{pos}$ Tregs and increased secretion of IL-10 (92, 97, 98). Several mechanisms have been proposed to explain, at least in part, the functional impairments of NK cells in advanced HCC. These include the increased expression on tumor infiltrating NK cell surface of inhibitory checkpoints [i.e., programmed cell death protein (PD-1) and NKG2A] as well as the higher surface levels of PD-1 ligands (PD-1Ls) and MHC-I on malignant cells. Both strategies simultaneously employed by HCC both on immune-effectors and targets have the same aim of evading human NK cell immune-surveillance, thus sparing tumor cells from NK cell killing (99–102). It has been also reported that in advanced HCC patients he-NK cells express a specific inhibitory NKp30 splice variant (Ih-NKp30), thus resulting in a deficiency of NKp30-mediated NK cell activation and function. Interestingly, the soluble form of NKp30 ligand (NKp30L) B7-H6 is increased in late stages of HCC (103). Another mechanism contributing to cNK cell impairment in HCC patients relies on their aberrant interactions with tumor infiltrating macrophages, inducing a rapid NK cell exhaustion both via the engagement of CD48/2B4 and NKp30 pathways (98, 104, 105). Additionally, several alterations in the cytokine milieu of neoplastic HCC tissue can influence cNK cell cytotoxicity and cytokine production. These include soluble immune-modulators such as TGF-β, prostaglandin E2 (PGE2) or indoleamine 2,3-dioxygenase (IDO) (105–107). More recently, it has been reported that IL-1R8 (TIR-8) can serve as another important checkpoint able to inhibit anti-tumor NK cell effector-functions in liver cancer murine models. Indeed, its blockade unleashes NK cell-mediated resistance to hepatic carcinogenesis and liver metastasis of CRC (108). Moreover, using a mouse model of cholangiocarcinoma (CCA), it has been demonstrated that adoptive NK cell transfer limits tumor growth and improves the prognosis of this aggressive liver cancer, although the related mechanisms associated to NK cell control of CCA have not yet been elucidated (109).

LIVER NK CELLS AND VIRAL INFECTION

HCV and HBV infections represent the main two infectious diseases inducing liver inflammation and failure (110). Controversial data are available regarding the immune status of he-NK cells in acute and chronic liver viral infections (**Figure 3**).

In acute HCV infection, he-NK cells show an increased expression of NKp46 and a high ability to degranulate and to produce IFN-γ following a strong activation by IFN-α/β and other cytokines (i.e., IL-12, IL-15, IL-18) (111, 112). Although intrahepatic $CD56^{bright}/NKp46^{high}$ he-NK cells contribute to a better control of HCV replication, their presence at high frequency is also associated with increased degrees of liver necrosis and fibrosis following acute infection (113). Interestingly, the livers of those patients experiencing self-limiting HCV-1 infection were not enriched of $CD56^{bright}/NKp46^{high}$ he-NK cells, but of NK cells are highly positive for CD57 and KIRs. These findings suggest that terminally-differentiated NK cells can better control HCV infection (114).

In the context of HBV infection, early cNK cell responses contribute to the initial control of infection and to the development of an efficient adaptive immune response through the secretion of IFN-γ, TNF-α, GM-CSF, and TGF-β able to inhibit viral replication or to induce the killing of infected cells (115–117). In this context, acute HBV infected patients showed an expansion of $CD56^{bright}$ cNK subset, but reduced frequencies of $CD56^{dim}$ NK cells. Notably, the inflamed lobular necrotic areas of HCV-infected livers from the same individuals were surrounded by $NKp46^{pos}$ NK cells (118). *In vivo* experimental studies also confirmed the presence of a strong activation of he-NK cells in response to acute HBV infection, a mechanism that limits viral replication. However, in these animal models the HCV-mediated priming of NK cells was not able to induce an antigen-specific T cells response (119–121).

When both HBV and HCV enter into their chronic stages, the frequencies of cNK cells remarkably decrease together with their ability of producing pro-inflammatory cytokines, such as IFN-γ and TNF-α (122–125). Although, he-NK cells maintain their cytotoxic potential in chronic HBV via the up-regulation of TRAIL (126, 127), however, they pathologically contribute to eliminate autologous HBV-specific $CD8^{pos}$ T cells expressing high levels of death receptor for TRAIL. Hence, this NK cell-mediated depletion of antigen-specific $CD8^{pos}$ T cells impairs adaptive antiviral immunity in chronic HBV-infected patients and contributes to viral persistence (128–130). Moreover, persistent viral infections have a remarkable impact on the cNK cell receptor repertoire and profoundly affect their

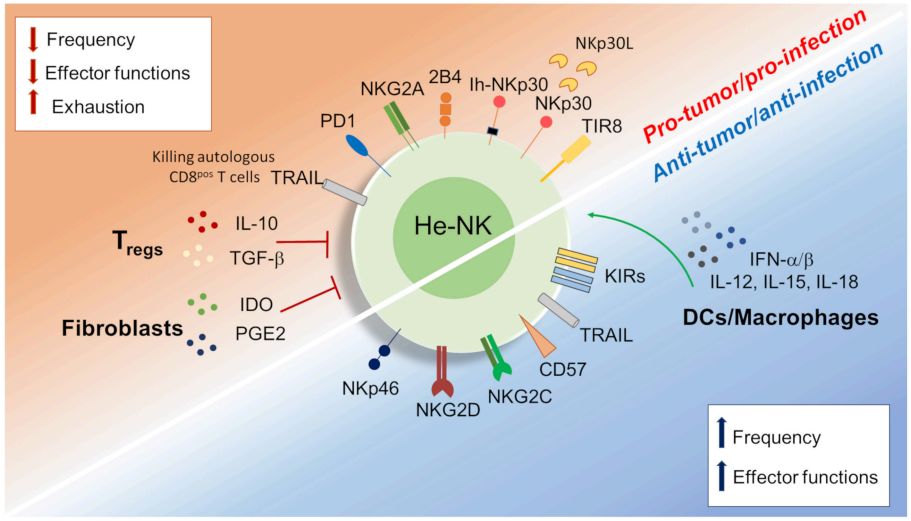

FIGURE 3 | Dichotomy in the he-NK cell response in tumor and viral infection. Different soluble factors and cell surface receptors contribute to positive or either negative he-NK cell response in both tumor and/or viral infection. Increased (vertical blue arrows) frequency and effector-functions of he-NK cells were observed in early tumor and acute viral response. On the other hand, NK cell dysfunction (vertical red arrows) in late tumor or chronic infection results with lower NK cells frequency, effector function inhibition and cell exhaustion. Arched green arrows show stimulatory connection and red lines inhibition.

effector-functions. Indeed, chronic exposure to HBV induces TGF-β production that, in turn, reduces the expression of NKG2D and 2B4, and their respectively, intracellular adaptor proteins DAP10 and SAP, thus further hampering their ability to eliminate viral infected cells (131). However, whether this immunosuppressive mechanism plays a role in shaping he-NK cells need to be further consolidated.

HEPATIC NK CELLS AS POTENTIAL THERAPEUTIC TARGETS

The possibility of tuning NK cell effector-functions represents an important therapeutic strategy for the treatment of several liver disorders, as demonstrated for infections and other malignancies (112, 113). Among the main methodological approaches developed in this context, there are protocols administering *in vivo* compounds targeting NK cell activation. Indeed, the use of several cytokines that can easily reach and activate liver endogenous NK cells has been extensively tested in several clinical and experimental trials. IL-12 and IL-18 have been shown to effectively inhibit liver carcinogenesis by boosting NK cell anti-tumor functions (132). Therapies with interferons showed anti-viral, anti-fibrotic and anti-tumor NK cell-mediated clinical outcomes (96, 133). Two cytokines widely adopted to enhance NK cell cytotoxicity are IL-2 and IL-15 (134, 135). In particular, IL-15 can rescue the anti-tumor activities of intrahepatic NK cells purified from HCC patients (136). Interestingly, the use of recombinant/modified IL-2 and IL-15 activates both NK and $CD8^{pos}$ T cells without stimulating Tregs and these cytokines are currently being tested also against hematological cancers (137–139). Agonists for several aNKRs expressed on lr-NK cells, such as NKG2D and NCRs, also represent a potential clinical therapeutic strategy. Moreover, the expression of NKRs can be also modulated at the transcriptional level. In this regard, the miR-182 has been shown to increase NK cells cytotoxicity in HCC patients by regulating the expression of NKG2D and NKG2A (95).

In a new era of cancer immunotherapy, several inhibitory checkpoints have been targeted also on NK cells through the development of blocking mAbs unleashing their anti-tumor effector-functions (13). In particular, anti-KIR mAbs are currently being tested in different hematological cancers alone or in combination with other treatments (140). Another important NK cell inhibitory checkpoint is anti-NKG2A, whose masking mAb is currently being tested in several solid tumors and hematologic diseases (23, 141–144). More recently, it has been also reported that NK cells can express PD-1 thus paving the ground in the future to target NK cells also with mAbs blocking PD-1/PD-L1 interactions (145). Further clinical trials are required to investigate the efficacy of these compounds in liver cancers.

Adoptive NK cell transfer therapies have been first introduced to improve the clinical outcome of patients affected by hematologic malignancies and undergone allogeneic hematopoietic stem cell transplantation (allo-HSCT) (146, 147). The great clinical outcome of this strategy in allo-HSCT together with newly available technologies made it possible to develop new protocols of adoptive NK cell therapies to treat both hematologic malignancies and solid tumor (148–151). More recently, the possibility of engineering NK cells with different technological approaches such as the so-called bi- and tri-specific killer engagers (BiKEs and TriKEs) (152) or chimeric antigen

receptors (CARs) (153) improved both tumor-specificity and the ability of NK cells to reach/infiltrate tumor tissues. Very little is known about the efficacies of adoptive NK cell transfer therapies in liver cancers, a gap that needs to be filled by new experimental and clinical trials.

CONCLUDING REMARKS

Despite a great number of studies that have been focusing on elucidating the role of he-NK cells in liver physiology and physiopathology, several questions still remain unanswered. In particular, given the high heterogeneity of NK cells in liver, further studies are needed to investigate their specific role in both homeostatic and pathological conditions. Indeed, understanding this high degree of diversity will likely explain the several and often opposite functions of he-NK cells. These include the different capacities of he-NK cells either to reside in the liver or to recirculate through this organ without being retained and their abilities to be tolerogenic toward foreign antigens while attacking viruses and tumors. This knowledge is key to understand and target those mechanisms participating in the onset of hepatic disorders.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by Associazione Italiana per la Ricerca sul Cancro (IG-14687 and IG 21567 to DM), Italian Ministry of Health (Bando Ricerca Finalizzata PE-2016-02363915) and Intramural Research Funding of Istituto Clinico Humanitas (to DM). CDV is recipient of the post-doctoral fellowships from the Fondazione Umberto Veronesi (2017-1464 and 2018-1974).

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

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frontiers in Immunology

ORIGINAL RESEARCH
published: 03 July 2019
doi: 10.3389/fimmu.2019.01502

Liver-Derived TGF-β Maintains the Eomes^{hi}Tbet^{lo} Phenotype of Liver Resident Natural Killer Cells

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OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Ennio Carbone, Università Degli Studi Magna Græcia di Catanzaro, Italy
Cai Zhang, Shandong University, China

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *03 December 2018*
Accepted: *17 June 2019*
Published: *03 July 2019*

Citation:
Harmon C, Jameson G, Almuaili D, Houlihan DD, Hoti E, Geoghegan J, Robinson MW and O'Farrelly C (2019) Liver-Derived TGF-β Maintains the Eomes^{hi}Tbet^{lo} Phenotype of Liver Resident Natural Killer Cells. Front. Immunol. 10:1502. doi: 10.3389/fimmu.2019.01502

The adult human liver hosts a complex repertoire of liver resident and transient natural killer (NK) cell populations with diverse phenotypes and functions. Liver resident NK cells are CD56^{bright} NK cells defined by a unique expression profile of transcription factors and cell surface markers (Eomes^{hi}Tbet^{lo}TIGIT^{+}CD69^{+}CXCR6^{+}CD49e^{-}). Despite extensive characterization of the phenotype of liver resident NK cells, it remains unclear how factors within the liver microenvironment induce and maintain this unique phenotype. In this study, we have explored the factors regulating the phenotype of liver resident NK cells. Isolation of healthy liver resident NK cells from donor liver perfusate and *in vitro* culture results in the gradual loss of the characteristic Tbet^{lo} phenotype, with the cells increasing Tbet expression significantly at day 7. This phenotypic loss could be halted through the dose-dependent addition of liver conditioned media (LCM), generated from the *ex vivo* culture of liver biopsies from healthy organ donors. TGF-β, but not IL-10, replicated the Tbet suppressive effects of LCM in both liver resident and peripheral blood NK cells. Furthermore, blocking TGF-β receptor signaling using the inhibitor SB431542, reversed the effect of LCM treatment on liver resident NK cells, causing the loss of tissue resident Eomes^{hi} Tbet^{lo} phenotype. Our findings identify liver-derived TGF-β as an important component of the liver microenvironment, which acts to regulate and maintain the phenotype of liver resident NK cells.

Keywords: TGF-β1, liver-resident NK cell, TBET, microenviroment, Eomes

INTRODUCTION

Large populations of tissue resident natural killer (NK) cells have been identified in a diverse range of tissues including liver, lung, lymph node, bone marrow, and uterus (1). However, the tissue-specific factors regulating resident NK cell phenotype and function remain largely unknown. The adult human liver hosts a diverse repertoire of liver resident and transient NK cell populations with differing phenotypes and functions, whose roles in liver homeostasis and disease remain poorly defined.

Liver resident NK cells are enriched in the CD56^{bright} NK cell subpopulation and are defined by their unique expression of the transcription factors Eomes and Tbet, and a range of cell surface markers including TIGIT, CD69, CXCR6, and the absence of CD49e (Eomes^{hi}Tbet^{lo}Hobit^{+}TIGIT^{+}CD69^{+}CXCR6^{+}CD49e^{-}) (2–6). This liver resident phenotype is absent in peripheral blood NK cell populations. Expression of the chemokine receptor CXCR6 is thought to be particularly important for the retention of resident populations within the liver due to constitutive expression of the ligand (CXCL16) in the liver sinusoid.

Despite extensive characterization of the phenotype of liver NK cells, it remains unclear how factors within the liver microenvironment contribute to the induction and maintenance of this unique phenotype. Our group has previously described the wide range of cytokines and growth factors present in healthy adult liver which can maintain and propagate NK cells (7–10). However, this work did not explore specific liver resident NK cell phenotypes. Previous research identified that liver resident NK cells produce significantly less IFN-γ compared with corresponding peripheral blood NK cell populations, and liver conditioned media (LCM) was sufficient to suppress IFN-γ in peripheral blood CD56^{bright} cells (2, 3), suggesting that liver-derived soluble factors are capable of regulating NK cell populations.

In this study we demonstrate that LCM can maintain the liver resident NK cell phenotype *ex vivo*, as well as suppress Tbet and Eomes expression in peripheral NK cells (which mirrors the Tbet phenotype observed in liver resident NK cells). Furthermore, we show TGF-β, produced in the liver microenvironment, suppresses Tbet and Eomes expression in liver resident NK cells and peripheral blood NK cells. Understanding how the liver microenvironment induces and maintains liver specific immune cell populations may identify novel therapeutic targets capable of regulating local immunity and tissue repair.

MATERIALS AND METHODS

Collection of Liver Perfusate During Orthotopic Liver Transplantation (OLT)

Samples were collected from donor livers ($n = 10$) during orthotopic liver transplantation at St. Vincent's University Hospital. During retrieval, the donor aorta and superior mesenteric vein were flushed with University of Wisconsin (UW) solution (Bristol-Myers Squibb, Uxbridge, UK) at the time of exsanguination. The liver was flushed again with UW solution after excision of the organ until all blood was removed and the perfusate appeared clear, at which time the liver was placed in a container with UW solution and packed on ice for transportation. Donor livers were transplanted within 12 h. At implantation, after completion of the upper inferior cava anastomosis, livers were flushed with normal saline through the portal vein to wash out the UW before reperfusion. This wash-out fluid was collected from the inferior vena cava; the UW transportation solution was also collected. A matched donor blood sample was taken at the time of organ retrieval. Peripheral blood was obtained from anonymised blood donors from the Irish Blood transfusion Board (IBTS). All protocols were approved by St. Vincent's University Hospital Ethics Committee and the Trinity College Dublin School of Medicine Research Ethics Committee, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Isolation of Hepatic Mononuclear Cells From Liver Perfusate and Peripheral Blood

Hepatic mononuclear cells (HMNCs) were isolated from donor liver perfusates, as previously described (11), by filtration through 70 μm filters (BD Biosciences, Erembodegem, Belgium) followed by centrifugation at 1,200 rpm for 10 min. The supernatant was aspirated and the cells resuspended in RPMI 1640 medium, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco, Wicklow, Ireland). HMNCs were separated from this suspension by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden) and residual red blood cells were removed by adding red cell lysis solution (Sigma, Wicklow, Ireland). Matched peripheral blood mononuclear cells (PBMCs) were also isolated by density centrifugation.

Preparation of Liver Conditioned Media

Wedge biopsies, taken at the time of transplantation, were used to generate tissue conditioned media. Tissue samples were measured and weighed. Tissue was then cut into sections measuring approximately 0.5 cm × 0.5 cm × 0.5 cm. These were placed in a 24-well-culture plate and 500 μl of X-VIVO (Lonza Biologics, Slough, UK) media was added to each well and incubated for 72 h at 37°C. Following incubation, the supernatant was centrifuged to remove cell debris and stored at −20°C until use.

Isolation of NK Cells From Liver Perfusate and Peripheral Blood

CD3^{-}CD56^{+} NK cells were isolated from fresh liver perfusates and healthy donor peripheral blood by negative selection using NK cell isolation kits (130-092-657; Miltenyi Biotech, Teterow, Germany, and 480054; BioLegend, CA, USA), as per the manufacturers protocol. Briefly, mononuclear cells were labeled with a biotin conjugated antibody cocktail against lineage specific targets. Anti-biotin microbeads were then added, and the NK cells were separated using a magnetic cell sorting (MACS) LS column. Cells were then cultured in RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. In addition, NK cells were sorted into two populations CD56^{bright} CD16$^{+/-}$ and CD56^{dim} CD16^{++} using a FACS Aria cell sorter (BD Biosciences).

Culture of Hepatic and Peripheral Blood NK Cells

Isolated NK cells were plated at 5 × 10^{5}/ml in round-bottomed 96 well-plates in RPMI 1640 supplemented with 10% Human AB serum and rhIL-15 (2 ng/ml) for 7 days in the presence or absence of liver conditioned media (5/10% v/v), TGF-β (5 ng/ml), or IL-10 (10 ng/ml). Media was changed every 2–3 days.

Phenotypic Analysis of NK Cells

HMNCs, PBMCs and cultured NK cells were stained with fluorescently labeled monoclonal antibodies to determine the phenotypic differences between hepatic and peripheral NK cells. The following antibodies were used: CD45 (HI30) BV510, CD3 (UCHT1) BV421 or Pacific Blue, CD56 (NCAM16.2) BV650 or APC, CD16 (3G8) PE-Cy7, CXCR6 PE-CF594. Intracellular staining was performed using FoxP3 staining buffer (00-5523-00, eBiosciences, San Diego, CA, USA) and the following antibodies were used: Eomes (WD1928) AF488 and T-bet (ebio4B10) PE (BioLegend). Dead cell exclusion was carried out using fixable viability stain 780 (BD Biosciences). Flow cytometric analysis was carried out using an LSR Fortessa (BD Biosciences) or a FACS Canto II (BD Biosciences) and data was analyzed using FlowJo (Version 7.6.5, Tree Star, Ashland, OR, USA).

Statistical Analysis

Statistical analysis was carried out using Prism GraphPad Version 5.0. For comparison of two unmatched groups, Mann Whitney *U*-test was used. Comparison of more than two unmatched groups was performed using Kruskal-Wallis test, with Dunn's multiple comparison test. For paired comparisons, Friedman test with Dunns multiple comparison tests was used for more than two groups, Wilcoxon signed rank test was used for comparison of two matched groups. Within an experiment, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively.

RESULTS

Liver Resident NK Cells Are Characterized by a Eomes^{hi}Tbet^{lo}CXCR6^{+} Phenotype, Which Is Gradually Lost Upon *ex vivo* Culture

NK cells from donor liver perfusate and matched peripheral blood were analyzed for expression of Eomes, Tbet, and CXCR6. As previously described, CD56^{bright} NK cells from liver perfusate displayed reduced expression of Tbet (LP: MFI 554 ± 69.6, PB: MFI 1074 ± 189.9, $p = 0.0024$, **Figures 1A,B**). Liver CD56^{bright} NK cells had increased expression of Eomes compared to matched blood cells (LP: MFI 2092 ± 337, PB: MFI 1126 ± 248, $p = 0.0005$, **Figure 1C**). Liver CD56^{bright} cells also express CXCR6, which is almost undetectable on peripheral blood counterparts (LP: 52.1 ± 10.1%, PB: 3.1 ± 1.9%, $p = 0.0223$, **Figure 1D**). In contrast, CD56^{dim} NK cells from liver perfusate resemble their peripheral blood counterparts with no significant differences in Eomes, Tbet, or CXCR6 (**Figures 1E–G**).

In order to determine whether the phenotype of hepatic NK cells is permanently altered by residing within the liver microenvironment, NK cells were isolated from liver perfusate and cultured in RPMI supplemented with human AB serum, to replicate the peripheral blood microenvironment. It was necessary to supplement this culture with IL-15 (2 ng/ml) in order to maintain NK cell viability and expression of CD56 (12, 13). NK cells were cultured for 7 days with the culture media replaced every 2–3 days.

Upon *ex vivo* culture the unique phenotype of CD56^{bright} hepatic NK cells is lost, with Tbet increasing significantly between day 0 (MFI 461.5 ± 255.5) and day 7 (MFI 1214 ± 572, $p = 0.042$, **Figures 2A,B**). Eomes expression increased but this did not reach statistical significance (day 0 MFI 2147 ± 632 and day 7 MFI 3497 ± 1500, $p = 0.3$, **Figure 2C**). Expression of the chemokine receptor, CXCR6, showed a trend toward decreasing on hepatic CD56^{bright} NK cells upon *ex vivo* culture, however this did not reach statistical significance (day 0, 71.8 ± 10.2%, and day 7, 40.3 ± 10.4%; $p = 0.052$, **Figure 2D**).

In CD56^{dim} hepatic NK cells, Tbet remained stable during culture with the MFI ranging between MFI 2007 at day 0 and MFI 1550 at day 7 ($p = 0.69$, **Figure 2E**). Eomes expression increased between day 0 (MFI 1463 ± 516) and day 7 (MFI 3364 ± 1807, **Figure 2F**) but this did not reach statistical significance. CXCR6 expression in CD56^{dim} hepatic NK cells increased marginally, however this increase was not statistically significant and even at day 7 only represents a small minority of the total CD56^{dim} population (Day 0 2.5 ± 1.3% vs. day 7 6.6 ± 2.4%; $p = 0.3$, **Figure 2G**).

The Eomes^{hi} Tbet^{lo} Phenotype of Liver Resident NK Cells Can be Maintained *ex vivo* by Supplementing With Liver Conditioned Media

Hepatic CD56^{bright} NK cells have a unique pattern of Eomes and Tbet expression, which is lost upon long term culture. In order to determine the effect of the liver microenvironment on these transcription factors, we assessed Eomes and Tbet expression after *ex vivo* culture supplemented with LCM ($n = 5$). LCM significantly reduced the expression of Tbet in liver resident NK cells, in a dose dependent manner, to a level similar to that seen at day 0. At day 7, the MFI of Tbet in untreated CD56^{bright} NK cells was 724.3 ± 170.4 MFI, rising from 323.7 ± 34.3 MFI at day 0. This was reduced to 391.0 ± 29.8 MFI with 5% LCM and further reduced to 261.7 ± 11.9 with 10% LCM ($p = 0.017$, **Figures 3A,B**). Similarly, the increase in Eomes expression upon *ex vivo* culture of CD56^{bright} NK cells was significantly reduced by treatment with LCM in a dose dependent manner. By day 7, Eomes MFI in untreated NK cells had risen to 3,149 ± 389.9 from 1,343 ± 204.4 at day 0. Treatment with 5% LCM reduced the MFI to 1,774 ± 630.6, with a further reduction to 983.3 ± 243.1 MFI with 10% LCM ($p = 0.0278$, **Figure 3C**). At day 7, CXCR6 was expressed on 45.5 ± 6.4% of CD56^{bright} NK cells. This level increased to 58.0 ± 15.5% and 65.3 ± 10.8% with 5 and 10% LCM, respectively ($p = 0.21$, **Figure 3D**).

Hepatic CD56^{dim} NK cells behave in a similar manner when treated with LCM although the effect is not as pronounced as in CD56^{bright} NK cells. Tbet expression is reduced in a dose dependent manner (untreated MFI 789.7 ± 171.8, 5% LCM MFI 720.7 ± 19.7, 10% LCM MFI 553.2 ± 7.8, $p = 0.18$, **Figure 3E**). A dose dependent decrease in Eomes expression was also seen, returning the MFI of Eomes to levels seen at day 0 (untreated MFI 2323 ± 4336, 5% LCM MFI 1157 ± 164.9, 10% LCM MFI 736 ± 118, $p = 0.0331$, **Figure 3F**). No significant change was seen in expression of CXCR6 on CD56^{dim} NK cells

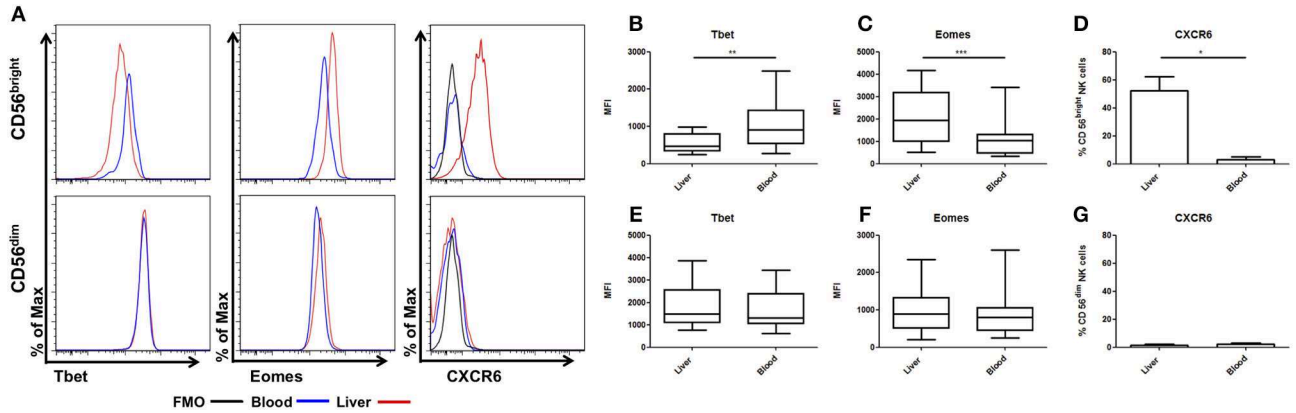

FIGURE 1 | CD56^{bright} NK cells have unique tissue resident phenotype. Mononuclear cells isolated from liver perfusate (LP) and matched peripheral blood (PB) were stained with monoclonal antibodies. NK cells were identified from the CD45^{+} lymphocyte gate as CD56^{+}CD3^{-}. CD56^{bright}CD16$^{-/+}$ and CD56^{dim}CD16^{+} subsets were then gated and non-viable cells were excluded by FVS780 staining. **(A)** Representative histograms of Tbet, Eomes and CXCR6 expression in LP and PB NK cell subsets. **(B)** The MFI of Tbet in CD56^{bright} NK cells. **(C)** The MFI of Eomes in CD56^{bright} NK cells. **(D)** Percentage of CXCR6 positive CD56^{bright} NK cells. **(E)** The MFI of Tbet in CD56^{dim} NK cells. **(F)** The MFI of Eomes in CD56^{dim} NK cells. **(G)** Percentage of CXCR6 positive CD56^{dim} NK cells. Data presented as mean ± SEM **(D,G)** or box and whisker plots with minimum and maximum values **(B,C,E,F)**. Data was analyzed using Wilcoxon matched pairs test ($n = 10$; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

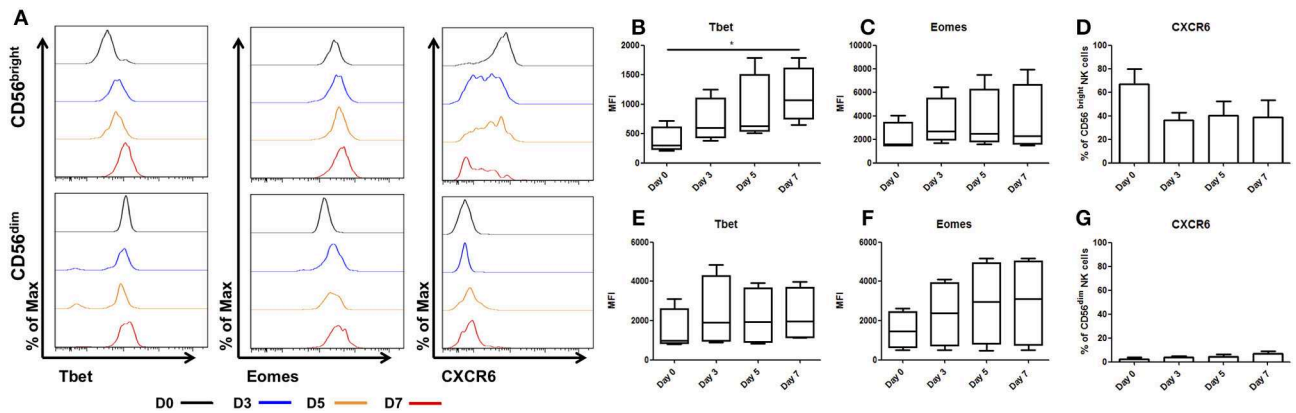

FIGURE 2 | Tbet expression increases in liver resident NK cells cultured *ex vivo*. NK cells isolated from liver perfusate (LP) were cultured for 7 days and stained with monoclonal antibodies to assess transcription factor expression. **(A)** Representative histograms of Tbet, Eomes and CXCR6 expression from cells at day 0 (black line), day 3 (blue line), day 5 (orange line), and day 7 (red line). **(B)** MFI of Tbet in CD56^{bright} NK cells from LP at day 0, 3, 5, and 7. **(C)** MFI of Eomes CD56^{bright} NK cells from LP at day 0, 3, 5, and 7. **(D)** Percentage of CXCR6 positive CD56^{bright} NK cells from LP at day 0, 3, 5, and 7. **(E)** MFI of Tbet in CD56^{dim} NK cells from LP at day 0, 3, 5, and 7. **(F)** MFI of Eomes in CD56^{dim} NK cells from LP at day 0, 3, 5, and 7. **(G)** Percentage of CXCR6 positive CD56^{dim} NK cells from LP at day 0, 3, 5, and 7. Data presented as mean ± SEM **(D,G)** or box and whisker plots with minimum and maximum values **(B,C,E,F)**. Data was analyzed using Friedman test, with Dunn's multiple comparison test ($n = 5$; $^{*}p < 0.05$).

(untreated 6.6 ± 2.4%, 5% LCM 3.6 ± 2.1%, 10% LCM 4.1 ± 2.6, $p = 0.72$, **Figure 3G**).

Treatment of Peripheral Blood NK Cells With LCM Suppresses Tbet and Eomes Expression but Fails to Induce CXCR6 Expression

In contrast to hepatic NK cells, blood CD56^{bright} NK cells do not significantly increase their expression of Tbet in culture (Day 0 954.7 ± 47.5 MFI, Day 7 973.5 ± 88.5 MFI, **Figures 4A,B**). However, treatment with LCM induces reduced Tbet expression after 7 days of treatment (Day 0 954.7 ± 47.5 MFI, Day 7 5% LCM 443 ± 91.2, 10% LCM 395.3 ± 25.2 MFI, $p = 0.033$, **Figure 4B**). At day 0 the MFI of Eomes was 1,514 ± 237.1 for CD56^{bright} cells, by day 7 the MFI had increased to 2,371 ± 267 (**Figure 4C**). This was reduced by addition of LCM to 1,177 ± 209 MFI (5% LCM) and 927.7 ± 34.2 MFI (10% LCM). Peripheral blood CD56^{bright} NK cells do not basally express CXCR6 and the receptor was not upregulated at any point during the experiment (**Figure 4D**).

CD56^{dim} NK cells also undergo a significant decrease in Tbet expression after LCM treatment (day 0 1198 ± 72.8 MFI, Day 7 untreated 1,012 ± 99.1 MFI, 5% LCM 418

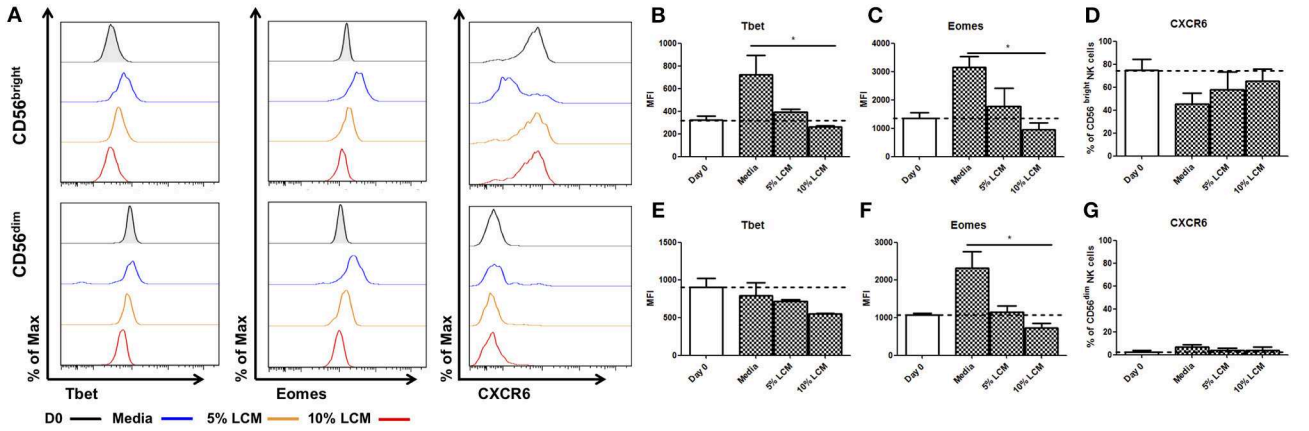

FIGURE 3 | Liver resident phenotype can be maintained by the addition of liver conditioned media. NK cells isolated from liver perfusate (LP) were cultured for 7 days, supplemented with 5 or 10% v/v liver conditioned media (LCM) and stained with monoclonal antibodies to assess transcription factor expression. **(A)** Representative histograms of Tbet, Eomes and CXCR6 expression from cells at day 0 (black line), day 7 untreated (blue line), day 7 5% LCM (orange line), and day 7 10% LCM (red line). **(B)** MFI of Tbet CD56^{bright} NK cells from LP at day 0 and 7 with LCM treatments. **(C)** MFI of Eomes in CD56^{bright} NK cells from LP at day 0 and 7 with LCM treatments. **(D)** Percentage of CXCR6 positive CD56^{bright} NK cells from LP at day 0 and 7 with LCM treatments. **(E)** MFI of Tbet in CD56^{dim} NK cells from LP at day 0 and 7 with LCM treatments. **(F)** MFI of Eomes in CD56^{dim} NK cells from LP at day 0 and 7 with LCM treatments. **(G)** Percentage of CXCR6 positive CD56^{dim} NK cells from LP at day 0 and 7 with LCM treatments. Data presented as mean ± SEM. Data was analyzed using Friedman test, with Dunn's multiple comparison test ($n = 5$; $^*p < 0.05$).

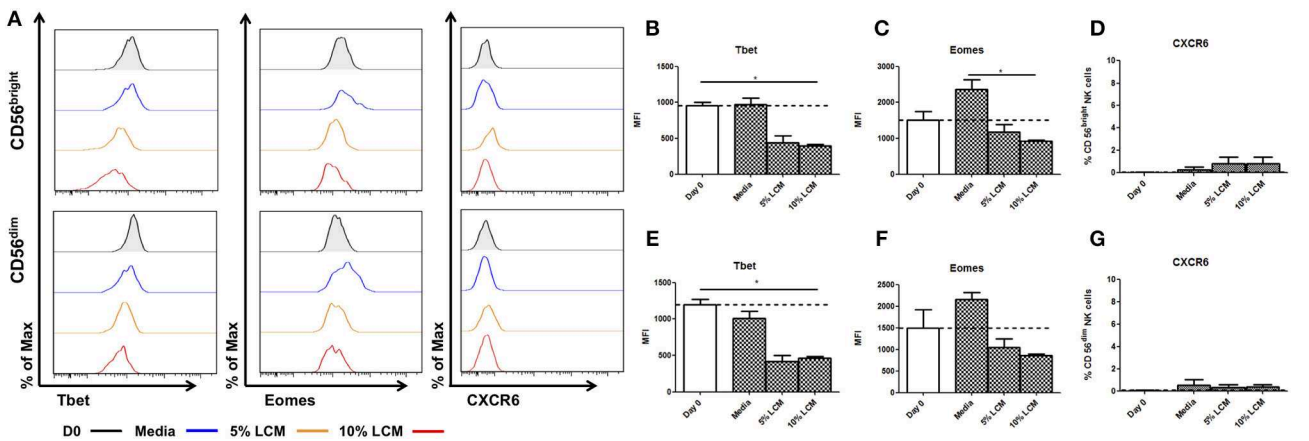

FIGURE 4 | Liver conditioned media suppresses Tbet expression in blood NK cells. NK cells isolated from peripheral blood (PB) were cultured for 7 days, supplemented with 5 or 10% v/v liver conditioned media (LCM) and stained with monoclonal antibodies to assess transcription factor expression. **(A)** Representative histograms of Tbet, Eomes and CXCR6 expression from cells at day 0 (black line), day 7 untreated (blue line), day 7 5% LCM (orange line), and day 7 10% LCM (red line). **(B)** MFI of Tbet in CD56^{bright} NK cells from PB at day 0 and 7 with LCM treatments. **(C)** MFI of Eomes in CD56^{bright} NK cells from PB at day 0 and 7 with LCM treatments. **(D)** Percentage of CXCR6 positive CD56^{bright} NK cells from PB at day 0 and 7 with LCM treatments. **(E)** MFI of Tbet in CD56^{dim} NK cells from PB at day 0 and 7 with LCM treatments. **(F)** MFI of Eomes in CD56^{dim} NK cells from PB at day 0 and 7 with LCM treatments. **(G)** Percentage of CXCR6 positive CD56^{dim} NK cells from PB at day 0 and 7 with LCM treatments. Data presented as mean ± SEM. Data was analyzed using Friedman test, with Dunn's multiple comparison test ($n = 5$; $^*p < 0.05$).

± 83.5 MFI, 10% LCM 464.7 ± 21.5 MFI, $p = 0.017$, **Figure 4E**). At day 0 the MFI of Eomes was 1,500 ± 420.5 for CD56^{dim} NK cells. At day 7 the MFI had increased to 2,155 ± 163.5, this was reduced to basal levels with the addition of LCM (5% LCM 1,040 ± 199.5 MFI, 10% LCM 859.9 ± 41 MFI, **Figure 4F**). Expression of CXCR6 on peripheral blood CD56^{dim} NK cells was negligible at all time points (**Figure 4G**).

TGF-β, Which Is Present in the Liver Microenvironment, Is Capable of Suppressing Tbet *ex vivo* in Liver Resident and Blood NK Cells

We next attempted to identify the factor present in LCM responsible for maintaining this liver resident phenotype. IL-10 and TFG-β are two immunosuppressive cytokines which can

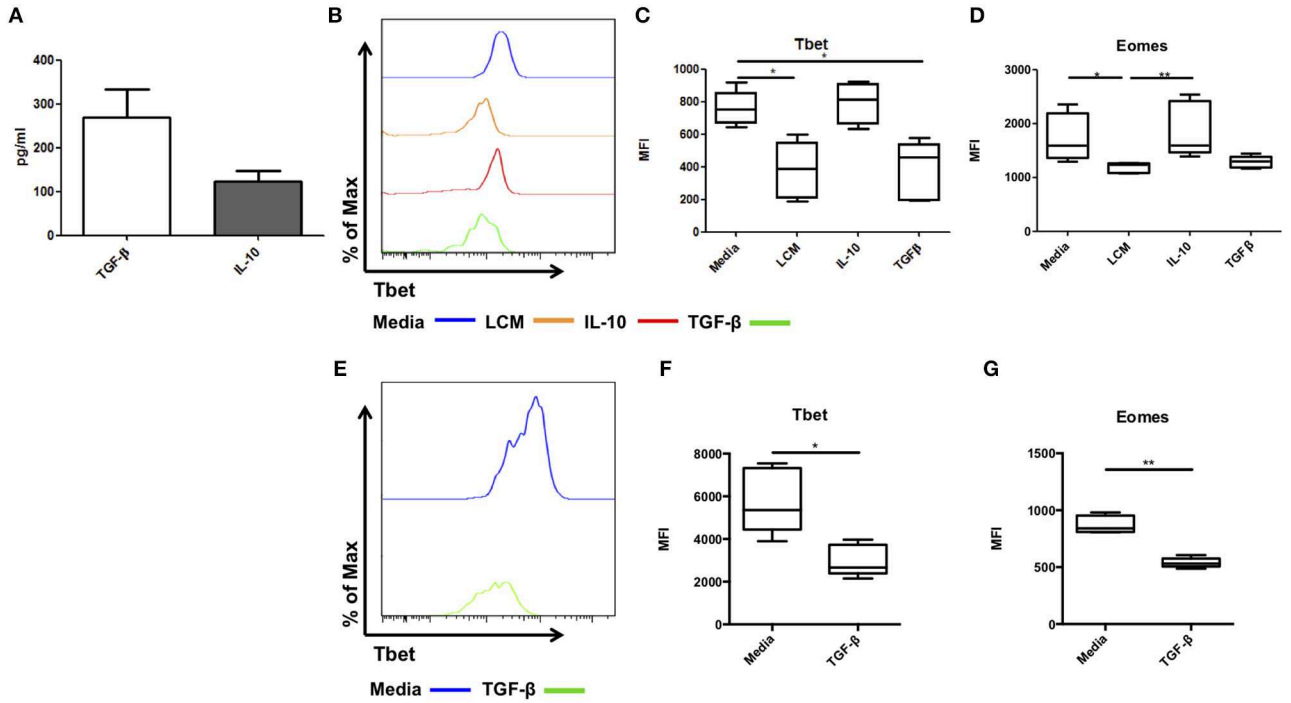

FIGURE 5 | TGF-β can regulate Tbet expression in liver resident and blood NK cells. Liver conditioned media (LCM) was generated from healthy donor liver biopsies. ELISA was performed for the cytokines TGF-β and IL-10. **(A)** Concentration of cytokines TGF-β and IL-10 from matched LCM samples ($n = 10$). CD56^{bright} Eomes^{hi} Tbet^{lo} NK cells FACS isolated from liver perfusate (LP) were cultured for 7 days, supplemented with 10% v/v liver conditioned media (LCM), IL-10 (10 ng/ml) or TGF-β (5 ng/ml) and acquired on a FACS Fortessa. **(B)** Representative histogram of Tbet expression from cells at day 7 untreated (blue line), 10% LCM (orange line), IL-10 (red line), and TGF-β (green line). **(C)** MFI of Tbet in CD56^{bright} NK cells from LP at day 7 with LCM, IL-10 or TGF-β treatments. **(D)** MFI of Eomes in CD56^{bright} NK cells from LP at day 7 with LCM, IL-10 or TGF-β treatments. **(E)** Representative histogram of Tbet expression from blood NK cells at day 7 untreated (blue line) or TGF-β (green line). **(F)** MFI of Tbet in PB CD56^{bright} NK cells at day 7 with or without TGF-β. **(G)** MFI of Eomes in PB CD56^{bright} NK cells at day 7 with or without TGF-β. Data presented as mean ± SEM **(A)** or box and whisker plots with minimum and maximum values **(C,D,F,G)**. Data was analyzed using Friedman test, with Dunn's multiple comparison test or paired *t*-test ($n = 5$; $^{*}p < 0.05$ and $^{**}p < 0.01$).

suppress IFN-γ production in NK cells and T cells (14–16). Our group has previously reported the presence of several NK related cytokines, including the essential IL-15, activatory IL-12 and IL-18, and regulatory IL-10 and TGF-β in healthy liver tissue (9). Secreted TGF-β and IL-10 were detected in all LCM samples at concentrations of 270.3 ± 63.1 pg/ml and 124.3 ± 22.3 pg/ml, respectively (**Figure 5A**). Next, we assessed the effect of IL-10 and TGF-β on the phenotype on hepatic NK cells in culture.

Hepatic CD56^{bright}Eomes^{hi}Tbet^{lo} NK cells were isolated by FACS sorting. They were cultured in RPMI supplemented with 10% human AB serum and IL-15 (2 ng/ml) for 7 days with or without LCM (10% v/v), IL-10 (10 ng/ml), or TGF-β (5 ng/ml). Media was replenished ever 2–3 days. As before Tbet and Eomes expression increased during culture and LCM significantly reduced the expression of Tbet and Eomes (**Figures 5B–D**). IL-10 had no effect on Tbet expression (MFI 792.4 ± 56.4, **Figure 5C**), however TGF-β significantly reduced the expression of Tbet compared to untreated cells (untreated MFI 760.5 ± 46.9, LCM 381.1 ± 78.2, TGF-β 385.2 ± 79.8, $p = 0.0014$, **Figure 5C**). IL-10 did not significantly alter the expression of Eomes, while TGF-β reduced Eomes expression to a similar level to LCM, however this did not reach statistical significance (**Figure 5D**).

Treatment of magnetic bead-purified peripheral blood NK cells with TGF-β for 7 days likewise resulted in a significant decrease in Tbet expression in CD56^{bright} NK cells (Day 7 untreated 5,776 ± 679 MFI, TGF-β treated 2,979 ± 328 MFI, $p = 0.0421$, **Figures 5E,F**). A significant decrease in Eomes expression upon TGF-β treatment was also observed (Day 7 untreated 873 ± 34 MFI, TGF-β treated 539 ± 19 MFI, $p = 0.0024$, **Figure 5G**).

Inhibiting TGF-β Signaling Partially Reverses the Effect of LCM Treatment on Liver Resident and Blood NK Cells

We next inhibited TGF-β receptor signaling using the inhibitor SB431542, which inhibits the TGF-β type I receptor activin receptor-like kinase (ALK)5 and related genes ALK4 and ALK7. Liver CD56^{bright} NK cells were pre-treated with SB431542 for 2 h before being treated with LCM. As before, treatment with LCM significantly reduced the expression of Tbet compared to untreated cells (**Figures 6A,B**). Pre-treatment with SB431542 prevented the reduction in Tbet expression (LCM: MFI 356 ± 49.6, LCM+SB431542: MFI 552.8 ± 69.6, $p = 0.0239$, **Figure 6B**). The addition of SB431542 also significantly

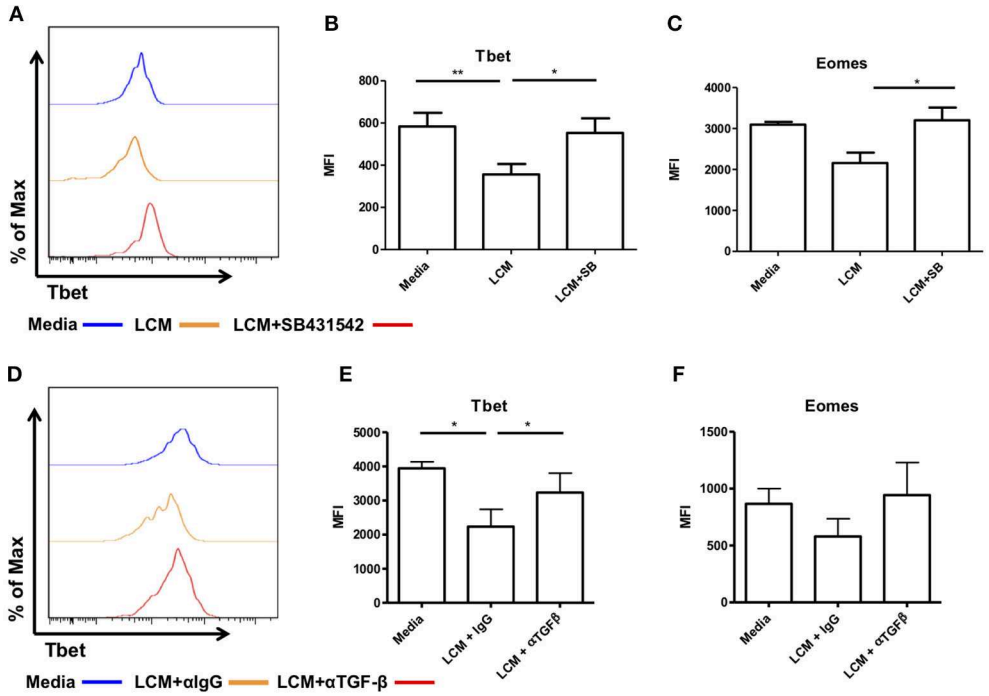

FIGURE 6 | Blocking TGF-β inhibits the ability of liver conditioned media to suppress Tbet and Eomes expression. CD56^{bright} Eomes^{hi} Tbet^{lo} NK cells FACS isolated from liver perfusate (LP) were cultured for 7 days, supplemented with 10% v/v liver conditioned media (LCM) with and without SMAD inhibitor SB431542 and acquired on a FACS Fortessa. **(A)** Representative histogram of Tbet expression from cells at day 7 untreated (blue line), 10% LCM (orange line) or LCM & SB431542 (red line). **(B)** MFI of Tbet in CD56^{bright} NK cells from LP at day 7 with LCM or LCM & SB431542. **(C)** MFI of Eomes in CD56^{bright} NK cells from LP at day 7 with LCM or LCM & SB431542. NK cells isolated from peripheral blood (PB) were cultured for 7 days, supplemented with 10% v/v LCM with 5 μg/mL anti-TGF-β1 blocking antibody or IgG isotype control and acquired on a FACS Canto II. **(D)** Representative histogram of Tbet expression in CD56^{bright} NK cells from peripheral blood at day 7 with LCM or LCM & TGF-β1 blocking antibody. **(E)** MFI of Tbet in CD56^{bright} NK cells from peripheral blood at day 7 with LCM or LCM & TGF-β1 blocking antibody. **(F)** MFI of Eomes in CD56^{bright} NK cells from peripheral blood at day 7 with LCM or LCM & TGF-β1 blocking antibody. Data presented as mean ± SEM. Data was analyzed using Friedman test, with Dunn's multiple comparison test or repeated measures one-way ANOVA ($n = 3$–8, $^{*}p < 0.05$ and $^{**}p < 0.01$).

increased the expression of Eomes compared to LCM treated cells (**Figure 6C**).

In order to confirm that this effect was specific to TGF-β receptor signaling we next treated magnetic bead-purified blood NK cells with LCM and 5 μg/ml of a TGF-β1 blocking antibody (clone 19D8, BioLegend) or isotype control (clone MOPC-21, BioLegend). Blocking TGF-β1 prevented the inhibition of Tbet in CD56^{bright} NK cells (**Figures 6D,E**). Blocking TGF-β1 also prevented the inhibition of Eomes by LCM although this did not reach statistical significance (**Figure 6F**).

DISCUSSION

In this study, we have shown that the liver microenvironment is essential for maintaining the unique Eomes^{hi}Tbet^{lo} phenotype of liver resident NK cells. NK cells isolated from liver perfusate lose their unique Eomes^{hi}Tbet^{lo} phenotype when cultured, but this can be reversed by the addition of LCM. The liver is rich in immunoregulatory cytokines, including TGF-β and IL-10, produced by Kupffer cells and immature dendritic cells (9, 17–19). Liver resident NK cells cultured with TGF-β, but not IL-10, maintain their Eomes^{hi}Tbet^{lo} phenotype, indicating that TGF–β is essential for maintaining liver resident populations. Blocking TGF-β signaling through pre-treatment with SB431542 reverses this effect and shifts the phenotype of liver resident NK cells to a peripheral blood-like state. Furthermore, Tbet expression in peripheral blood NK cells can be modulated by treatment with both LCM and TGF–β.

Culture with LCM can maintain CXCR6 expression, but it does not appear to induce expression of CXCR6 on peripheral blood NK cells. Cytokine stimulation (IL-12, IL-15) of peripheral blood NK cells can induce CXCR6 or CD49a expression, however the level of CXCR6 expression was highly variable and less than half of the stimulated NK cells showed expression, suggesting additional stimuli are required for sustained CXCR6 expression (20). NK cells may acquire CXCR6 expression in the periphery, through cytokine activation, and migrate to the liver. Here CXCL16, RANTES, and CCL3, present in the liver sinusoid, may retain activated NK cells (4, 21) and possibly sustain CXCR6 expression. Following recruitment to the liver sinusoid, TGF-β (produced by resident macrophages and dendritic cells) may alter transcription factor expression and repress the production of pro-inflammatory cytokines (IFN-γ).

Significant work has been performed investigating the effect of TGF-β on NK cells. TGF-β has been shown to have potent

immunosuppressive effects and alters NK cell development (15, 22). Furthermore, TGF-β has been shown to suppress glycolysis and inhibit the pro-inflammatory effector functions of $CD56^{bright}$ NK cells, such as IFN–γ production (23). The mechanism by which TGF-β inhibits Tbet and IFN-γ has previously been elucidated in T cells, where TGF-β signaling induces the expression of SHP-1 which in turn inhibits STAT1/4 and Tbet expression (24, 25). Recently, TGF-β has been shown to be involved in the conversion of NK cells to ILC1-like cells, via a JNK-dependent, Smad4-independent pathway resulting in reduced cytotoxicity in murine tumor models (26, 27). Why this process does not appear to occur in liver resident NK cells warrants further investigation. Intriguingly, Smad4 appears to suppress the JNK-dependent non-canonical TGF-β signaling associated with differentiation into ILC1-like cells within tissues, indicating that canonical TGF-β signals may be required to maintain NK cell phenotype. It is important to note there is a marked differences in the phenotype and function of liver resident NK cells between humans and mice (28). Furthermore, single cell RNA-seq analysis of human and murine NK cells have highlighted similarities but also significant differences in peripheral blood and splenic samples (29). Therefore, TGF-β may have divergent functions between species and care must be taken when comparing murine and human data.

In this study, we identified TGF-β as a factor required for the induction and maintenance of the tissue resident phenotype of liver resident NK cells in humans. Liver resident NK cells are unique in that they can provide immunosurveillance without producing large quantities of IFN-γ with its potential to drive tissue damaging pathology. In this context, liver resident NK cells can remove dysplastic or virally infected cells without perturbing the tolerogenic milieu of the liver, which so often leads to chronic inflammatory conditions. Further evidence for the importance of TGF-β in the establishment and maintenance of tissue resident NK cell populations in humans comes from analysis of uterine NK cells. Uterine NK cells share many phenotypic characteristics with liver resident NK cells ($CD56^{bright}Eomes^{hi}CD69^{+}$) (30). The local uterine microenvironment is rich in TGF-β and treatment of blood NK cells with either endometrial stromal cell supernatants or TGF-β alone can induce phenotypic changes resembling decidual NK cells (31).

Our results suggest that disruption of TGF–β levels in the liver microenvironment in disease could lead to a loss of local immune regulation and the promotion of tissue inflammation and damage. While the roles of liver resident NK cells in maintaining homeostasis or mediating liver disease have yet to be established in humans, a reduction in TGF-β levels during liver disease could drive resident NK cell populations to a more conventional pro-inflammatory phenotype and contribute to chronic inflammation. In this situation, restoring homeostatic cytokine levels would be essential to regulate local immunity and tissue repair in the liver. Interestingly, the use of a strong pro-inflammatory signal, such as PMA, appears to overcome this TGF-β mediated repression and restore IFN-γ production (2, 5). This appears similar to the mechanism by which TGF-β imprinted peripheral blood NK cells become hyper-producers of IFN-γ *in vitro* (32). While in healthy liver these resident NK cells have suppressed pro-inflammatory function, it appears this can be overcome with sufficient stimulation.

The liver is a naturally tolerogenic environment, which maintains unique anti-inflammatory status even in the presence of immune activating dietary antigens and bacterial components. It is therefore no surprise to find that liver resident NK cells are profoundly changed by residing in this microenvironment. TGF-β is one of the chief mediators of the tolerogenic hepatic environment and we have shown here that this regulation extends to the phenotype and function of NK cells. Through the suppression of Tbet, liver resident NK cells have reduced pro-inflammatory potential but maintain their ability to perform immunosurveillance in an organ prone to infection and malignancy.

ETHICS STATEMENT

All protocols were approved by St. Vincent's University Hospital Ethics Committee and the Trinity College Dublin School of Medicine Research Ethics Committee, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Patients were consented pre-operatively by DH, EH, or JG. All patients were supplied with a information packet informing them of the project aims, their rights to withdraw at any time and the length of time samples and data would be retained. All patients were over the age of 18 and had no conditions which would preclude them from giving informed consent.

AUTHOR CONTRIBUTIONS

CO, MR, and CH contributed to the conception and design of the study. DH, EH, and JG acquired data and managed clinical samples. CH, GJ, and DA designed and carried out experimental procedures. Statistical analysis was performed by CH, GJ, and MR. CO, MR, CH, DH, EH, and JG were involved in interpretation of data and manuscript preparation. Manuscript drafting was performed by CH, MR, and CO. All authors reviewed and approved the final manuscript.

FUNDING

This work was supported by grants from the Health Research Board of Ireland (RP 2008/189 and EIA-2017-013) and a Science Foundation Ireland Investigator Award (12/IA/1667).

ACKNOWLEDGMENTS

We wish to thank donor families for participating in this research project. The support of the whole liver transplant team at St. Vincent's University Hospital is gratefully acknowledged. The authors would also like to thank all members of the Comparative Immunology Group for helpful discussion.

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

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frontiers in Immunology

ORIGINAL RESEARCH
published: 04 June 2019
doi: 10.3389/fimmu.2019.01255

Retained NK Cell Phenotype and Functionality in Non-alcoholic Fatty Liver Disease

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OPEN ACCESS

Edited by:
Michael A. Caligiuri,
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Specialty section:
This article was submitted to
NK and Innate Lymphoid Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 03 December 2018
Accepted: 17 May 2019
Published: 04 June 2019

Citation:
Stiglund N, Strand K, Cornillet M,
Stål P, Thorell A, Zimmer CL,
Näslund E, Karlgren S, Nilsson H,
Mellgren G, Fernø J, Hagström H and
Björkström NK (2019) Retained NK
Cell Phenotype and Functionality in
Non-alcoholic Fatty Liver Disease.
Front. Immunol. 10:1255.
doi: 10.3389/fimmu.2019.01255

Non-alcoholic fatty liver disease (NAFLD), and the progressive stage non-alcoholic steatohepatitis (NASH), is the predominant cause of chronic liver disease globally. As part of the complex pathogenesis, natural killer (NK) cells have been implicated in the development of liver inflammation in experimental murine models of NASH. However, there is a lack of knowledge on how NK cells are affected in humans with this disease. Here, we explored the presence of disease-specific changes within circulating and tissue-resident NK cell populations, as well as within other major immune cell subsets, in patients with liver biopsy-confirmed NAFLD. Using 18-color-flow cytometry, substantial changes were observed in certain myeloid populations in patients as compared to controls. NK cell numbers, on the other hand, were not altered. Furthermore, only minor differences in expression of activating and inhibitory NK cell receptors were noted, with the exception of an increased expression of NKG2D on NK cells from patients with NASH. NK cell differentiation remained constant, and NK cells from these patients retain their ability to respond adequately upon stimulation. Instead, considerable alterations were observed between liver, adipose tissue, and peripheral blood NK cells, independently of disease status. Taken together, these results increase our understanding of the importance of the local microenvironment in shaping the NK cell compartment and stress the need for further studies exploring how NASH affects intrahepatic NK cells in humans.

Keywords: natural killer cells, liver immunology, adipose tissue immunology, NAFLD, obesity

INTRODUCTION

NK cells are an important part of innate immunity where they participate in the defense against viral infections and in tumor surveillance (1) Upon activation, NK cells perform cytotoxicity by the release of cytolytic granules. They can also contribute to a pro-inflammatory environment through the production of cytokines and chemokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (1). As part of the innate immune system, NK cells were believed to retain a static phenotype during their life span with little evidence of differentiation except for transition from

$CD56^{bright}$ to $CD56^{dim}$ NK cells (2). However, this view has been revised in the last decade and it is now clear that NK cells gradually undergo directed differentiation even after they have reached the $CD56^{dim}$ stage (2). In addition to their presence in the circulation, NK cells are found in numerous peripheral tissues and are especially enriched in the liver and uterus where they comprise up to 30 and 45%, respectively, of all lymphocytes (3, 4). However, compared to circulating NK cells, less is known regarding NK cells residing in tissues. In relation to the liver, studies have in recent years shown the importance of NK cells in the pathogenesis and clearance of chronic viral hepatitis infections in humans (5). However, the role of NK cells in many other liver diseases remains elusive.

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. A sub-group of NAFLD patients develop chronic inflammation in the liver, which over time can lead to liver fibrosis. This stage of the disease is known as non-alcoholic steatohepatitis (NASH) (6) and these patients are at risk of developing liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (7). The increased rates of obesity in many countries has contributed to the drastic global increase in NAFLD prevalence in recent years (8). Thus, NAFLD complications, such as NASH, liver cirrhosis, and HCC, are posing a significant challenge to health care systems worldwide (6). Several theories exist as to why liver inflammation develops in patients with NAFLD. In more detail, disease development is believed to be influenced by an interplay between genetic and environmental factors, ranging from disturbances in lipid storage and metabolism, changes in dietary patterns and microbiota, to perturbed immune activation (6). However, the exact mechanisms as to why some patients progress in their disease whilst others do not still remain elusive.

Interestingly, murine models have revealed the importance of innate immunity, and in particular NK cells, during NASH-development (9). Many NK cell ligands are up-regulated in the liver of mice with NASH and this is followed by influx of activated cytotoxic NK cells (10, 11). Furthermore, NK cells can, via Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) production, promote a pro-inflammatory state in the steatotic liver and by this mechanism contribute to progression toward steatohepatitis (10). In addition, NK cell activation in response to IL-15 promotes NASH-development in mice (12) and NK cells are also thought to play an important role in regulating fibrosis development in NASH (13, 14). In addition, obesity itself can also alter NK cell phenotype, metabolism, and function (15–18). Indeed, several recent studies in mice have suggested NK cells to be important for development of insulin resistance (19–21). However, there is a lack of knowledge on how NK cells are affected in humans with NASH, both with respect to circulating NK cells as well as to NK cells residing in metabolically active tissues such as liver and adipose tissue. To address this, we here performed an in-depth phenotypic and functional analysis of circulating NK cells in NAFLD patients as well as explored the NK cell compartment in liver and adipose tissue of these individuals.

Abbreviations: ALT, alanine transferase; AST, aspartate transferase; BMI, body mass index; HCC, hepatocellular carcinoma; HOMA-IR, homeostatic model assessment; HSC, hepatic stellate cell; IFN-γ, interferon-gamma; IL, interleukin; ILC1, innate lymphoid cell group 1; KIR, killer cell immunoglobulin-like receptor; MAIT, mucosa-associated T; mDC, myeloid dendritic cell; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NAS, NAFLD activity score; NK, natural killer; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; SDI, Simpson diversity index; SNE, stochastic neighbor embedding; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumor-necrosis factor.

MATERIALS AND METHODS

Clinical Cohorts

Several clinical cohorts were included in the current study. All studies were approved by the regional ethics committee in Stockholm (Dnr's: 2010/678-31/3, 2006/971-31/1, 2006/229-31/3, and 2014/979-31/1) and oral and written informed consent was obtained from all participants. First, peripheral blood samples were obtained from 26 patients with liver biopsy-confirmed NAFLD from the out-patient clinic at the Upper GI Tract Department, Karolinska University Hospital, Stockholm, Sweden. See **Table 1** for detailed patient characteristics. Secondly, as controls, peripheral blood from 15 healthy blood donors was collected from the blood bank at the Karolinska University Hospital, Stockholm, Sweden. Inclusion criteria in controls were normal body-mass index (BMI), normal liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], and no history of type 2 diabetes. Thirdly, in order to assess tissue resident NK cells, peripheral blood as well as liver and adipose tissue biopsies were collected from 26 patients undergoing laparoscopic gastric bypass surgery for morbid obesity at Danderyd and Ersta Hospitals in Stockholm. All patients had a BMI above 35 and had no previous history of liver disease. Included patients were not prescribed any low-calorie pre-surgery diet since this would influence degree of liver steatosis and possibly liver inflammation. A fraction of obtained liver biopsies was used for clinical scoring of liver histology according to the NAFLD activity score (NAS) and fibrosis stage. Based on the severity of the liver histology, patients were divided into three groups; patients with normal liver histology, patients with liver steatosis only (non-alcoholic fatty liver, NAFL), and patients with liver inflammation (NASH). See **Table 2** for more detailed characteristics on this patient cohort.

Isolation of PBMC From Blood Samples

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using density gradient centrifugation. Briefly, whole blood was diluted with phosphate buffered saline (PBS; Invitrogen, USA), carefully layered on top of Ficoll-Hypaque (GE Healthcare, UK), and centrifuged. The leukocyte layer was extracted, carefully washed, and cryopreserved in freezing medium [90% heat-inactivated fetal bovine serum (FBS; Sigma-Alderich, USA) and 10% dimethyl sulfoxide (DMSO; Life Technologies)] until flow cytometry experiments were performed.

TABLE 1 | NAFLD cohort characteristics.

	Healthy controls	NAFL[a]	NASH[b]
Subjects	15	11	15
Age, mean (years)	54	56	58
Male, n	10 (67%)	8 (73%)	9 (60%)
Female, n	5 (33%)	3 (27%)	6 (40%)
BMI[c] mean (kg/m^2)	24	35	31
ALT[d], mean (μkat/L)	NA	0.51	0.75
NAFLD Activity Score, median (range)	NA	3 (1–4)	5 (5–7)
Steatosis (0–3), median (range)	NA	1 (1–2)	2 (1–3)
Lobular inflammation (0–3), median (range)	NA	1 (1–3)	2 (1–3)
Hepatocyte ballooning (0–2), median (range)	NA	0 (0–1)	2 (1–2)
Fibrosis score (0–4)[e], median	NA	0 (0–1)	2 (0–3)

[a]*Non-alcoholic fatty liver.*
[b]*Non-alcoholic steatohepatitis.*
[c]*Body Mass Index.*
[d]*Alanine aminotransferase.*
[e]*Fibrosis score according to Kleiner et al. (22).*
NA, not applicable.

TABLE 2 | Bariatric surgery cohort characteristics.

	Non-NAFLD	NAFL[a]	NASH[b]
Subjects	8	10	5
Age (year), mean	43	45	43
Male	1 (12.5%)	3 (30%)	0 (0%)
Female	7 (87.5%)	7 (70%)	5 (100%)
BMI[c], mean (kg/m^2)	37	37	37
ALT[d] (μkat/L), mean	0.37	0.51	0.75
P-Insulin (IU[e]), mean	14.3	19.8	36.0
P-Glucose (mmol/L), mean	5.6	6.4	6.5
HOMA-IR[f], mean	3.6	5.7	10.5
NAFLD Activity Score, median (range)	0 (0)	3.5 (1–4)	5 (5)
Steatosis (0–3), median (range)	0 (0)	1 (1–3)	2 (1–2)
Lobular inflammation (0–3), median (range)	NA	1 (0–2)	2 (1–2)
Hepatocyte ballooning (0–2), median (range)	NA	1 (0–2)	1 (1–2)
Fibrosis score (0-4)[g], median (range)	1 (0–2)	1 (0–2)	2 (1–3)

[a]*Non-alcoholic fatty liver.*
[b]*Non-alcoholic steatohepatitis.*
[c]*Body Mass Index.*
[d]*Alanine aminotransferase.*
[e]*International Units.*
[f]*Homeostatic model assessment—insulin resistance.*
[g]*Fibrosis score according to Kleiner et al. (22).*

Isolation of Immune Cells From Liver and Adipose Tissue

The core liver biopsies were collected during surgery directly into complete cell medium [Hyclone RPMI (Invitrogen), 10% FBS, and 1 mM L-glutamine (Invitrogen)] and kept on ice until same-day processing. Tissue pieces were mechanically dissociated, followed by enzymatic digestion in collagenase II for 30 min at 37°C, and filtered through a 100 μm filter, before flow cytometry staining.

Antibody Staining Protocol

Cryopreserved PBMC were thawed in a 37°C water bath, immediately transferred to cell medium, washed, and resuspended in complete cell medium. Three million PBMC were stained in each test. Flow cytometry primary and secondary stainings were performed in flow buffer (PBS with 2 mM EDTA and 2% FBS) for 20 min in the dark at room temperature. After staining, cells were fixed for 15 min in the dark at room temperature using the Fix/Perm solution (eBioscience, USA). Finally, Fix/Perm solution was washed away, cells were resuspended in flow buffer, and kept at 4°C in the dark until they were acquired on the flow cytometer. For intracellular stainings, cells were permeabilized in Fix/Perm solution for 45 min and then stained for 30 min in permeabilization buffer (eBioscience, USA), diluted 1:10 with MQ water, before being washed and analyzed. All samples were run on an 18-color LSRFortessa (BD Biosciences, USA) equipped with 355, 405, 488, 561, and 639 nm lasers.

Functional Experiments

NK cell degranulation and cytokine production were assessed by co-culture experiments with target cells. PBMCs were pre-stimulated overnight with IL-12 (10 ng/ml, Peprotech) and IL-18 (100 ng/mL, Medical & Biological Laboratories) and then K562 cells were added at a 1:10 ratio. One hour after addition of target cells, Golgi plug (Brefeldin A, BD Biosciences) and Golgi stop (Monesin, BD Biosciences) were added and the assay was continued for an additional 5 h. NK cells were then stained for analysis using flow cytometry.

Microscopy

Liver biopsy specimens obtained from NAFL/NASH patients and patients undergoing gastric bypass surgery were stained with hematoxylin and eosin and graded in a blinded fashion by an experienced hepatologist according to the NAFLD activity score (NAS) (23). In addition, liver biopsies were stained with Sirius red to evaluate liver fibrosis and scored according to Kleiner on a 0–4 scale (23).

Flow Cytometry Analysis

To avoid bias from intra-experimental variability that could affect the flow cytometry analysis, samples from both healthy donors and patients were analyzed in each experiment. Acquired data was compensated using a compensation matrix generated based on antibody-stained control beads and analyzed using FlowJo Version 9.6.4 (Treestar, USA). Apart from conventional flow cytometry analysis, Barnes-Hut stochastic neighbor embedding (SNE) analysis, using an in-house built script (24) in R (The R Foundation for Statistical Computing), was performed in order to visualize potential differences not present in two-dimensional space. For SNE-analysis, 1,000 CD56^{dim} NK cells or 500 CD56^{bright} NK cells from each donor was included. The data was clustered based on median fluorescence intensity (MFI) of the following markers: CD16, CD25, CD44, CD49a, CD56, CD57, CD69, CD107a, HLA-DR, IFN-γ, KIRs, MIP-1β, NKG2A, NKG2C, and TNF.

Staining of Primary Hepatocyte

Primary human hepatocytes were isolated from three organ donors whose livers were not used for liver transplantation, acquired from the Karolinska University Hospital, using a protocol previously described (25). The cells were then stained fresh with antibodies against MICA, MICB, HLA-ABC, CD155, ULBP-1, ULBP-2, and ULBP-3 conjugated with PE and analyzed on a BD FACS Accuri.

Quantification of Soluble MICA and MICB by ELISA

MICA and MICB solid-phase sandwich ELISAs (enzyme-linked immunosorbent assay, Thermofisher) were performed according to the manufacturer instructions. Briefly, human sera were incubated 2, 5, and 2 h respectively diluted two times in provided buffers (capture phase). After washing, biotin-conjugated anti-MICA and MICB antibodies were incubated for 1 h and streptavidin-HRP was added after washing. Unbound streptavidin-HRP was removed by washing and substrate solution reactive with HRP was added. The reaction was stopped by acid after 30 min and absorbance measured at 450 nm.

Statistical Analysis

Data were analyzed using Prism Version 6.0 b (GraphPad Software Inc.; USA). The Mann–Whitney *U*-test or *t*-tests were used depending on if data sets were normally distributed, using D'Agostino-Pearson omnibus normality test. The threshold for statistical significance was set to $\alpha < 0.05$. For analysis of NK cell population diversity, Simpson Diversity Index (SDI) was calculated as previously described (26).

RESULTS

Global Assessment of Immune Cells in NAFL and NASH

To determine the impact of NAFL and NASH on the peripheral blood immune cell compartment, a broad profiling of major lymphoid and myeloid immune cells in patients and controls was initially performed (**Figure 1A**). Whereas, few differences were noted among CD4, CD8, and γδ T cells, a trend toward a decline in MAIT cells was observed in NASH patients as compared to NAFL patients and healthy controls (**Figure 1B**). A decrease of MAIT cells in NASH is in line with recent literature (27). Regarding NK cells, neither frequency (**Figure 1B**) nor absolute numbers (data not shown) were affected by the presence of obesity, NAFL, or NASH. With respect to the myeloid immune cell compartment few differences were observed for monocytes and myeloid DCs. However, a decline in the frequency of plasmacytoid DCs (pDCs) was noted in NASH patients (**Figure 1C**). Also, the absolute numbers of pDCs were decreased and the loss of pDCs correlated inversely with the degree of liver damage measured as serum alanine transferase levels (**Figure 1C**). Taken together, although alterations could be observed in certain innate immune cell subsets, the overall size of the peripheral blood NK cell compartment remained unaltered in NAFL and NASH.

Upregulation of NKG2D on NK Cells From NASH Patients

Since NK cells are far from a homogeneous population, a more in-depth immune-phenotyping of activating and inhibitory receptors on circulating NK cells was performed. The $CD56^{dim}$ to $CD56^{bright}$ NK cell relationship was unaffected in NAFL and NASH (**Figures 2A,B**). Next, we simultaneously assessed expression of 12 surface and intracellular markers on the NK cells (**Figure 2C**). As expected, $CD56^{dim}$ NK cells expressed higher levels of NKG2C, KIRs, and CD57, while $CD56^{bright}$ NK cells had a higher expression of NKG2A, CD161, CD44, and NKp46 (**Figures 2C,D**). Surprisingly, neither the degree of NAFLD disease severity (**Figure 2D**) nor presence of obesity (data not shown) had a detectable effect on the NK cell receptor repertoire on circulating NK cells, with the exception for expression of the activating receptor NKG2D. In more detail, both $CD56^{bright}$ and $CD56^{dim}$ NK cells from patients with NASH expressed significantly higher levels of NKG2D on their surface (**Figures 2E,F**). This was also observed when comparing normal weight with obese individuals (**Figure 2G**). However, since NK cells from NAFL patients had close to normal levels of NKG2D (**Figure 2F**), this would suggest that increased expression of NKG2D primarily associated with NASH. Furthermore, this increase was specific to NK cells since it was not observed on T cells from the same patients (data not shown). To dissect the role of NKG2D more in-depth in relation to the liver and NAFL we assessed presence of NKG2D-ligands. No difference in levels of soluble MICA and MICB was noted in patients as compared to controls (data not shown). Furthermore, primary human hepatocytes from healthy organ donors were negative for NKG2D-ligands whereas CD155 and HLA class I was expressed (**Supplementary Figure 1**).

Finally, we assessed NK cell differentiation, as determined by the expression of NKG2A, KIRs, and CD57, as well as NK cell diversity by calculating Simpson diversity index (SDI) (28, 29). However, both of these metrics for NK cell compartment composition remained unaltered in NAFL and NASH patients as compared to healthy controls (**Figures 2H,I**). In summary, the phenotype of the circulating NK cell population remains unaffected by NAFL and NASH with the exception of NKG2D being upregulated in patients with NASH.

Functional Capacity of Peripheral NK Cell Subsets in NAFL and NASH

Having determined the NK cell phenotype in peripheral blood, we next evaluated the functional capacity of NK cells in NAFL and NASH. To this end, NK cells were stimulated with cytokines (IL-12+IL-18) and/or K562 target cells and production of IFN-γ, TNF, MIP-1β, upregulation of CD107a, CD69, CD44, and CD25, as well as downregulating of CD16 as a consequence of activation was measured using flow cytometry (**Figure 3A**). As expected, stimulation with cytokines led to high levels of IFN-γ being produced as well as strong upregulation of CD25 and CD69 whereas K562 cell stimulation yielded a robust degranulation response and elevated levels of TNF and MIP-1β (**Figures 3A,B**). When assessing single functional responses, NK cells from

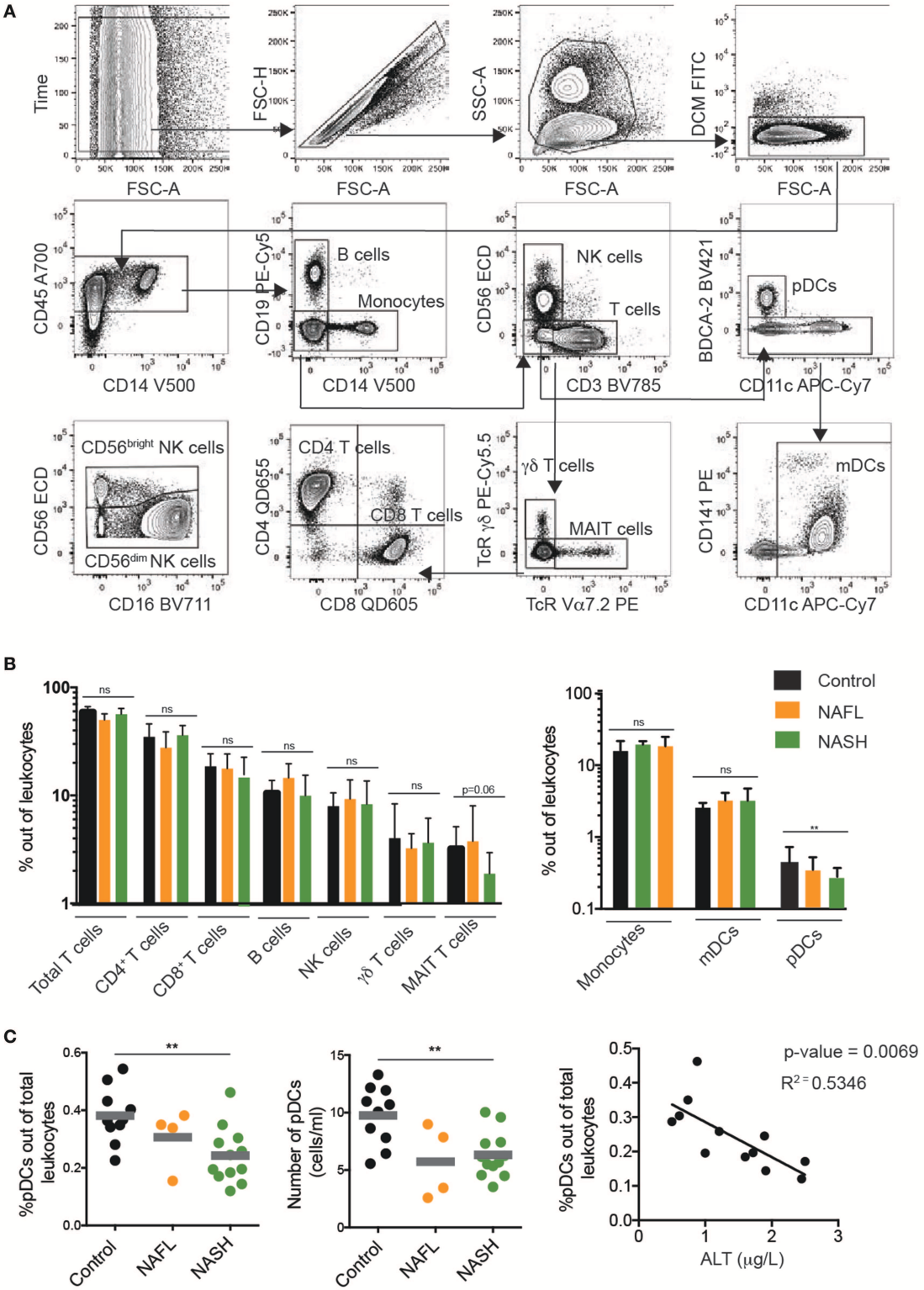

FIGURE 1 | Immunophenotyping of major peripheral blood immune cell subsets in NAFL and NASH. **(A)** Flow cytometry gating scheme used to identify the investigated immune cell subsets. Arrows indicate the sequence of gating. **(B)** Summary data for the frequency out of total leukocytes for the indicated immune cell populations in healthy controls ($n = 10$), NAFL ($n = 4$), and NASH ($n = 11$) patients. **(C)** Summary data of pDC frequency out of total leukocytes (left), absolute counts of pDCs (middle), and correlation between pDC frequency and ALT (right) in the indicated patient groups. Bars in **(B,C)** represent mean and error bars show SEM. $^{**}p < 0.01$.

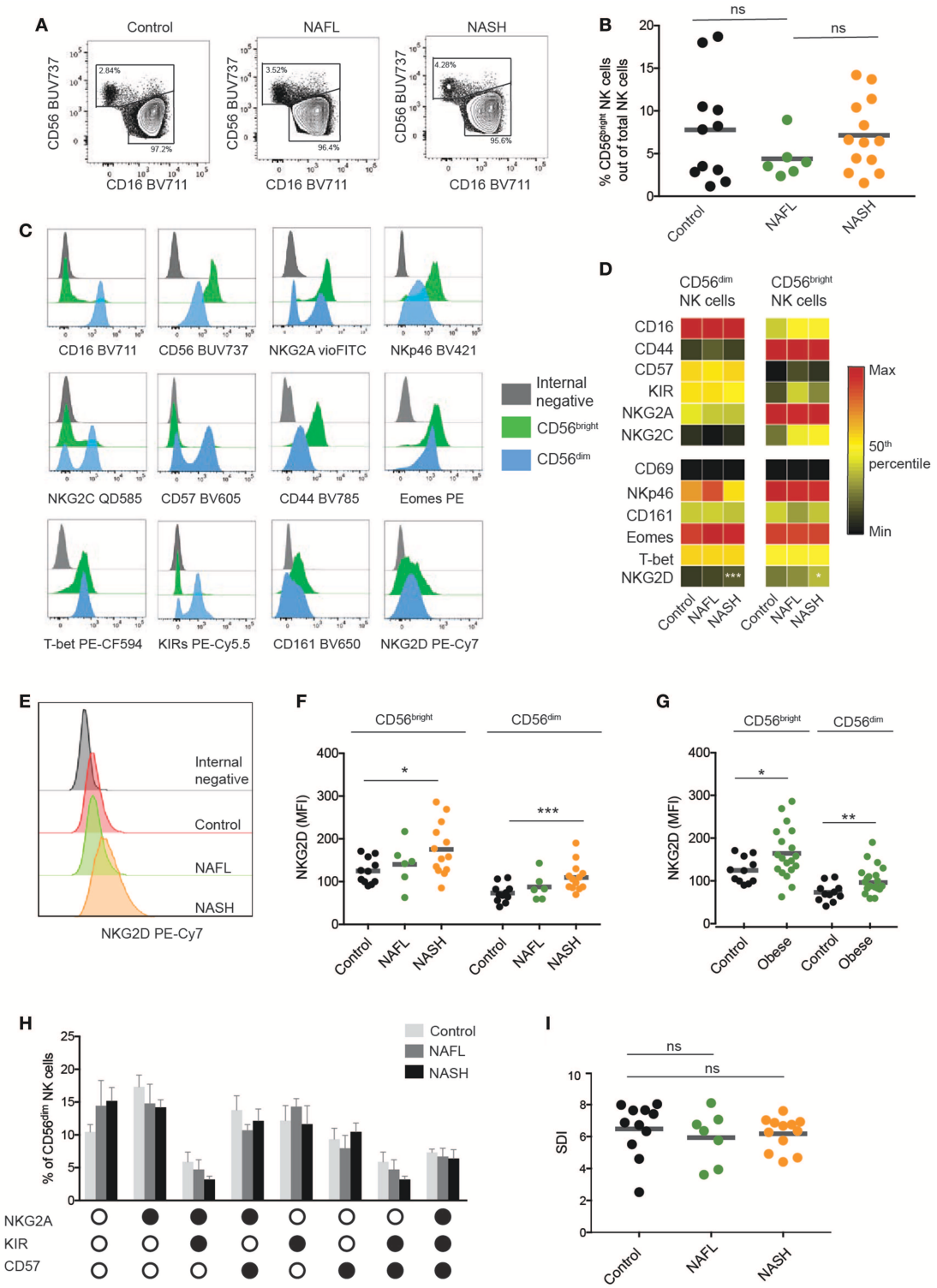

FIGURE 2 | Phenotypic characterization of circulating NK cells from NAFLD patients. **(A)** Representative flow cytometry plots of NK cells from healthy, NAFL, and NASH patients. **(B)** Frequency of CD56^{bright} NK cells out of total NK cells in peripheral blood of healthy controls (n = 13), NAFL patients (n = 9), and NASH patients (n = 16). **(C)** Representative histograms for the indicated markers on CD56^{bright} and CD56^{dim} NK cells as well as internal negative control. The plots represent stainings from one healthy donor. **(D)** Heat map depicting the mean frequency of NK cells expressing CD16, CD44, CD57, KIRs, NKG2A, and NKG2C as well as the mean MFI of CD69, NKp46, CD161, Eomes, T-bet, and NKG2D on CD56^{dim} and CD56^{bright} NK cells for the indicated groups. **(E)** Representative histogram of NKG2D

(Continued)

FIGURE 2 | expression on NK cells from healthy control, NAFL, and NASH patients respectively. **(F,G)** Scatter plots of NKG2D MFI on $CD56^{dim}$ and $CD56^{bright}$ NK cells from the indicated groups. In **(F)**, healthy controls ($n = 11$), NAFL ($n = 6$), and NASH ($n = 13$) patients, in **(G)** healthy controls ($n = 11$), obese individuals ($n = 18$). **(H)** Bar graph showing the frequency of $CD56^{dim}$ NK cells that express different combination of NKG2A, KIRs, and CD57. Black circles indicate presence of the marker and white circles no expression. **(I)** Inverse Simpson Diversity index (SDI) analysis for healthy ($n = 11$), NAFL ($n = 7$), and NASH patients ($n = 12$). The Mann–Whitney *U*-test was used for comparison between groups. Bars in **(B,F,G,I)** show mean, error bars in H represent SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

patients with NASH had a similar capacity to degranulate, produce cytokines, and upregulate activation markers as their NAFL counterparts and the healthy controls (**Figure 3B**). Also, the ability of NK cells to perform multiple functions was retained in both NAFL and NASH patients as compared to healthy controls (**Figure 3C**).

The NK cell compartment consists of many different subpopulations and NK cells can produce a multitude of functions in a variegated fashion. Although single or multifunctional analysis of NK cell functional responses revealed no considerable alterations in function when comparing NASH with healthy controls it is plausible that functional difference might exist in multivariate space not allowing identification by conventional flow cytometry gating. To address this, we performed a SNE analysis of the NK cell functional responses and first generated SNE maps of responding compared to non-responding NK cells (**Figure 3D**). These SNE maps segregated considerably (**Figure 3D**, residual map) and by projecting the differences onto the single parameters that formed the basis of the SNE map the pattern of NK cell responses in multidimensional space could be revealed (**Figure 3D**, lower panels). In more detail this shows how certain NK cell subpopulations responded with many functions and others only with single function. Together, this validated the analysis approach and allowed us to compare patients with healthy controls (**Figure 3E** and data not shown). Although the residual plots revealed difference when comparing responding NK cells from healthy controls with NAFL or NASH patients (data not shown) or NAFL patients with NASH patients (**Figure 3E**), these differences could not be attributed to a specific phenotype when the residual plot was projected onto the single parameters (data not shown). This suggests that also in multi-dimensional space, the NK cell response was unaltered in NAFL and NASH.

Characterization of NK Cells in Adipose and Liver Tissue

To this end, characteristics of circulating NK cells have been analyzed. However, NAFL and NASH are diseases of the liver and also tightly coupled to obesity, adipose tissue dysregulation, and reduced insulin sensitivity. Thus, the analysis was focused on NK cells from liver and adipose tissue. In line with previous literature (9), NK cells were enriched in the liver compared to peripheral blood (**Figures 4A,B**) and a sizeable population of NK cells could also be detected in visceral adipose tissue. Furthermore, both types of investigated tissue had a skewing in the distribution of $CD56^{bright}$ and $CD56^{dim}$ NK cells as compared to circulation with $CD56^{bright}$ NK cells representing up to half of all NK cells in liver and adipose tissue (**Figure 4B**). Also, whereas the differentiation status of adipose tissue NK cells, with respect to expression of NKG2A, KIRs, and CD57, mirrored that of circulating NK cells, differences were observed especially within liver $CD56^{dim}$ NK cells in expression of these markers (**Figures 4C,D**).

SNE-analysis of the NK cell compartment identified that NK cells derived from liver and adipose tissue displayed a unique phenotype compared to peripheral blood NK cells (**Figure 4E**). The major distinction between NK cells derived from circulation and NK cells derived from tissues was a higher expression of the tissue residency marker CD69 (**Figure 4E**). As a consequence of liver and adipose tissue containing larger populations of $CD56^{bright}$ NK cells, the SNE analysis also identified higher expression of NKp46, NKG2A, and CD56 but lower expression of CD16, CD57, and KIRs within the population enriched in the tissues compared to in circulation (**Figure 4D**). This is in line with the overall phenotypic differences observed when comparing $CD56^{bright}$ with $CD56^{dim}$ NK cells (**Figures 2C,D**). Finally, we confirmed expression of tissue residency markers on liver and adipose tissue by conventional flow cytometry gating (**Figures 4F,G**). As expected, the expression was primarily confined to the $CD56^{bright}$ NK cells. Interestingly, while CD69 was highly expressed in both liver and adipose tissue, CD49a was only found on a small fraction of $CD56^{bright}$ NK cells in the liver whereas higher expression was noted in adipose tissue (**Figures 4F–H**). These results highlight the importance of the organ-specific microenvironment in shaping the local NK cell population and emphasize the importance of studying the tissue-resident compartment when trying to understand the pathogenesis of diseases affecting peripheral organs.

The Phenotype of Tissue NK Cells Remains Unaltered in NAFL and NASH

Finally, we assessed the impact of NAFL and NASH disease stage and severity on the frequency and phenotype of liver and adipose tissue NK cells (**Figure 5**). Patients where data on tissue NK cells were available were stratified based on NAS score (non-NAFL which represented no steatosis present, NAFL, NASH), liver fibrosis (fibrosis score 0–1 vs. 2–3), and insulin sensitivity status (HOMA-IR <4.5 vs. >4.5). Although NK cells were more abundant in liver (**Figure 4B**) their frequency remained unaffected by clinical stage (**Figure 5C**). Also, the frequency of NK cells in adipose was unaltered by NAFLD disease activity, level of fibrosis, and insulin sensitivity (**Figure 5C**). Finally, the NK cell phenotype was assessed. Since $CD16^{-}$ and $CD16^{+}$ NK cells display distinct phenotypes (**Figures 2C,D**, **4E–G**), these were analyzed separately. For none of the investigated phenotypic markers, a clear link to the clinical parameters could be observed (**Figure 5D**).

Thus, whereas murine models suggest NK cells to play a distinct role in NAFL and NASH, both the human circulating and

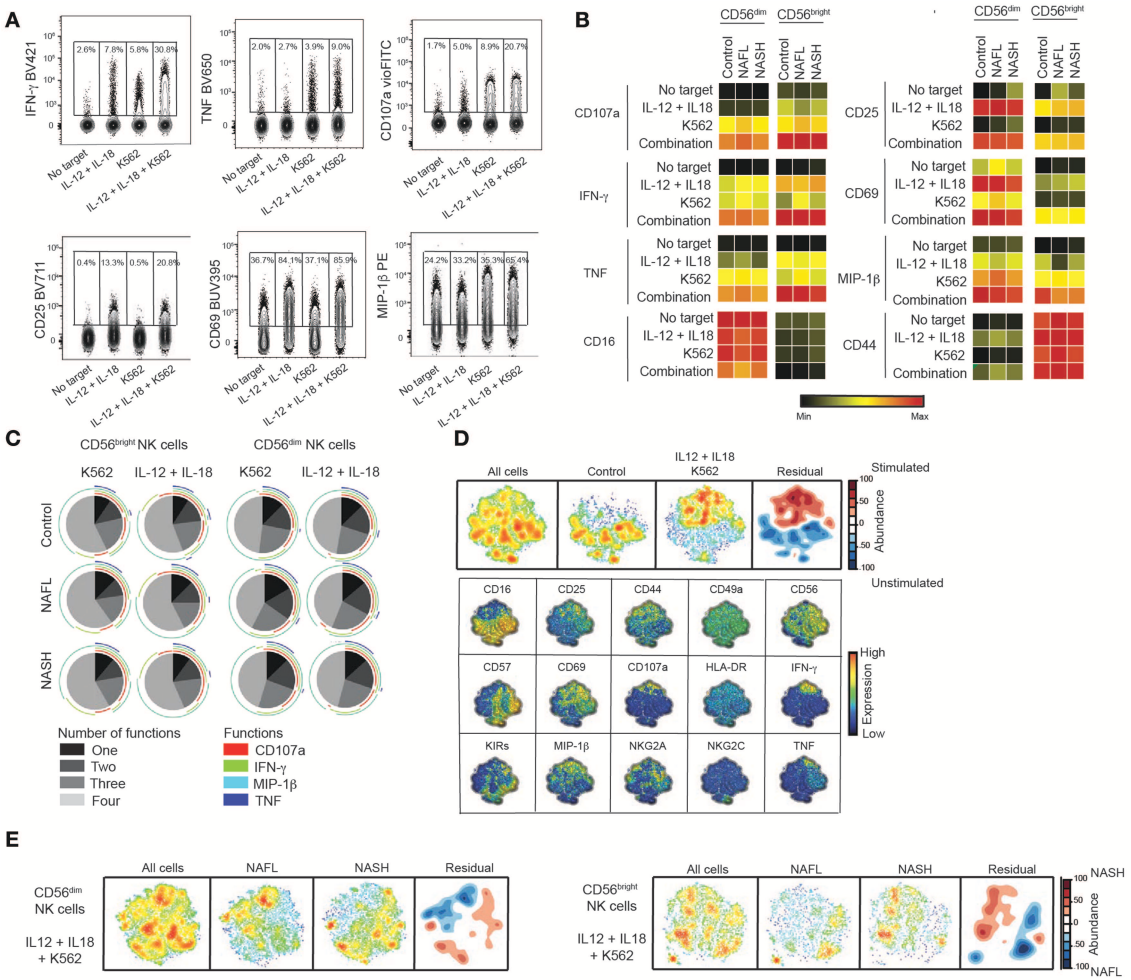

FIGURE 3 | Retained functional capacity of circulating NK cells in NAFLD. **(A)** Representative concatenated flow cytometry plots showing CD56^{dim} NK cell responses following the indicated stimulations. **(B)** Heat map summarizing the mean frequency of responding NK cells for the measured functions and indicated stimulations in healthy controls ($n = 15$), NAFL patients ($n = 10$), and NASH patients ($n = 16$). **(C)** Multifunctional analysis of the combination of degranulation (CD107a) and cytokine production (IFN-γ, MIP-1β, and TNF) responses for IL-12 + IL-18 stimulation and/or co-culture with K562 target cells. **(D)** SNE plots of total cells from healthy donors subsequently divided in stimulated and un-stimulated cells. The residual plot highlights the decreased (blue) or more highly expressed (red) areas in the stimulated cells. Underneath, individual plots of the expression of 15 single markers are shown. **(E)** SNE plots depicting the difference between CD56^{dim} (left) and CD56^{bright} (right) NK cells from NAFL and NASH patients after IL-12 + IL-18 + K562 stimulation where the density plots highlight the specific changes in NASH patients.

peripheral tissue NK cell compartments remain largely intact in these diseases.

DISCUSSION

NAFLD is the most common chronic liver disease worldwide and a subgroup of NAFLD patients develop chronic liver inflammation (NASH) with ensuing fibrosis and increased risk for HCC. Since little is known regarding NK cells in this disease and because NK cells are highly enriched in human liver, we here performed an extensive mapping of the NK cell compartment in NAFL/NASH using high-dimensional flow cytometry technology. We assessed the imprint of liver inflammation in NASH on circulating NK cells and show specific upregulation of the activating receptor NKG2D. In addition, by employing bariatric surgery as a human model, we also comprehensively mapped both liver and adipose tissue NK cells revealing substantial differences in NK cell population composition in-between tissues. However, no significant differences in the tissue-resident NK cell populations in patients with and without NAFL/NASH were detected.

NK cells have previously been investigated in other chronic liver diseases, especially in chronic viral hepatitis. These studies revealed that NK cells have decreased function in chronic hepatitis C (5, 30, 31). In obese individuals, decreased NK cell functionality combined with increased activation was reported (15, 16). A recent study showed that this reduced functionality was a result of metabolic paralysis of NK cells (18). However, our study detected no functional defects or changes in activation, despite both the in-depth and broad evaluation performed.

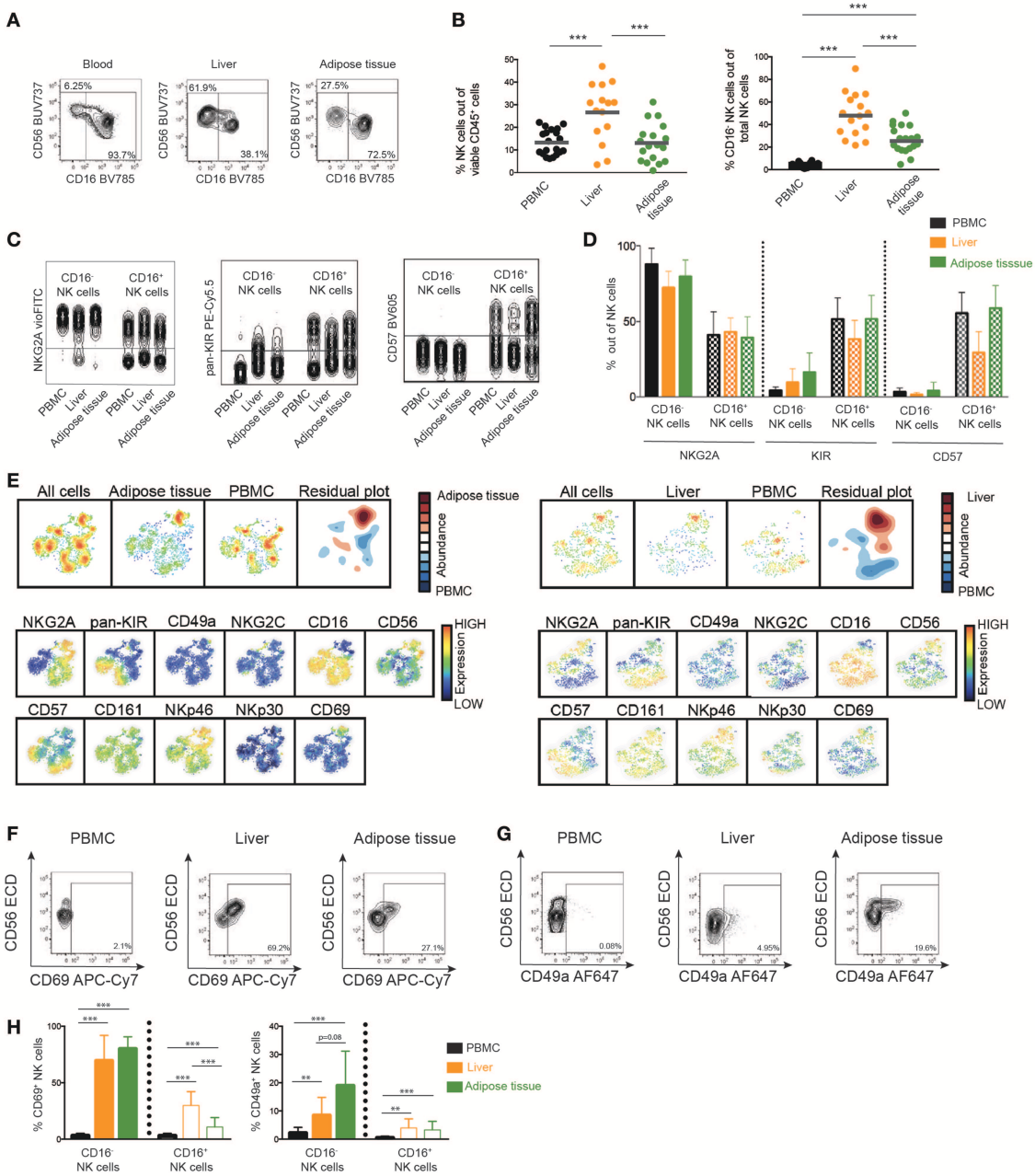

FIGURE 4 | Phenotype of NK cells in liver and adipose tissue. **(A)** Representative flow cytometry plots of NK cells derived from blood, liver, and adipose tissue. **(B)** Scatter plot of the frequency of total NK cells out of viable leukocytes and CD16$^-$ NK cells out of total NK cells in peripheral blood, liver, and adipose tissue. PBMC ($n = 22$), adipose tissue ($n = 19$), and liver ($n = 15$). **(C)** Representative concatenated flow cytometry plots showing expression of differentiation markers NKG2A, KIRs, and CD57 on CD16$^-$ or CD16$^+$ NK cells in blood, liver, and adipose tissue. **(D)** Bar graphs showing frequency of cells expressing the indicated differentiation markers in CD16$^-$ or CD16$^+$ NK cells from blood, liver, and adipose tissue. PBMC ($n = 22$), adipose tissue ($n = 19$), and liver ($n = 15$). **(E)** SNE plot of total NK cells from adipose tissue (left) or liver (right) compared to peripheral blood. The residual plot highlights the decreased (blue) or more highly expressed (red) areas within tissue NK cells. **(F,G)** Representative flow cytometry plots showing CD69 and CD49a expression on total NK cells from the indicated tissues. **(H)** Expression of CD69 and CD49a on CD16$^-$ and CD16$^+$ NK cells in peripheral blood, liver, and adipose tissue. In H, CD69 on PBMC ($n = 22$), adipose tissue ($n = 18$), and liver ($n = 15$), CD49a on PBMC ($n = 9$), adipose tissue ($n = 9$), and liver ($n = 7$). Bars in **(B,D,H)** represent mean and error bars in **(D,H)** show SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

These discrepancies might in part be based on methodological dissimilarities between the studies, with different target cells used but also on differing patient inclusion criteria. Indeed, our patients had, in general, mild fibrosis, with no patients suffering from cirrhosis, suggesting a more active inflammatory disease. This compared to other studies where many patients had cirrhosis (14) or higher BMI (15). Michelet et al identified functional defects as well as the loss of CD56^{bright} NK cells in

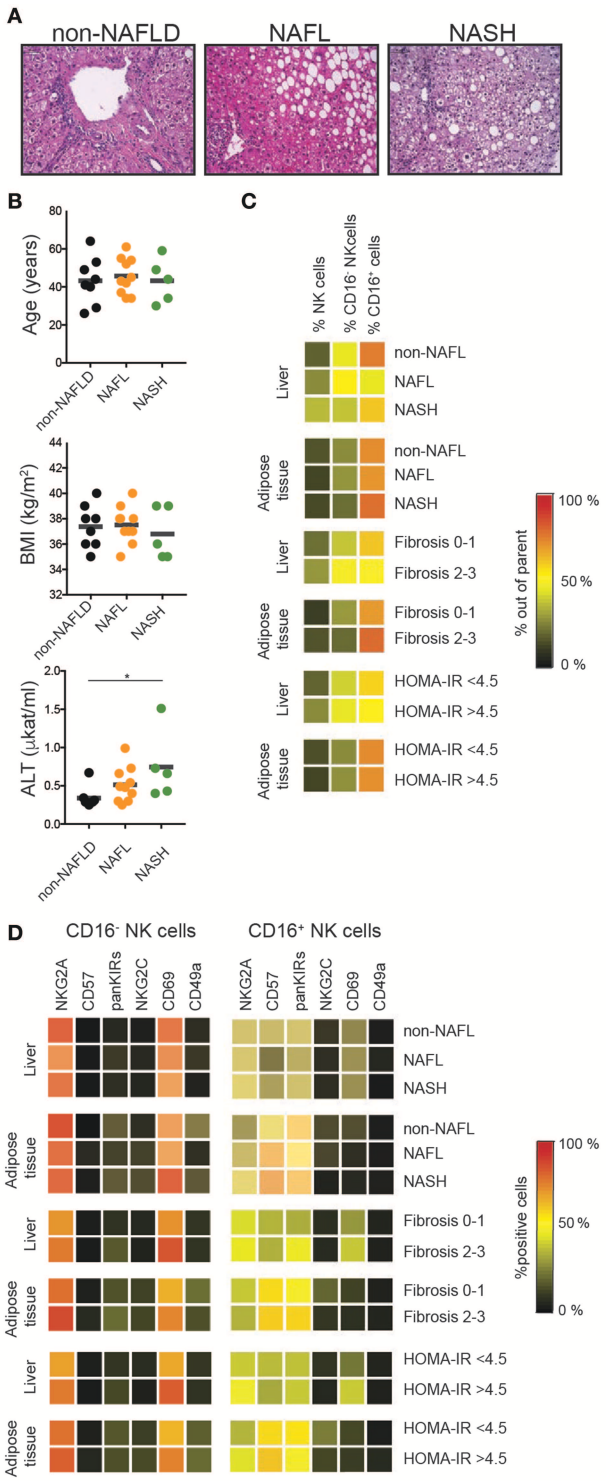

FIGURE 5 | Tissue NK cells in relation to patient disease characteristics. **(A)** Representative hematoxylin and eosin stainings on normal, NAFL, and NASH liver sections. **(B)** Distribution of the patients in relation to age, BMI, and levels of alanine transferase (ALT) as a proxy for liver damage. **(C)** Heat map showing mean frequency of total, $CD16^+$, and $CD16^-$ NK cells for the indicated groups. **(D)** Heat map showing mean frequencies of NKG2A, CD57, KIRs, NKG2C, CD69, and CD49a expression on $CD16^-$ and $CD16^+$ NK cells for the indicated groups. *$p < 0.05$.

obese patients (18). This is in line with a previous report (15), and common of these two studies was that included patients had BMI's of up to 50. Our cohort had a considerably lower average BMI of around 35. Together, this suggests that the functionality of NK cells and NK cell subset composition is retained in less severe obesity whereas alterations become evident in patients with morbid obesity. Atherosclerosis, hypertension, and adipose tissue inflammation are common comorbidities in NAFLD patients and thus potential confounding factors that should be controlled for. Our strategy to address this challenging issue was in one part to stratify patients into NAFL or NASH by the use of liver biopsies, considered the gold standard. In addition, we made a subgroup analysis based on the level of fibrosis and on insulin sensitivity. However, in none of these comparisons, neither in peripheral blood nor in liver or adipose tissue, any profound disease-related alterations in the NK cell populations could be observed. Of note, we focused our analysis on the major two subpopulations of NK cells: $CD56^{bright}$ and $CD56^{dim}$ NK cells. Apart from them, there are additional un-conventional NK cell subset, such as $CD56^-CD16^+$ and $CD56^{dim}CD16^-$ NK cells (32), that should be investigated in future studies.

A recent report demonstrated how chronic hepatitis C irreversibly causes decreased receptor repertoire diversity in circulating NK cells (26) Related changes have also been observed in chronic hepatitis D (33). Based on this, we wanted to investigate whether the chronic "non-infectious" inflammation in NAFLD could cause a similarly reduced diversity. However, NK cell repertoire diversity, determined by SDI, remained intact in NAFLD. Both NAFLD and chronic viral hepatitis are slowly developing liver diseases that take years to produce symptoms. However, many more changes in serum cytokines can be detected in hepatitis C patients as compared to NASH patients (34) and it is plausible that the general degree of inflammation in NASH is more low-grade as compared to chronic viral hepatitis. Thus, it might be that a larger inflammatory insult is needed in order to affect NK cell repertoire diversity.

In experimental models, NK cells have been shown to protect against liver fibrosis development, e.g., via NKp46-mediated macrophage activation (13) or by killing of hepatic stellate cells (HSCs) (35). Similar evidence exists in the human setting but primarily in relation to chronic viral hepatitis and fibrosis development (36). NKG2D is another NK cell surface receptor that may be protective against fibrosis development in mice by targeting activated HSCs (37). Furthermore, the NKG2D-ligands MICA and MICB are both upregulated in NASH-livers in mice (11). For NAFLD patients, level of fibrosis is an important predictor of long-term survival (38, 39). Interestingly, we could show that NK cells express higher levels of NKG2D in patients with NASH. This upregulated NKG2D expression could be a response to the increased hepatocyte stress, inflammation, and apoptosis that can be seen in NASH-livers (23). To determine if NKG2D influenced the degree of liver fibrosis in NAFLD patients, we compared levels of fibrosis to NKG2D expression on circulating NK cells but could not detect any association. This could be due to the fact that fibrosis is a dynamic process that occurs during many years in NAFLD. In the current study, we did not have the possibility to investigate NKG2D expression on

intrahepatic NK cells. Future studies should assess this and also specifically evaluate the capacity of NK cells to target HSCs (or hepatocytes) via NKG2D in NAFLD.

Apart from having a protective role in fibrosis development, adipose tissue NK cells, or ILC1-like cells, have also been shown to augment insulin resistance in experimental murine models (19, 20, 40). In more detail, it has been proposed that NK cells sense stressed cells in adipose tissue, respond with IFN-γ production, in turn causing macrophage polarization toward a pro-inflammatory phenotype, which subsequently leads to insulin resistance (19, 20, 40). However, little is known about human adipose tissue NK cells in general and also how they relate to obesity, insulin resistance, and liver disease. In this regard, we assessed visceral adipose tissue NK cell frequency and phenotype. Adipose tissue contained a similarly large population of NK cells as found in peripheral blood. However, adipose tissue was clearly enriched for $CD56^{bright}CD16^{-}$ NK cells expressing tissue residency markers. This profile with enrichment of $CD56^{bright}$ NK cells was similar to the phenotype of NK cells found in matched liver samples and also to NK cells in many other peripheral tissues in general (41). Interestingly, the specific subset of adipose tissue NK cells (or ILC1-like cells) that contribute to insulin resistance in mice express CD49a on their surface (21, 40). We here report that also human adipose tissue contains NK cells expressing CD49a. $CD49a^{+}$ NK cells had a $CD56^{bright}CD16^{-}$ phenotype, which is different from liver $CD49a^{+}$ NK cells (4), and were more prevalent in adipose tissue as compared to liver and peripheral blood. However, no link between the presence and levels of adipose tissue $CD49a^{+}$ NK cells and the presence of insulin resistance was noted in the investigated patients. Within the scope of this study, only CD49a and CD69 was studied as tissue residency markers. There are a number of additional surface markers as well as transcription factors that are linked to tissue residency, which should be investigated in future studies.

Our study design, with liver and adipose tissue biopsies acquired during laparoscopic surgery, enabled us to uniquely study NK cells from different peripheral tissues within the same individual. This analysis revealed interesting features, emphasizing the different phenotypes of tissue-resident cells from distinct tissues. We could show that NK cell differentiation status differed not only between liver and peripheral blood, in line with previous reports (41), but also between liver and adipose tissue-derived NK cells. The pattern of tissue residency marker expression was also distinct between liver and adipose tissue NK cells within the same individual. These data emphasize the importance of the specific local microenvironment in influencing the shape of the NK cell population. This also shows the importance of, although cumbersome, studying tissue NK cells in different human disorders.

Limitations of our study have also to be considered. First, the target cell line used in the functional experiments consist of the leukemia cell line K562. While being the gold standard for assessment of NK cells function, due to its lack of MHC class I, it is not representative for the NAFLD setting. However, it does express NKG2D-ligands (42), which primary hepatocytes derived from organ donors did not (**Supplementary Figure 1**). Ideally, future studies should assess NK cell responsiveness against primary target cells derived from livers of NAFLD patients, such as hepatic stellate cells or hepatocytes. Second, in addition to the target cells derived from NASH-livers, future work should focus on the function of tissue-derived NK cells since this study, due to practical reasons, only assessed the function of circulating NK cells. Third, within the scope of this study, NKG2D expression was only studied on NK cells derived from peripheral blood. This might not be representative of NKG2D expression on NK cells derived from liver and adipose tissue. Fourth, this study does not address the NKG2D-ligand expression in NASH livers which, in combination with the NKG2D expression on hepatic NK cells, could provide interesting insights into the disease mechanisms of NASH. Thus, the exact role of NKG2D in NAFLD still remains to be elucidated.

In summary, we here performed a comprehensive assessment of peripheral blood and tissue NK cells in relation to NAFLD, the most common chronic liver disease worldwide. Surprisingly, despite a substantial literature from experimental model systems suggesting a role for NK cells in NASH, we found a largely intact NK cell compartment in the human setting. Instead, our study reveals significant differences in composition of the NK cell compartment between human peripheral tissues and, thus, illustrates the importance of understanding the local microenvironment in shaping the NK cell repertoire.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Regional Ethics Committee of Stockholm, Stockholm, Sweden. All subjects gave oral and written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regional Ethical Review Board in Stockholm, Sweden.

AUTHOR CONTRIBUTIONS

NS designed the study, performed experiments, acquired and analyzed data, and drafted the manuscript. NB designed the study, performed data analysis, drafted the manuscript, and supervised the work. KS, MC, and CZ performed experiments and data analysis. JF and GM contributed to the data analysis, the discussion, and supervision. HH recruited patients and was instrumental in the histology assessments. PS and HH contributed with in-depth knowledge of NAFLD. AT, EN, SK, and HN recruited and sampled patients. All authors provided valuable contributions and insights into the manuscript.

FUNDING

This work was funded by the Swedish Research Council, the Swedish Cancer Society, the Swedish Foundation for Strategic Research, the Swedish Society for Medical Research, the Cancer Research Foundations of Radiumhemmet,

Knut and Alice Wallenberg Foundation, the Novo Nordisk Foundation, the Western Norway Regional Health Authority (Helse Vest RHF), the Center for Innovative Medicine at Karolinska Institutet, the Stockholm County Council, The Erling-Persson Family Foundation, SRP Diabetes Karolinska Institutet, and Karolinska Institutet.

ACKNOWLEDGMENTS

The authors would also like to thank the research nurses involved in the study, in particular Anette Bratt, Miriam Nordstedt, and Pia Loqvist as well as Lena Berglin. In addition, the authors thank Ewa Ellis for providing primary human hepatocytes.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01255/full#supplementary-material

Supplementary Figure 1 | Expression of NKG2D ligands on primary human hepatocytes. Representative histogram of flow cytometry stainings of NKG2D-ligands as well as HLA-ABC and CD155, ligand of DNAM-1, on primary hepatocytes. One representative staining out of three.

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

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frontiers in Immunology

REVIEW
published: 03 May 2019
doi: 10.3389/fimmu.2019.00961

Human Gut-Associated Natural Killer Cells in Health and Disease

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It is well established that natural killer (NK) cells are involved in both innate and adaptive immunity. Indeed, they can recognize molecules induced at the cell surface by stress signals and virus infections. The functions of NK cells in the gut are much more complex. Gut NK cells are not precisely organized in lymphoid aggregates but rather scattered in the epithelium or in the stroma, where they come in contact with a multitude of antigens derived from commensal or pathogenic microorganisms in addition to components of microbiota. Furthermore, NK cells in the bowel interact with several cell types, including epithelial cells, fibroblasts, macrophages, dendritic cells, and T lymphocytes, and contribute to the maintenance of immune homeostasis and development of efficient immune responses. NK cells have a key role in the response to intestinal bacterial infections, primarily through production of IFNγ, which can stimulate recruitment of additional NK cells from peripheral blood leading to amplification of the anti-bacterial immune response. Additionally, NK cells can have a role in the pathogenesis of gut autoimmune inflammatory bowel diseases (IBDs), such as Crohn's Disease and Ulcerative Colitis. These diseases are considered relevant to the generation of gastrointestinal malignancies. Indeed, the role of gut-associated NK cells in the immune response to bowel cancers is known. Thus, in the gut immune system, NK cells play a dual role, participating in both physiological and pathogenic processes. In this review, we will analyze the known functions of NK cells in the gut mucosa both in health and disease, focusing on the cross-talk among bowel microenvironment, epithelial barrier integrity, microbiota, and NK cells.

Keywords: gut-associated lymphoid tissues, natural killer cells, innate lymphoid cells, inflammatory bowel disease, colorectal carcinoma

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Kamalakannan Rajasekaran, Genentech, Inc., United States
William Garrow Kerr, Upstate Medical University, United States

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *07 December 2018*
Accepted: *15 April 2019*
Published: *03 May 2019*

Citation:
Poggi A, Benelli R, Venè R, Costa D, Ferrari N, Tosetti F and Zocchi MR (2019) Human Gut-Associated Natural Killer Cells in Health and Disease. Front. Immunol. 10:961. doi: 10.3389/fimmu.2019.00961

INTRODUCTION

Gut associated lymphoid tissue (GALT), the part of the mucosa-associated lymphoid tissue (MALT) found along the gastrointestinal tract (GI), is essential to understanding the reaction of the host to external environment components (1–3). The content of gut lumen is continuously shucked and changed from the birth through adulthood and into old age. These changes strongly influence the type of immune response elicited and, consequently, may generate gut diseases (4–6). The GALT should be able to distinguish pathogenic and harmful organisms and antigens from those that are beneficial and not dangerous. The innate arm of the immune system can be considered the first

line of defense against pathogenic microorganisms and their products; this arm can distinguish among microorganisms via pattern recognition receptors (PRRs) and Toll-like receptors (TLRs) present on macrophages, neutrophils, and dendritic cells (7–9). Also, natural killer (NK) cells can interact with gut microorganisms and influence and shape the adaptive T cell-mediated immune response by acting on professional antigen presenting cells (APCs), such as dendritic cells (DCs). The phenotypic and functional features of gut associated NK cells are different from those that are typical of NK cells isolated from peripheral blood. In addition, gut lymphoid cells expressing some markers of NK cells, termed innate lymphoid cells (ILC) (10–12), appear to be involved in the regulation of gut mucosa homeostasis and in the generation of gut-associated lymphoid structures (13–19). On this basis, it is conceivable that NK cells (ILC1) and other subsets of ILC (ILC2 and ILC3) are not only involved in the control of healthy gut but also in the pathogenesis and evolution of gut diseases, including inflammatory bowel disease (IBD) and colorectal carcinoma (CRC) (20–23).

NK CELL PHENOTYPIC AND FUNCTIONAL FEATURES IN HEALTHY GUT MUCOSA

It is well-established that NK cells represent a group of $CD3^{-}CD56^{+}$ innate immune cells (24). The majority of the findings on this topic is derived from studies on NK cells isolated from peripheral blood mononuclear cells (PBMCs) (24). Several comprehensive review articles have described the phenotypic and functional features of PB NK cells in detail (25–29). Briefly, PB NK cells can express the CD56 antigen at different intensities; indeed, $CD56^{bright}$ and $CD56^{dull}$ NK cells with predominant cytokine production or cytotoxic functions, respectively, have been identified. Several reports have stated that these two cell populations show a different functional role and a distinct array of receptors involved in the recognition of self-class I human histocompatibility antigens (HLA-I) (30–33). It is not clear whether these two NK cell subsets derive from the same or different NK cell precursors and whether they display some plasticity, converting from one into the other (34–36). Generally, lymphoid cells expressing CD56 isolated from the gut do not express CD16, i.e., the classical receptor for the crystallizable fragment of immunoglobulin (FcγRIIIa) that is usually found on majority of PB NK cells (24). It has been reported that $CD3^{-}CD56^{+}$ cells, like several other populations of tissue-resident innate cells (37–39), do not display a strong cytolytic activity if tested *in vitro* against conventional NK cell targets, but rather produce and release IFNγ *in vitro*. According to studies of human and murine NK cells, the reduction or lack of cytotoxicity can be considered as a marker of immaturity in the main function displayed by NK cells (40–42). More correctly, $CD3^{-}CD56^{+}CD16^{-}$ mucosal NK cells could be considered lymphoid effectors that have a relevant role in the regulation of gut homeostasis (11). In line with this interpretation, the production of IL22 by GALT NK cells is essential for modulating expression of many genes in mucosal epithelial cells to favor epithelial cell survival and remodeling (40, 43). Of note, IL22 is produced by mucosal T lymphocytes, and it is conceivable that the imbalance between IL22 and IL17 is relevant to the generation of IBD (44). Importantly, GALT NK cells produce IL22 in response to IL23, but not to IL12. The more conventional NK cells found in PB or in the gut mucosa respond to IL12 by producing IFNγ (45); these cytokines are involved in the response to infections (42–46). NK cells were found in lamina propria (LP) scattered in the middle of epithelial cells as intraepithelial lymphocytes (IELs), but not associated with lymphoid aggregates. This suggests that these cells do not participate as actively as T cells at antigen inductive sites present in the gut (41, 47).

SMALL AND LARGE INTESTINE MUCOSA HISTOLOGY AND FUNCTION

Herein, we focus mainly on the mucosa of the small intestine where the first interaction with lumen content takes place (**Figures 1A,B**). The surface epithelium and the underlying LP are arranged in villi and crypts, which give a velvet-like appearance to the mucosa. These structures amplify the absorbent surface of the gut and the crypts are surrounded and reinforced by a strong sheath of fibroblasts (FBs) (**Figure 1B**). Lymphoid and myeloid cells present within the gut mucosa can interact with epithelial absorptive cells (enterocytes), goblet cells that secrete ions, water and mucus, and a few endocrine cells that produce hormones and neuropeptides (47, 48). Goblet cells and enterocytes are derived from undifferentiated cells in a close cross-talk with pericryptal FBs and this relationship enhances the structural integrity and functional efficiency of the gut mucosa (49, 50). Importantly, LP penetrates the villi cores associated with blood vessels, connective tissue with different myeloid cells, smooth muscle cells, and blind-ended lacteals (**Figures 1A–C**, scheme in **Figure 1D**). The mucosa of large intestine is composed of the same cell types, but the structural organization is different; indeed, it is quite smooth without villi, and goblet cells are outnumbered by columnar absorptive cells. The principal protection for excluding undesired environmental factors, particularly harmful microorganisms, is provided by the GALT. GALT is composed of lymphoid aggregates (**Figure 1A**), mucosal LP lymphocytes, and intraepithelial lymphoid cells (**Figure 1C**) (51–53). Lymphoid aggregates increase in number along the small intestine and become confluent in the ileum, giving rise to Peyer's patches. These are un-encapsulated lymphoid structures, similar to lymph nodes (LN), with follicles composed of different cell types, including B, T, and accessory myeloid cells such as DCs (53, 54). Peyer's patches are important sites for the induction of the immune response, and the overlying epithelium contains multi-fenestrated or microfold cells (M cells). M cells take up different molecules from the gut lumen that, through transcytosis, come in contact with the underlying lymphoid cells (55). In the LP there are plasma cells, which mainly produce IgA, that protect from bacterial invasion, macrophages, T and B lymphocytes, and polymorphs. Intraepithelial lymphocytes are concentrated in the small intestine, reaching a maximal ratio of 20 lymphocytes to 100 enterocytes in the jejunum and a lower ratio in the

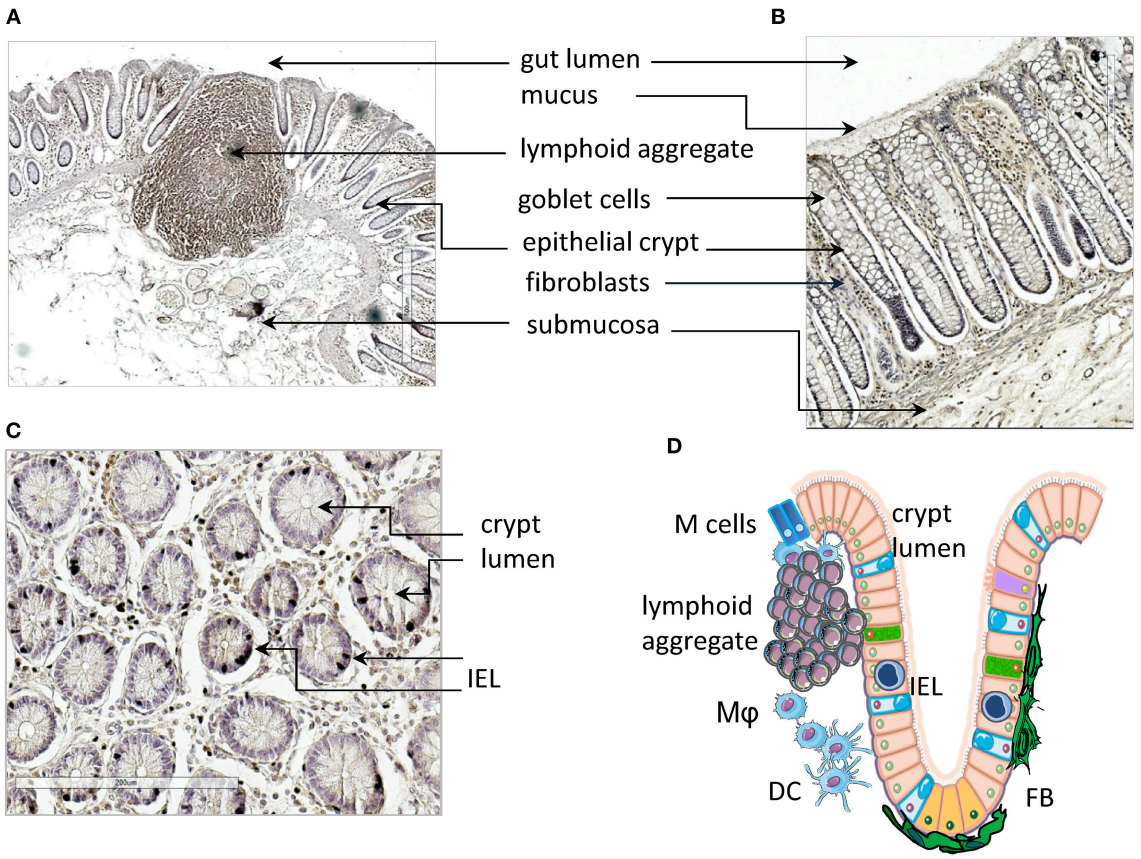

FIGURE 1 | Gut mucosa structure and gut-associated lymphoid tissue (GALT). **(A–C)** Longitudinal **(A,B)** and transversal (s) sections of healthy gut showing a lymphoid aggregate **(A)**, identified by immunostaining with an anti-CD45RO monoclonal antibody, epithelial crypts **(A–C)**, fibroblasts **(B)** and intraepithelial lymphocytes (IELs, **C**). **(D)** Schematic representation of the structure of gut mucosa and GALT. IELs, intraepithelial lymphocytes; Mϕ, macrophages; DC, dendritic cells; FBs, fibroblasts. Arrows in **(A–C)** indicate the microscopic anatomy of gut mucosa, with the indicated cells and structures. Pink: enterocytes; yellow-orange: LGR5^{+} stem cells; cyan, goblet cells; purple, tuft cells; green, neuroendocrine cells; blue, M cells; IELs, intraepithelial lymphocytes.

ileum (56). While the small intestine, less colonized by bacteria, shows both isolated lymphoid follicles (ILFs) and specialized Peyer's patches, only ILFs are observed in the colon. These ILFs are in variable positions but arise whenever bacterial products permeate the epithelial barrier. The assembly of ILFs has only been extensively elucidated in mouse models, where it is linked to lymphoid tissue inducer (LTi) cells. These cells are a subpopulation of ILC3 and depend on retinoic acid receptor-related orphan receptor gamma (RORγt) expression. It is likely that colorectal ILFs in humans can also arise *de novo*, as their density and grade of maturation vary considerably in any given area of mucosa (57).

NK CELL LOCALIZATION IN THE GUT

Although NK cells are present in the gut, it remains to be defined if these cells are resident or derive from PB circulating NK cells. It is well established that leukocytes can extravasate in a specific area under the chemotactic stimuli and action of adhesion molecules (58–61). For example, the lymphocyte function associated antigen 1 (LFA1, CD11a/CD18) can interact with the intercellular adhesion molecule (ICAM)-1 expressed on endothelial cells, while the CD29/CD49d integrin (formerly very late antigen-4) binds to the vascular cell adhesion molecule (VCAM)-1 (60, 61). Of note, ICAM1 and VCAM1 are usually absent in healthy endothelial cells; however, the effect of inflammatory cytokines, such as IL1β, IFNγ, and TNFα, in tissues where an immune response takes place, results in strong upregulation of both ICAM1 and VCAM1 (60–62). PB NK cells can express several chemokine receptors (63), some of which are more abundant on CD56^{bright} than on CD56^{dull} peripheral NK cells (64). It has been shown that *ex vivo* isolated NK cells bear CXCR1, CXCR3, and CXCR4, and contain subsets expressing CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6. More precisely, CD56^{dull} NK cells display a repertoire of chemokine receptors similar to that of neutrophils while this repertoire in CD56^{bright} is most similar to that of T-helper (Th) 1 cells. These findings suggest that the CD56^{dull} and the CD56^{bright} PBNK cells can migrate into tissues either at the beginning of the inflammatory reaction, which accompanies the immune response, or later (65). Of note, both CD56^{dull} and CD56^{bright} PB NK cells do not express the chemokine receptors needed

TABLE 1 | Main surface molecules involved in NK cell function in the gut.

Molecule	Family	Function	Ligands	Disease
CD56/NCAM	Immunoglobulins	Cell-cell, cell-matrix adhesion	HSPGs[a], L1, NCAM	CD, CRC
CD57/GA3S[b]	Carbohydrates	Adhesion, NK cell maturation	nd	CRC
NKRP1A/CD161	C-type lectin	Cell-cell, cell-matrix adhesion	LLT1[c]	CRC
NKG2D/CD314	C-type lectin	Recognition of infected/ transformed cells	MICs[d], ULBPs[e]	CD, UC, CRC
NKp30/CD337	NCR[f]	NK cell cytolytic activity	BAG6[g], B7-H6, Gal-3	CRC
NKp44/CD336	NCR	NK cell cytolytic activity	HSPGs, MLL5[h]	CD
NKp46/CD335	NCR	NK cell polarization	HSPGs, HAN[i]	CD, CRC
KIRs/CD158	Immunoglobulin like	Regulation of NK cell activity	HLA-A,-B, -C, -G	CD, CRC

[a]Heparansulfate proteoglycans; [b]Glucuronic acid-3 sulfate; [c]Lectin-like transcript-1; [d]MHC-related molecules; [e]UL16-binding proteins; [f]Natural Cytotoxicity Receptors; [g]BCL-2-associated athanogene-6; [h]Mixed-lineage leukemia-5; [i]Hemagglutinin-A neuraminidase.

to home to the small intestine, such as CCR6 and CCR9 (64–66). The lack of this homing capability would suggest that NK cells found in the gut are not derived from PB NK cells. However, some PB NK cells can express the CD161 antigen, also called NKRP1A (67, 68). This receptor is upregulated on NK cells upon stimulation with IL2 and, more importantly, it is expressed on majority of intestinal infiltrating lymphocytes (68, 69), including NK cells and some subsets of ILC (2, 5, 10). It has been demonstrated that CD161 can function as an adhesion molecule involved in the transmigration of PB $CD4^+$ T cells through endothelial cells (70). It is still unknown whether CD161 also plays a role in the transendothelial migration of PB NK cells, but it can be speculated that $CD161^+$ PB NK cells localize in the tissue upon the cooperative involvement of LFA1, and engagement of the platelet endothelial cell adhesion molecule-1 (PECAM1/CD31) on NK cells. Indeed, most NK cells express CD31, which allows a homophilic interaction with the CD31 present at the endothelial junction (71–74). CD161 might also regulate the speed of migration, as was shown for $CD4^+CD161^+$ T lymphocytes (70). The stromal derived factor 1 (SDF1, also named CXCL12), recognized by CXCR4, appears to favor tissue localization of NK cells, in particular of the $CD56^{bright}$ subset. However, NK cells, considered to be $NKp46^+$ lymphocytes, are not so represented in the gut, although several chemokines are detectable in bowel diseases, including CRC (75, 76). Collectively, these findings indicate that PB NK cells may localize into the gut, but their origin and the relative contribution of adhesion molecules and chemokine receptor-ligand interactions are yet to be established. **Table 1** summarizes the main surface molecules, and their respective ligands, involved in gut NK cell function.

INTESTINAL CRYPT NICHE AND IMMUNE SYSTEM CROSS-TALK AS THE FIRST LINE OF DEFENSE IN THE GUT

Intestinal crypts have the highest rate of tissue turnover in the body (3–5 days), a process that is strongly influenced by the products (mainly short-chain fatty acids and lactate) of commensal bacteria, (77, 78). As the crypt contains the proliferating component of mucosa (3–5 stem cells in each crypt), providing for self-renewal of the entire tissue, its niche is organized to limit mechanical, infectious, and inflammatory damage (**Figure 2**). LGR5-positive stem cells are positioned at the bottom of the crypt, mixed with Paneth cells in the small intestine. Paneth cells secrete both trophic (wnt3) and anti-microbial factors (alpha-defensins, lysozyme, RegIIIγ lectin) (**Figure 2B**). Goblet cells, mixed with maturating enterocytes inside the crypt, produce mucus and release antimicrobial molecules, thereby creating the main barrier to microbial infection. Under normal conditions, the inner mucus layer is almost microbe-free, thus neither epithelial nor immune cells are in contact with danger signals (79–81). TLRs act as the main sensors for pathogen invasion in intestinal epithelial cells (IECs). Most TLRs are localized in the basolateral membrane of enterocytes, although TLR2 and 9 are also expressed on the luminal surface (**Figure 2A**) (82–84). TLRs mediate enterocyte-driven reactions against viral and bacterial attacks. On the contrary, tuft cells which are rare, are specialized cells that act as sensors for parasites. When an infection occurs, tuft cells induce ILC2 activation and expansion by production of IL25. In turn, ILC2 secrete IL13 causing tuft cell proliferation, amplifying the signal (**Figure 2C**). The tuft cell-ILC2 cross-talk determines a type 2 cytokine response, activating goblet cells, macrophages, eosinophils, and other effectors (85, 86).

The cytokines that have a primary role in gut homeostasis and damage are summarized in **Table 2**. These cytokines, produced by NK cells and ILC, are involved in the regulation of these cell types, of their function and in integrity of gut mucosa. IEC behavior is modulated by several inflammatory cytokines that can increase tight junction permeability (TNFα, IFNγ, IL1β, IL6) (86), priming the immune system and triggering a chronic response against innocent targets. To counteract inflammation, IECs chronically release soluble IL1β receptor (sIL1RII) and thymic stromal lymphopoietin (TSLP), while inflammatory cytokines trigger TGFβ neosynthesis. Soluble IL1RII neutralizes IL1β, while TSLP and TGFβ condition dendritic cells, promoting Th2 and regulatory T cell (Treg) differentiation. Interestingly, sIL1RII is strongly downregulated during the active phases of Crohn's disease (CD) (87–89). A third regulatory mechanism is represented by the autocrine IL10 loop, activated in IECs in response to IFNγ, which re-establishes the mucosal barrier and maintains immunotolerance and acts through IL10R (90–92). Indeed, IL10 KO mice develop a gut permeability defect

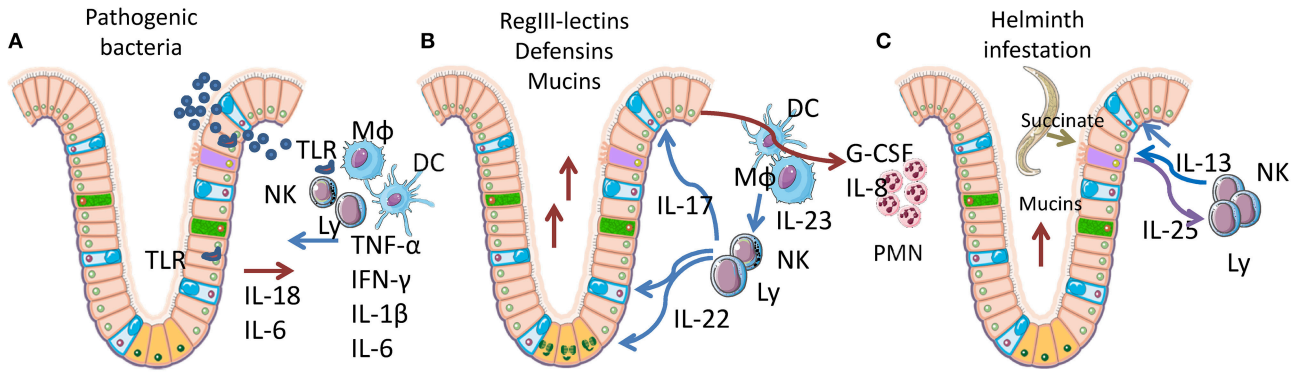

FIGURE 2 | Epithelial-immune cell cross-talk in gut mucosa. Simplified representation of the cytokine networks activated by the response to bacteria or parasites. Pink, enterocytes; yellow-orange, LGR5$^+$ stem cells; cyan, goblet cells; purple, tuft cells; green, neuroendocrine cells; Mϕ, macrophages; DC, dendritic cells; Ly, lymphocytes; NK, natural killer cells. **(A)** Acute inflammation caused by pathogenic bacteria is recognized through Toll-like receptors (TLR) and induces IL18 and IL6 release by IECs, followed by TNFα, IL1β, IFNγ, and IL6 release by lymphoid and myeloid cells. These cytokines trigger mucosal permeabilization and increase IEC proliferation and death. **(B)** Homeostatic inflammation, sustained by IL22 and IL17, secreted by lymphoid cells upon IL23 stimulation by myeloid cells. IL22 specifically targets IECs, restoring the epithelial barrier (tight junction formation), inducing the proliferation of LGR5$^+$ stem cells in the crypt bottom. IL22 also induces hyperplasia in goblet cells, with increased mucins production. IL17 induces G-CSF and IL8 expression in IECs, fibroblasts and leucocytes causing neutrophils recruitment. **(C)** Parasite infection sensed by tuft cells, which are able to recognize succinate that is released by many helminths. Tuft cells react by expressing IL25, which induces IL13 release and activation of ILC2 cells. IL13 mediates proliferation of tuft cells (amplifying the response) and goblet cell activation, finally determining a Th2 response.

that is associated with increased levels of TNFα and IFNγ, and exacerbates into a chronic colitis mediated by commensal bacteria (90–92).

In the gut, the specific subunit IL22R1 is almost exclusively expressed by IECs, making them a relevant target of IL22 produced by ILC, NK cells, CD4$^+$, CD8$^+$, and γδT lymphocytes. IL23, IL6, and IL1β are the main inducers of IL22, while TGFβ suppresses IL22 expression in all T cell subsets and induces IL17 expression. The main intestinal IL22-producing cells are NKp46 and RORγt positive (mouse), or NKp44 and RORC positive (human) (93). These do not express IL17 and lack typical NK cell effector functions (94). IL22 promotes the secretion of mucus-associated molecules, like MUC-1,−3, and−13 and other anti-microbial proteins, from goblet cells, reducing bacterial translocation across the epithelial barrier (95–100).

IL22 and IL17 frequently act in concert to limit bacterial invasion. The most common forms of IL17 are IL17A and IL17F, which share the same receptor but do not have completely overlapping activities. NKp46$^+$RORγt$^+$ NK and Tγδ innate immune cells are able to secrete IL17 and play an essential role before activation of a full Th17 response. The main source of IL17 is Th17 lymphocytes, which have differentiated from naïve CD4$^+$ T cells by APCs secreting TGFβ, IL6, and IL21 and are activated by IL23. The IL17 receptor is expressed by leucocytes, IECs, vascular endothelial cells and FBs, and increases G-CSF, IL6, and IL8 release favoring granulopoiesis and neutrophil recruitment (**Table 2** and **Figure 2B**). IL17 also contributes to strengthen the epithelial barrier by inducing tight junction formation in IECs. While an excess of IL17 contributes to CD, its presence is required to inhibit invasive bacteria (101, 102).

HUMAN NK CELLS AND IMMUNE RESPONSE TO GUT INFECTIONS

Most of what is known about the role of NK cells in response to gut infections comes from murine models (103–108). Murine NK cells appear to be relevant for *Listeria monocytogenes, Salmonella*, *Citrobacter rodentium*, and *Yersinia enterocolitica* infections (103–108). An efficient response to these infections mediated by NK cells is dependent on cytokines, such as IL15 and IFNγ. All molecular mechanisms involved in rodent gut immunity are very well reviewed elsewhere (108) and a specific analysis is beyond the scope of this review. It is conceivable that human NK cells in the gut can play a role in eliciting inflammation during bacterial infections that is independent of viral clearance and tumor control. Indeed, NK cells, like other innate cells, such as macrophages and neutrophils, can use different TLRs, mainly TLR2, TLR3, TLR4, and TLR9, to interact with bacteria-associated peptidoglycans, lipopolysaccharides, virus-derived dsRNA, and DNA with CpG motifs (also known as pathogen-associated molecular patterns, PAMPs) (109) to elicit an inflammatory response (**Figure 2A**). IL12 and IL18 produced by mucosa-associated macrophages are responsible for amplifying the immune response mediated by NK cells. In turn, IFNγ released by NK cells can trigger activation of myeloid cells to augment phagocytosis, respiratory burst and killing of bacteria (**Figure 2B**). These effects can further amplify the activation of NK cells and IFNγ toxicity, leading to systemic inflammation. Patients suffering from sepsis show dysfunction of several leukocyte subsets, including NK cells, that can cause a decreased host response against the primary bacteria and favor superinfections by other bacteria or latent viral reactivation, ultimately leading to fatal outcomes (110).

TABLE 2 | Cytokines involved in gut homeostasis and diseases.

Cytokine	Producer cells	Target cells	Physiology	Disease
IFNγ	NK, TL, ILC	Mϕ, BL, TL, IECs, END	Class II antigen expression, immunity, epithelial/endothelial permeabilization	CD, UC
IL1β	Mϕ, NK, PMN	Mϕ, BL, TL, IECs, END, FBs	Pyrogen, induces multiple inflammatory cytokines expression	Autoinflammatory disorders, CRC
IL6	Mϕ, IECs, END, FBs	BL, TL, FBs, HEP	Pyrogen, induces acute phase proteins, induces B and T cells maturation and platelet production	Chronic inflammation and autoimmunity, CRC
IL10	Mϕ, many tissues	most cells	Inhibits inflammatory and Th1 responses, stimulates BL, restores epithelial homeostasis	IBD, CRC
IL12	DCs, Mϕ, PMN	NK, TL, ILC, Tγδ	Th1 responses, TNFα and IFNγ secretion	IBD
IL13	NK, TL, ILC, EO	BL, Mϕ, IECs, FBs	Th2 responses against helminths, goblet cells activation and proliferation	CD, UC, fibrosis
IL15	Mϕ, DCs, IECs	NK, TL, ILC, Tγδ, IECs	Th1 responses, IEC death	CD, UD, coeliac disease
IL17	TL, NK, Tγδ, ILC	TL, IECs, END, FBs	Th17 responses, IEC barrier strength	CD
IL18	IECs	TL, NK, IECs	Limits Th17 differentiation, induces IFNγ secretion, sustains IEC regeneration, blocks goblet cells development	UC
IL22	ILC, NK, Tγδ, TL	IECs	Secretion of antibacterial peptides and mucins, $LGR5^+$ stem cells proliferation	CD, CRC
IL23	DCs	ILC, NK, Tγδ, TL	IL22 and IL17 expression, Th17 responses	CD, CRC
IL25	Tuft cells, TL, Mast cells	ILC2	Th2 responses against helminths	
TGFβ	DCs, IECs, FBs	LT, IECs, FBs	Inhibits Th1 and Th2 responses, induces Th17 and Tregs, IgA maturation, FB proliferation, IEC inhibition	CD, UC, CRC
TNFα	Mϕ, TL, NK, PMN, EO, IEC, END, FBs	Mϕ, TL, PMN, IEC, END, FBs	Pyrogen, induces acute phase proteins, pro-inflammatory, cytotoxic responses, epithelial permeability and IEC apoptosis	CD, autoimmunity

Mϕ, macrophages; NK, natural killer; TL, T lymphocytes; BL, B lymphocytes; ILC, innate lymphoid cells; Tγδ, gamma-delta T cells; DCs, dendritic cells; PMN, polymorphonucleated neutrophils; EO, eosinophils; IEC, intestinal epithelial cells; END, endothelial cells; FBs, fibroblasts; HEP, hepatocytes; CD, Crohn's Disease; UC, Ulcerative Colitis; CRC, Colorectal carcinoma; IBD, Inflammatory Bowel disease.

Focusing on NK cells in humans and NK cell-mediated anti-viral activity, interesting results analyzing the alteration of the mucosal distribution of NK cells during human immunodeficiency virus (HIV) infection have been reported (44). The frequency of NK cells is increased in HIV subjects with incomplete $CD4^+$ T cell recovery in PB upon long-term anti-retroviral therapy. More importantly, spontaneous HIV controllers with protective KIR/HLA genotypes (111, 112) showed higher numbers of IEL NK cells than those in subjects with non-protective genotypes. This suggests that a peculiar NK cell subset displaying CD57 may be involved in control of HIV replication at the rectosigmoid mucosal site. Also, Human Herpesvirus 6 (HHV-6) can influence the NK cell-mediated response in the large bowel (113, 114). Indeed, HHV-6 shows a wide cell tropism *in vivo* and, as similar to other herpesviruses, causes a lifelong latent infection in humans and can be found in the large bowel. Notably, the HHV-6 products U51A and U83A suppress the surface expression of NKG2D and NKp30, two relevant activating receptors of NK cells (114). This suppression can impair the ability of NK cells to counteract HHV-6 reactivation and to recruit adaptive immune cells for elimination of the virus. In addition, some early viral proteins downregulate NKG2D ligands and transcription of B7-H6 mRNA, the reported cellular ligand of the activating receptor NKp30 (115) (**Table 1**). These findings suggest that the inefficient NK cell response can eventually lead to impaired clearance of HHV-6 and determine the establishment of a persistent infection (116).

GUT MICROBIOTA AND IMMUNE RESPONSE: DIET AND PROBIOTICS

Disorders in the development or composition of bacterial microbiota (known as dysbiosis) result in immunological dysregulation, leading to altered immune responses that may underlie disorders such as IBD, allergies, and cancer. In turn, the term "probiotic" is used to describe dietary microbes that confer a health benefit to the host (117). In animal models, diet-modified microbiomes can rapidly promote obesity or reduce incidence of diabetes, in association with decreased pro-inflammatory cytokines IL17 and IL23 in colon mucosa (118).

Diet, gut microbiota, and immune responses are probable explanations for the expanding incidence of inflammatory/immune diseases such as asthma, type 1 diabetes, and IBD in people living in developed countries (119). A low fiber intake adversely affects the intestinal microbiota and leads to decreased production of immunomodulatory products, in

particular the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate, all of which are critically important for mucosal immune homeostasis and intestinal epithelial integrity (120). For example, butyrate has an anti-inflammatory effect by inhibiting the recruitment and pro-inflammatory activity of neutrophils, macrophages, and effector T cells, and increasing the number of Tregs. IBD patients have reduced SCFA-producing bacteria and reduced butyrate concentration linked to a marked increase in the number of pro-inflammatory immune cells in the gut mucosa (121).

Intestinal APCs protect the body against infections, and having co-evolved with microbiota, maintain immune tolerance to the normal gut microbiota. For example, DCs of Peyer's patches in mice produce high levels of IL10, and gut macrophages, located in close proximity to the intestinal microbiota, develop a non-inflammatory phenotype termed "inflammation anergy" when encountering microbial stimuli in homeostatic conditions (122).

Probiotic bacteria are considered to be "generally recognized as safe (GRAS)" organisms by the Food and Drug Administration in the United States (123). It is supposed that probiotic bacterial cells and/or their soluble factors exert immunomodulatory effects by activating TLR on gut DCs and macrophages, driving APCs to produce cytokines required for antigen-specific Th1 polarization, such as IL12, or leading to immunological tolerance. *Lactobacillus rhamnosus* GG (LGG) can relieve intestinal inflammation in patients with atopic dermatitis and food allergy by decreasing TNFα production and promoting IL10 expression (123, 124). In addition, LGG has also been shown to induce intestinal secretion of IgA in atopic children (124). A synbiotic (synergistic combinations of probiotic and prebiotic) comprising *Bifidobacterium longum* has been demonstrated to reduce CD in a randomized double-blind placebo-controlled study with an evident decrease in TNFα expression (125).

Along this line, *Lactobacillus plantarum* (*Lp*) can efficiently increase the expression of the natural cytotoxicity receptor (NCR) family and of IL22 in NK cells (126). Transfer of PB NK cells stimulated by *Lp* conferred protection against intestinal epithelial barrier damage induced by enterotoxigenic *Escherichia coli* (ETEC) in NCM460 cells *in vitro*. PB NK cells stimulated by *Lp* could partially offset the reduction in transepithelial electrical resistance (TEER) of NCM460 cell monolayers caused by ETEC. Furthermore, *Lp*-stimulated, compared to *Lp*-unstimulated, NK cells added to ETEC-infected NCM460 cells increased the expression of IL22R1, p-Stat3, and p-Tyk2 by NCM460 cells, which, together with ZO-1, claudin-1 and occludin, are known to play important roles in intestinal epithelial barrier function (127). Mechanistic experiments using polyclonal blocking anti-IL22 antibody showed that *Lp*-stimulated NK cells lost the ability to maintain TEER in NCM460 cells challenged with ETEC (127). These results suggest that *Lp* stimulation of NK cells could enhance IL22 production, which in turn provides defense against ETEC-induced damage to the intestinal epithelial barrier. Further studies demonstrated that treatment with different strains of *Lp* induces TRAIL on the cell surface of PBMCs. TRAIL production depended on IFNα and IFNγ and facilitated NK cell activity exerted by PBMCs against cancer cells (128).

Aging leads to a decline in immune function that adversely affects gut microbiota. In a prospective double-blind, randomized crossover study in 40 healthy elderly subjects (aged 60–80 years) consumption of *Lactobacillus rhamnosus* GG combined with soluble corn fiber increased NK cell activity, decreased the pro-inflammatory cytokine IL6 and decreased total and LDL cholesterol (129). In another phase II randomized, double-blinded, placebo controlled clinical trial, *Lactobacillus salivarius* treatment significantly increased the percentage of NK cells and monocytes, as well as the plasmatic levels of immunoglobulins and the regulatory cytokine IL10 (130).

The GI tract contains the largest endocrine organ in the body. Due to the strategic location of enteric endocrine cells in gut mucosa, interactions with the immune systems are very likely to play an important role in immune modulation (131). At least 14 different populations of enteric endocrine cells release biologically active compounds (132, 133). Although some data on the influence of these substances on Th1/Th2 responses in the gut are available (132, 133), to our knowledge information on NK cell recruitment and function is lacking.

INFLAMMATORY BOWEL DISEASE (IBD) AND NK CELLS

Overpowering cytokine production and chronic inflammation are typical of IBD, mainly represented by CD and ulcerative colitis (UC) (2, 134, 135). While the cause of IBD is still unknown, it is conceivable that associations between the altered immune response against intestinal flora and the genetic background of susceptible individuals could be responsible. Many studies have reported the linkage between the IBD3 region in the human leukocyte antigen (HLA) complex and CD or UC (136). Likewise, major histocompatibility complex (MHC) class I chain-related molecules (MIC) alleles and MICA polymorphisms have been associated with IBD [141, 142].

There is evidence that supports a major role for adaptive immunity in the pathogenesis of IBD, involving Th1 and Th2 cells, together with other subsets of T cells, namely Th17 and Tregs (134, 135). In particular, while it has long been thought that CD is due to an abnormal Th1 response with increased secretion of the pro-inflammatory cytokines IFNγ TNFα and IL1β (**Figure 3A**), UC has been associated with a non-conventional Th2 response that involves IL5, IL6, and IL13 (**Figure 3B**) (134, 135). Besides classical Th1 and Th2 responses, Th17 cells, which are a subset of T lymphocytes that expand in the presence of IL23, can also contribute to IBD pathogenesis through IL17 production (137). More recently it has been shown that the innate arm of immune response is relevant to the favoring of gut inflammation in IBD patients (137). While altered epithelial barrier function has been described in patients with CD and UC, it remains unclear whether these lesions represent the cause or the effect of chronic inflammation with intensified production of cytokines. Among the highly produced cytokines, IL23, besides amplifying the IL17 circuit, can also act on innate immune cells or unconventional T lymphocytes, such as $\gamma\delta$T lymphocytes which are good producers of IL17, or NK cells (138).

In this regard, the presence of killer (K) cells in mesenteric LN was described in CD patients long ago (139). The involvement of NK cells in IBD pathogenesis has been supported by a recent study on polymorphisms of killer immunoglobulin-like receptors (KIR) genes (140). KIR are NK cell surface receptors, which bind to the class I MHC and have inhibitory or activating effects on NK cells (**Table 1**). A meta-analysis of 432 UC and 1677 CD patients showed positive associations between 2DL5/2DS1 (members of KIR genes) and UC risk, and a negative association between 2DS3 and CD risk (140).

Crohn Disease (CD)

Aberrant innate immune responses, such as huge antimicrobial peptide production and enhanced innate microbial sensing and autophagy, are associated with CD pathogenesis (134). How NK cells contribute to this uncontrolled immune amplification is still unclear, nevertheless several groups have recently identified a unique subset of mucosal NK cells that contributes to local immunity. These mucosal NK cells in the human gut are distinct from conventional NK cells and are characterized by expression of the transcription factor retinoic acid-related orphan receptor C (RORC), CD127 (IL7Rα), and NKp44 or NKp46 (**Table 1**). Moreover, NKp44$^+$ NK cells produce IL22 (141), however whether they participate in pathologic or protective processes of chronic inflammation *in vivo* remains controversial. In humans, CD56$^+$CD127$^+$ NK cells are generated from LTi cells and produce little IFNγ, whereas CD56$^+$CD127$^-$ NK cells produce a large amount of this cytokine. NKp44 and NKp46 are expressed differentially on NK cells in the CD intestine, NKp46$^+$ NK cells predominate in intestinal mucosa of patients with CD compared with patients with UC and with controls. Upon interaction with intestinal inflammatory macrophages, NKp46$^+$NK cells from patients with CD are activated via IL23 and produce IFNγ (93).

Of note, genetic alterations in regulatory NK cell receptors have been reported. Indeed, KIR polymorphism is implicated in susceptibility to CD (140), with a significant association of the KIR2DL3/HLA-C1 genotype and CD (142), although the cellular mechanism of this genetic contribution is poorly defined. It has been described that the "licensing" of NK cells, determined by the presence of KIR2DL3 and homozygous HLA-C1 in the host genome, results in cytokine reprogramming that permits promotion of CD4$^+$ T cell activation and Th17 differentiation *ex vivo*. Licensed NK cells are more polarized to pro-inflammatory cytokine production than unlicensed NK cells. These cytokines, including IFNγ, TNFα, and IL6 (**Table 2**), augment CD4$^+$ T cell proliferation and IL17A/IL22 production. Interestingly, antibody blocking of these cytokines could reduce their effect (143), presenting a potential therapeutic target for CD and other IBD (144). However, due to the complexity of the cytokine network involved and the fact that Th17 cells may also have protective functions, neutralization of IL17A failed to induce any improvement in CD, at variance with other autoimmune disorders (145, 146).

An exploratory clinical trial to investigate the safety and efficacy of the humanized anti-IL6R mAb tocilizumab (also known as MRA) in patients with CD, showed promising results, with 20% of the patients entering remission and acute-phase responses normalized by a single MRA infusion (147). However, the gold standard IBD treatment, including CD, for many years has been based on the use of humanized or human anti-TNFα antibodies, despite many adverse effects—including the risk of tuberculosis (148).

Since CD pathogenesis has been linked with the IL12/23 pathway (138, 143, 149), a recent novel approach to interrupt this pathway has been proposed using ustekinumab, a therapeutic monoclonal antibody that blocks the p40 subunit of both IL12 and IL23 and prevents interactions with their receptors on T, NK, and APCs, has established efficacy in psoriasis (150).

Another important mechanism in the pathogenesis of CD is the expression of stress-related molecules belonging to the MIC family (136, 151). These molecules are recognized by NKG2D receptors (**Table 1**) expressed on T and NK cells and induce activation of these cytolytic cell types, thus contributing to mucosal cell damage (152, 153). A recent phase II clinical trial showed that an antibody against NKG2D induced clinical remission of CD in some patients, suggesting NKG2D and its ligands are attractive new targets for IBD therapies (152, 154).

Ulcerative Colitis (UC)

UC is characterized by contiguous inflammation of colonic LP wherein damage is triggered by an over-response to bacterial antigens, enhancement of DC and macrophage stimulation via TLRs (155, 156). In UC, the T-cell response to antigens is not Th1 dominant, as in the case of CD, but rather is either Th2 (IL4, IL13) dominant, or is mediated by specialized cells such as natural killer-like T (NKT) cells producing IL13 (**Figure 3B**). LP-NKT cells from UC patients produce significantly greater amounts of IL13. Thus, UC pathogenesis is considered to be an atypical Th2 response mediated by a distinct subset of NKT cells that produce IL13 and damage epithelial cells (156, 157). Along this line, decreasing IL13 production following treatment with IFNβ1a is associated with clinical improvement of UC symptoms (158). Other inflammatory cytokines (TNFα, IL1, IL6, IL9) play significant roles in worsening, while anti-inflammatory cytokines (TGFβ, IL10) delay disease progression (156).

In this context, the actual pathogenetic role of NK cell is still undefined. KIR polymorphism and positive associations between 2DL5/2DS1 KIR and UC risk has been reported (140, 159). In active UC, peripheral NK cells were decreased significantly compared to inactive UC. After anti-TNF treatment, peripheral NK cells in responsive IBD patients were significantly higher than in non-responsive UC (160). Intestinal LP NKG2D$^+$ NK cells have been investigated in UC, since it is thought that they play a role in regulating Th1/Th2 balance (**Figure 3B**). Severe UC patients have higher expression of mucosal NKG2D and its ligand MICA, and a lower number of LP NKG2D$^+$NK cells than mild to moderate UC. Furthermore, in bioinformatics analyses, mucosal Th1 cytokines, mainly TNFα, emerged as crucial to CD, but not UC, since anti-TNFα treatment proved less effective than in CD. This would suggest that NKG2D$^+$ NK cells play a regulatory role in UC by secreting Th1 cytokines that modulate the Th2-predominant Th1/Th2 imbalance (160). Although the precise role of NKG2D is less clear in UC pathogenesis than in CD, this molecule should be considered as a possible therapeutic

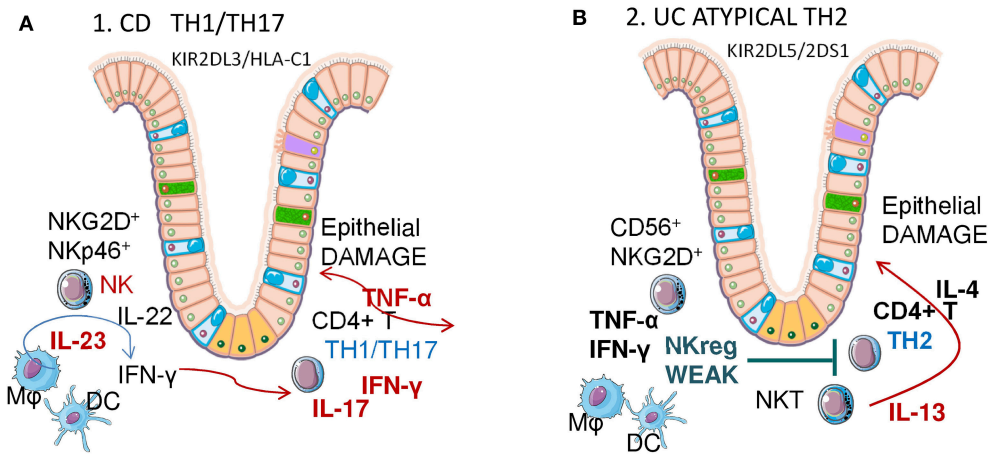

FIGURE 3 | Crohn Disease (CD) and Ulcerative colitis (UC) pathogenesis: possible role of NK cells. Different Th1/Th2 responses in CD and UC. Mϕ, macrophages; DC, dendritic cells; NK, natural killer cells; NKT, natural killer-like T cells. **(A)** Crohn's Disease is mainly a Th1/Th17 IBD, linked to a KIR2DL3/HLA-C1 genotype in NK cells. Mucosal NK cells are RORC, CD127 (IL7Rα), NKG2D and NKp46 positive, produce IL22 and IFNγ when activated via IL23 and contribute to the expansion of $CD4^+$ Th1/Th17 lymphocytes that, in turn, secrete both IFNγ and TNFα, in addition to IL17. **(B)** Ulcerative Colitis is mainly an atypical Th2 IBD, linked to a KIR2DL5/2DS1 genotype in NK cells. Atypical NKT produce IL13, which induces epithelial damage. In turn, NK cells are unable to induce an efficient TNFα IFNγ response to reduce the Th1/Th2 imbalance, contrasting excessive IL13 secretion.

target as well (153). Finally, NK cells can be targeted in CD by 6-mercaptopurine, a drug also used in UC treatment that causes NK cell apoptosis and depletion, which possibly limits the inflammatory response (161).

NK CELLS IN COLORECTAL CANCER (CRC)

CRC has been recently subdivided into four consensus molecular subtypes (CMS) on the basis of genetic and microenvironmental signatures. Three of these classes recall the well-known definitions of microsatellite instable (CMS1), sporadic (CMS2) and stromal rich (CMS4) CRC, while the class of metabolic CRC (CMS3) was recently introduced. In CMS2 and 3, comprising about 50% of all CRC, immune and stromal infiltration are limited. CMS1 (14%), due to microsatellite instability (MSI), has a high DNA mutation rate, causing the production of altered antigens that trigger the immune response. Accordingly, CMS1 shows a strong infiltration of innate and adaptive immune cells. It is not by chance that only MSI-high CRC showed a clinical response to anti-PD-1 and anti-PD-L1 immune checkpoint inhibitors therapy (162, 163). CMS4 (23%) defines a particular group of CRC showing a strong infiltration of stromal cells, such as FBs, an intermediate infiltration of immune cells and a minority of tumor cells. Despite the low prevalence of tumor cells, this CRC subtype has the worst prognosis. CMS4 is characterized by high TGFβ expression, determining an immunosuppressive microenvironment that is enriched in regulatory cells (164). In this regard, CRC-associated FBs co-cultured *in vitro* with NK cells can downregulate the expression of NKG2D and the NKG2D-mediated recognition of tumor target cells by NK cells (165).

CRC frequently shows diminished MHC class I expression, that would increase tumor susceptibility to attack by NK cells. In CRC with partial or total HLA class I loss, effector IELs are $CD8^+$ CTL, while NK ($CD56^+$) cells are only observed scattered in the stroma. On the contrary, in normal mucosa, $NKp46^+$ $CD3^-$ NK cells can also show intraepithelial localization and typically coexpress CD57 (46, 166) (**Figure 4**). To our knowledge, immunohistochemical studies on the NK cell population of CRC systematically used anti-NKp46 antibodies, thus no information about the $NKp44^+$ population is available. The quantification of $NKp44^+$ cells in the tumor infiltrate of CRC patients could be of great interest, as it has been shown that CRC-initiating cells preferentially express ligands for NKp30 and NKp44 (93, 167). CRC shows a reduced number of infiltrating NK ($NKp46^+$) cells compared to normal mucosa, also when $CD8^+$ T cell numbers are elevated, suggesting that CRC is able to limit NK cell infiltration. This limitation of NK cell recruitment is not mediated by the absence of homing chemokines as CXCL9, CXCL10, CCL3, CCL4 (active on $CD56^{bright}$ NK cells) and CXCL8, CXCL1, CXCL5, CXCL12 (active on $CD56^{dull}$ NK cells) show higher concentrations in the tumor than in the unaffected mucosa. Despite the reduced number in CRC, the $NKp46^+CD3^-$ NK population is enriched in $CD16^+CD56^+$ cells compared to normal mucosa, suggesting that ADCC could be elicited by humanized Ab targeting tumor cells (76, 165). The contemporary infiltration of $CD8^+$ T and NK cells in CRC is apparently linked to a better prognosis compared to the infiltration of $CD8^+$ T cells only. This effect could be due to direct involvement of NK effectors, or represent a less immunosuppressive microenvironment of the tumor (168). A proof of principle for the efficacy of NK cell reactions against CRC comes from a recent case-report describing a complete and sustained response

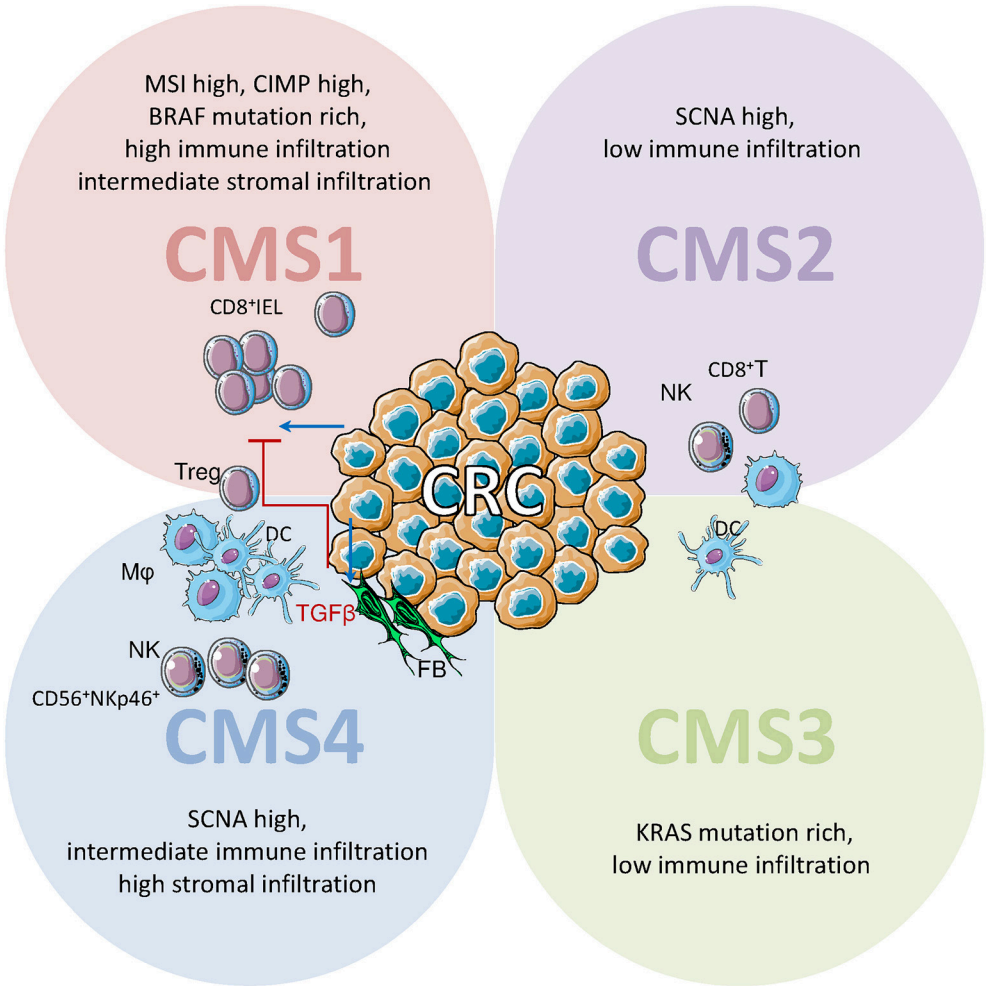

FIGURE 4 | Immune cell infiltrate in CRC: possible role of NK cells. Schematic representation of immune cell infiltrate in CRC defined on the basis of gene expression profiles in consensus molecular subtypes (CMS). IELs: intraepithelial lymphocytes; Mϕ, macrophages; DCs, dendritic cells; NK, natural killer cells; FBs, fibroblasts. IELs are $CD8^+$ cytolytic T lymphocytes (CTL), while $CD56^+NKp46^+$ NK cells are scattered in the stroma. CMS1 (14% of CRC, upper left) are characterized by high somatic copy number alteration (SCNA), high CpG island methylator phenotype CIMP), and a strong microsatellite instability (MSI); this high DNA mutation rate leads to altered antigens that trigger immune responses and causes a strong infiltration of innate and adaptive immune cells. In CMS2 and CMS3 (50% of CRC, upper right and lower right) immune and stromal infiltration are limited. CMS4 (23% of CRC, lower left) shows a strong infiltration of stromal cells (FBs) and intermediate infiltration of immune cells. In CMS4 high TGFβ expression determines an immunosuppressive microenvironment enriched in regulatory T cells.

mediated by NK cell activation, in a metastatic CRC patient (169). Indeed PVR (CD155), Nectin-2 (CD112), and MICA/B, the ligands for the activating NK receptors DNAM1 and NKG2D, are expressed in CRC, suggesting a possible target for NK effectors. Though CRC infiltrating NK cells show a partially reduced expression of DNAM1 and NKG2D, increased soluble (s)CD155, and sMICA/B have been detected in patient serum (170).

A TMA study on 462 primary colorectal tumors evaluated MIC, ULBP, RAET (NKG2D ligands) and the NK cell infiltration. The higher expression of all ligands was found in stage I (UICC-TNM) tumors, becoming less frequent in advanced stages. MIC levels correlated to NK infiltration. The contemporaneous high expression of MIC and RAET1G was linked to improved patient survival of 77 months over CRC expressing one ligand or low levels of both (171). According to these observations, the future clinical application of NK-based immunotherapies against CRC apparently depends on the identification and neutralization of the tumor-derived mediators that limit NK cell infiltration. As these mediators do not apparently influence $CD8^+$ T lymphocyte recruitment, they should probably be looked for among signaling molecules specific for NK cells. Among them, KIR and CD16 have been implicated in defining CRC genetic risk and clinical stage, although the matter is still under debate (172–174). Indeed, the allele frequency of KIR2DL2 and KIR2DS2, in the absence of their cognate HLA-C1 ligands, were significantly associated with reduced genetic risk of CRC. Conversely, CD16-48H polymorphism was associated with increased genetic risk of CRC (172). In 1990 Adachi et al. reported a higher

number of $CD57^+$ NK cells in draining CRC LN than in primary or metastatic lesions and suggested that these cells can limit tumor spreading (175). Indeed, acquisition of CD57 represents a shift toward a higher cytotoxic capacity, greater responsiveness to signaling via CD16 and NCR and decreased responsiveness to cytokines. This would be consistent with enhanced tumor surveillance/cytotoxicity of the mature, $CD57^+$ NK cell subset (46).

As discussed above, mucosal epithelium is a source and a target of several cytokines (**Table 2**) (176). In particular, IL18 is a typical cytokine produced by the normal mucosa and is decreased in CRC: its downregulation frequently correlates with a lack of IFNγ and FAS ligand, and formation of metastases. IL18 primes NK cells *in vivo* to produce IFNγ upon stimulation with IL12 and increases IFNγ neosynthesis in NK cells activated through CD16. In mouse models, IL18 limits the differentiation of Th17 cells and sustains epithelial regeneration upon inflammatory damage (177–179). IL22, produced by ILC, NK and T lymphocytes, binds only to IECs, representing a more defined target for CRC therapies. Indeed, IL22 has been associated with CRC growth, tumor cells protection from cytotoxic and apoptotic effects of chemotherapy and FOLFOX resistance in CRC patients (180–182).

IL12 is formed by IL12p35 and IL12p40 subunits. IL12p40 can also form a homodimer, antagonizing IL12 activity, or bind IL23p19 to form IL23; hence the overall effect of the single cytokine can be elusive. NK and γδ T cells express high levels of IL12 receptor. IL12 plays a central role in Th1 responses, triggering the activation of NK and $CD8^+$ T cells and inducing IFNγ production. In CRC patients, a low production of IL12 in DCs was associated with a poor prognosis (183–185). Despite the shared subunit with IL12p40, IL23 activates local inflammatory responses not involving Th1 effectors. IL23 is secreted by DCs, macrophages, and neutrophils during gut inflammation (**Table 2**). The main intestinal targets of IL23 are Th17, ILCs, and Tregs, inducing the production of IL22 and IL17. High IL23 levels, coupled with low SOCS3 expression in primary CRC, were predictive of increased risk of metastasis (186).

Despite the lack of data on NK cells and IL17, this cytokine is of interest as it shows a controversial influence on CRC. A genetic clustering of 125 CRC showed reduced survival of patients with a high Th17 signature. However, a tissue microarray evaluation of $IL17^+$ cells in 1148 CRC samples did not confirm this observation, as IL17 positive staining was correlated with neutrophils and $CD8^+$ cytotoxic lymphocytes infiltration, and intraepithelial Th17 lymphocytes were linked to a favorable prognosis. Thus, the overall effect of IL17 is apparently linked to the localization of IL17-producing cells. A further complication in the definition of the pro-tumor properties of IL17 comes from the IL17A polymorphism rs2275913 (G197A), with the AG and AA genotypes strongly associated to an increased CRC incidence (187–191).

NK/IMMUNE CELL-EXOSOMES CONNECTIONS: ROLE OF SIGNALS ORIGINATING IN GUT CELLS OR MICROBIOTA

The importance of extracellular nanovescicles (EVs), ranging from 50 to 1,000 nm in size, exosomes in particular (size 100–200 nm), as biological vehicles able to affect distant organs is well established. For example, salivary exosomes from patients with IBD, a condition predisposing toward UC and CRC, carry larger amounts of proteasome subunit alpha type 7 (PSMA7) than those in healthy subjects. High-throughput sequencing revealed ∼850 proteins in EVs secreted by intestinal cells and present in ascites of patients with CRC, showing that intestinal EVs may be transferred between organs and, in turn, modify the composition of EVs released by the target tissue (192). For a thorough examination of the state of the art of characterization and use of exosomal vesicles, we refer to recent comprehensive reviews (193, 194).

NK and organ cells apparently communicate in forward and backwards signaling loops where cell- and NK-derived exosomes exert reciprocal control in the circuit. The impact of intestinal physiology, depending also on the balance of the gut microbiota, on the immune system at the whole-body level has recently emerged as a crucial topic in cancer research and in research of related conditions (chronic inflammation, angiogenesis and metabolic syndrome, to cite a few). Responses to immunotherapy and metastatic dissemination seem to depend, at least in part, on the gut microflora-host interplay. In a remarkable *in vivo* preclinical study, the exodus of gut-primed immune cells in mice engineered with the fluorescent Kaede protein provided evidence of an intense two-way trafficking from defined colon tracts to lymphoid organs. Also specific LN at sites distant from the intestine, besides mesenteric LN, were the destination of Tregs, Th17, and other innate and adaptive immune cells involved in chronic intestinal and systemic inflammation (195). This work highlights the powerful potential of gut homeostasis and dysbiosis on control of health; however, a possible role for gut or immune cell EVs in these conditioning pathways was not examined. The influence of gut exosomes on the immune system is currently being intensely investigated, with efforts primarily focused on intestinal DC EVs and adaptive immunity cross-talk (196). Several experimental models have advanced knowledge of the effects of intestinal mucosa EVs on innate immune cells (mostly neutrophils, monocytes and macrophages), and vice versa, in inflammatory diseases of the GI tract (197).

Indeed, different preclinical studies have shown that tumor EVs promote tumor cell organotropism, favoring a pre-metastatic niche in the host microenvironment. A role for tumor EVs in metastatic progression has been reported for CRC, gastric (GC), and pancreatic ductal adenocarcinoma (PDAC) invading the liver microenvironment. Stromal cells seem to be the privileged targets of tumor exosomes in the metastatic organ, establishing a pro-inflammatory and immunosuppressive state (198).

Effects of Cellular Exosomes on NK Cell Function

The data reported focus primarily on NK cell activation induced by exosomes released by DCs, stressed cells, or tumor cells. EVs, particularly tumor EVs, are endowed with anti- or pro-tumor potential, and a dual immunostimulatory or immunosuppressive role. NK cell activation is only one of the multiple and contrasting functions of cellular EVs, and the final outcome at the level of the organ and the whole body, as well as on the disease state, comes from the prevailing effect.

DC-derived exosomes (Dexs) can boost NK effector functions through the binding of Dexs carrying TNFα to TNFR on NK cells, which stimulates the release of IFNγ and the cytotoxic response (199). Dexs have been shown to trigger NKG2D-dependent NK cell expansion and activation in lymphoid organs in mice (200). Moreover, Dexs express the nuclear/membrane protein BAG6, a ligand for the activating NCR NKp30 (**Table 1**). BAG6-expressing Dexs induce cytokine release by NK cells and their effector program (201). Dexs have the ability to directly kill T and NK cells, via Fas-L, TRAIL and other death receptors (199). As we will discuss later, Dexs can also kill tumor cells by similar mechanisms, opening new ways for anticancer therapies to target cancers.

A prominent immunosuppressive function for tumor EVs is apparently mediated by TGFβ1 and NKG2D ligands. It is well-established that intestinal epithelial cell EVs carrying abundant αvβ6 integrins can prime DCs and Tregs to produce active TGFβ from the latent form, thus acquiring a tolerogenic phenotype (202). Nevertheless, the stress-induced heat shock proteins (Hsps) are known to confer tumor immunogenicity and induce NK antitumor responses (203), and EVs expressing high levels of Hsp60 have been found in CRC. Exosomes carrying abundant Hsps have been shown to effectively limit liver metastasis in CRC and gastric cancer (204).

Pharmacological stress triggered by anticancer drugs, such as carboplatin and irinotecan, can also induce exosomal Hsps in hepatic carcinoma that can trigger NK antitumor activation. Membrane-bound Hsp70 is a tumor structure that enhances the cytotoxic attack by NK cells, improving their effectiveness. CRC EVs expressing Hsp70 can induce activating NK cell receptors, such as CD69, NKG2D, NKp44, and down-regulate the inhibitory NK receptor CD94, enhancing granzyme B-mediated NK cytotoxicity (204, 205). Tumor exosomes have been found to frequently exert immunostimulatory effects through the expression of BAG6/BAT3, a ligand for NK cell NKp30 activating receptor, normally expressed in DCs, as mentioned above.

The data available on the interactions between tissue or NK EVs in the gut are scarce. GI epithelial cells secrete EVs (206) and EV recipient cells include intestinal macrophages and DCs in the LP, which acquire information from the epithelial cells themselves or from luminal antigens provided by goblet cells. EVs secreted by intestinal mucosa, expressing intestinal epithelial-specific markers (A33, villin-1) and PGE_2, can deliver their content to APCs or NKT cells in the liver. This initiates an immunosuppressive program in the organ and an anergic-like state in NKT cells that can inhibit the anti-tumor response. The immunosuppressive effects of intestinal EVs, however, could also be exploited to limit liver autoimmune attack (207).

Effects of DCs and NK Exosomes on Neighboring Cells

Dexs, which have been thoroughly characterized, retain and expose proteins, ranging from presentation molecules (class I and class II MHC peptide complexes and CD1), costimulatory molecules (CD86, CD40), adhesion (ICAMs), and docking molecules (integrins), on the outer membrane. Clinical grade Dexs are of great value as more effective inducers of tumor-associated antigen (TAA)-specific T cell responses than DCs in novel immunotherapy strategies, and are currently being evaluated in clinical trials for vesicle-based cancer treatment (208).

Exosomes derived from NK cells were described in the context of immune surveillance against infectious agents and tumor cells by Lugini et al. (209, 210). Typical NK cells ($CD56^+CD16^+CD3^-$) constitutively release exosomes in resting and activated states. NK vesicles can express the basal NK cell markers CD56, NKG2D, and, to a lesser extent, the NCR NKp46, NKp44 and NKp30, and the cytotoxic machinery of perforins and FASL to kill cancer cells. FASL, however, was undetectable in circulating serum exosomes (209).

Jong et al. recently showed that EVs released by activated NK cells induce caspase-3,−7 and−9, exerting a cytotoxic against a panel of different tumor cell lines (211). Besides perforin, these vesicles also contain granulysin, granzymes A and B. In this scale-up isolation procedure, activation and expansion of NK cells was obtained by incubation of PBMCs with artificial APCs, K562-mbIL21, expressing a membrane variant of IL21. NK cell expansion offers opportunities for preclinical studies and possibly for clinical applications in the future. Interestingly, an observational clinical trial at the Ottawa Hospital is presently recruiting healthy donors and CRC patients after surgery to measure NK cell activity by the classical Cr^{51} cytotoxicity assay. The scope of the study is to investigate the activation state of NK cells, in order to boost clearance of tumor metastasis by NK cells (212).

The potential theranostic uses of EVs, regardless of the cell from which they are secreted, are far from being fully exploited. The term "exosomes," however, has been reported in 99 clinical studies, covering 47 observational studies aimed at defining disease-specific molecular signatures and intercellular signaling of exosomal vesicles. Further studies on exosomes are needed to develop their possible use as potential diagnostic markers or therapeutic tools for drug delivery. As an example, the exosomal form of KIR might be a valuable indicator of gut disease stage and/or progression.

FUTURE PERSPECTIVE TO STUDY NK CELLS IN HUMAN GUT

The evidence outlined herein underscores the intrinsic difficulty in studying either the physiological or pathogenic role of NK cells

in the human gut. Indeed, the complexity of the gut includes the cellular composition, the diet, and microbiota, the repertoire of hormones and cyto-chemokines, besides individual genetic background. On the other hand, NK cells and ILC are really rare and their precise identification is not easy. Nevertheless, the generation of organoids from colonic specimens and artificial scaffolds of the intestinal mucosa (213–215) suggest the possibility of studying the interactions of the NK-gut microenvironment in a more controlled experimental system. This approach might better define the relative contribution and relevance of NK cells and innate lymphoid cells in gut health and disease.

AUTHOR CONTRIBUTIONS

Although all the authors are responsible for and edited the whole content of this paper, some parts of the paper have been cured by a specific author. Briefly, AP wrote the introduction, NK cell features, mechanisms of localization of NK cells, some points on gut anatomy and CRC. RB, RV, and DC wrote the cytokines cross-talk, gut anatomy and CRC. NF wrote microbioma and endocrine influence on innate immunity, some mechanisms of antibacteria immune response. MRZ wrote the section on autoimmune diseases such as Chron Disease and Ulcerative Colitis. FT wrote the section on exosomes.

FUNDING

This work was partially supported by the 5x1000 2014 and 5x1000 2015 from the Italian Ministry of Health to AP, the Italian Ministry of Health RF-2013, GR-2013-02356568 to RV and the AIRC (Associazione Italiana per la Ricerca sul Cancro) IG 2018 Id.21648.

ACKNOWLEDGMENTS

The figures have been assembled using some modified slides of Servier Medical Art at the web site: https://smart.servier.com.

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

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frontiers in Immunology

MINI REVIEW
published: 26 March 2019
doi: 10.3389/fimmu.2019.00587

Natural Killer Cells in Kidney Health and Disease

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Natural killer (NK) cells are a specialized population of innate lymphocytes that have a major effector function in local immune responses. While their immunological functions in many inflammatory diseases are well established, comparatively little is still known about their roles in kidney homeostasis and disease. Our understanding of kidney NK cells is rapidly evolving, with murine studies highlighting the functional significance of NK cells in acute and chronic forms of renal disease. Recent progress has been made in translating these murine findings to human kidneys, with indications of NK cell subset-specific roles in disease progression in both native and allograft kidneys. Clearly, a better understanding of the molecular mechanisms driving NK cell activation and importantly, their downstream interactions with intrinsic renal cells and infiltrating immune cells is necessary for the development of targeted therapeutics to halt disease progression. In this review, we discuss the properties and potential functions of kidney NK cells.

Keywords: natural killer cells, acute kidney injury, glomerulonephritis, chronic kidney disease, transplantation

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Rafael Solana, Universidad de Córdoba, Spain
Bojan Polić, University of Rijeka, Croatia

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *10 December 2018*
Accepted: *05 March 2019*
Published: *26 March 2019*

Citation:
Turner J-E, Rickassel C, Healy H and Kassianos AJ (2019) Natural Killer Cells in Kidney Health and Disease. Front. Immunol. 10:587. doi: 10.3389/fimmu.2019.00587

INTRODUCTION

Kidney disease is a major public health problem, affecting ∼10% of populations in industrialized countries (1). Both acute kidney injury (AKI) and chronic kidney disease (CKD) are increasing worldwide (2). Progression of chronic kidney damage often leads to end stage renal disease with the need for renal replacement therapy (dialysis or transplantation), resulting in significant morbidity and mortality for affected patients.

Regardless of the diverse etiologies underlying AKI and CKD, the immune system is an important determinant in the initiation of most forms of kidney injury. Moreover, chronic inflammation in the kidney is a major driver of CKD progression, not only in autoimmune kidney disease and allograft rejection, but also in metabolic and ischemic renal injury (3, 4). A multitude of studies have provided convincing evidence that conventional T lymphocytes, reactive to classical major histocompatibility complex (MHC)-peptide antigen complexes, are important drivers of immune-mediated kidney pathology (5). In recent years, however, the role of innate and innate-like lymphocyte subsets in the progression of renal disease is beginning to be unraveled (4).

NK cells are one of the specialized subpopulation of innate lymphocytes that, in addition to being critical in anti-viral and tumor defense (6) play significant roles in regulating homeostasis and inflammatory processes in peripheral tissues (7). Although a number of reports point to an important role for NK cells in renal injury, our understanding of NK cell immuno-biology in kidney health and disease is still very limited. Here, we review the current knowledge of NK cells in different forms of acute and chronic kidney injury and, wherever possible, relate the functional evidence provided from studies of experimental animal models of renal disease to observations made in humans.

NK CELL SUBSETS

Human NK cells are classically defined as $CD3^-/CD56^+/CD335(NKp46)^+$ mononuclear cells that can be subcategorized based on expression levels of CD56 (neural cell adhesion molecule, NCAM) into low density ($CD56^{dim}$) and high density ($CD56^{bright}$) subsets (8). The two subsets are discriminated by their distribution, phenotype and function. $CD56^{dim}$ NK cells are the dominant subset in peripheral blood (9). They express high levels of CD16 (FcγRIII), can express CD57 (a marker of terminal differentiation) and behave as potent cytotoxic effector cells (10–12). In contrast, $CD56^{bright}$ NK cells are preferentially enriched in secondary lymphoid and peripheral tissues (7, 13). $CD56^{bright}$ NK cells are $CD16^{-/low}$ and mediate immune responses by secreting proinflammatory cytokines [e.g., interferon (IFN)-γ and tumor necrosis factor (TNF)-α] (10, 14).

Although murine NK cells show some similarities to the human system with regard to development, maturation, and activation, there are important differences that impact translation of functional studies of NK cell biology from mice to men (6). NK cells in mice are commonly defined by expression of NK cell receptors, such as NKp46 and/or CD161 (NK1.1), and presence of the transcription factors Eomesodermin (Eomes) and T-box expressed in T cells (T-bet). During maturation, they upregulate expression of CD11b and downregulate CD27, while the expression of distinct sets of inhibitory and activating Ly49 receptors marks functional licensing of mature NK cells (6). Similar to humans, distinct tissue-resident and recirculating, "conventional" NK cell subsets have been demonstrated in murine non-lymphoid organs, such as the liver and kidney, differing in transcriptional profile and functional capacity (15, 16). It is noteworthy that tissue-resident NK cell populations share important characteristics with group 1 innate lymphoid cells (ILC1s) that also express NK cell receptors, as well as the transcription factor T-bet, and produce IFN-γ upon stimulation. However, lack of Eomes expression and cytolytic activity separates ILC1s from closely related NK cell populations (17).

REGULATION OF NK CELL RESPONSES

NK cells are tightly controlled by a spectrum of both inhibitory and activating receptor:ligand interactions (as recently reviewed in the literature (6, 18). Under normal conditions, healthy cells express MHC class I molecules that engage the inhibitory receptors expressed on NK cells, delivering a negative signal and inhibiting NK cell activity. However, in diseased conditions, damaged and virally infected cells display reduced or aberrant MHC class I and/or express cellular stress ligands that engage with activating receptors on NK cells. In turn, these activation signals lead to NK cell production of inflammatory cytokines and cytotoxic activity (19). Another important pathway for NK cell activation is mediated by cytokines, predominantly derived from myeloid cells, e.g., dendritic cells and macrophages, during inflammatory responses. In particular, IL-12, acting in synergy with other cytokines, such as IL-18, IL-15, and IL-2, can stimulate NK cells to produce IFN-γ and other pro-inflammatory mediators and enhance their cytolytic activity (6).

KIDNEY NK CELLS UNDER STEADY-STATE CONDITIONS

Most of our knowledge regarding human NK cells is derived from studies in peripheral blood. However, it is now established that NK cells populate most healthy lymphoid and non-lymphoid organs of the human body, including the kidney. In fact, NK cells constitute a large fraction of total lymphocytes in healthy human kidneys (∼25% of lymphocytes) (13). Both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets have been identified within the NK cell compartment of healthy human kidneys (13, 20). In particular, a proportional enrichment of $CD56^{bright}$ NK cells is observed within healthy human kidneys (∼37% of total NK cells) as compared with peripheral blood (<10% of total NK cells) (13).

It still remains unclear whether these human NK cells represent a tissue-resident lymphocyte population permanently retained *in situ* or a circulating lymphocyte population that is transiently recruited to the kidney. In humans, the expression of CD69 (a C-lectin receptor) has been used to discriminate tissue-resident from circulating lymphocytes (21–23). Our group recently reported the expression of CD69 on human NK cells (predominantly on $CD56^{bright}$ NK cells) in healthy kidney tissue (20). Based on this initial indication of tissue residency, we speculate that human NK cells in healthy kidneys serve as sentinels to maintain barrier integrity and protect against pathogens, as has been suggested for tissue-resident NK cells in other human peripheral organs (7, 24–26).

The concept of a specialized NK cell subset that resides in the kidney tissue and is characterized by minimal exchange with its recirculating counterparts is supported by a recent study in mice. Using a parabiosis approach, a technique in which the blood circulations of two animals are surgically anastomosed, investigators showed that the murine kidney harbors two distinct populations of NK cells: tissue-resident (tr) NK cells with the surface marker combination $CD49a^+CD49b^-$, representing ∼20% of the total NK cell pool in the kidney, and conventional (c) NK cells which are $CD49a^-CD49b^+$ (16). The kidney-residing trNK cells displayed a surface marker profile distinct from cNK cells, did not require the cNK cell transcription factor NFIL3 for their development, partially depended on T-bet expression and, most importantly, were of functional relevance in a mouse model of ischemic AKI (see below) (16). However, whether these trNK cells play a role in maintaining kidney homeostasis in the steady-state or serve as a first line of defense against invading pathogens remains to be elucidated.

NK CELLS IN ISCHEMIC AKI

AKI is a clinical condition defined by acute impairment of kidney function, caused by heterogeneous etiologies including ischemia, sepsis and toxic insults. The most common morphology of (severe) AKI is acute tubular necrosis (ATN). Immunohistological examinations of NK cells in human ATN

are limited because clinical practice is not to biopsy when the impairment is expected to be time limited (27). Despite this, there is evidence that NK cells do indeed participate in AKI due to ATN in humans. Highlighting their potential pathogenic function, NK cells have been shown to directly kill human tubular epithelial cells (TECs) exposed to hypoxic conditions mimicking ischemic AKI *in vitro* (28). This cytotoxic function was dependent on the direct interaction of activating NKG2D receptor on NK cells and its ligand MICA expressed on TECs.

In mice, the kidney ischemia/reperfusion model has been used in several studies to investigate the role of NK cells in the induction and regeneration of ischemic ATN *in vivo*. In this model, $CD49b^+NKG2D^+$ cNK cells were shown to promote tubular injury induced by the ischemic insult, a finding that depended on NK cell expression of the cytotoxic effector molecule perforin, but was independent of IFN-γ production by NK cells (29). Mechanistically, in line with the human data, activated NK cells were able to directly kill TECs expressing the activating NKG2D ligand Rae-1 *in vitro* (29). It was further shown that ischemic injury of TECs *in vivo* upregulates their expression of Rae-1 and other stress molecules, such as the costimulatory molecule CD137L (30). Interaction of CD137L on TECs with $CD137^+$ NK cells resulted in the induction of CXCL2 expression in TECs, leading to neutrophil recruitment and immune-mediated progression of tubular damage (**Figure 1**) (30).

TECs are also instrumental in the initial recruitment of NK cells to the kidney in ischemic injury. By expressing molecules that induce NK cell chemotaxis, such as CCR5 ligands (e.g., CCL5) and osteopontin, TECs direct NK cells toward areas within the kidney tissue where they can engage in direct interaction with the damaged epithelium (31, 32). The production of CCR5 ligands by TECs was induced by TLR2 signaling, indicating that endogenous TLR2 ligands (damage-associated molecular patterns, DAMPs) released during cell death are sufficient to trigger this pro-inflammatory cascade (**Figure 1**) (31).

The question of which specific NK cell subset (trNK cells vs. cNK cells) in the mouse kidney possesses pathogenic potential in ischemic AKI was addressed by another recent study harnessing the differential expression of glycolipid asialo GM1 (AsGM1)—being highly expressed on cNK cells, but low on kidney trNK cells—to dissect the functional role of the two subsets (16). Accordingly, anti-AsGM1 antibody depletion preferentially targeted cNK cells, but spared a significant number of trNK cells in the kidney. By comparing total NK cell depletion (trNK cells and cNK cells by using an anti-NK1.1 antibody) to "selective" cNK cell depletion (using an anti-AsGM1 antibody), the authors concluded that predominantly trNK cells mediate tubular damage in ischemic AKI. Taken together, these findings demonstrate that after TEC-mediated recruitment of NK cells to the kidney, specific ligand-receptor interactions between damaged TECs and NK cells not only lead to direct perforin-mediated TEC killing, but initiate an innate inflammatory cascade that promotes immune-mediated injury in ischemic AKI (**Figure 1**).

NK CELLS IN GLOMERULONEPHRITIS AND OTHER FORMS OF CKD

CKD describes the progressive loss in renal function over a period of at least 3 months and is characterized, irrespective of its origins, by inflammatory injury and fibrosis within the tubulointerstitial compartment (33). Initial immunohistochemical (IHC)-based investigations reported the presence of interstitial NK cells ($CD56^+$, $CD57^+$ or $CD16^+$ cells) in native kidney biopsies from patients with IgA nephropathy (34) and crescentic glomerulonephritis (35). However, these early IHC studies were methodologically limited to single-antigen labeling assays. Indeed, single staining for these antigens lacks the specificity to identify human NK cells, given the broader expression of these individual markers on T cells in kidney tissue. For instance, Uchida et al. have recently suggested that human $CD56^+$ T cells are integral to the processes that mediate kidney injury (36). In addition, single-antigen IHC is insufficient to directly identify human NK cell subsets that can only be unequivocally identified by labeling multiple cell surface antigens. Therefore, our group has recently extended these earlier investigations by using a multi-parameter, flow-cytometric-based approach to evaluate NK cell subsets in a cohort of patients with different forms of CKD. In this study, Law et al. demonstrated significant correlations between tubulointerstitial NK cell numbers, in particular $CD56^{bright}$ NK cells, and the histological severity of interstitial fibrosis (20). This suggests that NK cells play a unique role in progression to CKD, regardless of the underlying etiology of kidney disease. Indeed, our group also examined the functional capabilities of these kidney NK cells, identifying $CD56^{bright}$ NK cells as an important source of proinflammatory cytokine IFN-γ in the fibrotic kidney (20). Recent evidence of strong expression of the activating NKG2D ligand MICA in TECs of patients with chronic lupus nephritis (37) provides a possible mechanistic pathway of kidney $CD56^{bright}$ NK cell activation in immune-mediated kidney disease (**Figure 2**).

Similar to the initial human studies, early investigations in rodent models were reliant on immunohistochemistry to discriminate NK cells in kidney leukocyte infiltrates in immune-mediated glomerular disease. While some studies suggested that NK cells were an important leukocyte subset infiltrating the kidney in glomerulonephritis models (38, 39), others could not confirm this finding (40–42). However, these discrepancies might easily be explained by technical limitations in detection of NK cells and variations in the models used. Another study reported an upregulation of the murine NKG2D ligand Rae-1 and its receptor, as well as increased numbers of $CD49b^+$ NK cells, in progressive glomerulosclerosis induced by injection of Adriamycin into BALB/c mice, a widely used mouse model for proteinuric CKD. However, in a functional approach, anti-AsGM1 antibody-mediated depletion of cNK cells or impaired function of NK cells in NOD-SCID mice did not alter disease severity in this model (43). Since we are now aware that the anti-AsGM1 antibody only effectively depletes cNK cells in the kidney, while sparing most of the trNK cells (16) it still remains

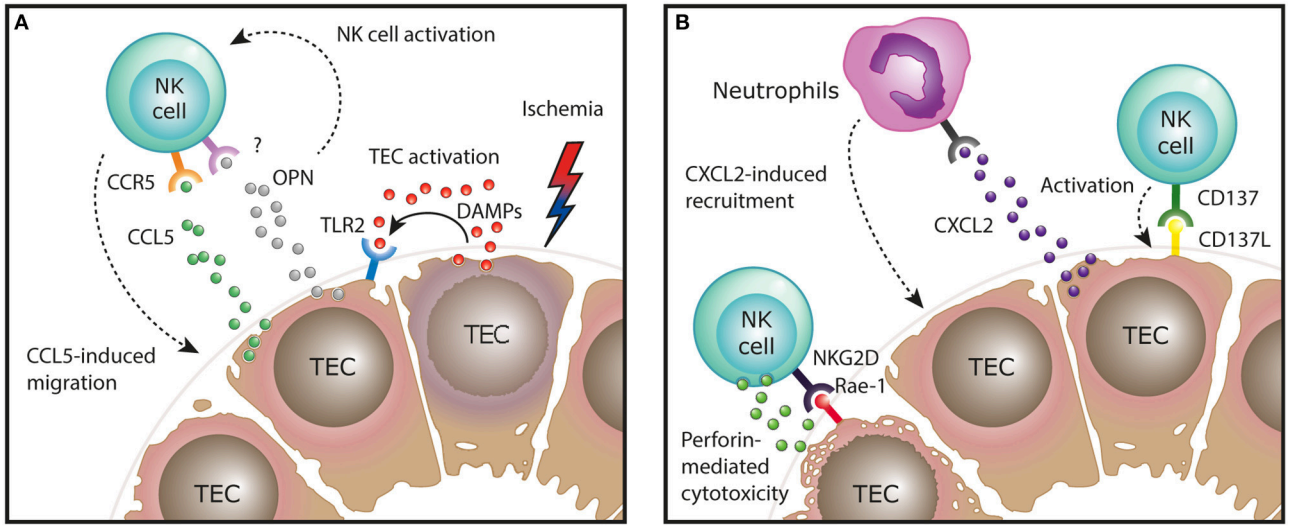

FIGURE 1 | Function of NK cells in the ischemia/reperfusion mouse model of AKI. **(A)** After ischemic injury, tubular epithelial cells (TECs) release endogenous damage-associated molecular pattern (DAMPs) that activate surrounding TECs via TLR2 to express CCR5 ligands, mediating NK cell recruitment. In addition, production of osteopontin (OPN) by injured TECs activates NK cells and indirectly regulates their recruitment, by a yet unknown mechanism. **(B)** After recruitment to the areas of ischemic injury, NK cells can engage in direct interaction with activating molecules expressed on the damaged epithelium. Activation of NK cells by these ligand: receptor interactions, such as NKG2D on NK cells and Rae-1 on TECs, results in perforin-dependent TEC killing. Interaction of CD137L on TECs with $CD137^+$ NK cells results in the induction of CXCL2 expression in TECs, leading to neutrophil recruitment and immune-mediated progression of tubular damage.

unclear whether the trNK subset might play a role in chronic glomerular scarring.

The involvement of NK cells in autoimmune glomerular disease was suggested by a more recent study investigating NK cell phenotype and activity in the MRL-*lpr* mouse model of lupus nephritis. Upregulation of NKG2D ligands was evident specifically in the glomeruli of mice with lupus nephritis and correlated with glomerular accumulation of $NKp46^+$ cells (37). The NK cells infiltrating the kidney in diseased MRL-*lpr* mice showed a mature/activated phenotype and were able to produce IFN-γ in response to IL-12/IL-15 stimulation, pointing to a potential proinflammatory role of NK cells in murine lupus nephritis.

In conclusion, while NK cells are likely to play a role in interstitial renal fibrosis in CKD (in humans), the evidence to support a substantial role of NK cells in glomerulonephritis is still scarce and detailed functional analyses of kidney NK cell subsets in glomerulonephritis with state-of-the-art methods are still missing.

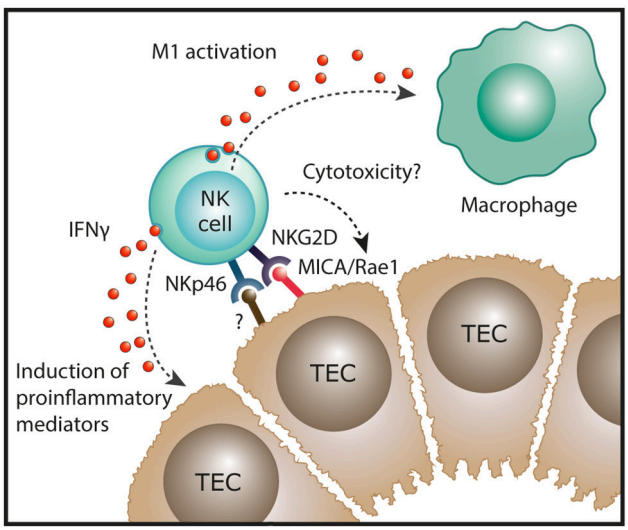

FIGURE 2 | Potential function of NK cells in chronic kidney disease. In human kidney fibrosis, NK cells reside in the tubulointerstitium and express the NK cell receptor NKp46 that can recognize stressed cells. In addition, the NKG2D ligand MICA is upregulated in tubular epithelial cell (TECs) of patients with lupus nephritis. In settings of chronic inflammation, NK cells could exert direct cytotoxic effects on damaged tubular epithelial cells. Moreover, kidney NK cells have been shown to produce IFN-γ in human CKD which could induce proinflammatory mediators in renal parenchymal cells and promote "classical" M1 activation of macrophages, both resulting in progression of renal inflammation.

NK CELLS IN KIDNEY ALLOGRAFT REJECTION

Kidney transplantation is the preferred treatment option for end-stage renal disease (ESRD) patients. However, despite improvements in immunosuppressive regimens, transplant rejection continues to contribute to loss of graft function and eventual graft loss (44). Kidney allograft rejection can be classified pathologically into two types: T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR) (45). TCMR is a tubulointerstitial process mediated by host alloreactive lymphocytes targeting donor human leukocyte antigen (HLA) molecules, whilst ABMR is a process of microvascular inflammation (glomerulitis, peritubular capillaritis) driven

by donor-specific antibodies (DSA) acting on the allograft endothelium (46–48). Human NK cells have attracted interest as key immunological players in both TCMR and ABMR.

Gene expression profiling of human kidney allografts identified high levels of NK cell transcripts in TCMR biopsies, suggesting a distinct role for NK cells in this tubulointerstitial disease process (49). These transcriptomic data have been validated by IHC-based investigations reporting significant associations between interstitial NK cells and acute TCMR (50, 51). However, these IHC-based studies have identified NK cells based solely on the expression of single antigens (CD56, CD57, or CD16) that cannot unequivocally define this innate lymphocyte population or allow the evaluation of discrete NK cell subsets. Future investigations incorporating multi-color flow cytometric approaches are essential to specifically identify and examine the function of NK cell subsets in human TCMR. A potential role for human NK cells in TCMR pathology may be to secrete proinflammatory cytokines (e.g., IFN-γ, TNF-α) that, in turn, are capable of: (1) inducing chemokines which recruit alloreactive T cells (52) and (2) up-regulating HLA alloantigens on target donor cells to make them more susceptible to cytotoxic killing (53).

Molecular assessments of ABMR in human kidney transplants have also been performed, with transcripts representing NK cells shown to be highly associated with ABMR pathology and the presence of DSA (49, 54–57). Single marker IHC-based evaluations have reported NK cells (identified as $CD56^+$ or $NKp46^+$ cells) in the peritubular capillaries of ABMR biopsies, consistent with an effector role for these innate cells in ABMR microvascular injury (50, 56, 57). In particular, Shin et al. observed that numbers of NK cells were significantly associated with chronic active ABMR, but not with acute ABMR (50). Emerging transcriptomic evidence indicates that NK cell activation in ABMR biopsies is specifically mediated via IgG Fc receptor CD16 triggering (58). Collectively, these findings have led to a proposed pathogenic function for NK cells in human ABMR, whereby DSA bound to allograft endothelial cells will engage with CD16 on NK cells to induce a mechanism of antibody-dependent cell-mediated cytotoxicity directed against the allograft (**Figure 3**). This model specifically implicates the CD16-expressing $CD56^{dim}$ NK cell subset in driving ABMR pathology and will be an area for future clinical investigation.

The experimental evidence from mouse studies also assigns an important role for NK cells in chronic renal allograft rejection. In a C57BL/6 parent to C57BL/6 x BALB/c F1 kidney transplant model, in which acute rejection is prevented by T cell tolerance to donor MHC, while chronic graft rejection still occurs, $CD49b^+$ cNK cells were shown to infiltrate the renal allograft. Intriguingly, chronic allograft injury in this model was still present in $Rag1^{-/-}$ mice, a model completely lacking T and B cells, but significantly ameliorated in $Rag1^{-/-}$ mice that were also depleted of cNK cells by injection of anti-AsGM1 antibody (59). Another recent study addressed the role of NK cells in a unique mouse model of ABMR, in which transplantation of MHC-mismatched kidney allografts into $Ccr5^{-/-}$ mice results in the development of DSA and signs of ABMR within 2–5 weeks (60). In this model, early depletion of the total NK cell pool by anti-NK1.1 therapy (in the absence of $CD8^+$ cytotoxic T cells in $Cd8^{-/-}Ccr5^{-/-}$ mice) resulted in reduction of IFN-γ, perforin, granzyme B, and other proinflammatory molecules in the renal transplants. Most importantly, however, with sustained depletion of NK cells in $Cd8^{-/-}Ccr5^{-/-}$ mice, long-term survival of about 40% of renal transplants could be achieved (61).

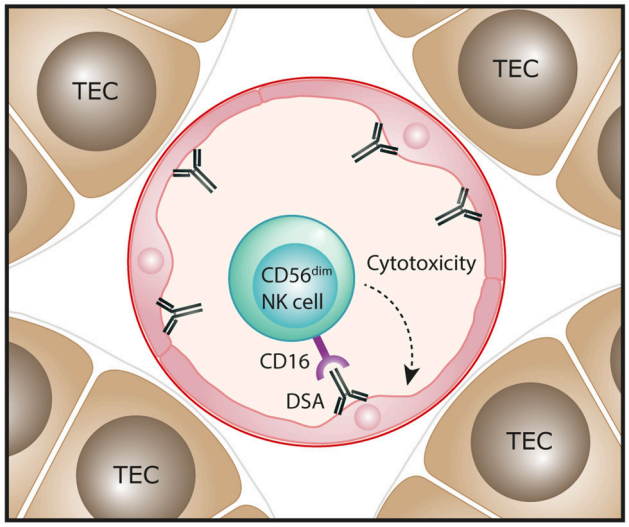

FIGURE 3 | Proposed function of NK cells in human antibody-mediated renal allograft rejection. In antibody-mediated rejection, donor-specific antibodies (DSA) bind to allograft endothelial cells. The interaction of these antibodies with the Fc receptor CD16 that is highly expressed on the $CD56^{dim}$ NK cell subset could trigger antibody-dependent cell-mediated cytotoxicity directed against endothelial cells, resulting in microvascular injury of the kidney allograft.

CONCLUDING REMARKS

The collective findings from experimental mouse models and cellular/molecular studies of human kidney specimens highlight the complex functions of kidney NK cells during homeostatic and pathological conditions. Indeed, we have presented evidence that NK cells in the kidney are a heterogeneous population of innate lymphocytes with subset-specific functional roles. Further evaluation of the kidney NK cell compartment in native and allograft models will enable the development of therapeutic approaches that specifically target the recruitment or triggering of discrete NK cell subsets dependent on the pathological conditions.

AUTHOR CONTRIBUTIONS

Each author has participated sufficiently in the work to take public responsibility for the content. J-ET, CR, HH, and AK drafted, revised and approved the final version of the manuscript.

FUNDING

AK and HH are supported by Pathology Queensland, a Royal Brisbane and Women's Hospital (RBWH) Research Grant, the

Kidney Research Foundation and a National Health and Medical Research Council (NHMRC) Project Grant GNT1099222. J-ET is supported by the Collaborative Research Center 1192 (TP A6) and an Emmy Noether Grant (TU 316/1-2) of the Deutsche Forschungsgemeinschaft. CR is supported by the Collaborative Research Center 1192.

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

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frontiers in Immunology

MINI REVIEW
published: 04 June 2019
doi: 10.3389/fimmu.2019.01263

NK Cells in the Human Lungs

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The lung offers one of the largest exchange surfaces of the individual with the elements of the environment. As a place of important interactions between self and non-self, the lung is richly endowed in various immune cells. As such, lung natural killer (NK) cells play major effector and immunoregulatory roles to ensure self-integrity. A better understanding of their abilities in health and diseases has been made possible over the past decade thanks to tremendous discoveries in humans and animals. By precisely distinguishing the different NK cell subsets and dissecting the ontogeny and differentiation of NK cells, both blood and tissue-resident NK populations now appear to be much more pleiotropic than previously thought. In light of these recent findings in healthy individuals, this review describes the different lung NK cell populations quantitatively, qualitatively, phenotypically, and functionally. Their identification, immunological diversity, and adaptive capacities are also addressed. For each of these elements, the impact of the mutual interactions of lung NK cells with environmental and microenvironmental factors are questioned in terms of functionality, competence, and adaptive capacities. As pulmonary diseases are major causes of morbidity and mortality worldwide, special attention is also given to the involvement of lung NK cells in various diseases, including infectious, inflammatory, autoimmune, and neoplastic lung diseases. In addition to providing a comprehensive overview of lung NK cell biology, this review also provides insight into the potential of NK cell immunotherapy and the development of targeted biologics.

Keywords: lung, NK cells, tissue-resident NK cells, CD49a, CD103

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *31 January 2019*
Accepted: *17 May 2019*
Published: *04 June 2019*

Citation:
Hervier B, Russick J, Cremer I and Vieillard V (2019) NK Cells in the Human Lungs. Front. Immunol. 10:1263. doi: 10.3389/fimmu.2019.01263

INTRODUCTION

The lung is faced daily with 10,000 liter of inhaled air containing a myriad of particles, potentially recognized as non-self. This constant exposure of one of the most important interfaces (>200 m^2) of the body requires a fine-tuned and rapidly acting immune system to immediately sense and protect the host at this intimate contact zone. For this purpose, the airways are endowed with a broad armamentarium of cellular and humoral host defense mechanisms, most of which belong to the innate arm of the immune system. The complex interplay between resident and infiltrating immune cells acting in concert with secreted proteins, such as defensins, mucins, or collectins, shapes the outcome of host-pathogen, host-allergen, and host-particle interactions within the airway microenvironment. Among the initial checkpoints that encounter inhaled antigens and trigger pro-inflammatory or tolerogenic/anti-inflammatory downstream immune responses, natural killer (NK) cells play a key role.

As innate lymphoid cells, NK cells provide a first line defense against infection and cancer. In comparison to their classic adaptive counterparts, NK cells are considered innate short-lived

effectors with a turnover time of approximately 2 weeks (1), compared to months or years for some T-cell subsets (2). Consistent with the critical nature of NK cell killing, impaired cytolysis is the primary diagnostic criterion in patients with functional NK cell deficiencies (3). NK cells also play regulatory roles via the release of cytokines and chemokines, and interactions with other immune cells. As such, they are also involved in various inflammatory and auto-immune diseases, in which they can act as protectors or promotors.

Under normal immune surveillance, NK cells express inhibitory receptors, including killer Ig-like receptors (KIRs), ILT-2, and the CD94:NKG2A heterodimer, which recognize primarily classical and non-classical major histocompatibility complex (MHC) class I molecules (4, 5). NK cell activation is possible when target cells lack expression of MHC-I molecules, a mechanism so called "missing-self" recognition. NK cell activity occurs also when stimulatory signals outweigh MHC class I inhibition. Several of these activating receptors have been characterized, including NKG2C, NKG2D, and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46, which ensure "stress-induced" recognition (4).

In addition to the description of this vast network of activating and inhibitory receptors, the knowledge of NK cell biology has improved in the past decades in terms of their maturation, diversity, and adaptive capacities (5), which are, at least in part, guided by the response to environmental factors, including non-fatal acute and chronic viral infections (6–8). More recently, following the identification of specific receptors related to tissue residency, a great step in understanding the critical role of NK cells in controlling self and non-self has been taken. Indeed, more than NK cells from peripheral blood, NK cells from tissues are directly interacting with normal and abnormal (micro)environments. As such, the lung contains a high reservoir of NK cells. Although still poorly understood, studies of NK cells within this organ, both in normal and pathological situations in humans, would tremendously increase the knowledge of NK cell biology. According to these recent advances, the development of new therapeutic targets could emerge, leading to a better management of respiratory diseases, which are one of the leading causes of death worldwide.

To this end, this mini-review will focus only on certain areas, with the aim of describing the specific roles of NK cells in the lung based on the most recent and exciting advances in health and disease.

NK CELLS IN THE NORMAL LUNG

Identification of NK Cell Populations

Despite a princeps study of NK cells in the human lungs in the 1980s (9), these cells have only recently been characterized in normal lungs (10, 11). The proportion of NK cells in this organ is roughly similar or even slightly higher than in peripheral blood, ranging from 5 to 20% of the $CD45^+$ lymphocytes (10). As shown in **Figure 1**, the vast majority (up to 80%) of lung NK cells display a mature $CD56^{dim}CD16^+$ phenotype (10, 11). The remaining subsets are composed of immature $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^-$ cells, this latter corresponding either to an intermediate stage of differentiation (12) or to recently activated NK cells that have lost cell-surface CD16 expression (13). These data contrast with NK cells from other tissues, including liver and secondary lymphoid organs, in which the $CD56^{bright}CD16^-$ subset largely predominates (14–16). Thus, in the lung, the different populations are present in similar proportions than in the peripheral blood, suggesting that most NK cells in the lungs are circulating cells. As a whole, this raises the question of the existence of resident lung NK cells *vs*. circulating cells, and of their identification.

By analogy with tissue resident T lymphocytes, resident lung NK cells were first identified by the cell surface expression of CD69 (17, 18), which is involved in maintaining immune cells within organs through inhibition of sphingosine-1-phosphate receptor. $CD69^+$ was differentially expressed in lung and matched peripheral blood NK cells (10). The subset of $CD69^+$ NK cells represents ∼25% of the total of lung NK cells. More recently, and in light of data regarding NK cells as well as T cells within other tissues (17), a more precise characterization of resident lung NK cells has been proposed. This identification is based on CD49a, known as a1-integrin (11, 19), which is not expressed by NK cells in the peripheral blood. Based on this definition, tissue resident lung NK cells reach up to 15% of lung NK cells. In their study, Cooper et al. (11) also analyzed the expression of CD69 and of a third marker of residency among NK cells, the aE-integrin also known as CD103. Both markers are differentially expressed by blood and lung NK cells. Not surprisingly, the $CD49a^+$ resident NK cells significantly express both CD69 and CD103 in much higher proportions than $CD49a^-$ NK cells. Of note, these different markers of lung residency are mostly expressed by the immature $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^-$ NK cell subsets, whereas they are only slightly expressed by mature $CD56^{dim}CD16^+$ NK cells. Based on this observation, it has been suggested that the small subset of triple positive $CD49a^+CD69^+CD103^+$ NK cells (**Figure 2**) could define resident NK cells more specifically (11).

From these definitions, it could be considered as a whole that resident NK cells represent the minority of lung NK cells (one-quarter of lung NK cells at most). Notably, this fraction in the lung is significantly smaller than that of other tissues, such as the liver in which resident NK cells represent 50% of their total (16). These data also indicate that the vast majority of lung NK cells (the remaining three-quarters) are circulating NK cells, which are mainly $CD56^{dim}CD16^+$ NK cells (10).

Phenotypical and Functional Characterization of Lung NK Cells

In-depth phenotypical analyses of lung NK cells have been performed among the different lung NK cell subpopulations to

Abbreviations: KIR, Killer Immunoglobulin-like Receptor; MHC-I, Major Histocompatibility Complex- I; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; NSCLC, Non-Squamous-Cell Lung Carcinoma; BALF, Broncho-Alveolar-Lavage Fluid; TME, Tumor Micro-Environment; ADCC, Antibody-Dependent Cellular Cytotoxicity; COPD, chronic obstructive pulmonary disease; TNF, Tumor-necrosis Factor; IFN, Interferon; PMA, Phorbol 12-Myristate 13-Acetate.

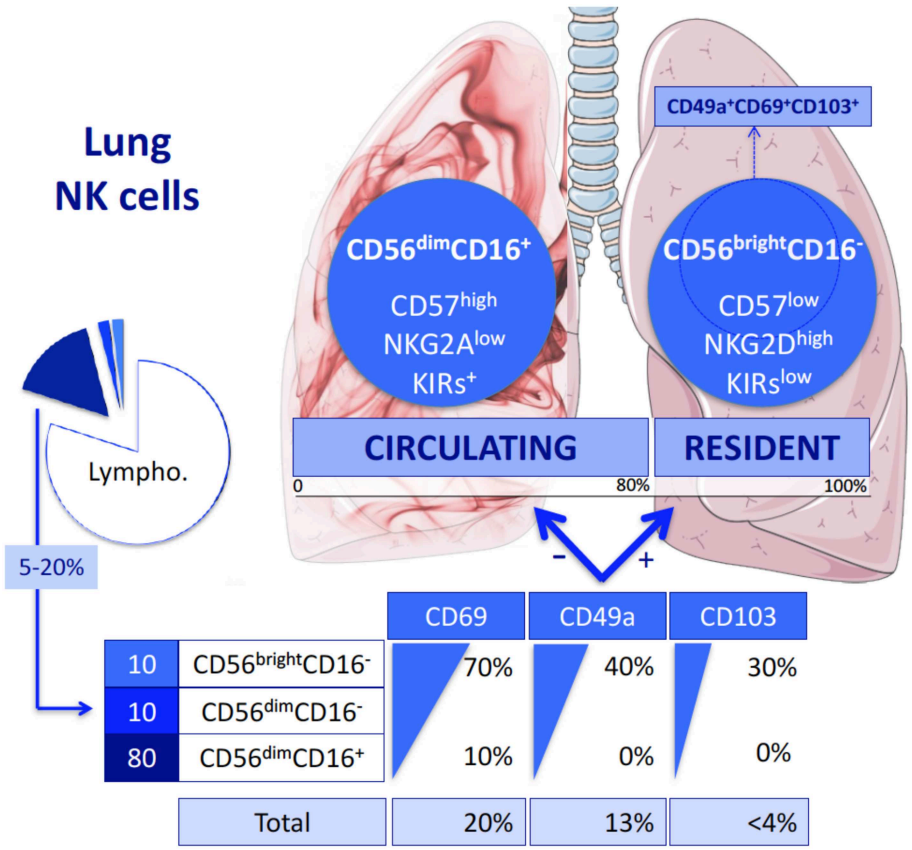

FIGURE 1 | Lung NK cell subpopulations. Like peripheral blood NK cells, lung NK cells represent 20% of all the Lymphocytes and are composed of three different subsets: $CD56^{dim}CD16^{+}$, $CD56^{dim}CD16^{-}$, and $CD56^{bright}CD16^{-}$ NK cells. Each subset expresses three markers of residency differentially. As a result, most of the lung NK cells do not express these markers: they form the circulating NK cells. They belong to the $CD56^{dim}CD16^{+}$ population and disclosed a terminally differentiated phenotype. In contrast, the cells expressing CD69, CD49a, and/or CD103 are considered as being resident NK cells. Almost all of them are $CD56^{bright}CD16^{-}$ or in a lesser extend $CD56^{dim}CD16^{-}$ NK cells. They display a less mature phenotype. Among them, triple positive $CD49a^{+}CD69^{+}CD103^{+}$ are thought to be more specifically the resident population, representing *in fine* <3% of the total lung NK cells.

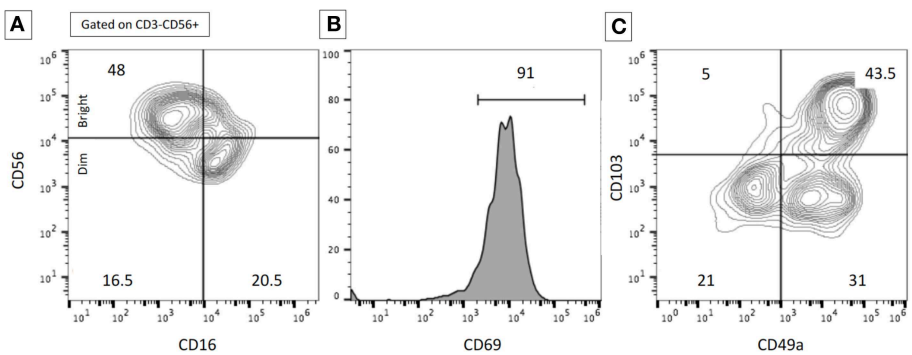

FIGURE 2 | Example of flow cytometry data illustrating the subset of resident lung NK cells. Flow cytometry analyses were performed on BALF in a patient with severe interstitial lung disease. The expression of the cell surface markers was performed after gating on $CD3^{-}CD56^{+}$ NK cells. **(A)** Proportions of $CD56^{dim/bright}$ and $CD16^{+/-}$ NK cells. **(B)** High expression of $CD69^{+}$ on NK cells. **(C)** Proportions of resident NK cells according to CD103 and CD49a expression. The proportion of resident lung NK cells was higher than expected on normal lung samples. Numbers represent the % of the different populations.

assess their maturation profile. This has been done according to previous studies showing that educated NK cells expressing KIRs and CD57 in association with low expression of NKG2A (12) would characterize the mature peripheral blood NK cell subset. It is difficult to perform such studies among each subpopulation (with respect to their resident or circulating characteristics)

because dissecting them generates small groups, although this may be possible in the future with the use of mass cytometry. To date, flow cytometry analyses have focused on lung $CD56^{dim}$ NK cells, considered herein as being mainly circulating lung NK cells, revealing that they disclose a terminally differentiated phenotype (10). It has not yet been clearly determined yet whether the proportion of these matured NK cells is enriched within the lungs or as frequent as observations made in the periphery (10, 11). In addition, these NK cells are hypofunctional against target cells when considering natural cytotoxicity, ADCC, and GM-CSF, spontaneously or following stimulation with PMA and Ionomycin or IFNα treatment (10, 11).

By contrast, when focusing on resident lung $CD69^{+}CD56^{dim}$ or $CD56^{bright}$ NK cells, it appears that these subpopulations have not matured to a terminal stage and are phenotypically similar to their blood counterpart (10). Interestingly, the $CD49a^{+}CD56^{bright}$ resident NK cells show a higher capacity to degranulate and to produce interferon (IFN)-γ when in contact with virally infected autologous macrophages *in vitro*, as compared to matched peripheral NK cells (11).

As a whole in the lung, the predominant circulating NK cells are highly differentiated but hypofunctional, while resident lung NK cells have the capacity to be hyperfunctional.

Diversity, Education, and Memory of Lung NK Cells

With recent technological advances, such as mass cytometry and single cell RNA sequencing (20), NK cell diversity has been extensively described and now appears dramatically much more important than previously expected; based on 28 surface markers, the NK cell repertoire is composed of up to 3×10^4 subpopulations (21). Globally, this diversity should be more important if the NK cell repertoire is settled at the different levels (22), including NK cell development, differentiation, and maturation (12, 21), and also the different functional capacities, to finally promote efficient innate immune response against a large variety of stress situations. While NK cell diversity is partly determined genetically (combination, number, and polymorphisms of KIRs), its modulation throughout life is mediated by interactions with the tissue microenvironment (23). As such, the lung offers one of largest interfaces with elements of the outside environment and with the microbiota. Although little is known to date regarding lung microbiota in humans, its impact on adaptive immunity and lung diseases have been suggested (24, 25). Despite correlations between microbiota and cytokines at least produced by NK cells, such as TNFα, a direct effect on lung NK cell activation or diversity has not yet been demonstrated (26). Furthermore, lung tissue consists of many different immune and non-immune cell types, thus offering many possibilities for acquiring NK cell diversity, both in normal and pathologic situations. Unlike other tissues, the lung NK cell diversity and its acquisition have been very little studied, especially regarding the resident lung populations. NK cell diversity is, however, perceptible even for the main resident population within the lung, namely $CD49a^{+}CD56^{bright}CD16^{-}$ NK cells. According to the residency markers CD69 and CD103, four different resident subpopulations may be distinguished. The $CD69^{+}CD103^{+}$ subset is the most important as compared to single positive or double negative subsets (11). The respective significance of these subsets in terms of ontogeny, differentiation, or functionality remains to be deeply studied.

Effector functions of NK cells are mainly governed by receptor interactions with MHC molecules. This process, so called "education," is essential for ensuring diversity and local immune surveillance in the lung against different stress situations, including cancer development (27). Phenotypical analyses of KIR expression by both circulating and resident NK cells in the normal lung have clearly demonstrated the presence of "educated" cells (10). These data suggest that the observed hypofunctionality of the circulating subset seems not to be related to a default in the process of education.

The identification of adaptive subpopulations among resident lung NK cells remains unknown, but could provide essential informations to search for the constitution of a memory NK cell signature in the lung. As previously described, most of the memory NK cells were derived from the expansion of adaptive $CD57^{+}NKG2C^{+}$ cells in a context of cytomegalovirus seropositivity (6, 7). Although NKG2C overexpression has not been demonstrated in lung NK cells from healthy donors (11), it could be hypothesized that, as being the site of many viral infections, the lung would be an interesting tissue in which to study the acquisition of NK cell memory (28).

LUNG NK CELLS IN DISEASES

Deciphering the distinct roles of lung NK cells in different pathological situations would help in understanding their complex functionality. However, studies distinguishing the roles played by resident vs. circulating lung NK cells in lung diseases, which requires matched and complex samples (including peripheral blood, BALF, and/or lung biopsy), have not yet been performed.

Quantitative Modulation of Lung NK Cells

Regarding the number of NK cells in the lung, one consideration that might be taken thus far is that the proportions of lung NK cells and their subpopulations do not appear to vary throughout life (11). By contrast, witnessing the possible impact of (micro)environmental factors, active cigarette smoking, and to a lesser extent, past smoking habit, decrease the number of lung NK cells (10, 11), whereas they are rapidly and dramatically increased during influenza virus lung infection. In different inflammatory diseases, including sarcoidosis, COPD, hypersensitivity pneumonitis (29), autoimmune diseases, and idiopathic pulmonary fibrosis, however, conflicting results have been found regarding the proportions and number of NK cells within the lungs (**Table 1**). Irrespective of their circulating or resident nature, NK cells might be increased or decreased during these diseases. These differences could have many causes, which may affect trafficking, homing, or local proliferation (10). Some have been slightly studied in mice (40, 41), but have not yet been explored in detail in humans.

TABLE 1 | Specificities of lung NK cells in non-neoplastic respiratory diseases.

Diseases		Quantitative observations	Qualitative considerations	References
Systemic Auto-immune Diseases	Sjögren Syndrome	Normal absolute count of Activated (HLA-DR^{+}) NK cells in BALF	nd	(30)
	Systemic Sclerosis	Normal absolute count of Activated (HLA-DR^{+}) NK cells in BALF	nd	(30)
	Anti-synthetase Syndrome	NK cells infiltration of all areas of lung fibrosis	NK cell expression of Granzyme B, Significant CD69 expression	(31, 32)
Inflammatory Diseases and/or Fibrosing diseases	Behçet Disease	Lower proportion of NK cells in BALF	Lower cytotoxicity	(33)
	Sarcoidosis	Increased number of CD56^{bright} NK cells in BALF	Immature phenotype with NKG2A^{high} & KIR^{low} predominant phenotype. Higher capacity to produce IFN-γ and TNF-α cytokines	(34–36)
	COPD	Normal count of CD56^{+}CD16^{+} lung NK cells	Decreased CD8 expression associated with poor outcome, Higher cytotoxicity. Normal expression of the activating receptor NKG2D but abnormal expression of its ligands MICA/B by lung epithelial cells.	(37)
	Idiopathic Pulmonary Fibrosis	Presence of NK cells in BALF	Predisposing factor involving NKG2D-MICA/B pathway	(38)
Allergy	Asthma	Decreased proportion of CD56^{dim} NK cells in BALF	Increased expression of Granzyme A	(39)
	Hypersensitivity Pneumonitis	Higher number of NK cells in BALF	nd	(29)
Infectious Diseases*	Influenza A Virus**	nd	Resident NK cells are hyperfunctional after *ex vivo* Infection, including degranulation, granzyme B expression and IFN-gamma expression	(11)
	HCMV°	Higher proportion of lung NKG2C+ NK cells (BALF) of patients with HCMV viremia following lung transplantation	NKG2C+ NK cells are more mature and have higher proliferation capacities. All abnormalities are associated with poor outcomes.	(28)

*HLA, Human Leukocyte Antigen; BALF, Broncho-alveolar lavage fluid; nd, not determined; HCMV, Human Cyto-megalo-virus; COPD, chronic obstructive pulmonary disease; *focuses on human studies although animal models exists for various infections (including Mycobacterium Tuberculosis, Klebsiella Pneumoniae…). **first infectious disease in which analyses have been performed according to the definition of resident lung NK cells, °studies are available only in the context of lung transplantation.*

Phenotypical and Functional Changes of Lung NK Cells in Inflammatory Diseases

In addition to their number, the phenotype and function of NK cells could also provide information regarding their involvement in diseases. As natural cytotoxicity has been shown to be influenced by cigarette smoke, it has been also hypothesized that the functionality of lung NK cells (10) could be influenced by environmental factors as well as by the lung microenvironment. Indeed, broncho-alveolar epithelial cells produce interleukin-15 during inflammation (42), whereas alveolar macrophages, the main population of immune cells within the lungs, are known to produce soluble factors likely to alter NK cell functions, such as transforming growth factor-β (43), following environmental toxin exposure.

Sarcoidosis (34–36) is a systemic granulomatosis of unknown origin commonly involving the lungs. During sarcoidosis, the analyses of lung NK cells from BALF showed an increased number of CD56^{bright} NK cells, disclosing an immature phenotype of NKG2A^{++}KIR^{low} NK cells. Following unspecific stimulation, these lung NK cells produce a large amount of Th1 cytokines (IFN-γ and TNF-α). Whether this population belongs to resident or circulating NK cells has not yet been determined. The consequences of these observed variations have not been explored either, especially in terms of fibrosis promotion (25).

COPD is closely associated with cigarette smoking, and is associated with recurrent infections, destruction of the lung parenchyma (emphysema), and/or airway obstruction. Both quantitative and qualitative lung NK cell abnormalities have been described in patients with COPD (37), but they have not been analyzed with respect to the recent resident or circulating definition. Despite effects opposite to those attributed to smoking, lung NK cell cytotoxicity could be enhanced in patients with COPD, especially against epithelial cells expressing the NKG2D stress ligands MICA/B. An association between enhanced stress-induced cytotoxicity and COPD severity has been observed, supporting a deleterious effect of lung NK cells in injuring self and promoting emphysema.

Deleterious involvement of stress-induced recognition could also play a role in the pathogenesis of pulmonary fibrosis: a possible predisposing factor involving the

NKG2D/MICA-B pathway has been identified in patients with idiopathic pulmonary fibrosis (38). Similarly, during anti-synthetase syndrome, an autoimmune connective tissue disease associated with interstitial lung disease, the NKp30 (NCR-3)/BAT-3 axis could promote the disease (31).

Further studies are required to precisely determine the respective roles of the different lung NK cell subsets (resident vs. circulating ones) in these phenomena. Increasing our understanding of the interactions between lung NK cells and the (micro)environment (44), as well as the role of resident vs. circulating lung NK cells in maintaining immune tolerance, could also lead to therapeutic strategies targeting NK cells in these pathological situations.

Lung NK Cells in Infectious Diseases and Cancer

Immune diversity, and NK cell diversity in particular, are essential to ensure effective recognition of the non-self. As an interface with the environment, the lung is the location of numerous infectious diseases, related to all types of pathogens. The impact of successive lung infections affecting individuals throughout life in terms of resident NK cell diversity and memory acquisition is one of the most challenging subjects of study to date. Unfortunately, no study of this kind is yet available in humans.

NK cell response to influenza virus has, however, been largely studied in mice, in which protective or detrimental effects were successively reported due to differences in influenza strain, dose, and genetic background of the mice. In humans, the majority of studies investigating NK cell response have used peripheral blood NK cells from patients or healthy donors following an *in vitro* infection (10). Notably, the specific response of resident lung $CD56^{bright}CD49a^{+}$ NK cells to influenza virus infection has been recently explored *in vitro* (11). In response to influenza infection, resident NK cells provided significant antiviral activity following contact with influenza-infected cells, natural cytotoxicity, and IFN-γ release. These data suggest that NK cell memory of influenza infection could exist within the human lung. The role of viral proteins, especially those which are bound by NKp46 and NKp44 (45), such as hemagglutinin, remains to be studied in light of the resident lung NK cell definition. Deciphering the mechanisms governing lung NK cell activation in this context, including cytokine signatures, activation pathways or transcription factors, would be of interest. In addition, both the diversity of the resident lung NK cell repertoire and the adaptive capacities of this specific lung NK cell population remain to be investigated.

Several lines of evidence also support the notion that NK cells play an important role in the control of tumor growth. Early studies dedicated to NK cell infiltration of the tumor microenvironment (TME) of non-small-cell lung carcinoma (NSCLC) suggested that NK cell density correlated with overall survival (46, 47). However, the most recent studies using the marker NKp46 (rather than the non-specific marker CD57) or the specific gene expression signature did not show any clear association between local NK cell infiltration and the clinical outcome (48–50). This could be explained by the ability of the TME to locally alter the intra-tumoral NK cell phenotype, as has been shown in different studies comparing them to matched normal lung NK cells and/or to peripheral blood NK cells. In humans, the NK cell population observed in NSCLC displayed profound alterations in the expression of relevant NK cell receptors, and more specifically, downregulation of expression of NKp30, NKp80, DNAM1, and CD16, as well as upregulation of NKG2A when compared to the normal counterpart (48, 51). Functionally, intra-tumoral NK cells displayed impaired ability to degranulate and to produce IFN-γ (48). The influence of the TME has been further confirmed by microarray analyses showing a modulation of the transcriptional profile and revealing a specific signature for intra-tumoral NK cells (52). Nevertheless, these conclusions were mainly drawn by considering NSCLC-infiltrating NK cells as a whole population. Although NK cells from the TME largely express CD69 (48), previously defined as a marker of residency, none of these studies suggested the possibility of specific modulation of tissue-resident NK cells. According to CD49a expression, such a comparison could now be more easily performed among the NK cell tumor infiltrate.

Apart from these lung residency considerations, it is also important to note that modulations of educated KIRs by intra-tumoral NK cells is a key element of tumor immune surveillance. Interestingly, the exposure of NK cells to exogenous MHC-I in mice led to upregulation of the activating receptors NKp46 and NKG2D and to downregulation of Ly49C/I inhibitors (the murine equivalent of KIRs inhibitors in humans) leading to a control of tumor growth (53). Thus, in addition to the recent development of anti-tumoral immunotherapies, which only partially affect NK cells, reversing the immunosuppressive TME to restore NK cell activity would increase the number of therapeutic strategies. Furthermore, diverse novel approaches, such as adoptive transfer of autologous, allogeneic, or engineered NK cells are also currently in development (54).

CONCLUSIONS AND PERSPECTIVES

In recent years, progress has been made in the characterization of NK cells in the lung; however, the concept of tissue resident NK cells has only recently been widely accepted, especially with the identification of residency markers, such as CD49a. These cells show important differences with the circulating NK cells in terms of phenotype and functions, which likely reflect the impact of the local micro-environment in shaping the tissue-specific characteristics of resident NK cells. The question of the ontogeny of tissue-resident NK cells remains complex and only partially explained (14), especially in the lung. While it is agreed that $CD34^{+}$ NK cell progenitors reside in the bone marrow, there is a less clear understanding of the mechanisms controlling seeding of NK cells within the tissues. Whether seeding of these cells into organs generates tissue-specific NK cell maturation, or whether predefined common lymphoid progenitors with specific

developmental and homing characteristics (55, 56) would exit under certain conditions from the bone marrow and specifically seed into the secondary lymphoid organs and finally into final sites of maturation remains unknown. Further analyses of the lung following human allogenic lung transplantation and/or graft vs. host disease in the lung following bone marrow transplantation would help improve our understanding of lung NK cell ontogeny. Armed with this knowledge, NK cell-based therapeutics (57–59) could be a promising avenue for the treatment of cancer and self/non-self-inflammation.

AUTHOR CONTRIBUTIONS

All authors were involved in reading bibliography and writing the article. All co-authors reviewed the article. BH drew the **Figure 1** and **Figure 2** which are original.

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

The reviewer FV and handling editor declared their shared affiliation at the time of review.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 17 May 2019
doi: 10.3389/fimmu.2019.01116

Influenza A Virus Infection Induces Hyperresponsiveness in Human Lung Tissue-Resident and Peripheral Blood NK Cells

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OPEN ACCESS

Edited by:
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Reviewed by:
Francisco Borrego,
BioCruces Health research Institute, Spain
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Columbia University, United States

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: 16 November 2018
Accepted: 01 May 2019
Published: 17 May 2019

Citation:
Scharenberg M, Vangeti S, Kekäläinen E, Bergman P, Al-Ameri M, Johansson N, Sondén K, Falck-Jones S, Färnert A, Ljunggren H-G, Michaëlsson J, Smed-Sörensen A and Marquardt N (2019) Influenza A Virus Infection Induces Hyperresponsiveness in Human Lung Tissue-Resident and Peripheral Blood NK Cells. Front. Immunol. 10:1116. doi: 10.3389/fimmu.2019.01116

NK cells in the human lung respond to influenza A virus- (IAV-) infected target cells. However, the detailed functional capacity of human lung and peripheral blood NK cells remains to be determined in IAV and other respiratory viral infections. Here, we investigated the effects of IAV infection on human lung and peripheral blood NK cells *in vitro* and *ex vivo* following clinical infection. IAV infection of lung- and peripheral blood-derived mononuclear cells *in vitro* induced NK cell hyperresponsiveness to K562 target cells, including increased degranulation and cytokine production particularly in the $CD56^{bright}CD16^{-}$ subset of NK cells. Furthermore, lung $CD16^{-}$ NK cells showed increased IAV-mediated but target cell-independent activation compared to $CD16^{+}$ lung NK cells or total NK cells in peripheral blood. IAV infection rendered peripheral blood NK cells responsive toward the normally NK cell-resistant lung epithelial cell line A549, indicating that NK cell activation during IAV infection could contribute to killing of surrounding non-infected epithelial cells. *In vivo*, peripheral blood $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells were primed during acute IAV infection, and a small subset of $CD16^{-}CD49a^{+}CXCR3^{+}$ NK cells could be identified, with CD49a and CXCR3 potentially promoting homing to and tissue-retention in the lung during acute infection. Together, we show that IAV respiratory viral infections prime otherwise hyporesponsive lung NK cells, indicating that both $CD16^{+}$ and $CD16^{-}$ NK cells including $CD16^{-}CD49a^{+}$ tissue-resident NK cells could contribute to host immunity but possibly also tissue damage in clinical IAV infection.

Keywords: human, lung, NK cells, influenza A virus, viral pathogenesis, respiratory infections

INTRODUCTION

Globally, acute respiratory infections cause 4 million deaths every year (1). Of these, infection with seasonal influenza and other respiratory viruses such as respiratory syncytial virus (RSV) constitute a major clinical and economic burden. In the case of influenza virus infections only, 3 to 5 million cases worldwide lead to hospitalization and 300,000 to 650,000 deaths annually[1]. Apart from the general respiratory symptoms, a major complication during different stages of respiratory viral infection is the severe tissue damage affecting the lung. The latter is caused not only by the viral infection and replication in target cells *per se*, but also by influx of and immunological reactions by immune cells including lymphocytes in the lung affecting non-infected cells.

Influenza A virus (IAV)-infected cells have recently been shown to be sensitive to natural killer (NK) cell-mediated cytotoxicity (2), indicating a role of NK cells in the lung upon IAV infection. However, little is known about how IAV infection affects human lung tissue-resident NK (trNK) cells and/or peripheral blood lung-infiltrating NK cells. While human NK cells are largely hyporesponsive in non-infected lungs (3), *in vitro* IAV infection of susceptible cells could readily induce lung NK cell degranulation and IFN-γ production (2, 4). Additionally, studies in murine models have shown that NK cells accumulate in the lung upon IAV infection, contributing to viral clearance (5, 6) and to shaping antiviral responses of cytotoxic T lymphocytes (7). In addition to changes in the lymphocyte composition in the lung, IAV also affects NK cells in other compartments such as the liver. For example, an influenza-specific adaptive-like NK cell subset has been shown to be present in mouse liver but not the lung following infection (8). Both in mice and in humans, a hallmark of hepatic adaptive-like NK cells is high expression of CD49a (9, 10), which is also a hallmark for trNK cells in diverse compartments including the human lung (2, 11) (Marquardt et al., unpublished observations).

IAV-mediated alteration of lung NK cell function and composition might be crucial for disease outcome. Moderate NK cell responses can be beneficial for restricting viral replication (6). However, lung tissue damage mediated by cytotoxic lymphocytes is a frequent complication during infection with RSV (12). Overproduction of NK cell-derived cytokines such as IFN-γ and TNF contributes to severe inflammation during IAV infection (13). It still, however, remains largely unknown how infection with IAV, as well as other respiratory viruses, affects human lung circulating and trNK cells.

Here, we performed a comprehensive assessment of the responsiveness of discrete NK cell subsets from human lung tissue and peripheral blood during *in vitro* and *in vivo* IAV infection. We show that, in particular, $CD16^-$ lung and peripheral blood NK cells are strongly primed following viral infection of lung cells. Activated lung trNK cells and NK cells which (re-)circulate to the infected lung likely contribute to host defense but may also exert significant tissue damage. A better understanding of how respiratory viral infections shape NK cell phenotype and function will help in improving and developing new therapeutic approaches for lung-specific pathologies including those caused by respiratory viruses.

Abbreviations: IAV, Influenza A virus; RSV, Respiratory syncytial virus; trNK cells, tissue-resident NK cells.

[1]http://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)

MATERIALS AND METHODS

Lung Tissue Collection and Influenza Patients

Clinical samples from seven patients undergoing lobectomy for suspected lung cancer were obtained for this study. None of the patients had received preoperative chemotherapy and/or radiotherapy. Patients had not undergone strong immunosuppressive medication and/or had any hematological malignancy. Clinical and demographic details of patients donating lung tissue are summarized in **Table 1**. The lung tissue was processed as described before (3).

Peripheral blood was collected from 12 patients with confirmed acute respiratory virus infection during acute phase of infection (1–8 days from onset of symptoms). Samples were also obtained from three patients (non-paired) during convalescence (around 4 weeks after acute virus infection). Of the acutely infected patients, two were receiving bronchial medications at the time of sampling (terbutaline, salbutamol: Short-acting bronchodilators; fluticasone-salmeterol combination therapy: Combination of inhaled cortisone and long-acting bronchodilator). Peripheral blood from 10 age-matched healthy controls was also collected.

The regional review board in Stockholm has approved all studies (lung tissue collection, peripheral blood collection from virus-infected patients and controls), and all donors had given informed written consent prior to collection of samples.

In vitro Infection of Cells With IAV

The influenza A/X31 strain (H3N2 laboratory-adapted strain) was propagated as described before (14). Total mononuclear cells were infected in RPMI1640 medium (Thermo Scientific), supplemented with 10% FCS (Thermo Scientific), 1 mM L-Glutamine (Invitrogen), 100 U/ml penicillin, and 50 μg/ml streptomycin (R10 medium) for 1 h with $5x10^5$ infectious particles of IAV per 10^6 cells (MOI 0.5), based on TCID50 studies with MDCK cells. Following the infection, cells were washed twice in complete R10 medium and rested or stimulated *in vitro* as described below.

Functional Analysis of NK Cells

IAV-infected or control mononuclear cells were either rested over night and subsequently cultured in the absence or presence of K562 or A549 target cells (E:T ratio 10:1 and 50:1, respectively) for 6 h, or stimulated with IL-12 (10 ng/ml) and IL-18 (100 ng/ml) for 24 h. During target cell stimulation, anti-CD107a (BV421, H4A3, BD Biosciences) was present throughout the stimulation period, and monensin (GolgiPlug, BD Biosciences) was added during the last 5 h of incubation. For cytokine stimulation, monensin and brefeldin A (GolgiPlug/Stop, BD Biosciences) were added during the last 6 h of incubation.

TABLE 1 | Clinical and demographic details of the lung cancer patients included in the study.

Patient	Diagnosis (TNM score)	Other comorbidities	Medication	Smoking (current/ex-smoker/never smoked)	Lung function (FEV1% predicted, normal >70)	Peripheral blood leukocyte count (normal 4–9 10^9/l)
59-yo female	Pulmonary adenocarcinoma (ALK positive, T1bN1M0)		None	Ex-smoker	107	4.1
83-yo female	Pulmonary adenocarcinoma (T2bN0M0)	Atrial fibrillation, atherosclerosis, breast cancer (6 yrs ago)	Furosemide, potassium chloride, ezetimibe, omeprazole, spironolactone, warfarin	Occasionally	106	8.8
71-yo female	Pulmonary adenocarcinoma (T1bN0M0)	Atherosclerosis	Acetylsalicylic acid, bisoprolol, felodipine, candesartane, simvastatin, escitalopram	Ex-smoker	93	6.4
69-yo female	Pulmonary adenocarcinoma (T2aN0M0)	Vulvar SQUAMOUS CELL CARCINOMA	None	Ex-smoker	111	5.6
77-yo female	Pulmonary adenocarcinoma (T3N0M0, two tumors in the same lobe, other minimally invasive)	Hypertension, asthma, minimal change nephropathy	Inhaled budesonide, occasional systemic low-dose corticosteroid	Ex-smoker	89	9.3
76-yo male	Squamous cell carcinoma and pulmonary adenocarcinoma (T1aN0M0)	diabetes mellitus type 2, hypertension	Sitagliptin/metformin, acetylsalicylic acid/dipyridamole, amiloride, felodipine/metoprolole, candesartane, simvastatin, finesterid, citalopram	Current smoker	94	7.4
53-yo female	Pulmonary adenocarcinoma (T1aN0M0)	Asthma	Tibolone, desloratadine	Ex-smoker	77	9.6

Flow Cytometry

Antibodies and clones against the following proteins were used: CD3 (UCHT1, PE-Cy5, Beckman Coulter), CD14 (MϕP9, Horizon V500, BD Biosciences), CD16 (3G8, Brilliant Violet 711 or Brilliant Violet 785, Biolegend), CD19 (HIB19, Horizon V500, BD Biosciences), CD38 (HIT2, Brilliant Violet 711, BD Biosciences), CD45 (HI30, Alexa Fluor 700, Biolegend), CD49a (TS2A, AlexaFluor 647, Biolegend), CD56 (N901, ECD, Beckman Coulter, or HCD56, Brilliant Violet 711, Biolegend), NKG2A (Z1991.10, APC-A780, Beckman Coulter), CD69 (TP1.55.3, ECD, Beckman Coulter), CXCR3 (G025H7, PE-Cy7, Biolegend). After washing twice, cells were stained with streptavidin Qdot 605 or Qdot 585 (both Invitrogen) and Live/Dead Aqua (Molecular probes, Life Technologies). After surface staining, PBMC were fixed and permeabilized using FoxP3/Transcription Factor staining kit (eBioscience). For intracellular staining, the following antibodies were used: granzyme B (GB-11, PE-CF594, BD Biosciences), IFN-γ-Brilliant Violet 570 (4S.B3, Brilliant Violet 570, Biolegend), Ki67 (B56, A700, BD Biosciences), perforin (dG9, PE, Biolegend), and TNF (MAb11, Brilliant Violet 421, Biolegend). IAV infection was monitored using anti-influenza A nucleoprotein-1 (431, Abcam). Samples were analyzed on a BD LSRFortessa equipped with 5 lasers (BD Biosciences), and data were analyzed using FlowJo versions 9.5.2 and 10.5.3 (Tree Star Inc).

Statistics

GraphPad Prism 6 (GraphPad Software) and SPICE software, version 5.35 (NIAID, NIH), were used for statistical analysis. Wilcoxon matched-pairs signed rank test was used for comparison of matched pairs of data or Mann-Whitney test for comparison of unmatched pairs.

RESULTS

IAV Infection Renders Human Lung NK Cells Hyperresponsive

Compared to peripheral blood NK cells, human lung NK cells are hyporesponsive to target cell stimulation (3). To test whether IAV infection could alter lung NK cell functionality including responsiveness to target cells, we infected mononuclear cells from paired human lung and peripheral blood with the influenza A strain X31 *in vitro*. IAV nucleoprotein 1 (NP1) was readily detectable in particular in lung $CD14^+$ cells but also, with lower expression, in NK cells and T cells in blood and lung after 24 h of infection at a MOI of 0.5, indicating that subsets of these cell populations were productively infected (**Figure 1A**). Despite the lack of productive infection in the vast majority of NK cells, *in vitro* infection with IAV induced a marked increase in CD69 expression on $CD56^{bright}$ and $CD56^{dim}$ NK cells in both blood and lung (**Figures 1B,C**), indicating that IAV infection of mononuclear cells activates NK cells. Notably, while CD69 indicates early activation of NK cells in peripheral blood (15), it is a hallmark marker for tissue-resident NK cells in diverse tissues including the lung (3). At baseline, ∼10% of $CD56^{dim}$ and 80% of $CD56^{bright}$ NK cells in the human lung express CD69 (3). Interestingly, following 24 hours of *in vitro* cell culture, ∼80% of $CD56^{dim}$ lung NK cells in most donors expressed CD69 even in the absence of IAV (**Figures 1B,C**), indicating that long-term cell culture is

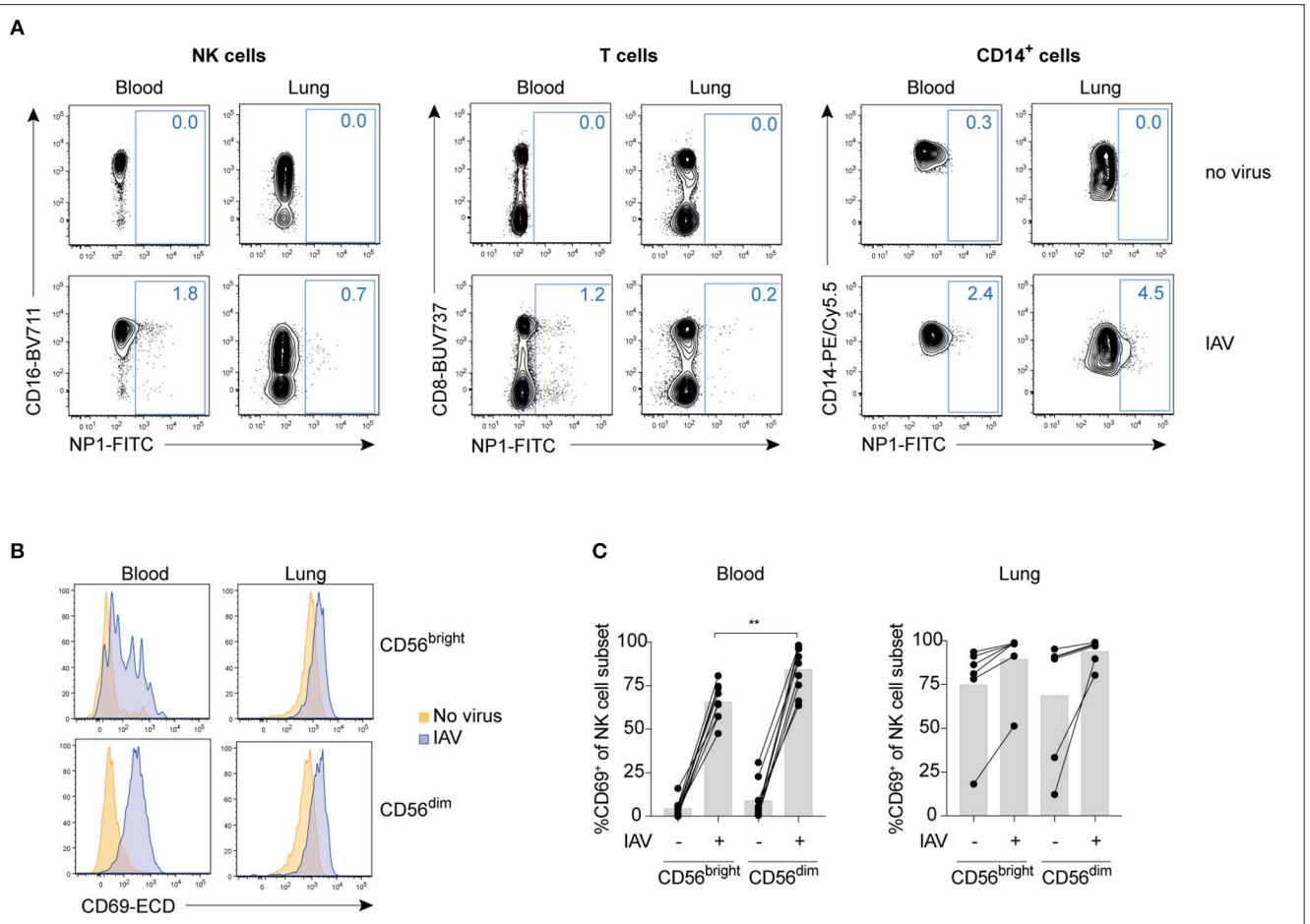

FIGURE 1 | IAV infection of total mononuclear cells activates blood and lung NK cells without infecting them. **(A)** Representative dot plots of NP1 staining in IAV-infected or non-infected blood or lung NK cells (NKG2A$^+$ OR CD16$^+$ out of live CD3$^-$CD14$^-$CD19$^-$CD45$^+$CD56$^+$ lymphocytes; left panel), T cells (live CD3$^+$CD14$^-$CD19$^-$CD45$^+$ lymphocytes; central panel), or CD14$^+$ cells (live CD3$^-$CD14$^+$CD19$^-$CD45$^+$ cells; right panel). **(B)** Representative histograms displaying CD69 expression in non-infected (orange) or IAV-infected (blue) CD56^{bright} (top) and CD56^{dim} (bottom) NK cells from blood (left) and lung (right). **(C)** Summary of data displaying CD69 expression on CD56^{bright}CD16$^-$ ("CD56^{bright}") and CD56^{dim}CD16$^+$ ("CD56^{dim}") NK cells in blood (left) and lung (right). $n = 9$ (blood), $n = 7$ (lung). Mann Whitney, $^{**}p < 0.01$.

sufficient to unspecifically induce CD69 on non-tissue-resident lung NK cells.

IAV infection also functionally activated human lung NK cells, as evidenced by increased sensitivity to target cells and cytokines (**Figure 2A** and **Figure S1A**). In both peripheral blood and lung, IAV induced strong NK cell responses with respect to degranulation following target cell stimulation in both CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells (**Figure 2B**). Notably, degranulation was if anything stronger in the CD56^{bright}CD16$^-$ NK cell subset than in the CD56^{dim}CD16$^+$ subset. IAV infection also induced degranulation even without any additional target cell stimulation in lung and peripheral blood NK cells, but only low levels of cytokine production (**Figures 2A–D**). IAV-mediated induction of cytokines including TNF and IFN-γ, however, could be boosted by additional stimulation such as with K562 (**Figures 2A–D**) or IL-12 and IL-18 (**Figure S1**), respectively.

In addition to CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells, CD56^{dim}CD16$^-$ NK cells can represent a substantial population in the human lung (2). In order to determine the full spectrum of NK cell responsiveness, we further compared NK cell functions in CD16$^-$ versus CD16$^+$ NK cells in lung and blood (**Figures 2C,D**). In comparison to CD56^{bright}CD16$^-$ NK cells (**Figures 2A,B**), total CD16$^-$ NK cells displayed a stronger CD107a$^+$ response in blood and lung (**Figures 2C,D**), indicating that CD56^{dim}CD16$^-$ NK cells contribute to the NK cell response.

Furthermore, the response to IAV infection alone without additional stimulus in CD16$^-$ lung NK cells was similar to that of CD16$^-$ lung NK cells stimulated with K562 cells in the absence of IAV infection (**Figure 2D**), thus showing that IAV-infection alone can drive a strong NK cell response. From a qualitative point of view, IAV infection induced a marked polyfunctional response in CD16$^-$ NK cells in the lung, both with and without additional stimulation by K562 cells (**Figure 2D**). In contrast, IAV infection mainly induced a stronger degranulation in CD16$^+$

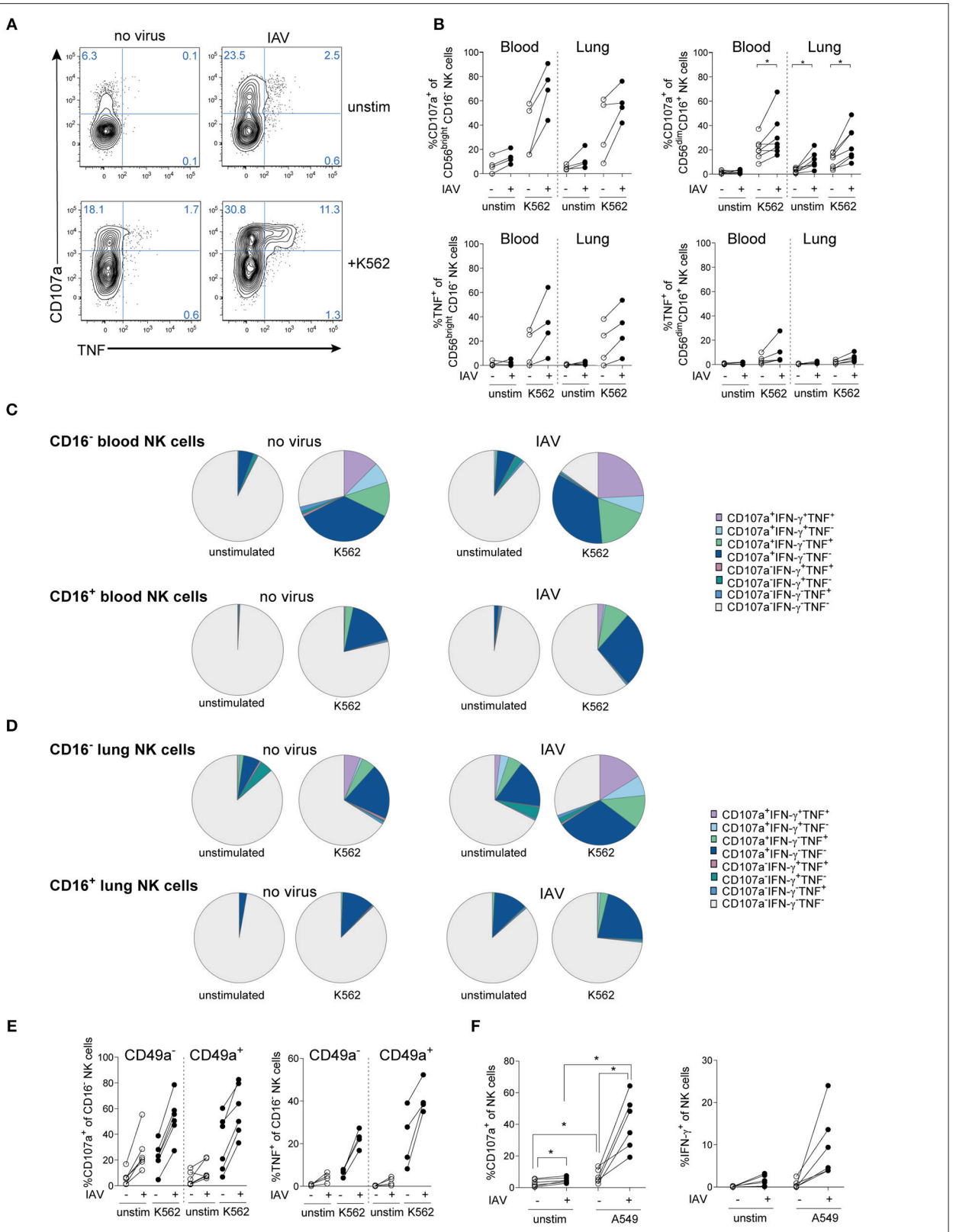

FIGURE 2 | IAV infection induces NK cell hyperresponsiveness in human lung and blood. **(A)** Representative dot plots and **(B)** summary of data displaying degranulation (CD107a expression) and TNF production of total lung NK cells or lung NK cell subsets following IAV infection of lung mononuclear cells and

(Continued)

FIGURE 2 | co-incubation with or without K562 target cells ($n = 4$ to 7), $^*p < 0.05$. **(C,D)** SPICE analysis of CD16$^-$ (upper panel) and CD16$^+$ (lower panel) NK cell responsiveness in **(C)** peripheral blood and **(D)** lung following infection with IAV and co-incubation with or without K562 target cells ($n = 4$). **(E)** Expression of CD107a (left) and TNF (right) in CD49a$^-$ and CD49a$^+$ CD16$^-$ lung NK cells following infection with IAV and co-incubation with or without K562 target cells ($n = 4$ to 7). **(F)** Expression of CD107a and IFN-γ in peripheral blood NK cells following *in vitro* IAV infection of PBMC and co-incubation with or without uninfected A549 cells ($n = 6$), $^*p < 0.05$.

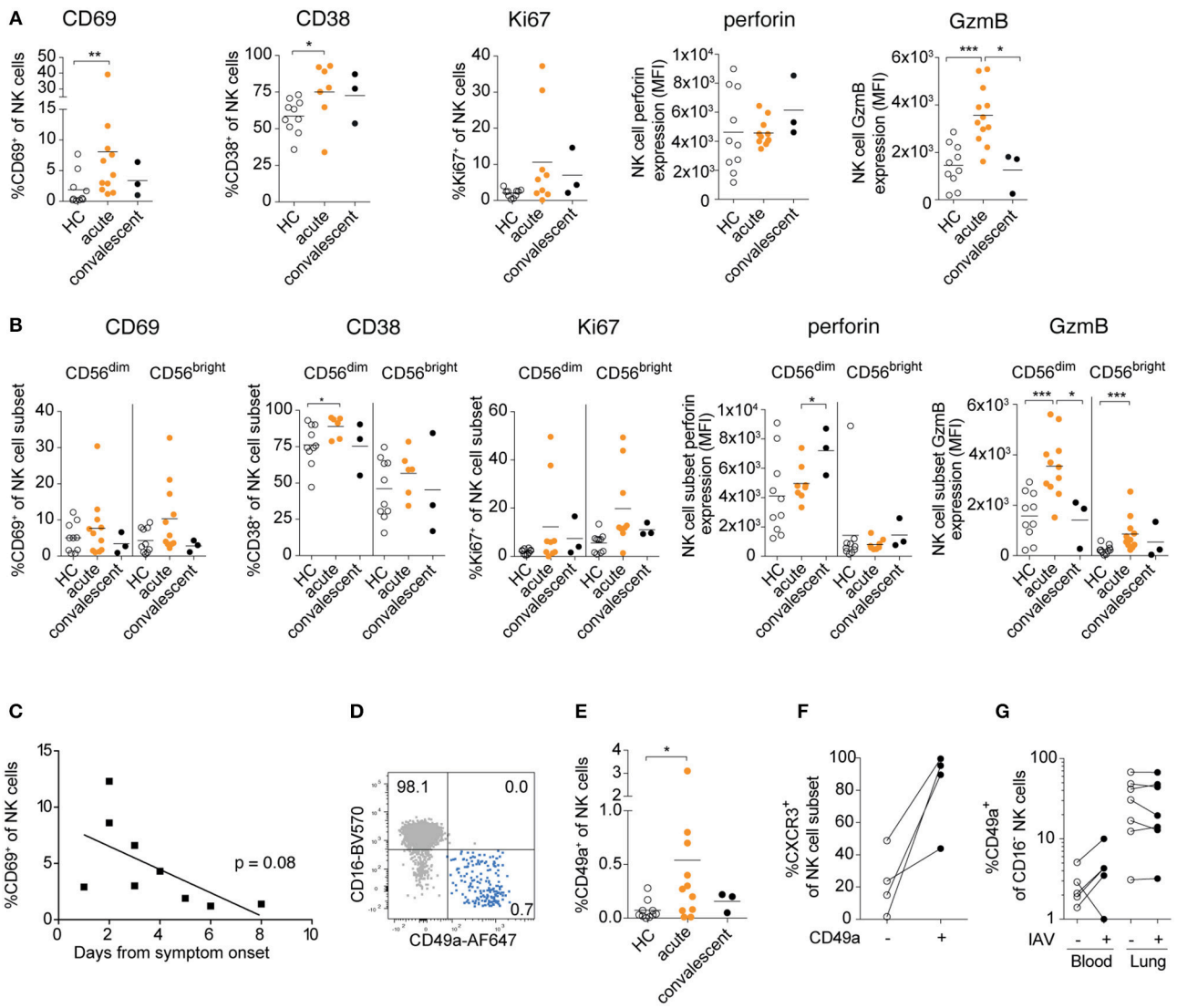

FIGURE 3 | Human peripheral blood NK cells are activated and upregulate CD49a upon acute IAV infection. **(A)** Frequencies of CD38$^+$, CD69$^+$, Ki67$^+$, and CXCR3$^+$ NK cells and expression levels of granzyme B and perforin in NK cells in age- and sex-matched healthy controls (HC). Patients were studied during the acute and convalescent phase of IAV infection. **(B)** Summary of data of CD38, CD69, Ki67, and granzyme B expression in CD56^{dim}CD16$^+$ and CD56^{bright}CD16$^-$ NK cells during acute and convalescence phase of IAV infection as compared to age- and sex-matched healthy controls. **(A,B)** Mann-Whitney, $n = 10$ (HC), $n = 6$ to 12 (IAV acute), $n = 3$ (IAV convalescent), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(C)** Frequency of CD69$^+$ NK cells during acute phase of IAV infection in relation to the days past from symptom onset, $n = 9$. **(D)** Representative dot plot displaying CD16 and CD49a expression on total peripheral blood NK cells during acute IAV infection. **(E)** *Ex vivo* expression of CD49a on NK cells from patients during acute and convalescent phase of IAV infection and healthy controls. Mann-Whitney, $n = 10$ (HC), $n = 11$ (IAV acute), $n = 3$ (IAV convalescent), $^*p < 0.05$. **(F)** *Ex vivo* expression of CXCR3 on CD49a$^-$ and CD49a$^+$ blood NK cells during acute IAV infection, $n = 4$. **(G)** Expression of CD49a on CD16-blood and lung NK cells following *in vitro* IAV infection, $n = 5$ (blood), $n = 7$ (lung).

NK cells, with less induction of TNF and IFN-γ compared to CD16$^-$ NK cells (**Figure 2D**). Thus, IAV infection affects mainly CD16$^-$ NK cells in human lung and peripheral blood. In the human lung, CD16$^-$ NK cells are partly constituted by trNK cells as identified by expression of CD69, CD49a, and/or CD103 (2, 3). Due to their location, trNK cells are likely to be directly

exposed to virus-infected neighboring tissue cells including epithelial cells and alveolar macrophages upon respiratory virus infection *in vivo*. Within the CD16$^-$ NK cell subset, CD49a$^+$ trNK cells were functionally competent and were strongly primed by IAV infection *in vitro*, with a trend toward increased TNF production by CD49a$^+$ NK cells as compared to CD49a$^-$ NK cells (**Figure 2E**). Activation of discrete NK cell subsets in lung and peripheral blood by respiratory virus infection might have physiological consequences upon infection, as indicated by RSV infection in mice (12). In line with that, IAV-primed peripheral blood NK cells became strongly sensitive to A549 cells, a lung epithelial cell line which is normally not lysed by resting NK cells (**Figure 2F**). This suggest a potential contribution of blood NK cells to host responses but potentially also to lung tissue-damage during IAV infection, since circulating NK cells constitute the vast majority of NK cells in the human lung (3).

IAV Infection Activates Human Peripheral Blood NK Cell Subsets *in vivo*

In order to determine the extent to which IAV infection promotes activation of circulating NK cells *in vivo*, we assessed the activation of peripheral blood NK cells in patients with confirmed IAV infection during different stages of the disease (**Figure 3**). During the acute phase of the infection, increased expression of CD69, CD38, Ki67, and granzyme B was observed on NK cells indicating activation (**Figure 3A**).

While both CD56^{dim} and CD56^{bright} NK cells displayed signs of activation, the two subsets differed in their responses: Expression of CD69 and Ki67 were particularly elevated in CD56^{bright} NK cells, whereas granzyme B expression was higher in CD56^{dim} NK cells (**Figure 3B**). Interestingly, the expression levels of perforin increased in the CD56^{dim} NK cells even in the convalescent phase, in contrast to all other activation markers analyzed, indicating potential long-term imprinting by respiratory virus infection (**Figure 3B**). Together, our data reveal that IAV infection *in vivo* activates NK cells in the acute stages of infection, with distinct responses in CD56^{bright} and CD56^{dim} NK cells.

As described above, IAV infection activated peripheral blood NK cells *in vivo* as indicated by CD69 expression, and we detected a trend toward increased activation levels at the earliest stages of acute infection (2–4 days after symptom onset) (**Figure 3C**). It remains to be determined, however, whether human peripheral blood NK cells have been locally activated in the lung tissue or whether they have been activated systemically. Notably, a small but detectable population of CD16$^-$CD49a$^+$ NK cells was identified in the blood during the acute phase of infection, but not in the convalescent phase or in healthy donors (**Figures 3D,E**). These CD49a$^+$ NK cells strongly co-expressed the lung-homing receptor CXCR3 (**Figure 3F**). Induction of CD49a on blood NK cells could also be observed upon *in vitro* IAV infection, whereas CD49a expression was not further increased on lung NK cells (**Figure 3G**). Elevated expression of CD69 and CD49a might facilitate the infiltration of NK cells into the lung tissue upon infection, suggesting that circulating activated NK cells could extend the pool of trNK cells in the human lung, further contributing to host immune responses but potentially also to tissue damage.

DISCUSSION

NK cells are the most frequent innate lymphoid cells in human lung tissue (3), and the vast majority of NK cells in the human lung are circulating between the organ and perhipheral blood (3). Respiratory virus infections such as IAV and RSV are likely to affect not only NK cells residing in the lung but also NK cells in the circulation or cells circulating between the lung and peripheral blood. Futhermore, both circulating and lung trNK cells likely contribute considerably not only to host protection but also to possible adverse effects in the tissue during respiratory infection. Here, we characterized the NK cell response to IAV infection in human lung and blood, both *in vitro* and *ex vivo*, and demonstrate marked viral infection-induced hyperresponsiveness, particularly in CD56^{bright}CD16$^-$ NK cells.

While confirming that IAV infection is able to override lung NK cell hyporesponsiveness (2), Cooper et *al*. found no functional differences between CD56^{dim} and CD56^{bright} NK cells following IAV infection in lung tissue explants. The difference between their results and the present results might possibly be due to differences in infection and cell activation in cell suspensions vs. tissue explants. In the tissue explants, limited exposure of lung NK cells to IAV and activated accessory cells and/or co-localization with alveolar macrophages might result in lower NK cell activation potential. While usage of tissue explants might be more physiological due to maintaining the tissue structure and lymphocyte localization in the tissue, *in vitro* infection in single cell suspensions allows investigations into the overall response capacity of the total NK cell population present in the tissue. Adding to these results, our findings demonstrate that IAV infection not only activates human lung NK cells but also peripheral blood NK cells, which are recruited in high numbers to the lung upon viral infection (5, 6) and are likely to considerably contribute to lung tissue damage.

In acutely infected IAV patients, serum/plasma levels of IL-6, IL-8, IL-15, and CXCL10 (IP-10) are elevated (13, 16, 17), and airway epithelial cells and alveolar macrophages release CXCL10 and IL-15 in the lung upon stimulation with IFN-γ, viral infection, or other pulmonary diseases (18–22). Together, this altered cytokine mileu in infected lung tissue is likely to induce priming specifically of CD56^{bright}CD16$^-$ NK cells. In addition, direct cell contact with infected target cells can contribute to subset-specific NK cell activation (2, 23). However, IAV infection increased peripheral blood NK cell responsiveness also toward non-infected lung epithelial cells which are normally NK cell resistant, indicating that healthy non-infected lung epithelial cells can be targeted by human NK cells upon respiratory viral infection, consistent with studies in mice (12).

In the lung tissue, a subset of CD56^{bright}CD16$^-$ NK cells can be identified as trNK cells, co-expressing CD69, CD49a, and/or CD103 (2, 3). *In vitro* IAV infection strongly primed

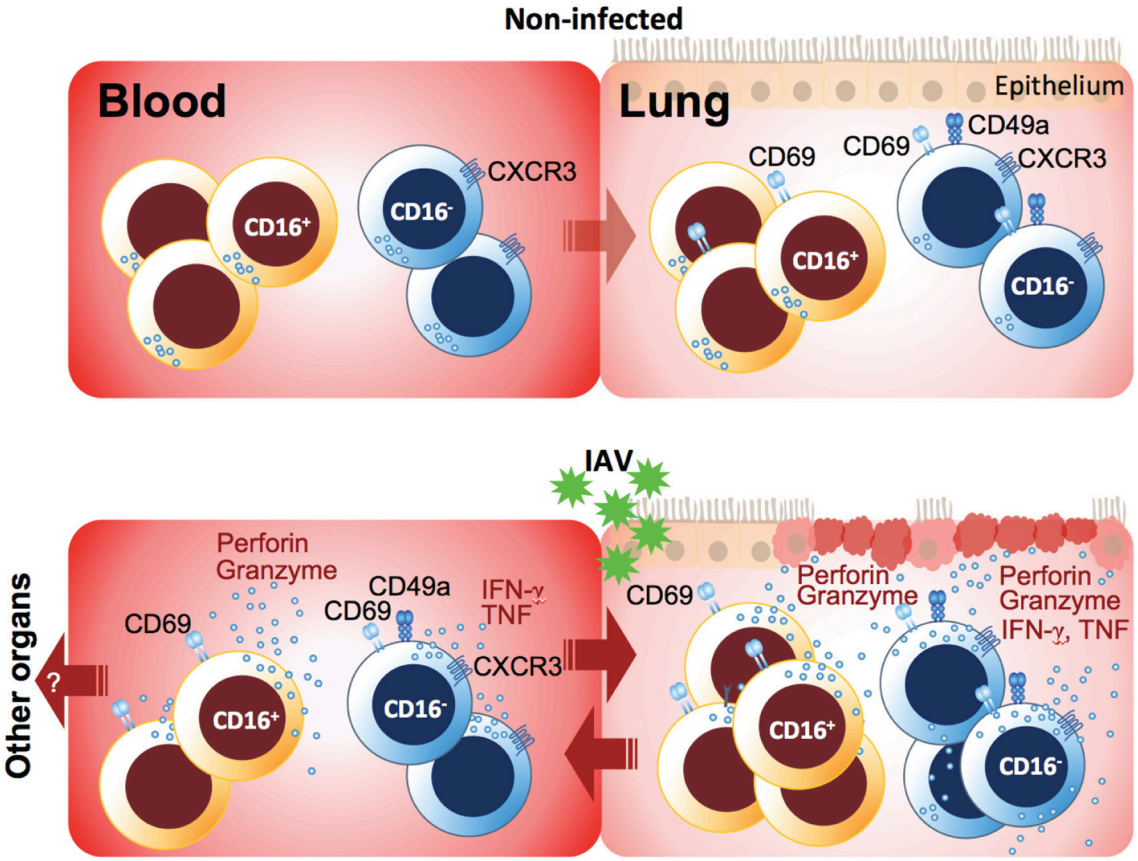

FIGURE 4 | Model of peripheral blood and lung NK cell activation in acute respiratory IAV infection. While NK cells in non-infected lung are hyporesponsive, IAV infection induced rapid upregulation of CD69 and unspecific degranulation in particular, but not exclusively, in CD16$^-$ NK cells both in blood and lung during the acute phase of infection. The presence of target cells or cytokines boosted these NK cell responses and, in addition, induced cytokine production. In peripheral blood, CD16$^+$ NK cells displayed mainly cyotoxic characteristics including increased levels of effector molecules, whereas a small subset of CD16$^-$ NK cells co-expressing the integrin CD49a and the lung-homing chemokine receptor CXCR3 emerged during the acute phase of infection. CD49a and CXCR3 likely facilitate tissue-homing and establishment of tissue-residency in the IAV-infected lung but potentially also in other organs. Finally, functionally primed NK cells accumulate in the lung, and both recruited peripheral blood NK cells but also tissue-resident NK cells may contribute to tissue damage through unspecific and/or specific lysis of lung epithelial cells.

CD16$^-$CD49a$^+$ trNK cells toward target cell-responsiveness, indicating that not only activated circulating NK cells entering the lung but also trNK cells get activated and, hence, might contribute to host response and potential tissue damage. Furthermore, despite overall low expression, CD49a was upregulated *in vivo* on a subset of blood NK cells during the acute phase of IAV infection. These CD49a$^+$ NK cells strongly co-expressed CXCR3, which might facilitate specific recruitment of this subset to the lung. Several studies have demonstrated elevated levels of CXCR3 ligands in the context of airway inflammation in chronic obstructive pulmonary disease (24), asthma (25, 26) and chronic lung allograft dysfunction (27, 28). Moreover, elevated levels of CXCL10 are present in the circulation during acute IAV infection (16) and are linked to pathogenicity of severe IAV infection (29). In mice, recruitment of NK cells to the lung upon IAV infection was shown to be partially dependent on expression of CXCR3 (30). Additionally, IAV-activated blood NK cells might also be recruited to other compartments than the lung, similar to recruitment of lung-specific T cells to the small intestine upon respiratory influenza infection in mice, causing tissue injury and intestinal disease (31). Thus, elevated expression of receptors promoting tissue-invasion of peripheral blood NK cells might contribute to host response and possible disease-associated pathology. Lastly, elevated levels of CD49a on CD16$^-$ blood but not lung NK cells suggests either egress of activated trNK cells from the lung into the circulation upon acute infection, or cytokine-induced upregulation of CD49a expression in blood, lung, or other compartments (32).

Together, our data demonstrate that NK cells in blood and lung are tightly regulated and linked to each other upon respiratory viral infection (**Figure 4**). IAV infection-mediated hyperresponsiveness of peripheral blood and lung NK cells might considerably contribute to tissue injury in the lung, promoted by increased tissue-homing capacity upon infection. Identifying and targeting these hyperresponsive NK cells in viral infections might be a future therapeutic approach for reducing respiratory virus-induced tissue pathologies.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the regional ethical review board in Stockholm with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the regional ethical review board in Stockholm.

AUTHOR CONTRIBUTIONS

MS, JM, and NM: conceptualization. SV, JM, and NM: methodology. MS, EK, and NM: investigation. NJ, KS, SF-J, AF, AS-S, MA-A, and PB: resources. MS and NM: writing—original draft. MS, JM, NM, and H-GL: writing—review and editing. NM: visualization. AS-S, JM, NM, and H-GL: funding acquisition.

ACKNOWLEDGMENTS

We want to thank all donors for participating in the study and A.-C. Orre, V. Jackson, and S. Hylander for administrative and clinical help. This work was supported by the Eva och Oscar Ahréns Stiftelse, Stiftelsen Tornspiran, Åke Wibergs Stiftelse (M18-0183), the Swedish Cancer Society (2017/505), the Swedish Research Council (2015-02499, 2017-01026), the Swedish Foundation for Strategic Research (SB12-0003), the Swedish Heart and Lung Foundation (20160243, 20160246, 20180073, 20170869), and Karolinska Institutet (2018-01593, 2018-01663).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01116/full#supplementary-material

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frontiers in Immunology

ORIGINAL RESEARCH
published: 12 July 2019
doi: 10.3389/fimmu.2019.01503

Symptomatic Carotid Atherosclerotic Plaques Are Associated With Increased Infiltration of Natural Killer (NK) Cells and Higher Serum Levels of NK Activating Receptor Ligands

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OPEN ACCESS

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: 08 March 2019
Accepted: 17 June 2019
Published: 12 July 2019

Citation:
Bonaccorsi I, Spinelli D, Cantoni C, Barillà C, Pipitò N, De Pasquale C, Oliveri D, Cavaliere R, Carrega P, Benedetto F and Ferlazzo G (2019) Symptomatic Carotid Atherosclerotic Plaques Are Associated With Increased Infiltration of Natural Killer (NK) Cells and Higher Serum Levels of NK Activating Receptor Ligands. Front. Immunol. 10:1503. doi: 10.3389/fimmu.2019.01503

A wide array of immune cells, including lymphocytes, is known to be present and to play a pathogenetic role in atherosclerotic lesions. However, limited information is currently available regarding the presence of Natural Killer (NK) cell subsets within vessel plaque, and more in general, regarding their role in human atherosclerosis. We evaluated the distribution of NK cells in human carotid atherosclerotic plaques, dissecting asymptomatic and symptomatic patients (identified as affected by stroke, transient ischemic attack, or amaurosis fugax within 6 months) with the aim of shedding light on the putative contribution of NK cells to the pathogenic process that leads to plaque instability and subsequent clinical complications. We observed that carotid plaques were consistently infiltrated by NK cells and, among them, $CD56^{bright}perforin^{low}$ NK cells were abundantly present and displayed different markers of tissue residency (i.e., CD103 CD69 and CD49a). Interestingly, carotid atherosclerotic plaques of symptomatic patients showed a higher content of NK cells and an increased ratio between $CD56^{bright}perforin^{low}$ NK cells and their $CD56^{dim}perforin^{high}$ counterpart. NK cells isolated from plaques of symptomatic patients were also stronger producers of IFN-γ. Analysis of the expression of NK activating receptor ligands (including MICA/B, ULBP-3, and B7-H6) in atherosclerotic carotid plaques revealed that they were abundantly expressed by a $HLA\text{-}DR^{+}CD11c^{+}$ myeloid cell population resident in the plaques. Remarkably, sera of symptomatic patients contained significant higher levels of soluble ligands for NK activating receptors. Our observations indicate that $CD56^{bright}$ NK cells accumulate within human atherosclerotic lesions and suggest a possible contribution of NK cells to the process determining plaque instability.

Keywords: natural killer cells, atherosclerosis, carotid atherosclerotic plaques, natural killer cell activating receptors, interferon-γ, metalloproteases

INTRODUCTION

Carotid atherosclerosisis is strongly associated with stroke and cerebrovascular disease. Nevertheless, some carotid atherosclerotic plaques (CAP) are stable and unlikely to produce symptoms, whereas others are unstable and may induce increased incidence of vascular complications. Symptomatic plaques generally reveal intima rupture, a thinner fibrous cap and a large necrotic core infiltrated by macrophages and lymphocytes. However, the complex molecular and cellular events underlying plaque destabilization are not completely elucidated and different immunological/inflammatory mechanisms are probably responsible for the clinical complications associated with unstable atherosclerotic plaques (AP). To further clarify the multiple causes of carotid plaque destabilization, it would be desirable to define a pathogenetic signature by characterizing phenotypic and functional features of cells taking part not only to plaque formation but also to the occurrence of patient's symptoms.

Immune cells, including innate lymphocytes such as Natural Killer (NK) cells, have been previously reported to infiltrate atherosclerotic lesions (1–3). NK cells are large granular lymphocytes, divided in two main subsets: $CD56^{dim}CD16^{+}$cells are the majority in human peripheral blood (PB) (≥95%) (4), while $CD56^{bright}CD16^{-}$ cells account for around 5% of NK cells in PB but represent the majority of NK cells in several peripheral tissues and in secondary lymphoid organs (5–7). $CD56^{dim}CD16^{+}$NK cells display potent cytolytic activity, while the $CD56^{bright}CD16^{-}$ counterpart is mainly responsible for cytokine production, primarily IFN-γ and TNF-α, but is poorly cytolytic (6, 8). Although NK cells were mainly described in PB and, more recently, in lymphoid tissues (8–10), it is now well-established that NK cells can be recruited at inflammatory sites (8, 10–12) and even enduringly reside in peripheral tissues at steady state (13–15).

The presence of NK cells in APs has been previously reported (1, 16–18) and it has also been shown that NKG2D, a potent immune-activating receptor mainly expressed by cytotoxic lymphocytes, including NK cells, plays a role in the vicious cycle of chronic inflammation that promotes atherosclerosis, since blocking NKG2D in mice reduced inflammation and plaque progression in atherosclerotic lesions of aorta (19).

It is noteworthy that Major Histocompatibility Complex (MHC) class I-related chains A and B (MICA/B), which represent major ligands of NKG2D receptor, are highly expressed in foam cells infiltrating the fibrous cap as well as in the intra-plaque hemorrhage space in atherosclerotic aortas of patients with type 2 diabetes mellitus (19). In agreement with these results, the expression of MICA/B in human phagocytes was also confirmed *in vitro* on foam cells derived from macrophages exposed to modified low density lipoproteins (20). All these findings suggest a role for NKG2D in enhancing the inflammatory state in atherosclerosis disease and support the hypothesis that NK cells might be actively engaged in atherosclerotic lesions.

Given that other ligands for activating receptors specifically expressed by NK cells, such as the NKp30 ligand B7-H6, have been shown to be present on macrophage surface under inflammatory conditions (21–23), we can hypothesize that induction of these ligands might even occur in the context of the inflammatory network of atherosclerosis.

Thus, on the basis of a possible contribution played by NK cells in the pathogenesis of atherosclerosis, the purpose of this study was to investigate whether the frequency and functions of NK cells infiltrating CAP might correlate with clinical complications occurring in the patients.

Abbreviations: KIR, killer Ig-like receptors; MMPs, metalloproteases; NCRs, natural cytotoxicity receptors; NK, natural killer; AP, atherosclerotic plaques; CAP, carotid atherosclerotic plaques; MMP, metalloprotease; IHC, immunohystochemistry; IFN-γ, interferon-gamma.

MATERIALS AND METHODS

Patients and Samples

Fifty patients undergoing carotid endarterectomy at the Vascular Surgery Unit of the University Hospital *G. Martino* of Messina were enrolled in the study.

Patients were selected for invasive treatment according to the European Society for Vascular Surgery (ESVS) guidelines (24). Patients were evaluated with duplex-ultrasound. The risk of stroke was predicted estimating the diameter reduction of internal carotid artery (ICA) and evaluating plaque morphology (25). Patients with symptoms of stroke, transient ischemic attack (TIA) or amaurosis fugax within 6 months since diagnosis of carotid artery disease were defined symptomatic (26–28).

All patients were admitted in the Vascular Surgery ward of the University Hospital *G. Martino* of Messina 1 day before the intervention. Carotid plaques were removed by eversion carotid endarterectomy technique (24, 29) and pre-operative blood samples were obtained from all patients. As control, PB samples were obtained from age-matched individuals with a similar gender distribution and <40% of carotid stenosis assessed by ultrasonographic study. The study was approved by our Institutional Ethics Committee and all patients gave their written informed consent according to the Declaration of Helsinki.

Carotid Plaque and Blood Sample Processing

After surgical removal, carotid plaques were extensively washed in phosphate-buffered saline (PBS) to remove cell debris and red blood cells (RBC) aggregates. Samples were then mechanically minced by scissors to obtain small fragments. In order to minimize blood contamination, tissue specimens were extensively rinsed after initial tissue slashing in small fragments. Then, samples were enzymatically digested with a mixture containing DNAse (100 μg/ml; Roche Diagnostics International Ltd., Rotkreuz, Switzerland) and collagenase (1 mg/ml; Worthington, Lakewood, NJ) in RPMI 1640 for 60 min at 37°C. The suspension was then filtered through a cell strainer, and, subsequently, washed by

centrifugation in PBS to remove residual enzyme. To obtain mononuclear cells (MNCs), plaque cell suspensions or blood underwent Ficoll-Hypaque (Sigma-Aldrich, St. Louis, Missouri) density-gradient centrifugation.

Flow Cytometry

The following mouse anti-human mAbs were used in our study: anti-CD3 PerCp Cy5.5 FITC (clone UCHT1), -CD16 PE-Cy7 (clone 3G8), -CD56 APC (clone NCAM 16.2), -CD94 FITC (clone HP-3D9), -HLA-DR FITC/APC-H7/BV421 (clone G46-6), -CD11c PerCP-Cy5.5 (clone B-ly6), -CD45 APC-H7 (clone 2D1), -Perforin FITC (clone δG9), -CD103 BV421(clone Ber-ACT8), -CD49a PE (clone SR84), -CD69 APC/APC-H7 (clone FN50), -CD57 PE (clone NK-1), -CD19 FITC (clone HIB19) from BD Biosciences (San Jose, CA); -perforin FITC (clone delta G9) from Ancell (Stillwater, MN); -CD3 VioGreen (clone REA 613), -NKG2A PE-Vio770 (clone REA 110), -NKG2C VioBright-FITC (clone REA205), -CD11c (clone MJ4-27G12), and –MICA/B PE-Vio770 (clone 6D4), -BDCA-2 FITC (clone AC144), -NKp30-PE (clone AF29-4D12) from MiltenyiBiotec (Bergisch Gladbach, Germany); -ULBP-3 PE (clone 166510), -B7-H6 APC (clone 875001) from R&D (Minneapolis, MN); -CD16 Pacific Blue (clone 3G8) from Beckman Coulter (Brea, CA). After incubation with the relevant mAbs for 20 min at 4°C, cells were then washed and analyzed by flow cytometry. Negative controls included isotype-matched irrelevant Abs. Intracellular staining with anti-human perforin-FITC was performed using the Fix/Perm kit from BD Biosciences (San Jose, CA) according to the manufacturer's indications. Samples were then acquired using FACSCantoII (BD Biosciences, San Jose, CA) and analyzed by FlowJo 9.0.2 software (Tree Star).

Cytokine Production Assay

For the analysis of cytokine production, NK cells from carotid plaques or autologous PB were stimulated with 10 ng/mL phorbolmyristate acetate (PMA) and 500 ng/mL ionomycin (both from Sigma-Aldrich, St. Louis, Missouri). After 45 min, Golgi Stop and Golgi Plug (both from BD Bioscience, San Jose, CA) were added and stimulation was allowed to continue for a total of 4 h. At the end of the stimulation, cells were first stained for relevant surface markers and finally fixed and permeabilized for intracellular detection of IFN-γ expression by specific mAb (BD Biosciences, San Josè, CA).

RT-PCR Analysis

Total RNA was extracted from carotid plaques. Briefly, samples were homogenized with Tissue Ruptor (Qiagen, Hilden, Germany) and subsequently processed using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared using the Real Master Script Super Mix Kit (5-PRIME) following manufacturer's instructions. Amplifications were performed for 30 or 35 cycles utilizing Platinum TAQ DNA Polymerase (ThermoFisher, Waltham, Massachusetts) with an annealing T of 58°C. Primers used were: β-actin for 5′ ACTCCATCATGAAGT GTGACG and β-actin rev 5′ CATACTCCTGCTTGCTGATCC; MICA for 5′TACGATGGGGAGCTCTTC and MICA rev 5′ GACCCTCTGCAGCTGATG; MICB for 5′ TCCCGGCATT TCTACTAC and MICB rev 5′ TGCATCCCTGTGGTCTCC; H6 for 5′ TGCTGTGGGCGCTGACGA and H6 rev 5′ GGTA GAACCCACTTGACTCA; ULBP-1 for 5′ TGCAGGCCAGG ATGTCTTGT and ULBP-1 rev 5′ CATCCCTGTTCTTCTCC CACTTC; ULBP-2 for 5′ CCCTGGGGAAGAAACTAAATGTC and ULBP-2 rev 5′ ACTGAACTGCCAAGATCCACTGCT; ULBP-3 for 5′ ATTCTTCCGTACCTGCTATT and ULBP-3 rev 5′ GCTATCCTTCTCCCACTTCT. PCR products (249 bp fragment for β-actin, 618 bp for MICA, 698 bp for MICB, 462 bp for B7-H6, 171 bp for ULBP-1, 199 bp for ULBP-2, and 492 bp for ULBP-3) were separated by electrophoresis on a 1.5% (w/v) agarose gel and visualized by ethidium bromide staining.

ELISA

Sera were processed using a serum separator tube (SST) and samples were allowed to clot for 2 h at room temperature before centrifugation for 15 min at 1,000 × g. Samples were stored at −20°C prior analysis. To analyze the soluble ligands of NK activating receptor MICA, ULBP-3 and B7-H6 (sMICA, sULBP-3, and sB7-H6), ELISA was performed following manufacturer's instructions using the following reagents: ULBP-3 ELISA KIT (Cusabio, Hubei Province, CN), MICA Duo ELISA Set (R&D, Minneapolis, MN); B7-H6 Elisa Kit (MyBioSource, Inc., San Diego, CA).

Statistical Analysis

We applied Kruskal-Wallis tests for evaluating quantitative variables adjusting for multiple comparison using Dunn's correction. Graphic representation and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software La Jolla, CA). Pearson correlation index was used to calculate correlation for pair-wise continuous variables.

RESULTS

Clinical Characteristics of Patients

Out of 50 patients 16 were women (32%). Mean age was 71 ± 10 years (range 46–85). Twenty-one patients (42%) were diabetic, 28 (56%) were smokers, 35 (70%) had hypertension, and 31 (62%) had hyperlipidemia. Thirty-one patients were asymptomatic (62%) and 19 (38%) were symptomatic. Clinical characteristics of patients and control individuals are summarized in **Table 1**.

Symptomatic Carotid Plaques Are Infiltrated by a Higher Number of NK Cells

To evaluate the presence of NK cells, we performed flow cytometric analysis of cell suspensions obtained upon dissociation of CAP from both symptomatic and asymptomatic patients. NK cells isolated from CAP of 41 patients affected by atherosclerotic disease of carotid artery (19 symptomatic and 22 asymptomatic) were analyzed in comparison to NK cells from autologous PB. Since NK cells share their CD56 expression with group 3 innate lymphoid cells (ILC3) (30), this latter subset was excluded from the analyses on the basis of the absence

TABLE 1 | Clinical characteristics of atherosclerotic patients and controls.

	Symptomatic ($n = 19$)	Asymptomatic ($n = 31$)	Controls ($n = 15$)
Age	72.6y (4.4)	71.4y (5.8)	69.0y (6.7)
Women	5 (26.3%)	11 (35.4%)	5 (33%)
Stroke	15 (78.9%)	0	0
TIA	3 (15.7%)	0	0
Amaurosis fugax	1 (5.2%)	0	0
Smoker	8 (42.1%)	20 (64.5%)	7 (47%)
Hypertension	15 (78.9%)	20 (64.5%)	8 (54%)
Diabetes status	7 (36.8%)	14 (45.1%)	5 (33%)
Hyperlipidemia	16 (84.2%)	15 (48.3%)	7 (47%)
Ischemic: heart disease	11 (57.8%)	8 (25.8%)	0
Plaque stenosis			
70–80%	2 (10.5%)	3 (9.6%)	0
>80%	17 (89.4)	28 (90.3)	0

Categorical variables were reported as count (percentage). Continuous variable was reported as mean ± standard deviation.

of CD94 and perforin (15, 30, 31). NK cells were consistently detected in all the examined samples and we assessed the relative frequency of the two main NK cell subsets, namely $CD56^{bright}perforin^{low}$ and $CD56^{dim}perforin^{high}$ NK cells. Due to the possible downregulation of CD16 on NK cells extracted from solid tissues (32, 33), we distinguished $CD56^{dim}$ from $CD56^{bright}$ NK cells on the basis of perforin expression (**Figure 1A**). NK cell frequency was higher in CAP isolated from symptomatic patients whereas CAP from asymptomatic patients showed NK cell frequency similar to PB (**Figure 1B**). NK cells isolated from CAP of both symptomatic (sCAP) and asymptomatic (aCAP) patients displayed a higher frequency of $CD56^{bright}perforin^{low}$ NK cells when compared to autologous PB, and noteworthy, sCAP showed the highest frequency of $CD56^{bright}$ $perforin^{low}$ NK cells (**Figure 1C**). As previously reported (16), no significant difference in the frequency of NK cell subsets was observed between PB of atherosclerotic patients and controls. In line with their constitutive low expression of intracellular perforin, $CD56^{bright}$ NK cells also presented low or undetectable surface expression of CD16 and CD57, while they were typically positive for NKG2A (**Figure 2**). Both NKG2D and NKp30 activating receptors were homogenously expressed in all the samples analyzed. Within the $CD56^{dim}$ subset a consistent fraction of NK cells expressed NKG2C (**Figure 2A** and **Figure S1A**), while markers of lymphocyte residency, such as CD103 (also known as αE integrin), CD49a (also known as α1 integrin), and CD69 (14), appeared confined to the $CD56^{bright}$ NK cell subset (**Figure 2B**), suggesting a preferential migration of $CD56^{bright}$ NK cells into CAP. However, no significant differences were observed for these molecules between symptomatic and asymptomatic patients (**Figure S1B**).

Carotid Plaque Tissues Express Ligands for NK Cell Activating Receptors

Having observed the presence of distinct populations of NK cells in CAP, a main question remained whether ligands for their activating receptors were expressed by CAP tissues. In agreement with previous reports (19), we found CAP tissues consistently expressed significant levels of mRNA for MICA, a main ligand for NKG2D receptor. In addition, we found that B7-H6, a main cellular ligand for NKp30 receptor (34), was also expressed in carotid plaques (**Figure 3A**). MICB and ULBPs mRNA were detectable only in a fraction of carotid plaques analyzed, and, among ULBP molecules, ULBP-3 was the most frequently observed (**Figure 3A** and **Figure S2**).

Thus, considering these results, we processed CAP tissues and analyzed by flow cytometry, on the isolated cells, the protein expression of the most represented ligands, revealing that MICA/B (6 pts out of 10), ULBP-3 (4 pts out of 10), and B7-H6 (6 pts out of 10) were detectable in a $CD45^{+}HLA\text{-}DR^{+}CD11c^{+}lin$ $(CD3, CD19, CD94)^{neg}$ cell population resident within CAP (**Figure 3B**).

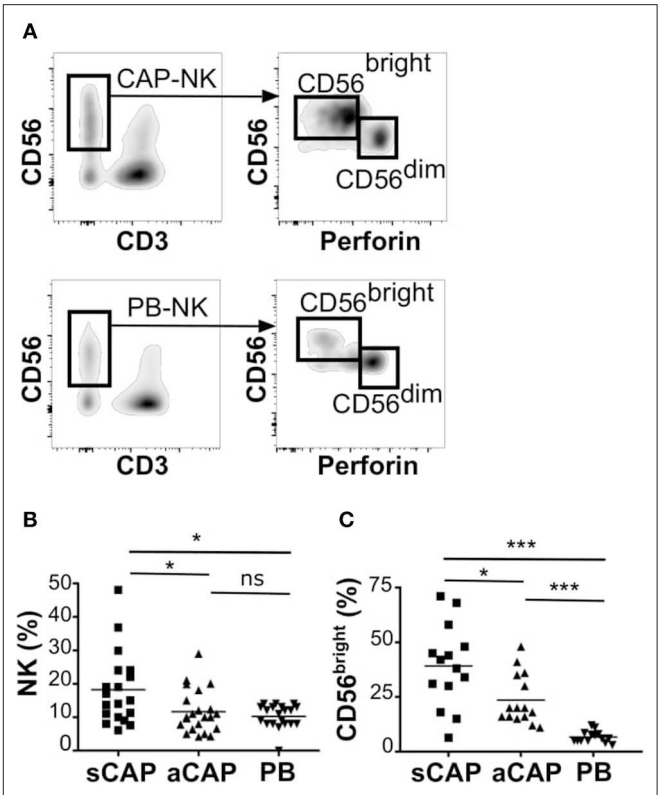

FIGURE 1 | Characterization of NK cells from carotid atherosclerotic plaques (CAP-NK). After processing carotid plaques, atheroma-resident mononuclear cells were stained with relevant mAbs and analyzed by flow cytometry gating on $CD45^{+}$ lymphocytes. **(A)** Comparative analysis of total NK cells and of NK cell subsets (i.e., $CD56^{bright}perforin^{low}$ and $CD56^{dim}perforin^{high}$ cells) from carotid atherosclerotic plaques (CAP-NK) and autologous PB (PB-NK). **(B)** Percentage of total NK cells present in carotid plaques gating on $CD45^{+}$ lymphocytes from symptomatic (sCAP-NK) and asymptomatic (aCAP-NK) patients. **(C)** Percentage of $CD56^{bright}$ NK cells present in carotid plaques gating on total NK cells. In **(B)** and in **(C)** NK cells from autologous peripheral blood (PB-NK) were also analyzed as control. Kruskal-Wallis test was used to determine statistical significance for group comparison. n.s., non-significant; $^{*}p < 0.05$ and $^{***}p < 0.001$.

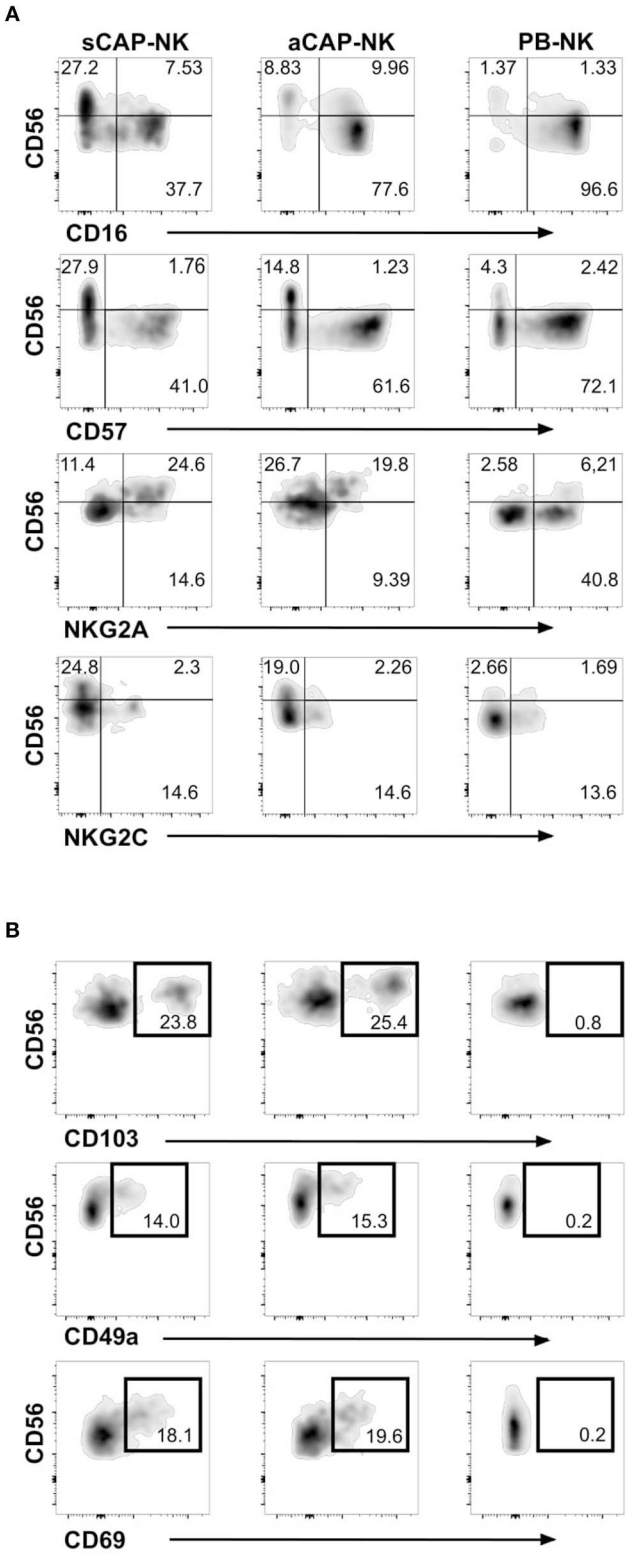

FIGURE 2 | Expression of NK cell receptors and tissue resident markers on NK cells from carotid atherosclerotic plaques (CAP-NK). **(A)** Expression of different NK cell surface molecules was analyzed on carotid plaque NK cells from symptomatic patients (sCAP-NK), asymptomatic patients (aCAP-NK), and on peripheral blood NK cells (PB-NK) of the same patients by flow cytometry. Data shown are representative of at least five carotid plaques showing similar results. No main differences were observed in PB of symptomatic and asymptomatic patients. **(B)** Expression of tissue resident markers (i.e., CD103, CD49a, and CD69) was analyzed on NK cells from sCAP-NK, aCAP-NK, and PB-NK. Data shown are representative of at least five carotid plaques showing similar results.

It is worth to note that although mRNA expression of these ligands was detectable in the majority of the samples, protein expression was detected in a smaller fraction, which might reflect technical impediments associated to CAP tissue processing.

Sera of Symptomatic CAP Patients Contain Higher Levels of Soluble Ligands for Activating NK Cell Receptors

Metalloproteases (MPPs) have been reported to play a role in inducing AP instability (35, 36). Because these enzymes can also cause the shedding of cellular ligands for NK activating receptors (37–39), we investigated whether symptomatic CAP could be associated with the release of soluble forms of NK activating receptor ligands detected in CAP.

Soluble forms of the ligands were consistently increased in the serum of atherosclerotic patients and sera levels of both sMICA and sULBP-3 were significantly higher in symptomatic patients (fold of increase CAP/Controls: 10 and 5.7 for sMICA and sULBP-3, respectively) (**Figure 4**). In addition, symptomatic patients displayed significant levels of sB7-H6, which were undetectable in both asymptomatic patients and controls (**Figure 4**).

IFN-γ Production by CAP-NK Is Associated With Serum Levels of Soluble MICA

MMPs have been reported to be induced by IFN-γ (40), a major cytokine produced by activated NK cells. Having observed that CAP as well as sera of atherosclerotic patients contained the ligands for NK activating receptors, we investigated whether IFN-γ production by CAP-NK cells might be associated with either plaque stability or release of soluble NK activating ligands. We observed that NK cells isolated from sCAP were more prone to produce IFN-γ (**Figure 5A**). Remarkably, the frequency of IFN-γ^+ NK cells in CAP positively correlated with serum levels of sMICA (**Figure 5B**), although no significant correlation was observed for other soluble ligands.

DISCUSSION

Although previous studies demonstrated a functional role of NK cells in different experimental models of atherosclerosis, recent data derived from hypercolesterolemic mice failed to find a definite role for NK cells in atherosclerosis development. Nevertheless, they also demonstrated that NK cells might exacerbate atherosclerosis in case of activation induced by viral

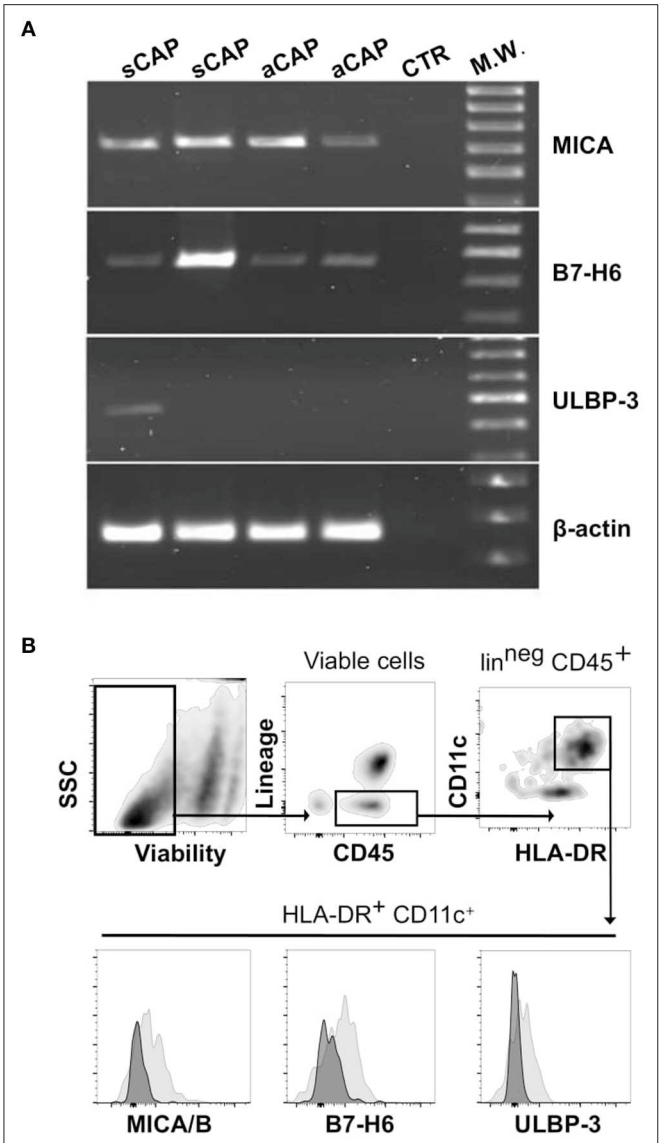

FIGURE 3 | Expression of cellular ligands for NK cell activating receptors within carotid atherosclerotic plaques (CAP). **(A)** mRNA expression of MICA, B7-H6, and ULBP-3 was analyzed in CAP-specimens by PCR. Primers specific for β-actin were utilized as positive control. Data shown are from atheroma specimens obtained from four different patients, two symptomatic (sCAP) and two asymptomatic (aCAP), and are representative of results obtained with 10 different patients. **(B)** After processing CAP to single cell suspension, mononuclear cells were analyzed by flow cytometry for the expression of the main NK activating receptor ligands, which were detected on a $CD45^+HLA\text{-}DR^+CD11c^+lin\ (CD3, CD19, CD94)^{neg}$ cell population (gray histograms). Negative controls included isotype-matched irrelevant mAbs (dark histograms).

infections (17), an event frequently occurring throughout the whole life course. In agreement with this previous report, chronic viral infections by CMV, which strongly activate NK cells, have been shown to contribute to the inflammatory process leading to the formation of AP (41–43) as well as to increase the risk of vascular complications. Thus, it is conceivable that activation of NK cells in these inflammatory contexts might contribute to exacerbate atherosclerosis.

Because most of the current knowledge on human NK cells in AP has been derived from investigations of atherosclerotic lesions by immunohistochemistry, information on phenotypic and functional features of NK cells involved in this pathology was so far limited. Given the substantial functional differences between the two main human circulating NK cell subsets, the lack of information regarding the relative distribution of $CD56^{bright}$ and $CD56^{dim}$ NK cells has so far represented a major limitation for a comprehensive understanding of the functional role of NK cells within the immune network ruling CAP development. In this study, we identified NK cells in CAP by multiparametric flow cytometric analysis, also corroborating previous results obtained by IHC (1, 44, 45).

The analysis of NK cell subsets revealed that CAP were enriched in $CD56^{bright}$ $perforin^{low/neg}$ NK cells as compared with autologous PB. The frequency of $CD56^{bright}$ NK cells was even higher in plaques of symptomatic patients compared to that of asymptomatic ones, suggesting their preferential accumulation in the microenvironment of unstable plaques or, on the other hand, their potential contribution in plaque destabilization. This finding is in line with the distribution of chemokine receptor ligands leading to NK cell migration in AP, as it has been recently demonstrated that CCL19 and CCL21, the major chemokines dictating CCR7-dependent migration of $CD56^{bright}$ NK cells, are upregulated in atherosclerotic carotid plaques of symptomatic patients as compared to asymptomatic ones (46, 47). Remarkably, we observed that $CD56^{bright}$ CAP-NK cells selectively express variable levels of tissue resident markers, i.e., CD103, CD49a, CD69, which are absent in the $CD56^{dim}$subset as well as in PB-NK cells, suggesting that $CD56^{bright}$ NK cells might preferentially be recruited to the plaque and then up-regulate markers of tissue residency under the influence of local inflammation. Our current observations are also in agreement with former studies showing that $CD56^{bright}$ NK cells, a cell subset particularly prone to cytokine production, often represent the main NK cell population in other inflamed tissues (48).

In line with previous reports showing the expression of MICA/B in atherosclerotic lesion (19) and of B7-H6 under inflammatory conditions (21), we observed that MICA/B, B7-H6, and ULBP-3 were expressed in CAP by large/scattered cells of myeloid origin compatible with a macrophagic/dendritic cell phenotype. It is likely that foam cells, derived from monocytes recruited upon inflammatory conditions within the intima of atherosclerotic lesions, might express ligands for activating NK receptors, since this event has been shown to occur, at least for some NK activating receptor ligands, in the presence of oxidized low density lipoproteins (20, 21). However, we could not exclude that other cell subsets residing within carotid plaques or endothelial cells might also express these ligands in the inflammatory microenvironment of the vessel. Further analyses are needed to explore in deeper details AP cellular components that might represent cell targets for NK cells.

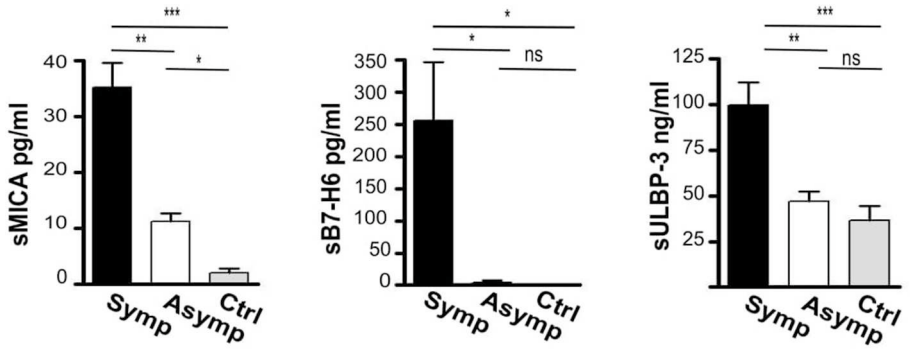

FIGURE 4 | Assessment of soluble ligands for NK cell activating receptors in the sera of patients affected by carotid atherosclerotic plaques. Soluble ligands for NK activating receptors (sMICA, sB7-H6, sULBP-3) were assessed in the serum of symptomatic (Symp, black bars), of asymptomatic (Asymp, white bars) patients and of controls (Ctrl, gray bars) by ELISA; Bars represent mean values ± SD from 10 symptomatic, 19 asymptomatic patients and 10 control individuals. Kruskal-Wallis text was the used to determine statistical significance for group comparison. n.s., non-significant; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

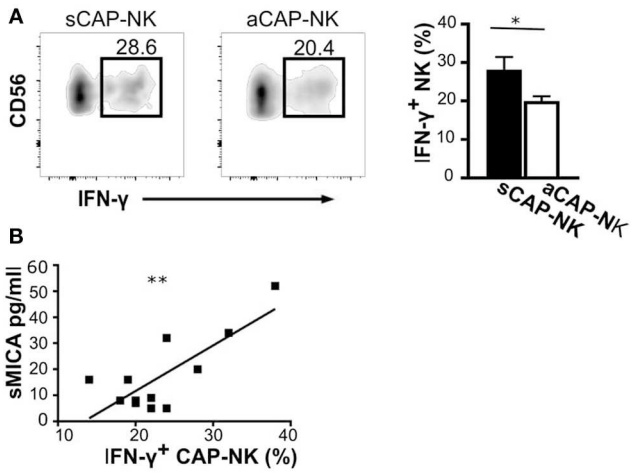

FIGURE 5 | IFN-γ production by NK cells isolated from carotid plaques (CAP-NK). **(A)** IFN-γ production was assessed in NK cells from carotid plaques of symptomatic (sCAP-NK) and asymptomatic (aCAP-NK) patients after 4 h of stimulation with PMA and ionomycin. Dot plots shown are representative of 12 patients (five symptomatic and seven asymptomatic). Kruskal-Wallis test was used to determine statistical significance for group comparison. Bars represent mean value ± SD of the frequency of IFN-γ^+ NK cells in carotid plaques from symptomatic (white bar) and asymptomatic patients (black bar). **(B)** The Pearson correlation coefficient was calculated between serum levels of soluble MICA and IFN-γ^+ NK cells isolated from CAP. $*p < 0.05$ and $**p < 0.01$.

Overall, our observations might suggest the existence, within the atherosclerotic lesions, of an inflammatory pathway sustained by NK cells recognizing their activating ligands on macrophage-like cells or other cells expressing the ligands in CAP tissue, with a consequent release of IFN-γ able to boost the inflammatory process. This assumption is also supported by previous reports describing in AP direct contacts between CD56$^+$ cells, assumed to be *bona fide* NK cells, and macrophages (1). Thus, because CAP-NK cells constitutively express both NKp30 and NKG2D at levels similar to PB-NK (**Figure S3**), they can be actively triggered by target cells expressing ligands for these receptors within CAP.

Remarkably, NK cells in symptomatic patients were stronger IFN-γ producers. Given that IFN-γ has been shown to behave as a pro-atherogenic cytokine, also causing plaque destabilization through either the induction of smooth muscle cell apoptosis or the release of MMPs (40), it might be envisaged that CAP-resident NK cells might contribute to both plaque formation and destabilization by massively secreting this cytokine. IFN-γ produced by CAP-NK cells, by inducing MMP production, might also contribute to determine ligand shedding. This hypothesis is further sustained by the observation that the frequency of IFN-γ^+ NK cells correlated with serum levels of sMICA.

In conclusions, our data indicate that CD56^{bright} NK cells might play a role in the progression of the atherosclerotic process within carotid plaques by exerting a pro-inflammatory effect upon recognition of cell targets within CAP. The evidence that soluble forms of NK activating receptor ligands are both increased in symptomatic patients and associated with the amount of CAP IFN-γ^+ NK cells, also favors the hypothesis that NK cell-derived IFN-γ might contribute to the production, within AP, of MMPs able, on the one hand, to shed these soluble forms (38, 39) and, on the other, to critically affect CAP stability (35, 36) and hence its clinical consequences.

DATA AVAILABILITY

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

ETHICS STATEMENT

This study was approved by local Ethical Committee (Comitato Etico di Messina prot. 59/18).

AUTHOR CONTRIBUTIONS

IB planned and performed most experiments, analyzed data, and wrote the manuscript. DS, CB, NP, and FB

provided critical biological samples and patient data. CC established new methods, performed experiment, analyzed the data, and revised the manuscript. CD and DO collected human samples and performed experiments. RC provided unique reagents and advised on their use. DS, PC, and FB supervised the project, analyzed data, and revised the manuscript. GF planned experiments, supervised the project, analyzed data, and wrote the manuscript.

FUNDING

Research in our lab is supported by Italian Ministry of Health (RF-2018-12367242).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01503/full#supplementary-material

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

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ORIGINAL RESEARCH
published: 21 August 2019
doi: 10.3389/fimmu.2019.01963

Different Features of Tumor-Associated NK Cells in Patients With Low-Grade or High-Grade Peritoneal Carcinomatosis

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Edited by:
Miguel López-Botet, Mar Institute of Medical Research (IMIM), Spain

Reviewed by:
Jacques Zimmer, Luxembourg Institute of Health (LIH), Luxembourg
Hun Sik Kim, University of Ulsan, South Korea

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *13 May 2019*
Accepted: *05 August 2019*
Published: *21 August 2019*

Citation:
Pesce S, Belgrano V, Greppi M, Carlomagno S, Squillario M, Barla A, Della Chiesa M, Di Domenico S, Mavilio D, Moretta L, Candiani S, Sivori S, De Cian F and Marcenaro E (2019) Different Features of Tumor-Associated NK Cells in Patients With Low-Grade or High-Grade Peritoneal Carcinomatosis. Front. Immunol. 10:1963. doi: 10.3389/fimmu.2019.01963

Peritoneal carcinomatosis (PC) is a rare disease defined as diffused implantation of neoplastic cells in the peritoneal cavity. This clinical picture occurs during the evolution of peritoneal tumors, and it is the main cause of morbidity and mortality of patients affected by these pathologies, though cytoreductive surgery with heated intra-peritoneal chemotherapy (CRS/HIPEC) is yielding promising results. In the present study, we evaluated whether the tumor microenvironment of low-grade and high-grade PC could affect the phenotypic and functional features and thus the anti-tumor potential of NK cells. We show that while in the peritoneal fluid (PF) of low-grade PC most CD56dim NK cells show a relatively immature phenotype (NKG2A+KIR–CD57–CD16dim), in the PF of high-grade PC NK cells are, in large majority, mature (CD56dimKIR+CD57+CD16bright). Furthermore, in low-grade PC, PF-NK cells are characterized by a sharp down-regulation of some activating receptors, primarily NKp30 and DNAM-1, while, in high-grade PC, PF-NK cells display a higher expression of the PD-1 inhibitory checkpoint. The compromised phenotype observed in low-grade PC patients corresponds to a functional impairment. On the other hand, in the high-grade PC patients PF-NK cells show much more important defects that only partially reflect the compromised phenotype detected. These data suggest that the PC microenvironment may contribute to tumor escape from immune surveillance by inducing different NK cell impaired features leading to altered anti-tumor activity. Notably, after CRS/HIPEC treatment, the altered NK cell phenotype of a patient

with a low-grade disease and favorable prognosis was reverted to a normal one. Our present data offer a clue for the development of new immunotherapeutic strategies capable of restoring the NK-mediated anti-tumor responses in association with the CRS/HIPEC treatment to increase the effectiveness of the current therapy.

Keywords: human NK cells, peritoneal carcinomatosis, pseudomyxoma peritonei, NK cell receptors, immune escape, immune checkpoint, PD-1/PD-L, NKp30

INTRODUCTION

Peritoneal carcinomatosis (PC) is a histologically heterogeneous, progressive malignant extremely rare disease. It is characterized by the accumulation of tumor tissue in the peritoneal cavity that ranges in biologic behavior from benign to highly malignant (1–4).

Low-grade peritoneal diseases include well-differentiated papillary mesothelioma, a rare subtype of epithelioid mesothelioma, arising from the mesothelial layers of the peritoneum and pseudomyxoma peritonei, a mucinous adenocarcinoma of the appendix spread to the peritoneal cavity which is characterized by recurrent voluminous mucinous ascites (4). These tumors grow along the peritoneal surfaces (feature that makes them susceptible to surgical debulking) (1). Although frequently characterized by an indolent course, they are not a harmless condition, and are fatal if untreated. A second category of peritoneal diseases includes peritoneal mesothelioma, an aggressive malignancy arising from mesothelial cells within the serosal lining of the peritoneum, and peritoneal mucinous carcinomatosis, an invasive, high-grade, poorly differentiated carcinoma with large extracellular pools of mucin. This second type originates from mucinous carcinomas of the gastrointestinal tract (5), gallbladder (6), pancreas, or ovary (7), thus representing the metastatic spread of a primary cancer resulting in peritoneal carcinomatosis. Similar to high-grade peritoneal mesotheliomas, it is always associated with poor patients survival.

In view of the poor efficacy of classical therapies, alternative treatments have increasingly been applied, including the cytoreductive surgery (CRS) associated with intraoperative intraperitoneal chemohyperthermia (HIPEC) (1, 8, 9). In general, in low-grade histologic variants this treatment gives excellent long-lasting results, while responses in high-grade peritoneal mucinous carcinomatosis are limited. Currently, there is no marker that allows to predict which patients may benefit from these aggressive treatments. Moreover, the cellular and molecular mechanisms responsible for the proliferative potential and the resistance to therapy by peritoneal carcinomatosis tumor cells have not been elucidated. In this context, however, it became clear that immunological alterations occurring in the tumor microenvironment seem to favor tumor growth and implantation, thus resulting in a poorer prognosis. Indeed, PC tumor cells can develop different mechanisms of immune escape capable of suppressing the activity of the immune system, including induction of PD-1 expression on T cells and release of pro-tumoral/immunosuppressive cytokines (such as IL6, IL10, and TGF-beta1) that may either regulate tumor growth or modify the anti-tumor immune responses (10–12).

Natural Killer (NK) cells play a fundamental role in the immune response against tumor cells (13, 14). For this reason, immunotherapies exploiting anti-tumor NK cell activity are the most promising in the treatment of so far incurable tumors (15–20). NK cell functions (21), including potent anti-tumor cytolytic activity, cytokine production, and cross-talk with different immune cells (22), are controlled by a balance of several inhibitory and activating receptors (13, 23). In humans, the inhibitory receptors include HLA-I-specific and non-HLA-I specific receptors. The HLA-I-specific inhibitory receptors are represented by the well-known killer immunoglobulin-like receptors (KIR) that recognize the polymorphic HLA-A, -B, and -C molecules, the immunoglobulin-like receptor 1 (LIR-1/ILT-2) that is specific for different HLA-class I molecules, and the CD94/NKG2A heterodimer that recognizes HLA-E, a non-polymorphic non-classical HLA molecule (24–26). The non-HLA-I-specific inhibitory receptors include the immune checkpoint PD-1 that was originally described on T cells (27), but recently shown also on a subset of peripheral blood (PB) NK cells from healthy HCMV+ individuals and in tumor patients (28–30). The PD-1+ NK cell subset is mainly composed of fully mature cells, expressing the CD56dimKIR+LIR−1+NKG2A–CD57+CD16bright surface phenotype (29). The main non-HLA-I-specific activating NK receptors are NKp46, NKp30, NKp44 (called "natural cytotoxicity receptors," NCR), NKG2D, and DNAM-1 (31, 32). Many of the activating NK receptor ligands have been identified and shown to be variably expressed by tumors (14).

Given the importance of NK cells in anti-tumor responses, it is not surprising that tumors use a wide range of mechanisms to avoid recognition by NK cells (33). Many mechanisms have been described, such as expression of tumor ligands for inhibitory receptors, induction/up-regulation of inhibitory receptors expression by NK cells, release of soluble ligands for activating NK receptors, downregulation of activating receptors on NK cells (19, 28, 34–36), and release of pro-tumoral/immunosuppressive soluble factors. These mechanisms lead to a suppression of anti-tumor NK cell activity and to an uncontrolled tumor growth.

In the present study, we analyzed PB and peritoneal fluid (PF)/ascites derived NK cells in patients with low-grade or high-grade PC and the ability of the PC tumor microenvironment to shape the NK cell compartment. We show that both low-grade and high-grade PC patients show an impaired NK cell phenotype, but with defects of different nature (e.g., downregulation of the

main activating NK cell receptors vs. up-regulation/induction of inhibitory receptors such as PD-1). In addition, we observed different functional impairments in low-grade and high-grade PC patients. This analysis indicates that, based on the tumor grade, PC microenvironment may differently contribute to the tumor escape from immune surveillance.

Notably, after CRS/HIPEC treatment, the altered NK cell phenotype of a patient with a low-grade disease and favorable prognosis was reverted to a normal one (37).

Although in this study we examined a small group of patients, especially because of the rarity of this disease, our observations represent an important basis to carry on further investigations and to better understand the mechanisms underlying the development of PC and the possible intervention increasing the effectiveness of the CRS/HIPEC treatment by restoring also the NK-mediated anti-tumor responses.

METHODS

Patients and Samples

This study included 8 patients with peritoneal carcinomatosis (PC) who had tumor surgery on PC between 2015 and 2018:

- 2 patients with well-differentiated papillary mesothelioma of the peritoneum (Pt. 6, Pt. 7)
- 3 patients with low-grade pseudomyxoma peritonei (Pt. 1, Pt. 8, Pt. 9)
- 2 patients with mucinous peritoneal carcinoma arising from epithelial ovarian cancer (Pt. 2, Pt. 3)
- 1 patient with mucinous PC arising from colorectal cancer (Pt. 4)

Samples of PB and PF/ascites from all the patients were collected before CRS/HIPEC treatment (time 0) and, when possible, at different time points after treatment (time 1: 48 h, time 2: 7 days) and analyzed without further processing or culturing with the exception of primary cell lines and functional experiments (see below).

Mononuclear cells from heparinized PB and from PF were obtained by density gradient centrifugation over Ficoll (Sigma, St. Louis, MO), and then resuspended in RPMI 1640 medium, supplemented with 2 mM glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated FCS (Fetal Calf Serum, Biochrom Ltd). Buffy-coats (healthy controls) were collected from volunteer blood donors admitted at the blood transfusion center of IRCCS Ospedale Policlinico San Martino, Genova, Italy.

In some cases, we obtained primary cell lines by culturing PF free cells for 2 weeks in the presence of PF derived from the matched patients.

Ethical Statements

This study was carried out in accordance with the recommendations of the ethical standards of the institutional and/or national research committee. The protocol was approved by the ethics committee of the Liguria Region, Genova, Italy (no. 428Reg2014 for PC patients and no. 39/2012 for healthy donors). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Monoclonal Antibodies

The following mAbs generated in the Laboratory of Molecular Immunology, DIMES, University of Genoa were used in this study:

A6/136 (IgM, anti-HLA-1); L14 (IgG2A, anti-Nectin-2); 5A10 (IgG1, anti-PVR); 11PB6 (IgG1, anti- KIR2DL1/S1); GL183 (IgG1, anti-KIR2DL2/L3/S2); AZ158 (IGg2A, anti-KIR3DL1/S1/L2); C227 (IgG1, anti-CD69); BAB281 (IgG1, anti-NKp46); AZ20 (IgG1, anti-NKp30); QA79 (IgG1, anti-p75); F278 (IgG1 anti-LIR1); ECM217 (IgG2b, anti-NKG2D); GN18 (IgG3, anti-DNAM-1); PP97 (IgM, anti-CD45).

The following purchased mAbs were used in this study: anti-ULBP1 (clone M295), anti-ULBP2 (clone M310), anti-ULBP3 (clone M550) (Amgen, Seattle, WA); anti-CXCR4-APC (R&D Systems, MN, USA); anti-ESA, IgG1 (Novocastra Laboratories Ltd.); anti-CD90, IgG1 (BD Biosciences, Pharmingen, CA, USA); anti-CD16-PerCP5.5 (clone 3G8) (BD Biosciences, Pharmingen, CA, USA); anti-CD107-PE (clone H4A3) (BD Biosciences, Pharmingen, CA, USA); anti-NKG2A-APC (clone Z199) (Beckman Coulter/Immunotech, Marseille, France); anti-PD-1 PE (clone 1.3.1.3) (Miltenyi Biotec, Bergisch Gladbach, Germany); anti-CD57-Vioblue (Miltenyi Biotec, Bergisch Gladbach, Germany); anti-B7-H6 (clone MAB7144) (R&D Systems, MN, USA); anti-PD-L1 (clone 27A2) (MBL, Woburn, MA); anti-PD-L2 (clone 176611) (R&D Systems, MN, USA); anti-CK7 (clone OV-TL 12/30) (Abcam, Cambridge, Regno Unito); anti-MUC2 (clone Ccp58) (Abcam, Cambridge, Regno Unito); anti-CD56-PC7 (clone c218) (Beckman Coulter/Immunotech, Marseille, France); anti-CD3-Viogreen (BW264/56 clone), anti-CD19-VioGreen (LT20 clone), anti-CD14-Viogreen (TÜK4 clone) (Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow Cytometer Analysis

Analyses were performed using a FACSCalibur or a FACSVerse flow cytometer (BD) and data were analyzed using the CellQuestPro software or the FacsSuite software, respectively (Becton Dickinson, Mountain View, CA). We also used FlowJo v10 for visualization of the unbiased t-distributed stochastic neighbor-embedding (t-SNE) algorithm. Analysis of NK cells was made on CD56dimCD3-CD19-CD16+/– gated cells. For KIR analysis, we used a pool of anti-KIR2DL1/S1, anti-KIR2DL2/L3/S2, and anti-KIR3DL1/S1/L2 mAbs. For analysis of CD45neg primary cell lines derived from PF cells, we analyzed the surface expression of different markers (including CD90, ESA, CK7, MUC2) and a series of ligands for NK cell receptors (including HLA-I, PVR, Nectin-2, B7-H6, ULBPs, PD-L1, and PD-L2 molecules) (38). In degranulation experiments, performed with frozen cells, the 7AAD (BD Biosciences, Pharmingen, CA, USA) nucleic acid dye was used for the exclusion of non-viable cells in flow cytofluorimetric assays.

Cytokine Production

To analyze the presence of a panel of different soluble factors, we used two different kits (Bio-Plex Pro Human Cytokine 48-plex Assay, Bio-Plex Pro Human TGF-beta1 and TGF-beta2 Assay from Bio-Rad) (Hercules, CA, USA) and all assays were

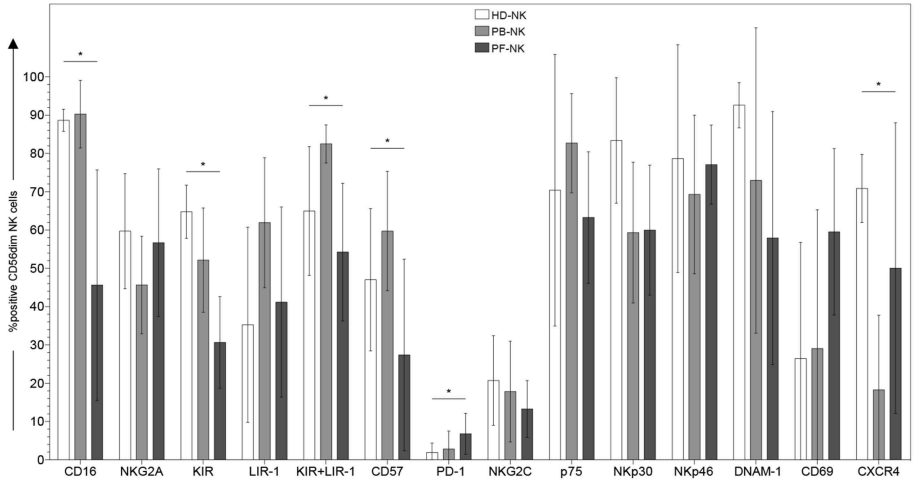

FIGURE 1 | Surface phenotype of CD56dim NK cells derived from peripheral blood or peritoneal fluid of patients affected by PC. Cytofluorimetric analysis of the expression of different cell surface markers on CD56dim-gated NK cells from peripheral blood of healthy donors (HD-NK) (□ bars), peripheral blood of PC patients (PB-NK) (■ bars) ($n = 8$: Pt. 1, Pt. 2, Pt. 3, Pt. 4, Pt. 6, Pt. 7, Pt. 8, Pt. 9) and peritoneal fluid of PC patients (PF-NK) (■ bars) ($n = 6$: Pt. 1, Pt. 2, Pt. 3, Pt. 4, Pt. 6, Pt. 8). Histograms indicate the percent ±SD of HD-/PB-/PF-NK cells positive for the indicated receptors. In order to compare the expression of the three groups simultaneously (i.e., HD-NK, PB-NK, and PF-NK), we computed the Krustall rank sum tests for each cell surface marker analyzed. *$P < 0.05$.

performed by Bioclarma s.r.l. (Torino, Italy) according to Biorad kit procedures. Soluble B7-H6 (sB7-H6) was measured in cell supernatants by enzyme-linked immunosorbent assay (Human B7-H6 DuoSet ELISA, R&D Systems, MN, USA).

Cell Line and Degranulation Assays

The target cell line used in this study was the human erythroleukemia K562 cell line.

For degranulation assay, PB and PF lymphocytes were cultured O.N. in the presence of sub-doses of IL15 (0.5 ng/ml) and then co-incubated with K562 target cells at an E/T ratio of 1:4 in a final volume of 200 μl in round-bottomed 96-well plates at 37°C and 5% CO_2 for 3 h in culture medium supplemented with anti-CD107a-PE mAb. Surface staining was done by incubating the cells with anti-CD3, anti-CD56 anti-CD19, and anti-CD16 for 30 min at 4°C. The cells were washed and analyzed by flow cytometry (FACSVerse, Becton Dickinson). Analysis of NK cells was made on CD56+ CD3− CD19− gated cells.

Statistical Analysis

The unsupervised hierarchical clustering was performed by using the online tool MORPHEUS (https://software.broadinstitute.org/morpheus/).

In order to compare the expression of the three groups simultaneously (i.e., HD-NK, PB-NK, and PF-NK), we computed the Krustall rank sum tests for each cell surface marker analyzed.

These analyses were performed using R software (**Figures 1**, **2**).

In order to compare the expressions of low-grade and high-grade PF-NK, we performed the Power Analysis for Two-group Independent sample *t*-test, using the R package pwr. In this test "d" represents the *Cohen's d* that is an effect size used to indicate the standardized difference between two means. *Cohen's d* equal to 0.2, or to 0.5, or to 0.8 were considered a small, medium and large effect size. Each test was considered valid if the significance level was ≤ 0.05 and if the power was ≥ 0.80 (**Figure 3**).

RESULTS

Phenotypic Analysis of Peripheral Blood and Peritoneal Fluid/Ascites NK Cells From Patients Affected by Peritoneal Carcinomatosis

To analyze the impact of the PC tumor microenvironment on NK cells, we characterized fresh NK cells derived from the peripheral blood (hereafter termed as PB-NK) and from the peritoneal fluid/ascites (hereafter termed as PF-NK) of patients affected by low-grade and high-grade PC by flow cytometry for the expression of a large panel of cell surface markers. The results were compared with those obtained using NK cells derived from peripheral blood of healthy donors (hereafter termed HD-NK). CD56bright NK cell subset on PF-NK cells showed phenotypic features similar to those of classical CD56bright PB-NK cells although the proportion of this subset was higher in the PF compartment (PF average score: 39%; PB average score: 9%). On the other hand, CD56dim PB-, and PF-NK cells derived from the same patients and HD-NK cells showed some differences in the expression of both activating and inhibitory NK cell receptors. In particular, CD56dim PF-NK cells displayed lower levels of KIR and CD57 (a marker typical of terminally differentiated NK cells) and higher level of PD-1 as compared to CD56dim HD- and PB-NK cells (**Figure 1**). Interestingly, most CD56dim PF-NK cells displayed a significant down-regulation of CD16, the Fc-gamma receptor responsible for antibody-dependent cellular

cytotoxicity (ADCC), as compared to CD56dim HD- and PB-NK cells (**Figure 1**). Moreover, the CD56dim PF-NK cell subset showed a trend of increment in CD69 expression, thus indicating that activated NK cells represented a large fraction of PF-NK cells (**Figure 1**).

We also observed that the expression of CXCR4 was strongly downregulated in PB-NK cells as compared to autologous PF-NK cells as well as to HD-NK cells, suggesting a possible role of this chemokine receptor in the recruitment of CD56dim NK cells from the periphery to the tumor microenvironment. In this context, we analyzed PF and plasma from matched patients for the presence of up to 50 soluble factors using a Multiplex Assay, in order to identify mediators potentially involved in the shaping of the phenotypic/functional characteristics of PF-NK cells (**Table 1**). This analysis revealed that PF is generally enriched with the SDF-1alpha chemokine (the ligand for the CXCR4 receptor) as compared to plasma derived from the same patients. In some cases, this increase was >70%.

Phenotypic Comparison Between PB- and PF-NK Cells Derived From Low-Grade or High-Grade PC Patients

To understand if the differences between PB- and PF-NK cells were features common to all patients or were dependent on the tumor grade, we divided the patients into two groups: one characterized by a low-grade disease (which included the well-differentiated mesothelioma and the low-grade pseudomyxoma) and the other characterized by high-grade disease (which included peritoneal metastasis of primary colon or ovarian carcinomas). By comparing the PB- and PF-NK cells of low-grade PC patients, many differences highlighted in **Figure 1** were confirmed and even more evident. In particular, we observed that the low-grade PC microenvironment was enriched with more immature NK cells, characterized by lower expression of CD16, KIR, LIR-1, and CD57 and higher expression of NKG2A, as compared to autologous PB (**Figures 2A,C** and **Supplementary Figures 1, 2**). In this regard, it is important to consider that a CD16 downregulation was previously described also on PF-NK cells from ovarian cancer patients, mainly due to soluble factors, including IL-18 and TGF-beta (34, 35), present in this compartment. In addition, a distinct CD56dim NK cell subset, characterized by lower expression of CD16 and KIR and undetectable levels of CD57, was described by different research groups in different pathological conditions (39–41). Moreover, the CD56dimCD16dim NK cell subset expressed higher levels of CXCR4 (39).

In our case, the CD56dim PF-NK cell subset of low-grade patients is characterized by the same features of the subset previously described, including higher levels of CXCR4 as compared to autologous PB (**Figure 2** and **Supplementary Figures 1, 2**).

On the other hand, no significant differences were detectable between PB- and PF-NK cells from high-grade PC patients (**Figures 2B,C** and **Supplementary Figures 1, 2**).

Comparison of PF-NK Cells Derived From Low-Grade and High-Grade PC Patients

To fully understand if, in low-grade and high-grade PC microenvironment, distinct features characterize NK cells, we performed further analyses comparing PF-NK cells derived from low-grade or high-grade PC.

Interestingly, in the PF of low-grade PC patients a large fraction of CD56dim NK cells showed features of immature NK cells, characterized by the NKG2A+KIR-CD57-CD16dim phenotype, whereas the percentage of CD56dim NK cells expressing KIR (**Figures 3A,B**) and LIR-1 (**Figure 3A**) was increased in the PF of high-grade PC patients. In addition, PF of low-grade PC patients contained a substantially higher percentage of CD56bright NK cells than PF of high-grade PC patients (average score: 48 vs. 27%).

By using the t-SNE algorithm, we observed that the co-expression pattern of markers on NK cells generated specific clusters associated with selected NK cell receptors (CD16, NKG2A, and KIR), in the PF-NK cells of low-grade and high-grade PC patients (**Figure 3C**). Furthermore, this approach allowed to better visualize the different distribution of these NK cell receptors in the PF-NK cells of these two groups of patients.

Moreover, an impaired expression of some activating NK receptors, primarily NKp30 and DNAM-1, was shown on the PF-NK cells from low-grade PC patients (**Figure 3A**). Previous studies demonstrated that the defective expression and function of activating NK cell receptors, including NKp30 and/or DNAM-1, may be induced by the chronic engagement of these receptors by their ligands in soluble form or expressed on tumor cells. In this context, it is important to consider that CD45neg primary cell lines derived from PF expressed some activating NK receptor ligands including PVR and Nectin-2 (DNAM-1 ligands) (35), and B7-H6 (NKp30 ligand) (34), although, in some of the cases analyzed, this last molecule was only detectable in the cytoplasm and not at the cell surface (**Supplementary Figure 3**). In addition, PF microenvironment of low-grade PC patients contained a much higher concentration of TGF-beta1 (an increment over 80%) as compared to high-grade PC patients. Considering that TGF-beta1 is known to induce down-regulation of some activating NK cell receptors, including NKp30 (42), it is likely that the downmodulation of NKp30 observed in low-grade PC patients may be the result of the combined action of soluble B7-H6 (sB7-H6) and TGF-beta1. Further analysis indicated that a relationship between the level of NKp30 downmodulation on PF-NK cells and the concentration of TGF-beta1 and sB7-H6 in the PF could be established. More specifically, low-grade PC patients displaying an important decrease in the expression of NKp30 exhibited also a high concentration of TGF-beta1 and sB7-H6 in the PF. On the other hand, high-grade patients displaying higher levels of surface NKp30 on PF-NK were characterized by very low concentration of TGF-beta1 and sB7-H6 in the PF (**Figures 4A,B**).

Notably, TGF-beta1 could also be involved in the CD16 impairment (43, 44) detected in the PF-NK of low-grade PC patients and in the induction of NKG2A expression, as previously described in other tumor types (45, 46). Indeed, it

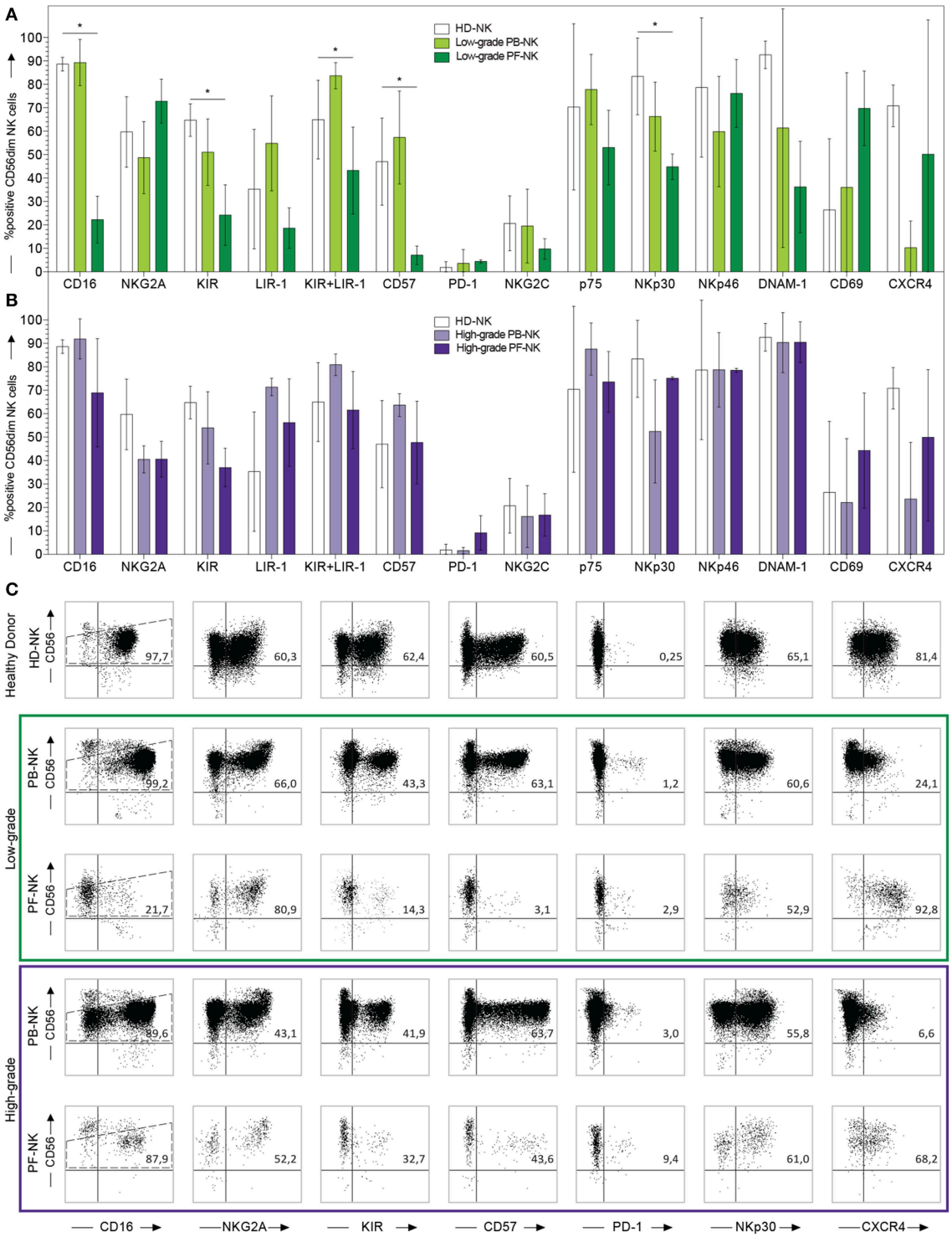

FIGURE 2 | Comparison between PB- and PF-NK cells derived from low-grade and high-grade PC patients. Cytofluorimetric analysis of the expression of a panel of cell surface markers on HD-NK (□ bars) ($n = 6$), PB-NK of low-grade PC patients (■ bars) ($n = 5$: Pt. 1, Pt. 6, Pt. 7, Pt. 8, Pt. 9) and PF-NK of low-grade PC patients (■ bars) ($n = 3$: Pt. 1, Pt. 6, Pt. 8) **(A)**. Cytofluorimetric analysis of the expression of a panel of cell surface markers on HD-NK (□ bars) ($n = 6$), PB-NK of high-grade PC patients (■ bars) ($n = 3$: Pt. 2, Pt. 3, Pt. 4) and PF-NK of high-grade PC patients (■ bars) ($n = 3$: Pt. 2, Pt. 3, Pt. 4) **(B)**. Cells are gated on CD56dim NK cells. In order to compare the expression of the three groups simultaneously (i.e., HD-NK, PB-NK, and PF-NK), we computed the Krustall rank sum tests for each cell surface marker analyzed. *$P < 0.05$. **(A,B)** Dot plots derived from a representative healthy donor (HD-NK), a representative low-grade PC patient (PB-NK/PF-NK) and a representative high-grade PC patient (PB-NK/PF-NK) are shown. Percentages of positive NK cells (gated on CD56dim subset as indicated by the dotted line) for the indicated receptors are reported in the upper right quadrant of each dot plot **(C)**.

TABLE 1 | Soluble factors detected in plasma and peritoneal fluid (PF) of low-grade and high-grade PC patients compared with soluble factors detected in healthy donors (HD) plasma.

Patient	Sample	VEGF	TRAIL	TNF-b	TNF-a	SDF-1a	SCGF-b	SCF	RANTES	PDGF-bb	MIP-1b	MIP-1a	MIG	MIF	MCP-3	MCP-1 (MCAF)	M-CSF
HD 1	Plasma	10.28	160.055	460.8	88.565	695.965	202488.01	32.35	ND	20667.39	258.245	1.96	2364.995	1895.435	0.22	11.435	30.97
HD 2	Plasma	199.075	138.935	396.9	111.785	789.445	124500.015	52.735	ND	23018.27	280.365	1.17	339.63	2471.425	0	24.26	58.335
Pt. 1	Plasma	173.225	49.87	233.2	20.44	351.98	38700.6	6.91	2152.38	175.59	131.345	0.865	476.08	25.89	0	29.38	6.29
Pt. 2	Plasma	26.26	27.05	85.2375	2.855	113.3075	22372.235	7.2675	1001.33	52.9575	59.7825	1.01	240.6425	138.35	0.11	51.6375	1.5825
Pt. 3	Plasma	0	11.875	38.81	0.94	24.585	116900.705	7.74	296.505	84.65	59.86	1.91	822.31	194.72	1.82	580.605	13.38
Pt. 4	Plasma	182.73	101.465	289.27	29.755	471.935	61759.515	16.055	2810.5	217.905	159.76	0.85	669.645	1420.815	0	114.295	5.475
Pt. 6	Plasma	0	132.93	246.78	56.61	606.365	79799.94	69.865	6180.09	304.16	144.87	3.085	4405.87	853.22	2.415	137.95	48.335
Pt. 7	Plasma	149.805	119.5	280.45	24.905	461.575	42892.27	23.43	6357.5	748.89	172.025	0.96	665.3	940.995	0.42	96.775	19.64
Pt. 8	Plasma	0	107.385	285.975	36.42	534.265	83015.745	26.33	11953.38	403.195	169.9	1.315	418.4	1305.49	0	68.695	0
Pt. 1	PF	243.795	32.4	45.635	40.81	1312.535	204341.7	51.31	309.675	94.265	60.275	6.34	1723.75	361.75	3.515	66.62	21.815
Pt. 2	PF	0	20.395	36.855	9.275	156.17	2373.91	5.37	327.12	39.58	28.25	1.235	85.095	1631.79	0	19.845	0.88
Pt. 6	PF	206.17	161.195	70.05	272.79	1939.845	180303.03	71.175	75.25	170.66	244.47	133.45	10117.56	17280.6	454.985	ND	41.475
Pt. 8	PF	131.145	25.785	39.775	57.16	1165.935	76851.885	38.05	171.555	56.12	88.06	12.32	488.88	5486.46	6.9	145.96	4.87

Patient	Sample	LIF	IP-10	IL-9	IL-8	IL-7	IL-6	IL-5	IL-4	IL-3	IL-2Ra	IL-2	IL-1ra	IL-1b	IL-1a	IL-18	IL-17
HD 1	Plasma	4.23	1918.885	421.4	233.155	51.89	2.21	0	4.74	0	118.125	0	426.365	2.12	9.32	101.44	4.71
HD 2	Plasma	3.14	2095.385	352.525	97.61	146.895	6.38	0	9.055	0	69.145	0	1751.08	6.075	8.505	100.32	14.895
Pt. 1	Plasma	13.995	400.91	223.48	4.66	12.615	0.955	0	2.265	0	43	0	0	0.645	5.435	9.695	0
Pt. 2	Plasma	5.5275	186.65	88.1125	7.0725	2.26	0.53	0	1.405	0	23.8325	0	33.89	0.635	2.4325	7.44	0
Pt. 3	Plasma	6.375	0	34.63	60.44	0	277.05	0	1.025	0	60.31	0	259.99	0.71	4.865	18.775	0
Pt. 4	Plasma	0	788.26	266.22	50.34	0.605	21.285	0	4.47	0	45.6	0	375.455	1.11	0	13.435	0
Pt. 6	Plasma	0.58	1033.445	222.565	19.42	0	19.555	7.855	2.79	0	539.595	0	127.415	0.825	8.28	98.205	1.065
Pt. 7	Plasma	0	93.41	296.04	33.58	36.3	25.775	8.875	1.635	0	51.59	0	31.2	0.8	4.865	14.865	0
Pt. 8	Plasma	110.9	440.87	262.735	14.55	9.07	0.845	0	1.205	0	81.025	0	116.585	0.075	1.035	18.46	0
Pt. 1	PF	30.495	5694.53	42.955	118.26	7.73	657.55	9.18	2.405	0	509.645	1.24	39.36	0.835	9.725	14.21	3.96
Pt. 2	PF	6.17	221.97	42.48	6.35	2.645	1.87	0	0.8	0	8.215	0	51.89	0.295	1.215	2.065	1.44
Pt. 6	PF	117.065	#DIV/0!	74.45	9170.92	15.59	5494.13	55.24	11.35	1.17	1067.33	6.665	4488.69	4.62	27.755	73.68	22.25
Pt. 8	PF	60.945	1760.75	41.615	395.75	15.505	649.12	11.445	3.63	0	252.995	0.105	166.42	0.72	3.72	16.01	6.125

Patient	Sample	IL-16	IL-15	IL-13	IL-12p40	IL-12(p70)	IL-10	IFN-g	IFN-a2	HGF	GROa	GM-CSF	G-CSF	FGF b	Eotaxin	CTACK	b-NGF
HD 1	Plasma	327.035	6.97	0	33.205	0.07	1.465	0	3.02	2074.565	526.38	0	48.84	8.225	40.745	1716.99	1.345
HD 2	Plasma	908.425	15.375	0	30.5	0	1.465	40.335	2.33	2042.815	952.65	5.325	113.035	13.66	405.53	955.815	3.4
Pt. 1	Plasma	29.68	12.625	0	2.65	0.07	0.02	0	0	238.33	299.14	0	50.745	0.825	83.525	989.48	1.345
Pt. 2	Plasma	34.69	0	0	3.0775	0	1.245	0	0	180.8	123.745	0	33.79	1.2375	31.3125	465.12	0
Pt. 3	Plasma	6.45	0	0	12.35	0.07	0	125.35	4.74	2187.025	0	0	54.435	2.475	21.865	79.455	0
Pt. 4	Plasma	55.1	0	0	0	0.88	0	8.825	0	424.265	317.125	0	95.745	2.475	72.93	1791.72	0
Pt. 6	Plasma	171.58	329.125	0	3.57	0.07	0	91.275	1.585	902.7	389.41	5.705	114.815	4.265	133.575	1452.625	5.895
Pt. 7	Plasma	81.985	194.27	0	11.07	2.985	273.685	0	0	443.885	443.59	4.31	65.075	3.645	129.18	755.355	9.065
Pt. 8	Plasma	84.785	0	0	0	0	1.465	6.32	0.955	612.41	290.065	0	59.03	0.825	134.885	924.92	0
Pt. 1	PF	219.89	3.39	0.145	60.135	0.59	22.765	78.11	3.7	3570.565	125.21	0	145.975	5.49	15.27	1370.125	5.345
Pt. 2	PF	62.965	0	0	4.51	0.02	0.54	0	1.495	148.58	54.315	0	23.845	41.905	8.92	115.165	0.175
Pt. 6	PF	867.895	48.965	3.055	93.975	1.715	28.525	441.335	22.48	2151.51	622.24	3.625	1747.37	13.415	59.71	1081.29	7.54
Pt. 8	PF	224.455	0	1.07	51.775	0.22	69.325	54.42	5.105	2120.91	195.885	0.315	273.885	15.695	58.47	1013.39	6.225

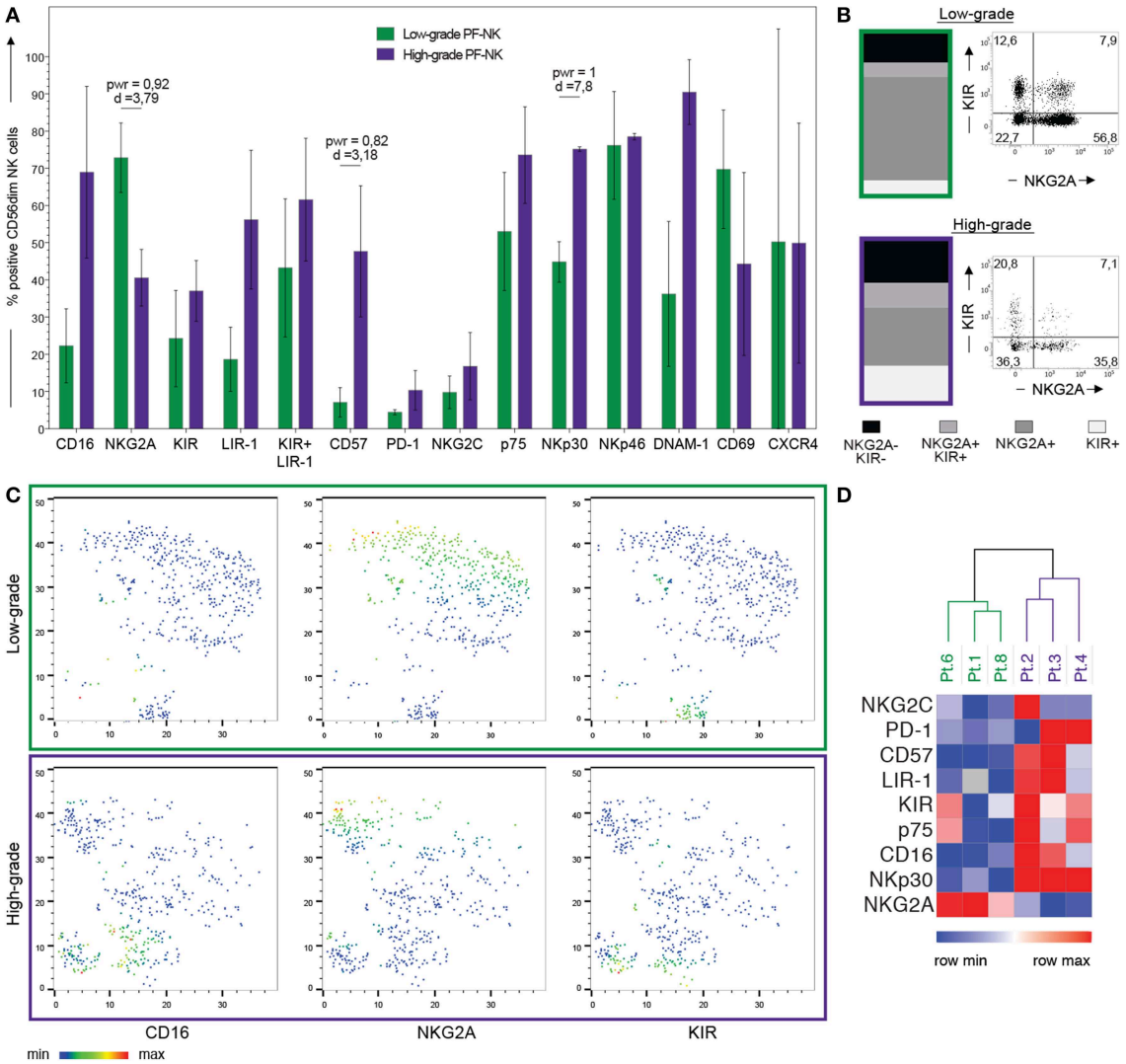

FIGURE 3 | Comparison of PF-NK cells derived from low-grade and high-grade PC patients. Cytofluorimetric analysis of the expression of a panel of cell surface markers on PF-NK derived from low-grade PC patients (low-grade PF-NK) (■ bars) (n = 3:Pt. 1, Pt. 6, Pt. 8) and PF-NK derived from high-grade PC patients (high-grade PF-NK) (■ bars) (n = 3: Pt. 2, Pt. 3, Pt. 4). The power (pwr) and Cohen's distance (d), calculated with the Power Analysis for Two-group Independent sample t-test, of the pair are indicated when significant (power ≥ 0,8) **(A)**. Distribution of KIR and NKG2A receptors on gated CD56dim NK cells of low-grade PC patients and high-grade PC patients. A representative donor is shown for each group of patients **(B)**. t-SNE clusterization of CD56dim PF-NK cells derived from a representative low-grade PC patient (Pt. 6) and a representative high-grade PC patient (Pt. 4). The color scale represents the different distribution of CD16, NKG2A, and KIR receptors evaluated through the variation of Geo-mean **(C)**. Heatmap showing the clustering of low-grade (Pt. 1, Pt. 6, Pt. 8) and high-grade (Pt. 2, Pt. 3, Pt. 4) PC patients based on the differential expression of the indicated NK cell receptors on gated CD56dim NK cells. The color scale goes from blue (low relative expression) to red (high relative expression) and it is based on the variation of each single receptor through the six patients **(D)**.

was possible to observe a relationship between the level of CD16 downmodulation on PF-NK cells and TGF-beta1 concentration in the PF (**Figure 4A**).

Thus, the impaired expression of both activating and inhibitory receptors on PF-NK cells from low-grade PC patients induced by tumor cells and/or soluble factors present in the PF microenvironment may contribute to the development of escape mechanisms from immune surveillance.

Regarding the PF-NK cells from high-grade PC patients, we observed a trend of increment in the expression of the PD-1 inhibitory checkpoint. The fact that this difference is not clearly significant may depend on the small number of PC patient samples analyzed, due to the rarity of this disease. Considering that the expression of this inhibitory receptor on NK cells can compromise their anti-tumor activity, and that PF-derived primary cell lines express both PD-L1 and PD-L2 (**Supplementary Figure 3**), the increased PD-1 expression may play a role in inducing NK cell impairment in tumor control.

To support our findings regarding the significant differences between PF-NK cells derived from low-grade and high-grade PC patients, we perform hierarchical clustering in

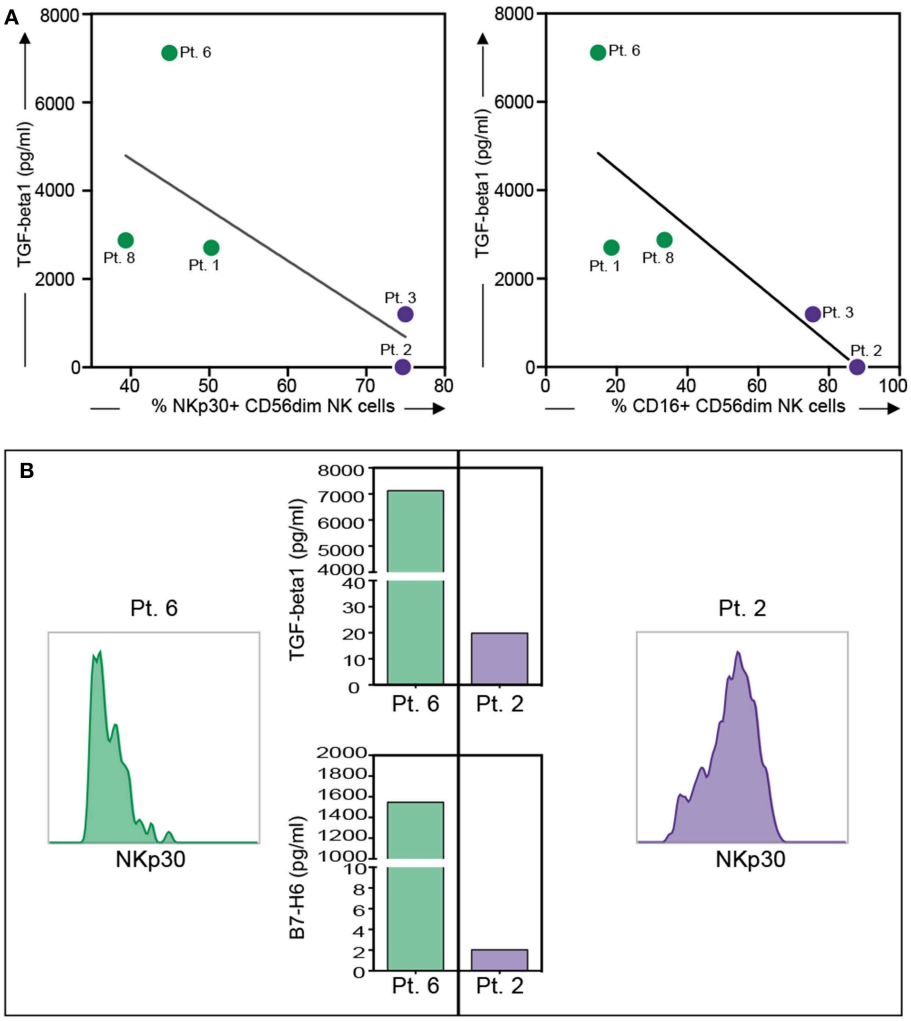

FIGURE 4 | Relationship between NKp30 or CD16 expression on PF-NK cells and soluble factor's concentration in the PF of low-grade and high-grade PC patients. Relationship between NKp30 or CD16 receptor downregulation on PF-NK cells and TGF-beta1 concentration in the PF of low-grade PC patients (● dots, $n = 3$: Pt. 1, Pt. 6, Pt. 8) or high-grade PC patients (● dots, $n = 2$: Pt. 2, Pt.3). The linear regression line is shown (NKp30 vs. TGF-beta1: $R = 0.52$ and $p = 0.17$; CD16 vs. TGF-beta1: $R = 0.68$ and $p = 0.09$) **(A)**. Histograms showing the expression of NKp30 on PF-NK cells of a representative low-grade PC patient (Pt. 6) and a representative high-grade PC patient (Pt. 2) and TGF-beta1/sB7-H6 concentration in PF of the same patients **(B)**.

order to verify the presence of particular patterns in our data. In **Figure 3D**, the two-color heatmap plots show the unsupervised hierarchical clustering of NK samples derived from PF of PC patients. This analysis clearly separates low-grade from high-grade samples, based on the different expression of a small group of classical NK cell molecules, including the activating and inhibitory receptors appointed above.

Thus, the low-grade or high-grade PC tumor microenvironment may differently contribute to the tumor escape from immune surveillance, by inducing different shaping in the tumor-associated NK cell receptors repertoire (e.g., down-regulation of activating receptors vs. up-regulation of inhibitory ones).

Functional Comparison Between PB- and PF-NK Cells Derived From Low-Grade or High-Grade PC Patients

To support our results regarding the impaired expression of some activating NK receptors (primarily NKp30) on the PF-NK cells from low-grade PC patients, degranulation assays against K562, a HLA-I negative cell line previously shown to express B7-H6, were performed in the absence or in the presence of the anti-NKp30 blocking mAb. The results were compared with those obtained using HD-NK cells.

As shown in **Figure 5**, PB-NK cells derived from a representative low-grade PC patient or a representative high-grade PC patient as well as HD-NK cells displayed ability to degranulate following exposure to K562. These functional

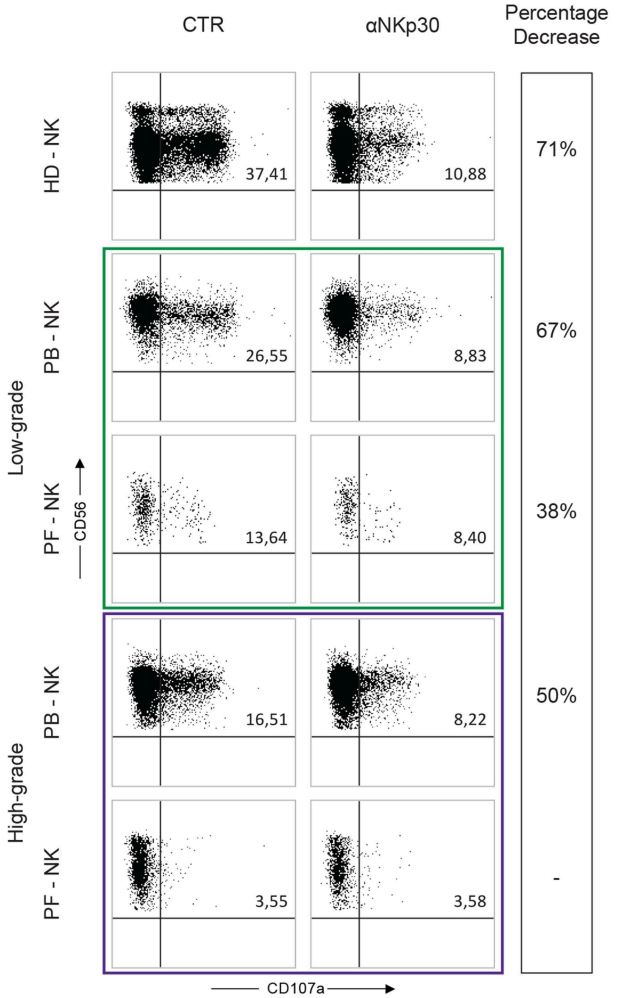

FIGURE 5 | Degranulation assay of PB- and PF-NK cells derived from low-grade and high-grade PC patients. Degranulation assay (CD107a expression) of PB- and PF-NK cells from a representative low-grade PC patient (Pt. 6), a representative high-grade PC patient (Pt. 3) and a representative HD stimulated by K562 tumor target cells, in the absence or in the presence of anti-NKp30 mAb. Percentages of CD107a+ NK cells are reported in the upper right quadrant of each dot plot.

activities were inhibited in the presence of anti-NKp30 mAb, capable of disrupting the NKp30/B7-H6 interaction.

On the contrary, under the same conditions, PF-NK cells derived from both types of PC patients were characterized by substantial differences in terms of degranulation. In particular, as expected, the degranulation of PF-NK cells derived from the low-grade PC patient (characterized by a compromised expression of NKp30) was lower than that of HD-NK and autologous PB-NK cells (expressing normal levels of NKp30). Again the degranulation was partially inhibited in the presence of anti-NKp30 mAb.

On the other hand, unexpectedly, the degranulation activity of PF-NK cells derived from the high-grade PC patient was deeply impaired, either in the absence or in the presence of anti-NKp30 (**Figure 5**), despite the normal expression of NKp30. The same result was obtained by using PD-L+ tumor cell lines (including OVCAR5), either in the absence or in the presence of anti-PD-1 mAb (not shown). These data suggest that PF-NK cells derived from the high-grade PC patient present additional impairments (other than PD-1-mediated block) still to be defined. In this context, it is important to consider that in the PF the ratio between NK cells and tumor cells is clearly unbalanced toward tumor cells, probably due to a defective migration of NK cells within this compartment. This event can decrease the chances that NK cells can efficiently meet and kill cancer cells even in *in vitro* experiments.

Impact of a CRS/HIPEC Successful Treatment on NK Cell Compartment: A Case Presentation

In order to acquire insights on the effect of the CRS/HIPEC therapy, we characterized PB- and PF-NK cells derived from Patient 1, affected by pseudomyxoma peritonei, before (time 0), and at different time points (time 1 and time 2) after therapy. At time 0, PF-NK cells, but not PB-NK cells, showed a highly compromised phenotype in terms of anti-tumor potential. In fact, PF-NK cells were characterized by a strong down-regulation of the main NK cell-activating receptors, in particular NKp46, DNAM-1, NKp30, and CD16 (**Figure 6**). In addition, differently from autologous PB, a higher fraction of PF-NK cells was NKG2A+, whereas only a minor cell subset expressed KIR. Notably, starting from time 1, the percentages and surface density of NKp46 and NKp30 were greatly increased, whereas those of DNAM-1 were only slightly increased. At time 2, the expression of all activating receptors, including CD16, was almost completely recovered and comparable to that of the patient's PB (and to a HD-NK, see **Figures 1**, **2A,C**). Furthermore, a re-balancing in the distribution of KIR and NKG2A could be observed. In fact, if at time 0 almost all PF-NK cells were NKG2A+ KIR-, at time 2 the percentages of KIR+ NK cells were increased by 60% and the percentages of NKG2A+ NK cells were decreased by 25%. This result means that the CRS/HIPEC therapy in PC tumor patients can induce phenotypic changes on NK cells, thus reverting tumor-induced NK cell suppression, although the molecular mechanisms of this effect still need to be fully clarified. Importantly, the prognosis of patient 1 was very favorable. To date 4 years after treatment, this patient is in good health, with no evidence of disease recurrence.

DISCUSSION

Peritoneal carcinomatosis (PC) is the site of histologically heterogeneous rare primitive diseases (including pseudomixoma peritonei and mesothelioma) and one of the problematic sites of metastases for abdominal malignancies, including gastrointestinal, and ovarian cancers (1–4). Its presentation is usually associated with a significantly reduced quality of life and a very poor prognosis. To date, PC remains among the most common causes of death from abdominal cancers.

Intraperitoneal chemotherapy with surgical debulking is associated with higher survival rates than systemic

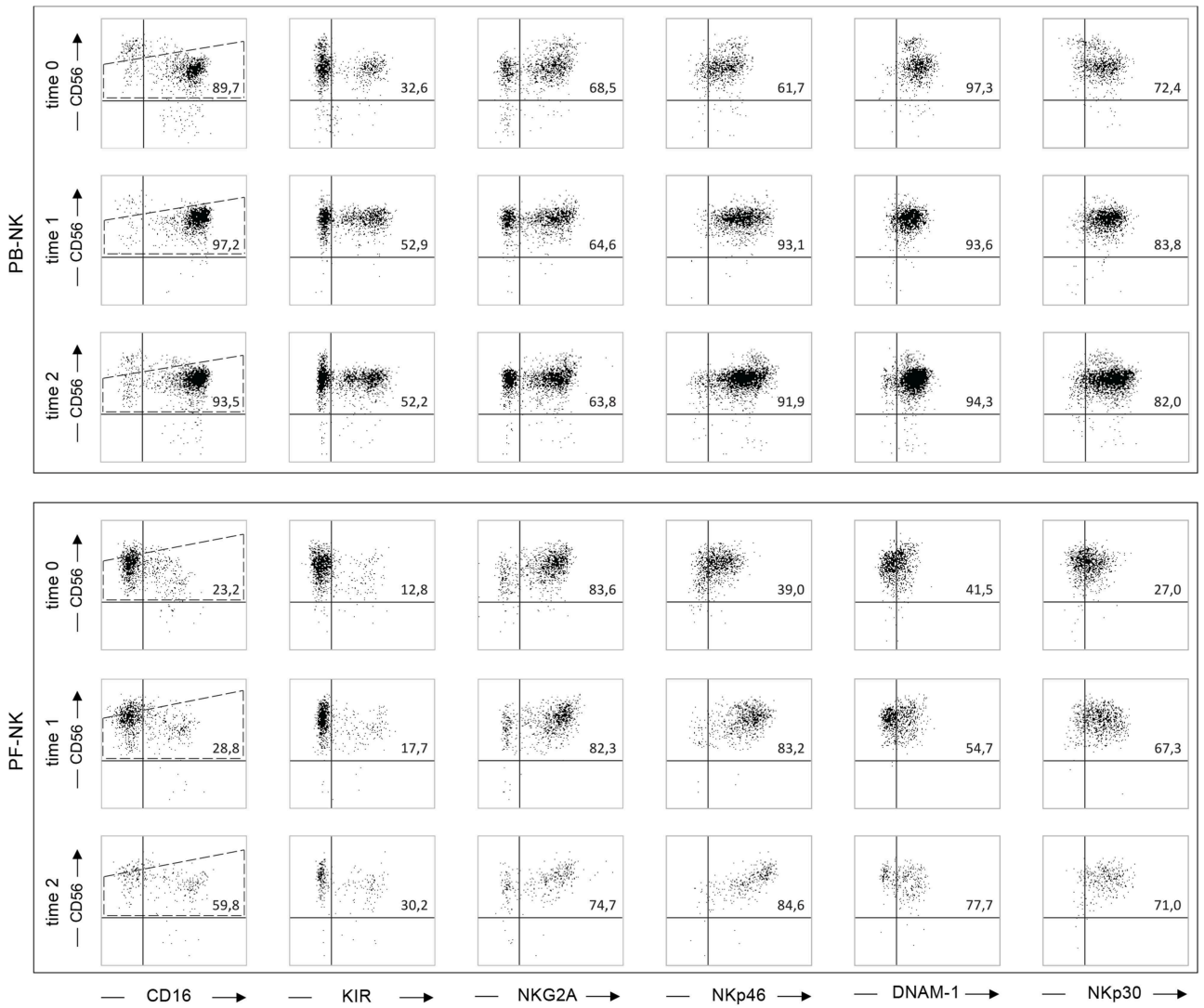

FIGURE 6 | Reversion of the altered NK cell phenotype in a low-grade PC patient after the CRS/HIPEC treatment. Dot plots illustrating the expression of different NK cell receptors on PB-NK and PF-NK derived from a low-grade PC patient (Pt. 1), before treatment (time 0), and 48 h (time 1), or 7 days (time 2) after CRS/HIPEC treatment. Percentages of NK cells (gated on CD56dim subset as indicated by the dotted line) positive for the indicated receptors are reported in the upper right quadrant of each dot plot.

chemotherapy, but results are still disappointing, and treatment is rarely curative (3). Several independent groups have reported that immune modulating agents can provide a significant therapeutic benefit in preclinical models of PC (47–50). These promising results suggest that strategies based on increasing the anti-tumor immune response within the peritoneal cavity should be pursued in PC treatment.

NK cells are a major component of the anti-tumor immune response and are involved in controlling tumor progression and metastases. However, tumors have evolved mechanisms of immunoevasion, including production of pro-tumoral/immunosuppressive cytokines, expression of ligands for inhibitory receptors, or loss of ligands for activating receptors in order to escape from the NK cell-mediated attack. Here, we show that in PC patients, tumor-associated NK cells are characterized by phenotypic and functional dysfunctions. Interestingly, the NK cell impairment is different between patients with low-grade or high-grade PC. In particular, we observed a large fraction of immature NKG2A+ CD57- NK cells displaying a strong downregulation of the main activating NK cell receptors (such as NKp30, DNAM-1, and CD16) in low-grade PC patients vs. a most mature KIR+ CD57+ NK cell fraction showing an up-regulation/induction of the immune checkpoint PD-1 in high-grade PC patients. In both cases, PF-NK cells displayed functional defects, however, while in low-grade PC patients these defects reflected the compromised phenotype, in the high-grade PC patients PF-NK cells showed much more important defects that only partially reflected the

compromised phenotype detected. This suggests that further impairments, still to be highlighted, exist at the level of this NK cell population.

Thus, based on the tumor grade, PC microenvironment may be characterized by structural and functional reorganization by inducing different tumor escape mechanisms from immune surveillance. In this context, it has been previously demonstrated that metastatic PC from primary colorectal cancer (pCRC) is characterized by modification in tumor microenvironment and immune cell reaction. These changes include senescence in PC tumor cells, and enhancement of pro-tumoral soluble factors, including VEGF-A and TGF-beta, that promote neovascularization in metastatic niche and tumor growth/invasion/metastasis formation, respectively. The authors also found high amount of the NK cell-regulating cytokine IL15 and a significantly increased numbers of mature (CD57+) NK cells expressing CD107a on the surface and releasing high levels of IFN-gamma and TNF (47). Another research group has recently demonstrated that recruitment of cytotoxic, IFN-gamma-secreting, NK cells is associated with reduced tumor burden, and improved survival in a colon cancer model of PC (48). All these data suggest that NK cells can play a crucial immunosurveillance role in PC. Thus, the balance between anti-tumor and pro-tumor effects can deeply influence the anti-tumor activity of these immune cells and the metastasis formation.

Understanding the mechanisms by which PC tumor microenvironment works in inducing immune cells suppression is a complex issue, also considering that these types of tumors are uncommon and histologically heterogeneous, ranging from benign to highly malignant.

By analyzing the phenotype of NK cells derived from PF of low-grade and high-grade PC patients, we found that the PF compartment of low-grade disease is enriched in most immature NKG2A+KIR-CD57-CD16dim NK cells. A NK cell subset characterized by a similar phenotype and endowed with multifunctional activity (including potent killer and IFN-gamma producing capacity) was found in the bone marrow both of healthy children and of pediatric leukemic patients and it has been suggested that this NK cell subset can represent an intermediate differentiation stage between CD56brightCD16neg/dim and CD56dimCD16bright NK cells (39). Interestingly, these immature PF-NK cells are characterized by a strong downregulation of the main activating NK cell receptors (primarily NKp30 and DNAM-1) that may be a consequence of their engagement by specific ligands expressed on tumor cell surface or present in soluble form together with immunomodulatory soluble factors in the PF. In this context, it has been previously demonstrated that chronic receptor-ligand interactions may dampen the surface expression of some activating NK receptors, thus affecting the ability of NK cells to kill tumor cells expressing ligands for those receptors. In particular, a soluble form of B7-H6 (sB7-H6), the main NKp30 ligand, was found in the PF-microenvironment of ovarian cancer patients (34). A high amount of sB7-H6 is correlated with a greater downmodulation of NKp30. These NK cells display impaired IFN-gamma production and cytolytic function, thereby showing poor NK cell-mediated elimination of B7-H6+ ovarian cancer cells (34). Moreover, the activating NK cell receptor DNAM-1 can also be downmodulated by the chronic exposure to the ligand expressed on the surface of ovarian tumor cells (35). In addition, TGF-beta can also contribute to the downmodulation of the NKp30 activating receptor (42). Consistent with these observations, we detected the expression of ligands for NKp30 (B7-H6) and DNAM-1 (PVR, Nectin-2) on PF-derived tumor cells and several soluble factors, including sB7-H6, and TGF-beta, in the PF. These factors are certainly involved in the induction of the compromised phenotype observed in the PF-NK cells of low-grade PC patients.

With disease progression (high-grade PC tumors), we found that expression of activating NK cell receptors (including NKp30, DNAM-1, and CD16) was recovered, and the expression of inhibitory receptors (such as KIRs, LIR-1, and PD-1) increased together with the maturation state of NK cells.

Thus, low-grade and high-grade PC tumors shape their environment differently in order to evade NK cell anti-tumor immunity. Along this line, PF-NK cells from both low-grade and high-grade PC patients showed decreased NK cell degranulation function. Importantly, this impairment was apparently more pronounced in NK cells from high-grade PC patients, even if, in this case, it is not clear which mechanisms lead to the lack of NK cell degranulation capacity. Indeed, this defect also occurs with respect to target cells (i.e., K562) that do not express ligands for inhibitory receptors (i.e., HLA-I and PD-Ls molecules) up-regulated on PF-NK cells, but express ligands for activating receptors (e.g., B7-H6) that are normally expressed on these cells. However, because the percentage of NK cells was severely reduced in the PF of the high-grade PC, the fact that PF-NK cells from high-grade PC tumor were unable to fulfill their degranulation function is not surprising considering their low proportion within the tumor. Further investigations will be necessary to clarify this aspect.

Thus, cancer cells and the tumor milieu can deeply alter the functional composition of anti-tumor effector cells, including cytotoxic NK cells. This consideration highlights the importance of developing future therapies able to restore NK cell cytotoxicity and limit/prevent tumor escape from anti-tumor immunity.

Remarkably, we show that in a low-grade PC patient, with no evidence of disease recurrence after 4 years after treatment, CRS/HIPEC treatment could restore the NK cells impaired phenotype, thus reverting tumor-induced NK cell suppression. In this scenario, we can hypothesize that removal of the tumor (CRS) combined with HIPEC procedure may improve survival in low-grade PC patients by reducing the tumor factors involved in immune cell suppression (37, 51).

On the other hand, since most high-grade PC remains refractory or responds only partially to the CRS/HIPEC treatment, new therapeutic approaches aimed at increasing the effectiveness of the current therapy are necessary (52, 53). For example, immunotherapeutic reagents capable of blocking inhibitory receptors expressed by NK cells

or strengthening the ADCC mechanism (mainly in high-grade PC patients with normal CD16 expression) could be associated to the CRS/HIPEC treatment in order to enhance the anti-tumor response.

In summary, this study contributes toward identification of possible markers of prognosis or candidates that may represent possible targets in immunotherapeutic approaches aiming to strengthen the anti-tumor activity of NK cells in PC tumors (15–18, 54, 55), a disease that has not been adequately investigated yet due to its rarity.

DATA AVAILABILITY

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

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethical standards of the institutional and/or national research committee. The protocol was approved by the ethics committee of the Liguria Region, Genova, Italy (no. 428Reg2014 for PC patients and no. 39/2012 for healthy donors). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

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

FUNDING

Supported by grants awarded by Fondazione Associazione Italiana per la Ricerca sul Cancro (AIRC)-IG 2017 Id. 20312 (SS, EM, MD, SCar, SP, MG, and SCan); Fondazione Associazione Italiana per la Ricerca sul Cancro (AIRC)-Special Program Metastatic disease: the key unmet need in oncology 5 per mille 2018 Id. 21147 (SS, EM, MD, SP, MG, SCan, and LM); Progetto Roche per la Ricerca 2017 (SP and EM). SP is recipient of a fellowship awarded by Fondazione Umberto Veronesi.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01963/full#supplementary-material

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

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frontiers in Immunology

ORIGINAL RESEARCH
published: 16 October 2018
doi: 10.3389/fimmu.2018.02358

Hypoxia Modifies the Transcriptome of Human NK Cells, Modulates Their Immunoregulatory Profile, and Influences NK Cell Subset Migration

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Hypoxia, which characterizes most tumor tissues, can alter the function of different immune cell types, favoring tumor escape mechanisms. In this study, we show that hypoxia profoundly acts on NK cells by influencing their transcriptome, affecting their immunoregulatory functions, and changing the chemotactic responses of different NK cell subsets. Exposure of human peripheral blood NK cells to hypoxia for 16 or 96 h caused significant changes in the expression of 729 or 1,100 genes, respectively. Gene Set Enrichment Analysis demonstrated that these changes followed a consensus hypoxia transcriptional profile. As assessed by Gene Ontology annotation, hypoxia-targeted genes were implicated in several biological processes: metabolism, cell cycle, differentiation, apoptosis, cell stress, and cytoskeleton organization. The hypoxic transcriptome also showed changes in genes with immunological relevance including those coding for proinflammatory cytokines, chemokines, and chemokine-receptors. Quantitative RT-PCR analysis confirmed the modulation of several immune-related genes, prompting further immunophenotypic and functional studies. Multiplex ELISA demonstrated that hypoxia could variably reduce NK cell ability to release IFNγ, TNFα, GM-CSF, CCL3, and CCL5 following PMA+Ionomycin or IL15+IL18 stimulation, while it poorly affected the response to IL12+IL18. Cytofluorimetric analysis showed that hypoxia could influence NK chemokine receptor pattern by sustaining the expression of CCR7 and CXCR4. Remarkably, this effect occurred selectively (CCR7) or preferentially (CXCR4) on $CD56^{bright}$ NK cells, which indeed showed higher chemotaxis to CCL19, CCL21, or CXCL12. Collectively, our data suggest that the hypoxic environment may profoundly influence the nature of the NK cell infiltrate and its effects on immune-mediated responses within tumor tissues.

Keywords: NK cells, hypoxia, tumor immunology, cytokines/chemokines, chemokine receptors, CD56bright cells, tumor infiltration, transcriptome

OPEN ACCESS

Edited by:
Marina Cella, Washington University in St. Louis, United States

Reviewed by:
Kerry S. Campbell, Fox Chase Cancer Center, United States
Emily Mace, Columbia University, United States

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *06 August 2018*
Accepted: *24 September 2018*
Published: *16 October 2018*

Citation:
Parodi M, Raggi F, Cangelosi D, Manzini C, Balsamo M, Blengio F, Eva A, Varesio L, Pietra G, Moretta L, Mingari MC, Vitale M and Bosco MC (2018) Hypoxia Modifies the Transcriptome of Human NK Cells, Modulates Their Immunoregulatory Profile, and Influences NK Cell Subset Migration. Front. Immunol. 9:2358. doi: 10.3389/fimmu.2018.02358

INTRODUCTION

NK cells are powerful effectors of the innate immunity with anti-tumor activity (1–5). They are endowed with a unique pattern of receptors sensing changes in MHC-I expression levels (which are often decreased in tumor cells) or recognizing ligands induced by tumor transformation, cell stress, and DNA damage (1, 5–8). By these receptors NK cells can direct their potent lytic machinery to target and eliminate many tumor cell types (6). In addition, NK cells can release pro-inflammatory cytokines and various chemotactic factors (IFNγ, TNFα, GM-CSF, CCL3/CCL4) potentially amplifying immune responses to the tumor (1, 6, 9–11). The cytolytic function and the capability of releasing cytokines and chemokines appear to be differently represented in the two major subsets of peripheral blood (PB)-NK cells, characterized by the $CD56^{dim}/CD16^{bright}$ ($CD56^{dim}$) or the $CD56^{bright}/CD16^{dim/neg}$ ($CD56^{bright}$) phenotype (1, 12–14). The $CD56^{dim}$ cells are strongly cytotoxic and can also produce cytokines in response to specific stimuli. These cells represent the large majority of the PB-NK cell population and express chemokine receptors, mainly CXCR1 and CX_3CR1, that enable their recruitment to inflamed tissues (1, 9, 11, 15, 16). Conversely, $CD56^{bright}$ cells are poorly cytotoxic and release high amount of cytokines, especially in response to monokines. According to their expression of CCR7 and CD62L, these cells are mainly located within secondary lymphoid compartments while they account for only 10% of PB-NK cells (1, 9, 11, 15).

The increasing interest on NK cells as potential tools for immunotherapy has recently inspired many studies aimed at defining how their anti-tumor activity can be influenced by the tumor microenvironment. Along this line, different suppressive interactions mediated by tumor cells, tumor-associated fibroblasts, or regulatory immune cells have been described and characterized (17–22). In addition, it has been shown that tumor cells can escape NK cell attack by modulating the surface expression of various NK-receptor ligands (2, 23–27). In spite of these important advances in the field, a crucial issue that still remains to be investigated for an effective exploitation of NK cells in the therapy of solid tumors is the recruitment of NK cells to tumor tissues. Few recent studies have shown that higher NK cell infiltration correlates with a better prognosis of the disease (23, 28, 29), but have also indicated that the NK cell infiltrate in tumor tissues is often poor and, in some cases, mostly represented by poorly cytotoxic $CD56^{bright}$ cells (5, 30, 31). Specific chemokine milieus, or alteration of chemokine receptor patterns, may account for these findings. However, an exhaustive explanation on how the tumor microenvironment can influence NK cell infiltration has not yet been achieved.

Reduced partial O_2 tension (pO_2, 0–20 mm Hg, hypoxia), which often affects tumor tissues, may play role in this context. Hypoxia is an important driver of malignant progression and resistance to therapy (23, 32, 33). It can influence the function of different cell types within the tumor lesion and affect the recruitment of immune cells, favoring tumor escape mechanisms (33). Indeed, exposure to hypoxia can induce different immune and non-immune cells to change the expression of pro-angiogenetic factors, cytokines, and chemokines (including VEGF, SPP1, IL-1β, MIF, CXCL12, and CXCL8), or chemokine receptors (including CXCR4, CCR2, and CCR5) (34–38). In spite of many studies on this issue, limited information is currently available on the impact of hypoxia on NK cells and their subsets, notably on their ability to respond to specific chemotactic stimuli or to release immune-active soluble factors (39–41). We have previously shown that, in NK cells exposed to IL-2, hypoxia can down-regulate expression and function of most NK cell receptors that activate cytolytic activity against tumor or virally infected cells, but preserves NK cell ability to kill targets via ADCC (39), suggesting that NK cells may be effective even in hypoxic niches in the context of combined immunotherapeutic approaches. In this study, we integrate previous data and provide an overview of the effect of hypoxia on NK cells stimulated with IL-2. Moreover, we indicate some clues on how the composition and the function of NK cell infiltrate may be influenced by the hypoxic environment in tumor tissues.

Abbreviations: PB, peripheral blood; pO_2, low oxygen tension; GSEA, Gene Set Enrichment Analysis; qRT-PCR, Real time PCR; Hy-NK, hypoxic NK cells; HIF, hypoxia-inducible factor; NES, normalized enrichment score; FDR *q*-val, false discovery rate *q*-values; NOM *p*-val, nominal *p*-values; LEA, Leading Edge Analysis; GO, gene ontology; HMGs, hypoxia-modulated genes; MDMs, monocyte-derived macrophages; iDCs, immature DCs; mDCs, mature DCs; IONO, ionomycin.

MATERIALS AND METHODS

NK Cells Isolation and Culture

NK cells were obtained from PB of healthy donors provided by the transfusion center of the Ospedale Policlinico San Martino following approved internal operational procedures (IOH78). Written informed consent from the donors was provided according to the Declaration of Helsinki. Briefly: NK cells from healthy donors were isolated from PB mononuclear cells using RosetteSep NK Cell Enrichment Cocktail (StemCell Technologies, 15025 Vancouver, Canada). Only preparations displaying more than 95% of CD56+ CD3– CD14– NK cells were selected for the experiments. After isolation, NK cells were cultured for the indicated time points in RPMI 1640 (Lonza Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (FBS, Voden Medical S.p.a. Meda MB, Italy), antibiotic mixture (0.05 mg/mL penicillin, 0.05 mg/mL streptomycin Lonza, Verviers, Belgium), and 100 U/mL recombinant human IL-2 (Proleukin, Novartis Basilea, Switzerland) at 2X 10^6 cells/mL in round bottom 96-well microtiter plates. The cultures were performed either under normoxic conditions in a humidified incubator containing 20% O_2, 5% CO_2, and 75% N_2 or under hypoxic conditions. Hypoxic conditions were obtained by culturing cells in a sealed anaerobic workstation incubator (Ruskinn, $INVIVO_2$ 400, CARLI Biotec, Roma, Italy), incorporating a gas mixing system (Ruskinn Gas Mixer Q) and flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2.

RNA Isolation and cRNA Synthesis

Total RNA was purified from NK cells derived from three healthy donors using the RNeasy MiniKit from Qiagen (Milano,

Italy). RNA was controlled for integrity by nanoelectrophoresis with an Agilent 2100 Bioanalyzer (Agilent Technologies Europe, Waldbroon, Germany), quantified by spectrophotometry using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA), and reverse-transcribed into double-stranded cDNA on a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Milano) using the one-cycle cDNA synthesis kit (Affymetrix, Milano). cDNA derived from three donors/time point was purified and biotin labeled using the GeneChip IVT kit (Affymetrix). Labeled cRNA was fragmented according to Affymetrix's instructions.

GeneChip Hybridization and Array Data Analysis

Gene expression profiling was performed as described previously (42). Briefly, Fragmented cRNA was hybridized on the Affymetrix HG-U133 plus 2.0 GeneChips (Genopolis Corporation, Milano) containing 54,000 probe sets (coding for 47,000 transcripts and variants, including 38,500 unique human genes) on a single array. Chips were stained with streptavidin-phycoerythrin (Invitrogen Life Technologies, Milano) and scanned using an Affymetrix GeneChip Scanner 3000, as described. Data were processed by RMA normalization utilizing the "Affy" R package. Statistical analysis using paired *t*-test was performed to identify differentially expressed genes. We corrected the *p*-value for multiple hypothesis testing by Benjamini–Hochberg method to false discovery rate control. Only gene differences that passed the test at a confidence level of 95% ($P < 0.05$) and a false discovery rate of 0.05% were considered significant. Fold-change (FC) was calculated as the ratio between the average expression level under hypoxia and normoxia. Genes were defined as being differentially regulated by hypoxia if they exhibited more than 2-fold increase in gene expression or down-regulated if they showed <0.5-fold change compared with normoxic cultures. We converted the Affymetrix probe sets into the corresponding gene symbol by Netaffx tool. When multiple probe sets were associated with the same gene symbol, the probe set with the highest expression signal was considered. The full set of data from each microarray experiment has been deposited in the Gene Expression Omnibus public repository at NCBI (www.ncbi.nlm.nih.gov) and is accessible through GEO (Accession number GSE116660). Biological processes were assessed by DAVID Gene Ontology (GO) enrichment analysis (http://david.niaid.nih.gov). The significant GO terms were defined as $p < 0.05$ and FDR < 0.05.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed on all probe sets of the Affymetrix HG-U133 Plus 2.0 GeneChip, as described previously (43). An enrichment score (ES) and a normalized enrichment score (NES) were calculated for every gene set. The statistical significance of NES was estimated by an empirical test using 1,000 gene set permutations to obtain the nominal *p*-value. A false discovery rate (FDR) q value was estimated to control the probability that a NES could represent a false positive finding. The gene sets used in the GSEA belong to the C2.CGP collection of the Broad Institute Molecular Signature v5 Database (MSigDB) (44). The analysis used Signal-to-Noise metric and considered gene sets containing at least 15 and up to 250 probe sets. An enrichment with FDR q-values lower than 0.05 and nominal $p < 0.05$ was considered significant. Leading Edge Analysis (LEA) of enriched gene sets was used to identify key genes related to NK response to hypoxia.

Real-Time RT-PCR

cDNA was prepared from purified total RNA using SuperScript Double-Stranded cDNA synthesis kit (Invitrogen). Real time PCR (qRT-PCR) was performed on a 7500 Real Time PCR System (Applied) in triplicate for each target transcript using SYBR Green PCR Master Mix and sense/antisense oligonucleotide primers synthesized by TIBMolbiol (Genova) or purchased from Quiagen, as detailed before (45). Expression data were normalized on the values obtained in parallel for three reference genes (actin related protein 2/3 complex subunit 1B, ARCP1B; ribosomal proteins S18, RSP18; and RSP19), using the Bestkeeper software, and relative expression values were calculated using Q-gene software, as detailed (45).

mAbs and Flow Cytofluorimetric Analysis

The following mAbs were used in this study: anti-CCR1 (R&D System, MAB 145-100, Minneapolis U.S.A.), anti-CCR5 (R&D System, MAB 182-100 Minneapolis U.S.A.), anti-CCR7 (R&D System, MAB 197-100 Minneapolis U.S.A.), anti-CXCR1/IL-8 RA (R&D System, MAB 173-100 Minneapolis U.S.A.), anti-CXCR3 (R&D System, MAB 160-100), anti-CXCR4 (R&D System, MAB 173-100), PE-conjugated anti-CX_3CR1 (Medical & Biological Laboratories Co., LTD, D070-5), FITC-conjugated anti-CD3 (eBioscience, 11-0038-42 Thermofisher scientific, Waltham, Massachusetts, Stati Uniti), PE-cyanine 7-conjugated anti-CD56 (Beckman Coulter, A21692, Brea, California U.S.A.), PE-conjugated anti-CD16 (130-106-704, Miltenyi Biotec Bergisch Gladbach, Germany). The staining with the appropriate unlabeled mAbs are followed by PE-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL, U.S.A.), and fluorescence was quantified on a Gallios™ Flow Cytometer (Beckman Coulter, Brea, California U.S.A.).

Multiplex ELISA Analyses

Freshly isolated NK cells were cultured for 20 h at 5X 10^5/mL in flat bottom 96-well microtiter plates in the presence of the following recombinant human cytokines: IL-2, IL-12+IL-18, or IL-15+IL-18. The cytokine concentrations were: 100 U/mL IL-2 (Proleukin, Novartis Basilea, Switzerland); 2.5 ng/mL IL-12 (Peprotech, 200-12 London, UK); 20 ng/mL IL-15 (Peprotech, 200-15 London, UK); 200 ng/mL IL-18 (Medical & Biological Laboratories Co. LTD, B001-5, Japan). In the "PMA+IONO IL-2" condition, NK cells were cultured in the presence of IL-2 for 26 h, and 100 ng/mL PMA (Phorbol 12-myristate 13 acetate, SIGMA-Aldrich Saint Louis, Missouri, U.S.A.) and 500 ng/mL IONO (Ionomycin, SIGMA-Aldrich, Missouri, U.S.A.) were added to the cultures for the last 6 h. The cultures were performed

in parallel under normoxic and hypoxic conditions (see above). Culture supernatants were then collected and analyzed for their cytokine content by MAGPIX® System (Luminex® xMAP® Technology, Merck Millipore, Germany).

Chemotaxis Assay

NK cells freshly isolated from peripheral blood and then cultured for different time points (24, 48, and 96 h) with IL-2 under hypoxic or normoxic conditions were seeded at 2.5×10^6/mL in the upper chamber of a Transwell system (3 mm pore size; Corning Costar, 3415). 10% FBS RPMI 1640 medium alone or supplemented with recombinant human CXCL12 [100 ng/mL] (Peprotech, 300-28A), or CCL19 [0.3 μg/mL], or CCL21 [0.6 μg/mL] was added to the lower compartment. Cells were allowed to migrate for 2 h at 37°C under normoxic condition. Cells migrated in the lower chamber were collected and counted using the MACSQuant Analyzer (Miltenyi Biotec Bergisch Gladbach, Germany) or analyzed with the Gallios™ Flow Cytometer after surface double staining of CD56/CD16 markers. Cells migrated in the lower chamber containing medium alone (w/o chemokines) represented spontaneous migration due to unspecific cell motility. Chemotactic response was assessed as percentage of spontaneous migration and was calculated as follows: (number of migrated cells in the presence of chemotactic stimulus/number of migrated cells in the absence of stimulus) × 100. The chemotactic response of $CD56^{bright}CD16^{dim/neg}$ NK cells to CCL19, CCL21, and CXCL12 was assessed as enrichment of this specific cell subset within migrated cells and was calculated as follows: $CD56^{bright}CD16^{dim/neg}$ cell percentage within cells migrated in response to chemokines/$CD56^{bright}CD16^{dim/neg}$ cell percentage within spontaneously migrated cells.

Statistical Analysis

Statistical analyses were performed using the Prism software package (GraphPad Software). Data are expressed as the mean ± SEM of at least three independent experiments, unless differently specified. Statistical significance was evaluated by two-tailed paired Student's *t*-test. A $p < 0.05$ (*), <0.01(**), or <0.001(***) was considered statistically significant.

RESULTS

Gene Expression Profile of Hypoxic NK Cells

To obtain an overview of NK cell response to hypoxia, we assessed the gene expression profile of NK cells isolated from the PB of three independent healthy donors and cultured with IL-2 for 16 or 96 h under hypoxic (1% O_2) or normoxic (20% O_2) conditions. mRNA was individually hybridized to human Affymetrix HG-U133 plus 2.0 GeneChips, obtaining three biological replicates for each experimental condition. Raw data were processed as described in the section Materials and Methods. We used GSEA to determine the enrichment of the published C2.CGP gene set collection (44) in the 16 and 96 h transcriptomes of hypoxic NK cells (Hy-NK) as compared to their normoxic counterparts. We selected 25 gene sets using "hypoxia" and "hypoxia-inducible factor (HIF)" as keywords (see section Materials and Methods for details). The list of gene sets, their normalized enrichment score (NES), false discovery rate *q*-values (FDR q-val), and nominal *p*-values (NOM p-val) are reported in **Table 1**. Among selected gene sets, 20 were significantly enriched in both Hy-NK cell transcriptomes ($p < 0.05$; FDR $q < 0.05$), 1 and 3 additional gene sets were specifically enriched in the 16 h (upregulated) and the 96 h (downregulated) hypoxic transcriptomes, respectively, and only 1 gene set (upregulated) was not significantly enriched at either time points. Representative 16 and 96 h plots showing clear enrichment of the gene sets at the top or the bottom of the ranked list are presented in **Figure 1A** for a visual inspection of the GSEA results (46). These data demonstrate that gene expression changes in Hy-NK cells follow a consensus hypoxia transcriptional profile.

Gene transcriptional activation by hypoxia is mediated primarily by HIF, a heterodimer of a constitutive HIF-1β subunit and an O_2-sensitive α-subunit (HIF-1α or HIF-2α) (32, 34). Interestingly, some of the selected gene sets were from cells undergoing HIF-1α or HIF-2α silencing (46). The reported sets of down- or up-regulated genes were found inversely enriched in the Hy-NK cell transcriptomes (**Table 1**—gene sets 1, 2, 19, 20—and **Figure 1B**), suggesting that HIF-1α and HIF-2α and their target genes could play an important role in NK cell response to hypoxia.

Leading Edge Analysis (LEA) applied to the significantly enriched gene sets allowed to define the subsets of hypoxia-related genes with the highest impact on the enrichment score (referred to as the leading edge subset) at 16 or 96 h (**Figure S1**). These subsets include genes involved in glycolysis, gluconeogenesis, and glucose transport (ALDOA, ALDOC, ENO1, ENO2, GAPDH, GPI, HK1, HK2, LDHA, PDK1, PGK1, SLC2A1, TPI1), non-glycolytic metabolism and ion transport (P4HA1, P4HA2, PAM, VLDLR), apoptosis, stress response, and proliferation (BNIP3, BNIP3L, CCNG2, DDIT3, EGLN1, EGLN3, NDRG1), transcription and signaling activity (FOSL2, JAK2, JUN, MXI1, SOCS2). These results extend to Hy-NK cells the expression of a large cluster of hypoxia-related genes previously identified in other cell types, including tumor and immune cells (32, 34, 47–49).

Functional Assessment of Genes Modulated by Hypoxia in NK Cells

To identify novel genes affected by hypoxia in NK cells, we performed differential expression analysis of microarray data. We filtered transcripts for a differential expression of at least 2-fold changes and a $p \leq 0.05$. Using these selection criteria, we identified a total of 1,474 transcripts that were significantly modulated under hypoxic vs. normoxic conditions, with expression changes ranging from 106-fold upregulation to 25-fold downregulation (**Table S1**). The majority of differentially-expressed transcripts were identified as unique genes named in the GenBank™, whereas the remaining transcripts were either unnamed expressed sequence tags or hypothetical. As shown by the Venn diagram in **Figure 2A**, 355 transcripts were up-[179] or down-[176] regulated at both time points, whereas 374

TABLE 1 | Hypoxia- and HIF-related gene sets enriched in the 16 and 96 h hy-NK cell transcriptomes.

GSEA term[a]	16 h up				96 h up			
	Size[b]	NES[c]	FDR *q*-val[d]	Nom *p*-val[e]	Size	NES	FDR *q*-val	Nom *p*-val
ELVIDGE_HIF1A_TARGETS_DN	69	3.26	<0.001	<0.001	69	2.98	<0.001	<0.001
ELVIDGE_HIF1A_AND_HIF2A_TARGETS_DN	75	3.17	<0.001	<0.001	75	2.96	<0.001	<0.001
MENSE_HYPOXIA_UP	75	3.16	<0.001	<0.001	75	2.70	<0.001	<0.001
ELVIDGE_HYPOXIA_UP	130	3.06	<0.001	<0.001	130	2.78	<0.001	<0.001
ELVIDGE_HYPOXIA_BY_DMOG_UP	102	3.03	<0.001	<0.001	102	2.85	<0.001	<0.001
FARDIN_HYPOXIA_11	25	2.92	<0.001	<0.001	25	2.75	<0.001	<0.001
SEMENZA_HIF1_TARGETS	28	2.70	<0.001	<0.001	28	2.09	0.003	<0.001
LEONARD_HYPOXIA	33	2.67	<0.001	<0.001	33	2.55	<0.001	<0.001
KIM_HYPOXIA	18	2.62	<0.001	<0.001	18	2.02	0.005	<0.001
HARRIS_HYPOXIA	63	2.60	<0.001	<0.001	63	1.91	0.018	<0.001
GROSS_HIF1A_TARGETS_DN	17	2.59	<0.001	<0.001	17	1.75	0.064	0.006
WINTER_HYPOXIA_METAGENE	184	2.55	<0.001	<0.001	184	2.21	<0.001	<0.001
QI_HYPOXIA	107	2.47	<0.001	<0.001	107	2.01	0.005	<0.001
MANALO_HYPOXIA_UP	163	2.34	<0.001	<0.001	163	2.63	<0.001	<0.001
GROSS_HYPOXIA_VIA_ELK3_AND_HIF1A_UP	108	2.15	0.001	<0.001	108	1.96	0.01	<0.001
GROSS_HYPOXIA_VIA_ELK3_DN	125	1.52	0.1934	0.000	125	1.18	0.5	1
GSEA term	**16h down**				**96h down**			
	Size	**NES**	**FDR *q*-val**	**Nom *p*-val**	**Size**	**NES**	**FDR *q*-val**	**Nom *p*-val**
MANALO_HYPOXIA_DN	236	−3.20	<0.001	<0.001	236	−3.34	<0.001	<0.001
ELVIDGE_HYPOXIA_DN	123	−2.84	<0.001	<0.001	123	−2.14	<0.001	<0.001
ELVIDGE_HIF1A_TARGETS_UP	56	−2.71	<0.001	<0.001	56	−1.88	0.003	<0.001
ELVIDGE_HIF1A_AND_HIF2A_TARGETS_UP	37	−2.62	<0.001	<0.001	37	−2.00	<0.001	<0.001
ELVIDGE_HYPOXIA_BY_DMOG_DN	49	−2.42	<0.001	<0.001	49	−2.12	<0.001	<0.001
GROSS_HYPOXIA_VIA_HIF1A_UP	60	−2.11	<0.001	<0.001	60	−1.81	0.007	<0.001
GROSS_HYPOXIA_VIA_ELK3_AND_HIF1A_DN	77	−1.57	0.0678	0.0051	77	−1.87	0.003	<0.001
GROSS_HYPOXIA_VIA_ELK3_UP	166	−1.43	0.1474	0.0118	166	−2.28	<0.001	<0.001
GROSS_HYPOXIA_VIA_ELK3_ONLY_DN					37	−1.85	0.004	<0.001

Microarray analysis was carried out on NK cells from 3 different donors cultured under normoxic (20% O2) and hypoxic (1% O2) conditions for 16 and 96 h. Differentially expressed transcripts were ranked by level of hypoxia-mediated up- or down-regulation. The ranked gene lists were then compared with published gene sets for hypoxia-regulated genes or for genes previously shown to be HIF targets in other cell types by GSEA.

[a]Gene sets enriched in the GSEA analysis. Gene sets belonged to the C2.CGP collection of the MSigDB and were selected using the keywords "hypoxia" and "HIF" and filtering out those having <15 probe sets and more than 250 probe sets. "Up" indicates genes enriched in the hypoxia transcriptomes (i.e., up-regulated in hypoxic NK cells); "down" indicates genes enriched in the normoxia transcriptomes (i.e., down-regulated in hypoxic NK cells).

[b]Relative number of probe sets in the gene sets.

[c]Normalized enrichment score of the gene sets. Gene sets are listed in decreasing order of NES.

[d]FDR q-value of the false discovery rate. Values ≤0.05 are considered acceptable.

[e]NOM p-value of the normalized enrichment score. Values ≤0.05 are considered significant.

(139 induced and 235 repressed) and 745 (334 induced and 411 repressed) transcripts were specifically modulated at 16 or 96 h, respectively. These results provide the first indication that Hy-NK cell signature varies with the duration of exposure to hypoxia.

To gain insights into the biological processes modulated by hypoxia, we carried out a Gene Ontology (GO) enrichment analysis on the lists of up- and down-regulated transcripts. We identified 28 biological processes containing a statistically significant enrichment of hypoxia-modulated genes (HMGs) (**Figure 2B**). Most processes were represented at both time points, although with variable HMG enrichment. Metabolism and biosynthesis resulted as the most enriched processes (in both up- and down-regulated genes), followed by response to stimulus. Additionally, Hy-NK cell transcriptional profile was related to regulation of apoptosis, and response to stress, but also to cell proliferation, signaling, and chromatine/chromosome organization. Certain processes, including regulation of gene transcription and expression, and cell differentiation, were selectively enriched in upregulated genes, whereas processes related to cell cycle, cellular component organization, and DNA replication and repair were exclusively enriched in downregulated genes at both time-points.

Importantly, different immune-related processes, including immune system development/response, hemopoiesis, leukocyte activation and differentiation, angiogenesis, regulation of cell motility and communication, and

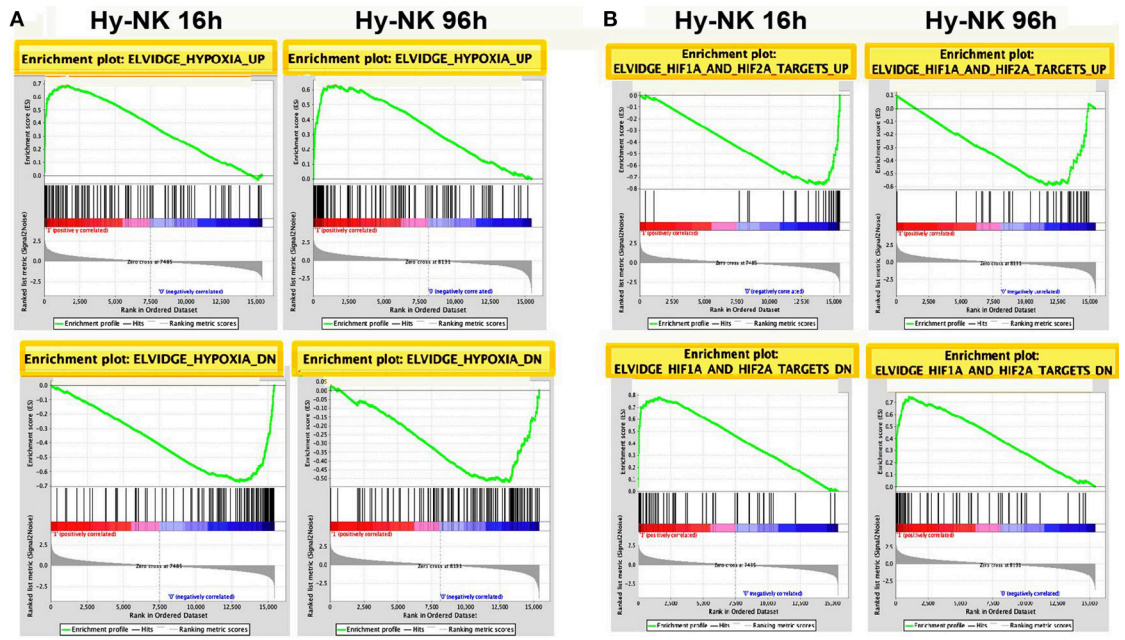

FIGURE 1 | Gene Set Enrichment Analysis (GSEA) Plots for representative hypoxia- or HIF1α/2α-related gene sets in Hy-NK cell transcriptomes. The transcripts identified by microarray analysis in NK cells were ranked by level of hypoxia-mediated up- or down-regulation. The ranked gene lists were then compared by GSEA with previously published gene sets for hypoxia-regulated genes or for genes previously shown to be HIF targets in other cell types. **(A)** GSEA plots of representative sets of up- or down-regulated genes from cells exposed to hypoxia (ELVIDGE_HYPOXIA_UP or _DN, respectively). **(B)** GSEA plots of representative sets of up- or down-regulated genes from cells undergoing HIF-1α and HIF-2α silencing (Elvidge_HIF1A_and_HIF2A_TARGETS_UP or _DN, respectively). Note that in the case HIF1α/2α-silencing the sets of up- or down-regulated genes resulted inversely enriched in the Hy-NK cell transcriptomes. The enrichment score is calculated by walking down a list of genes ranked by their correlation with the phenotype, increasing a running-sum statistic when a gene in that gene set is encountered (each black vertical line underneath the enrichment plot) and decreasing it when a gene that isn't in the gene set is encountered. The enrichment score is the maximum deviation from zero encountered in the walk.

immune effector processes, were enriched in a statistically significant percentage of HMGs, suggesting regulatory effects of hypoxia on NK cell-mediated immune responses.

Characterization of Cytokine/Chemokine and Receptor Gene Modulation in Hy-NK Cells

As the effect of hypoxia on the NK cell capability of killing target cells has been previously assessed (39), we focused our analysis on genes involved in immunoregulation and migration. The evaluation of immune-related gene clusters highlighted by GO analysis led to the identification of 43 HMGs coding for cytokines and chemokines, their receptors, and/or associated signaling molecules (**Table 2**). Some of these genes (26) were rapidly modulated by hypoxia (at 16 h time-point), while the remaining genes (17) were modulated after longer exposure (96 h).

Compared to their normoxic counterparts, Hy-NK cells showed increased expression of genes coding for molecules with a primary role in angiogenesis (VEGFA, VEGFB, ADM, SPP1, FGF11) and promotion of inflammation or lymphocye cytotoxic responses (SPP1, SPP2, VEGF,TNFRSF11A and 12A, IGFBP2, FGFBP2, PTAFR), but also in apoptosis inhibition (IGF1R, TNFRSF10D, 11A, and 12A), tumor progression (IGFBP2, SPP1, MIF, PDGFD, TGFβ2, FGF11), and immunosuppression (MIF and TGFβ2) (34, 37, 48, 50–55). On the other hand, hypoxia inhibited mRNAs coding for cytokines and/or receptors mainly involved in anti-tumor and anti-viral immune responses including IFNγ, IFI30, IFI44, IL-17RC, IL1RL1, and various components of the tumor necrosis factor (TNF) superfamily, such as TNFα, LTA, LTB,TNFSF10,11,14, and TNFRSF18 (55–63). The only exception was represented by the inhibition of mRNA coding for LIF, a pleiotropic factor involved in the regulation of inflammation.

The Hy-NK transcriptome was also characterized by the differential modulation of genes coding for chemokines and chemokine receptors (11, 15). Specifically, we observed hypoxia-dependent upregulation of the mRNA for CXCL8 (which also has proangiogenic properties) (11, 37, 52), and downregulation of mRNAs for CXCL10, CCL3, and XCL1 (9, 11, 52, 64). Regarding the chemokine receptors known to be important for NK cell migratory activity mRNAs coding for CXCR4 and CX_3CR1 were selectively up-regulated, whereas those coding for CXCR1, CCR1, CCR5, and CXCR3 were downregulated.

Microarray results were validated by qRT-PCR analysis of a subset of HMGs (17 belonging to the 16 h transcriptome and 9 to the 96 h transcriptome). As shown in **Figure 3** and **Table 2** there was almost full concordance between qRT-PCR and Affymetrix

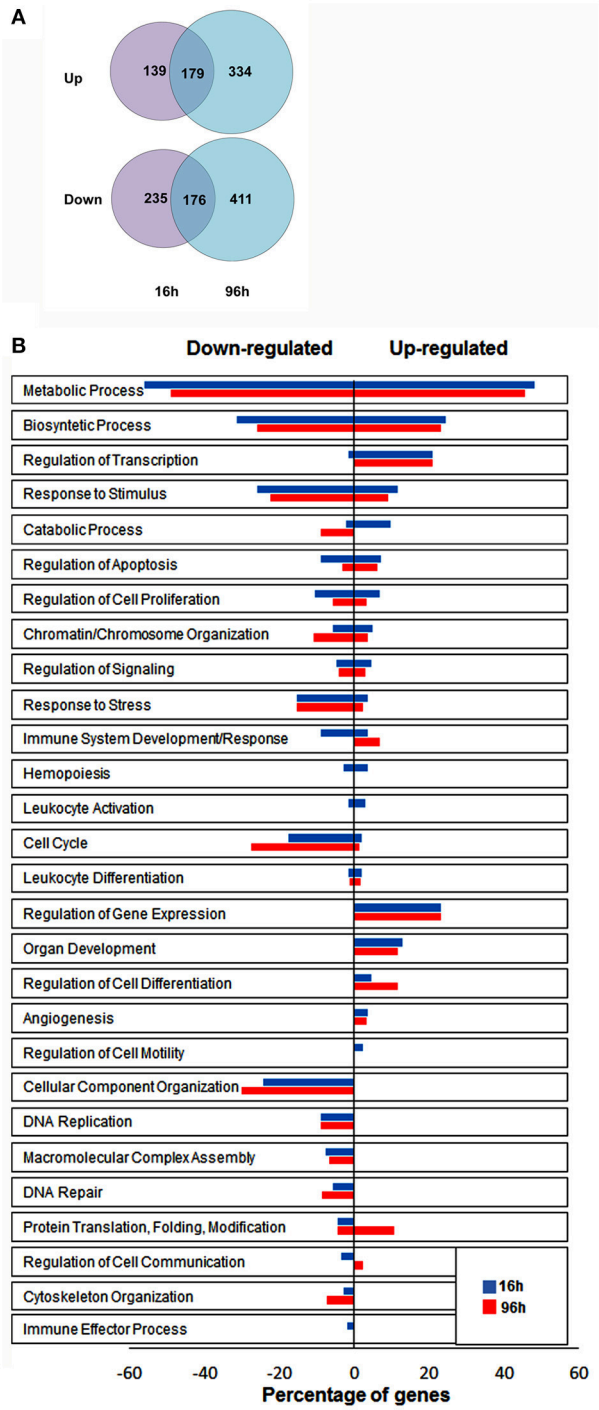

FIGURE 2 | Identification of genes significantly modulated by hypoxia in NK cells by differential expression analysis. **(A)** Graphical representation of transcripts differentially expressed in hypoxic vs. normoxic NK cells. The gene expression profile of NK cells isolated from 3 different donors and exposed to hypoxia for 16 h (top) or 96 h (bottom) was analyzed by microarray analysis, as described in the section Materials and Methods. The Venn diagram depicts the number of transcripts exhibiting ≥2 fold up- or down-regulation in hypoxic vs. normoxic cells at the two time points. About 24% of differentially expressed transcripts are common to the 16 and 96 h transcriptomes.

(Continued)

FIGURE 2 | (B) Functional assessment of hypoxia-responsive genes by GO enrichment analysis. Unique genes showing at least 2-fold change in expression levels between Hy-NK and NK cells were clustered into different biological processes using the DAVID GO enrichment analysis. Based on this classification scheme, genes were placed in more than one biological process if more than one function of the encoded protein was established. The y-axis shows the GO terms. The x-axis shows the percentage of genes within each process relative to the total amount of genes belonging to that process: bars on the right of the y axis represent upregulated genes; bars on the left of the y axis represent downregulated genes. The blue columns represent genes modulated at 16 h whereas the red column represent genes modulated at 96 h.

data with respect to the direction of the expression changes, with the only exception of CX_3CR1 whose upregulation by hypoxia was not confirmed by qRT-PCR. For about half of validated genes, the extent of modulation was also comparable to that shown by microarray data, whereas it was higher for nine genes and lower for three genes. Such discrepancies, however, are consistent with previous findings showing that these techniques can often differently estimate the extent of gene regulation (42, 48).

A literature survey indicated that some of the HMGs in NK cells were targeted by hypoxia in different cell types including T lymphocytes (49), primary monocytes (48), monocyte-derived macrophages (MDMs) (38, 65, 66), immature (i)DCs (67–70), and mature (m)DCs (37, 68) (**Table 2**). In particular, MIF- and VEGFA-coding genes were upregulated by hypoxia in all the immune cell types analyzed, ADM and SPP1 were increased in the innate immune cells, while CXCL8 and CXCR4 upregulation was reported in T cells and in some mononuclear phagocyte populations. Other genes were variably modulated depending on the analyzed cell type. To our knowledge, a consistent part of the cytokine/chemokine- and receptor-coding genes identified in Hy-NK cells have not been reported to be modulated by hypoxia in other immune cell populations analyzed.

Taken together, these data indicate that hypoxia can induce a specific cytokine/chemokine and receptor gene signature in NK cells with possible functional consequences. To assess this possibility, we proceeded with the overall evaluation of the effects of hypoxia on NK cell immune-regulatory and migratory functions.

Effects of Hypoxia on NK Cell-Mediated Release of Chemokines And Cytokines

To assess the effect of hypoxia on cytokine/chemokine release, PB-NK cells were freshly isolated from additional donors and cultured in the presence of IL-2 under hypoxic or normoxic conditions. After 20 h, supernatants were collected and analyzed by multiplex immunoassay for the content of IFNγ, TNFα, CCL3, GM-CSF, CCL5, CXCL8, VEGF, and MIF (**Figure 4**), namely those factors that are typically released by NK cells (6, 12) and/or that were shown to be transcriptionally affected in microarray analysis (**Table 2**). As shown in **Figure 4A**, upon exposure to IL-2 NK cells released low levels of different factors, including IFNγ, CCL3, GM-CSF, CCL5, and MIF. Under hypoxic

TABLE 2 | Relative expression of genes encoding cytokines/chemokines and their receptors in H-NK vs. NK cells∧.

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
16 h										
Up-regulated										
NM_000597	IGFBP2	Insulin-like growth factor binding protein 2	Member of a regulatory network controlling cell proliferation, migration, and apoptosis; functions as a transporter of IGFs from the circulation into tissues; has growth stimulatory effect on tumor cells; contributes to T-cell activation and proliferation	71.7	/	/	/	/	/	
NM_00102539	VEGFA	Vascular endothelial growth factor A	Member of the PDGF/VEGF growth factor family; mitogen for endothelial cell (EC); plays a central role in driving angiogenesis and vasculogenesis via stimulation of EC survival, proliferation and migration and inhibition of apoptosis; promotes monocytic cell recruitment/activation	28.7	Up	Up	Up	Up	Up	(37, 45, 46, 63, 65–67, 69)
NM_001124	ADM	Adrenomedullin	Angiogenic peptide vasodilator that belongs to the calcitonin/calcitonin gene-related peptide (CGRP)/amylin peptide family; may function as a hormone in circulation control; acts as a multifunctional regulatory peptide	12.9	Up	Up	Up	Up	/	(45, 63, 66)
NM_00104008	SPP1/ OPN	Secreted phosphoprotein (Osteopontin)	Pleiotropic cytokine/extracellular matrix phosphoprotein; plays a critical role in the activation of type I immunity and tumor growth, progression, and spread; endowed with EC adhesive capacity and chemotactic activity for EC, monocytes, and T lymphocytes	11.6	Up	Up	Up	Up	/	(37, 45, 66)
NM_000584	CXCL8	Chemokine (C-X-C motif) ligand 6 (IL-8)	Member of the a-chemokine family; functions as a neutrophil chemotactic and activating factor, potent angiogenic factor and major mediator of the inflammatory response	4.9	/	Up	Up	Up	Up	(37, 46, 63, 65–67)
NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4	Receptor of the stromal cell-derived factor-1; key regulator of cell motility and migration; critical for retention and homing of hematopoietic cells, including NK cells, in the BM; plays important roles in tumor cell metastatization in many types of cancers	3.4	Up	Up	Up	/	Up	(45, 46, 63, 65, 67, 68)
NM_003377	VEGFB	Vascular endothelial growth factor B	Member of the PDGF/VEGF growth factor family; ligand for VEGF receptor 1 and neuropilin-1; regulates the formation of blood vessels and is involved in EC physiology controlling uptake of fatty acids	3.2	/	/	/	/	/	
NM_003840	TNFRSF10D (TRAILR4)	Tumor necrosis factor receptor superfamily, member 10d	Member of the TNF-receptor superfamily; receptor of TNF-related apoptosis-inducing ligand (TRAIL); plays an inhibitory role on TRAIL-induced cell apoptosis	2.5	/	Up	/	Up	/	(37)
NM_000875	IGF1R	Insulin-like growth factor 1 receptor	High affinity receptor of insulin-like growth factor; functions as an anti-apoptotic agent by enhancing cell survival; mediates regulation of NK cell development and cytotoxicity by IGF-1	2.4	/	/	/	/	/	

(Continued)

TABLE 2 | Continued

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
NM_00113559	TGFB2	Transforming growth factor, beta 2	Member of the TGFB family of cytokines; interferes with antitumor immune responses; has suppressive effects of interleukin-2 dependent T-cell growth; responsible for the down-regulation of the activating immunoreceptor NKG2D expression in CD8(+) T and NK cells and suppression of their lytic functions; may act directly as a tumor progression factor	2.3	/	/	/	/	/	
NM_002415.1	MIF	Macrophage migration inhibitory factor	Pleiotropic cytokine with multiple effects in immunoregulation and inflammation; promoter of tumor cell proliferation, migration, metastasis, and tumor angiogenesis; suppressor of p53-mediated apoptosis and antitumor immunity; inhibitor of macrophage motility and NK cell cytotoxicity against tumor cells by down-regulating NKG2D receptor	2.1	Up	Up	Up	Up	Up	(37, 45, 46, 63, 65, 66)
Down-regulated										
NM_002309	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	Member of the IL6 family of cytokines; displays pleiotropic effects on various cell types and organs; involved in the induction of myeloid hematopoietic and neuronal cell differentiation; regulator of mesenchymal to epithelial conversion; plays a key anti-inflammatory role in cutaneous inflammation; promotes Treg development and adaptive immune tolerance; involved in immune tolerance at the maternal-fetal interface	0.21	/	/	/	/	/	
NM_003701.3	TNFSF11 (RANKL)	Tumor necrosis factor (ligand) superfamily, member 11	Member of the TNF cytokine family; functions as a key factor for osteoclast differentiation and activation; involved in the regulation of T cell immune response and DC survival; may have a role in the regulation of apoptosis; involved in oncogenesis, tumor progression, and metastatization	0.27	/	/	/	/	/	
NM_00115970	LTA (TNFSF1)	Lymphotoxin alpha (TNF superfamily, member 1)	Member of the TNF family, forms heterotrimers with lymphotoxin-beta; mediates inflammatory, immunostimulatory, and antiviral responses; involved in secondary lymphoid organ formation during development; plays a role in tumor apoptotic killing byNK cells	0.33	/	/	/	/	/	
NM_002341.1	LTB (TNFSF3)	Lymphotoxin beta (TNF superfamily, member 3)	Type II membrane protein of the TNF family; anchors lymphotoxin-alpha to the cell surface through heterotrimer formation; inducer of inflammatory responses; involved in development of lymphoid tissue; plays a role in tumor apoptotic killing by NK cells and in IL-12 production	0.34	/	/	/	/	/	

(Continued)

TABLE 2 | Continued

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
NM_002995	XCL1 (ATAC)	Chemokine (C motif) ligand 1 (Lymphotactin)	Member of the C-chemokine subfamily produced by T, NK, and NKT cells during infectious and inflammatory responses; functions in inflammatory and Th1-type immune responses; induces T cell migration and activation, DC-mediated cytotoxic activity; regulates thymic establishment of self-tolerance and generation of Treg	0.34	/	/	/	Up	/	(37)
NM_004195	TNFRSF18	Tumor necrosis factor receptor superfamily, member 18 (GITR)	Member of the TNF-receptor superfamily, binds TNFSF18; modulates T-lymphocyte survival in peripheral tissues, important for interactions between activated T-lymphocytes and ECs; participates in the development of autoimmune/inflammatory responses and graft-vs-host disease and potentiates response to infection and tumors through activation of effector T-cells, inhibition of regulatory T (Treg) cells, NK-cell co-activation, activation of macrophages, modulation of DC function	0.38	/	/	/	Up	/	(37)
NM_000619	IFNG	Interferon, gamma	Member of the type II interferon family; endowed with antiviral, immunoregulatory, anti-angiogenic, anti-proliferative, and pro-apoptotic properties; promotes tumor immunogenicity; potent activator of macrophages; critical for NK-mediated CTL activation and Th1 cell development	0.39	/	/	/	/	Down	(69)
NM_000594	TNFA (TNFSF2)	Tumor necrosis factor-alpha	Multifunctional proinflammatory cytokine that belongs to the TNF superfamily; involved in the regulation of several biological processes including immune cell proliferation, differentiation, migration, and activation, apoptosis, necrosis, lipid metabolism, and coagulation; implicated in autoimmune diseases, insulin resistance, and tumor immune surveillance	0.42	Up	Up	Down	/	Down	(45, 46, 63, 67)
NM_00100147	CCL3 (MIP-1a)	Chemokine (C-C motif) ligand 3	Member of the beta-chemokine subfamily; plays a role in inflammatory responses; induces monocyte, activated T cell, immature DC, and NK cell chemotaxis; inhibits HIV replication; plays a role in NK cell cytolytic and antiviral activity	0.43	/	Up	/	Up	Up	(37, 46, 63)
NM_172014	TNFSF14 (LIGHT)	Tumor necrosis factor (ligand) superfamily, member 14	Member of the TNF ligand family, binds to TNFRSF14 and LTβR on hematopoietic and stromal cells; functions as a costimulatory factor for lymphoid cells and as a deterrent to infection by herpesvirus; stimulates T cell proliferation; triggers tumor cell apoptosis; plays a critical role in NK activation/expansion and activated NK cell and DC priming of CD8+ T cells; increases effector cell priming, recruitment, and retention at tumor sites	0.43	Up	/	Up	/	Down	(45, 46, 65)

(Continued)

TABLE 2 | Continued

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
NM_000634	CXCR1	Chemokine (C-X-C motif) receptor 1	Member of the G-protein-coupled receptor family; high affinity receptor of CXCL8; mediates NK cell trafficking in the bone marrow (BM) under physiological conditions and recruitment to inflammatory sites	0.45	/	/	/	/	/	
NM_001295	CCR1	Chemokine (C-C motif) receptor 1	Member of the betachemokine receptor family, binds CCL3, CCL5, CCL7, and CCL23; mediates NK cell trafficking in the BM under homeostatic conditions and recruitment of BM-derived NK cells to inflammatory and tumor sites; increases NK cells cytolitic activity	0.49	Down	/	/	/	/	(45)
NM_000579	CCR5	Chemokine (C-C motif) receptor 5	Member of the beta-chemokine receptor family, binds CCL3, CCL4, CCL5, and CCL8; plays a role in NK cell proliferation and circulation under physiological conditions; mediates recruitment of BM-derived NK cells to sites of inflammation and tumors; enhances NK cell cytolitic activity; co-receptor for macrophage-tropic virus, including HIV	0.49	Down	Down	Up	Up	Up	(45, 46, 64, 66–68)
NM_001565	CXCL10 (IP-10)	Chemokine (C-X-C motif) ligand 10	Member of the alpha-chemokine subfamily; ligand for the receptor CXCR3; IFNg-inducible chemokine endowed with pleiotropic effects; inducer of monocytes, NK and T-cell migration; inhibitor of angiogenesis; modulator of adhesion molecule expression	0.50	/	/	Up	/	/	67
NM_001504	CXCR3	Chemokine (C-X-C motif) receptor 3	G protein-coupled receptor with selectivity for CXCL9, CXCL10, and CXCL11; induces integrin activation, cytoskeletal changes, and chemotactic migration; promotes NK cell trafficking in the BM and accumulation into tumors; enhances NK cell antitumor activity	0.50	/	/	/	/	/	
96 h										
Up-regulated										
NM_001124	ADM	Adrenomedullin	Angiogenic peptide vasodilator that belongs to the calcitonin/calcitonin gene-related peptide (CGRP)/amylin peptide family; may function as a hormone in circulation control; acts as a multifunctional regulatory peptide	9.2	Up	Up	Up	Up	/	(45, 63, 66)
NM_006944	SPP2	Secreted phosphoprotein 2, 24kDa	Secreted phosphoprotein member of the cystatin superfamily; NFkappaB-dependent gene with a pro-inflammatory role	7.4	/	/	/	/	/	
NM_00102539	VEGFA	Vascular endothelial growth factor A	Member of the PDGF/VEGF growth factor family; mitogen for endothelial cell (EC); plays a central role in driving angiogenesis and vasculogenesis via stimulation of EC survival, proliferation and migration and inhibition of apoptosis; promotes monocytic cell recruitment/activation	7.2	Up	Up	Up	Up	Up	(37, 45, 46, 63, 65–67)

(Continued)

TABLE 2 | Continued

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
NM_000584	CXCL8	Chemokine (C-X-C motif) ligand 6 (IL-8)	Member of the a-chemokine family; functions as a neutrophil chemotactic and activating factor, potent angiogenic factor and major mediator of the inflammatory response	6.4	/	Up	Up	Up	Up	(37, 46, 63, 65–67)
NM_004112	FGF11	Fibroblast growth factor 11	Member of the FGF family with mitogenic, cell survival,proangiogenic, and tumorigenic functions	6.0	/	/	/	/	/	
NM_000597	IGFBP2	Insulin-like growth factor binding protein 2	Member of a regulatory network controlling cell proliferation, migration, and apoptosis; functions as a transporter of IGFs from the circulation into tissues; has growth stimulatory effect on tumor cells; contributes to T-cell activation and proliferation	5.8	/	/	/	/	/	
NM_00101383	PTAFR	Platelet-activating factor receptor	Receptor for platelet-activating factor with pleiotropic functions; regulator of cell motility, smooth muscle contraction, leukocyte migration, and cytokine/chemokine production; implicated in allergy, asthma, septic shock, arterial thrombosis, and inflammatory processes; involved in immunosuppression	3.5	/	/	/	/	/	
NM_001616	ACVR2A	Activin A receptor, type IIA	Receptor of activins, which are members of the TGF-beta superfamily involved in several biological processes.	3.1	/	/	/	/	/	
NM_000875	IGF1R	Insulin-like growth factor 1 receptor	High affinity receptor of insulin-like growth factor; functions as an anti-apoptotic agent by enhancing cell survival; mediates IGF-1-dependent promotion of NK cell development and cytotoxicity	3.1	/	/	/	/	/	
NM_003840	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10 d (TRAILR4)	Member of the TNF-receptor superfamily; receptor of TRAIL; plays an inhibitory role in TRAIL-induced cell apoptosis	3.0	/	/	/	/	/	
NM_002185	IL7R	Interleukin 7 receptor	Receptor of interleukine 7; essential for T lymphocyte development, survival, and proliferation	2.8	/	/	/	/	/	
NM_003839	TNFRSF11A (RANK)	Tumor necrosis factor receptor superfamily, member 11a	Member of the TNF-receptor superfamily; can interact with various TNF-receptor associated factor (TRAF) family proteins, activator of the PI3K-Akt pathway; regulator of T/DC interaction; essential mediator of osteoclast differentiation and activation	2.6	/	/	Down	/	/	(65)
NM_016639	TNFRSF12A (TWEAKR)	Tumor necrosis factor receptor superfamily, member 12A	Member of the TNF-receptor superfamily; activator of the NF-kB and PI3K-Akt signaling pathways; mediator of inflammation and tissue remodeling; plays a role in the pathogenesis of inflammatory and systemic autoimmune diseases	2.5	/	/	Up	/	/	(65)

(Continued)

TABLE 2 | Continued

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
NM_00114531	CX_3CR1	Chemokine (C-X3-C motif) receptor 1	Receptor for fractalkine/CX3CL1; plays a role in NK cell adhesion, egress from BM, and migration to sites of disease mediated by CX3CL1 and CCL26; involved in NK cell cytotoxicity against tumor cells expressing CX3CL1	2.4	up	/	Up	/	/	(45, 65)
NM_033135	PDGFD	Platelet derived growth factor D	Member of the PDGF family; potent pro-tumorigenic and angiogenic factor; stimulates matrix metalloprotease activities and monocyte/macrophage migration; contributes to epithelial-mesenchymal transition (EMT)	2.2	/	/	/	/	/	
NM_031950	FGFBP2	Fibroblast growth factor binding protein 2	Member of the FGF binding protein family selectively secreted by cytotoxic lymphocytes and involved in cytotoxic lymphocyte-mediated immunity	2.2	/	/	/	/	/	
Down-regulated										
NM_002309	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	Member of the IL6 family of cytokines; displays pleiotropic effects on various cell types and organs; involved in the induction of myeloid hematopoietic and neuronal cell differentiation; regulator of mesenchymal to epithelial conversion; plays a key anti-inflammatory role in cutaneous inflammation; promotes Treg development and adaptive immune tolerance; involved in immune tolerance at the maternal-fetal interface	0.17	/	/	/	/	/	
NM_153460	IL17RC	Interleukin 17 receptor C	Component of theIL-17R complex with IL-17RA; mediates the functions of both IL-17A and IL-17F proinflammatory cytokines; involved in host defense, innate immunity, tissue remodeling, and acute phase responses; implicated in the progression of inflammatory and autoimmune diseases; enhances IFNg secretion by NK cells	0.19	/	/	/	/	/	
NM_003701.3	TNFSF11 (RANKL)	Tumor necrosis factor (ligand) superfamily, member 11	Member of the TNF cytokine family; functions as a key factor for osteoclast differentiation and activation; involved in the regulation of T cell immune response and DC survival; may have a role in the regulation of apoptosis; involved in oncogenesis, tumor progression, and metastatization	0.21	/	/	/	/	/	
NM_003856	IL1RL1 (IL-33R)	interleukin 1 receptor-like 1	Member of the interleukin 1 receptor family; receptor for IL-33; promotes both Th1 and Th2 immune responses; activates IFNg production from NK cells	0.36	/	/	/	/	/	
NM_006417.4	IFI44	interferon-induced protein 44	Microtubule-associated protein; member of the family of type I interferon-inducible genes; mediator of interferon alpha/beta antiviral and antiproliferative activities	0.36						

(Continued)

TABLE 2 | Continued

Ref Seq RNA	Gene symbol	Full Name	Main function(s) of gene product	Fold Change§	Modulated in*					References
					Monocytes	MDM	iDCs	mDCs	T cells	
NM_006332.4	IFI30	interferon, gamma-inducible protein 30	Gamma-interferon-inducible lysosomal thiol reductase; enhances antigen presentation of a subset of MHC class II-restricted epitopes from disulfide bond-containing antigens.	0.39						
NM_000579	CCR5	chemokine (C-C motif) receptor 5	Member of the beta-chemokine receptor family, binds CCL3, CCL4, CCL5, and CCL8; plays a role in NK cell proliferation and circulation under physiological conditions; mediates recruitment of BM-derived NK cells to sites of inflammation and tumors; enhances NK cell cytolitic activity; co-receptor for macrophage-tropic virus, including HIV	0.44	Down	Down	Up	Up	Up	(45, 46, 64, 66–68)
NM_003810	TNFSF10 (TRAIL)	Tumor necrosis factor (ligand) superfamily, member 10	Member of the TNF ligand family; binds to several members of TNF receptor superfamily; induces apoptosis in transformed and tumor cells triggering activation of MAPK8/JNK, caspase 8, and caspase 3; plays a role as an effector molecule in NK cell tumoricidal activity; implicated in immunosuppressive, immunoregulatory and immune-effector functions; plays a role in immune response to viral infections and tumor immune surveillance	0.49	Down	/	/	/	Down	(45, 46)

^NK cells were cultured under normoxic (20% O2) and hypoxic (1% O2) conditions for 16 and 96 h, and gene expression profiling was then carried out independently by microarray analysis on the RNA purified from three independent preparations. Comparative analysis of gene expression differences between the two experimental conditions was conducted as described in the section Materials and Methods. A GeneBank accession number, a common gene symbol, a full name, a brief description of the gene product main functions, and the fold change value (≥2-fold increase, ≤0.5-fold decrease) are specified for each gene. Genes in each group are ordered by fold change. Those validated by qRT-PCR are underlined.

§Results are expressed as ratio of fold differences between hypoxic and normoxic samples (mean of expression level of three experiments). Genes up/downmodulated by ≥2-fold are shown.

**Comparison of microarray results with data previously obtained in monocytes, monocyte-derived macrophages, immature and mature dendritic cells, and T cells exposed to hypoxia.*

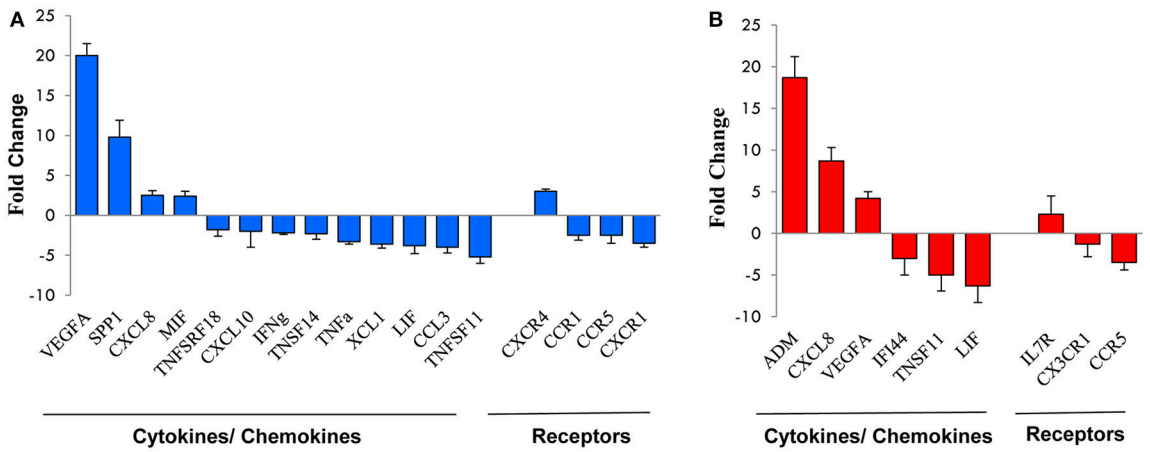

FIGURE 3 | qRT-PCR validation of genes selected from the microarray profile. Total RNA from the NK cell preparations analyzed by microarray was subjected to qRT-PCR for the expression of a subset of genes randomly selected from those up- or down-modulated at 16 h **(A)** or 96 h **(B)**. Expression changes were evaluated in relation to the values obtained for three reference genes, as detailed in the section Materials and Methods. Results are expressed as fold-changes (Hy-NK relative to NK cells) and represent the mean of three determinations for each transcript. Positive values indicate that the mRNA levels of a specific gene was up-regulated, whereas negative values indicate that the transcript was down-regulated. Genes are ordered by fold-change within each group.

conditions, release of IFNγ, CCL3, GM-CSF, and CCL5 was decreased (although differences reached statistical significance only for CCL3 and GM-CSF). Since IL-2 has typically limited direct effects on cytokine release while it primes NK cells to respond to other stimuli, we assessed the effect of hypoxia on NK cells cultured for 20 h with IL-2 and stimulated for further 6 h with PMA + Ionomycin (PMA+IONO). As shown in **Figure 4B**, an inhibitory effect on CCL3 and (slightly) on GM-CSF release was induced by hypoxia also on PMA+IONO-stimulated NK cells.

We next assessed the effect of hypoxia on NK cells cultured with other classical NK-activating stimuli, such as the monokines, IL-12, IL-15, and IL-18. In particular, we set monokine combinations known to potently stimulate NK cell cytokine secretion (i.e., IL-12+IL-18, and IL-15+IL-18). As shown in **Figure 4C**, NK cells cultured in the presence of IL-12 + IL-18 for 20 h released into the culture supernatant very high amounts of IFNγ and moderate to low amounts of CCL3, GM-CSF, TNFα, and CCL5. Hypoxia did not modify significantly NK cell ability to release cytokines in response to IL-12+IL-18, although a trend toward inhibition was observed for CCL3, GM-CSF, and TNFα release. Compared to IL-12+IL-18, IL-15+IL-18 stimulation induced lower release of IFNγ and TNFα and higher secreted levels of CCL3, GM-CSF, and CCL5 under normoxic conditions (**Figure 4D**). Exposure to hypoxic conditions resulted in the significant inhibition of IFNγ, TNFα, CCL3, GM-CSF, and CCL5 release.

We conclude from these data that hypoxia can differently affect cytokine/chemokine release depending on the type of stimulus. Specifically, it can modulate the release of only a few cytokines/chemokines in NK cells cultured in the presence of IL-2 or IL-2+PMA+IONO, exert a more general inhibition of cytokine/chemokine secretion on NK cells exposed to IL-15+IL-18, while it has no significant effects on NK cells exposed to IL-12+IL-18.

Effect of Hypoxia on Chemokine Receptor Expression

We next assessed whether hypoxia could modulate chemokine receptor expression on NK cell surface. To this end, freshly isolated PB-NK cells were cultured in the presence of IL-2 under hypoxic or normoxic conditions and analyzed by flow cytometry for the expression of CCR5, CCR7, CCR1, CX_3CR1, CXCR1, CXCR4, and CXCR3 immediately after isolation or after 24, 48, or 96 h of culture. Upon exposure to IL-2 (under normoxic conditions), NK cells progressively down-regulated expression of CXCR1, up-regulated that of CXCR3 and (transiently) that of CXCR4, while they minimally modified the expression of CCR5, CCR1, and CX_3CR1. Hypoxia further significantly increased the up-regulation of CXCR4 expression, slightly decreased CCR5 expression, while it did not substantially modify the expression trend of CCR1, CX_3CR1, CXCR1, and CXCR3 (**Figures 5A,C**).

The analysis of CCR7 on the whole PB-NK cell population didn't give meaningful data, as CCR7 expression is generally confined to the small fraction of $CD56^{bright}$ cells (12, 17) (**Figures 5A,C**). On the other hand, $CD56^{bright}$ NK cells showed progressive decrease of CCR7 expression during culture with IL-2. Hypoxia significantly reversed such effect sustaining CCR7 expression on $CD56^{bright}$ cells (**Figures 5B,C**). Remarkably, a careful analysis of such NK cell subset revealed that also CXCR4 expression could be sustained by hypoxia in $CD56^{bright}$ cells (**Figures 5B,C**).

Effects of Hypoxia on NK Cell Chemotaxis

Experiments were then carried out to assess whether hypoxia-induced changes of CCR7 and CXCR4 expression could affect specific chemotactic activity of NK cells. To this end, NK

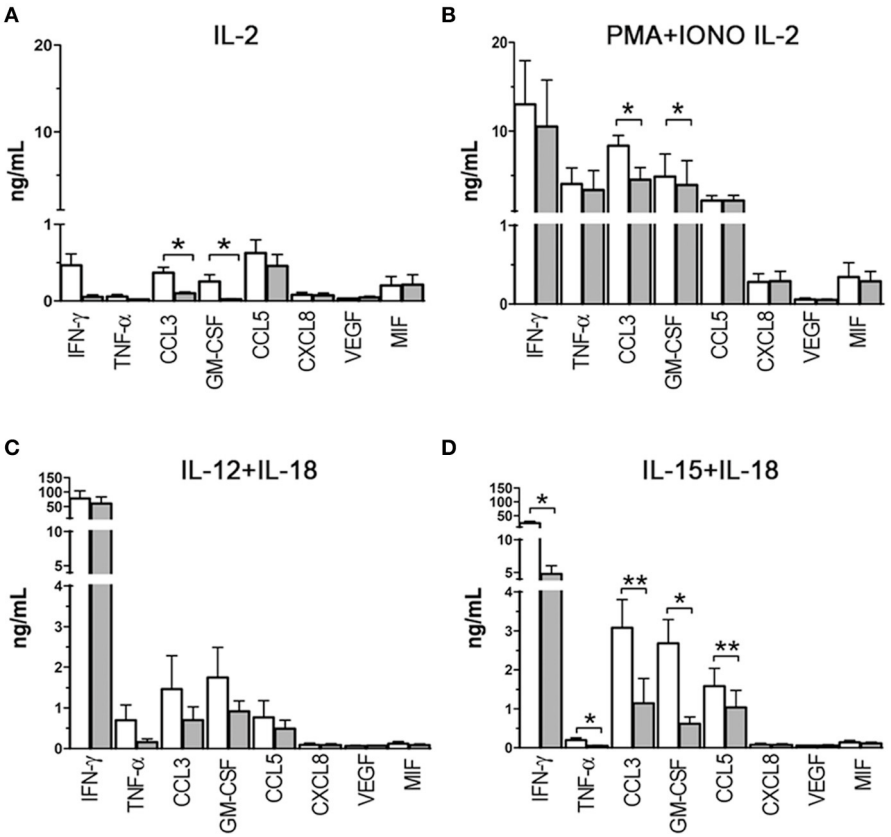

FIGURE 4 | Cytokine/chemokine release capability of NK cells exposed to hypoxia or normoxia. Cell-free supernatants from NK cells cultured in the presence of IL-2 **(A)**, IL-2 and PMA+ionomycin **(B)**, IL-12+IL-18 **(C)**, IL-15+IL-18 **(D)** under normoxic (white columns) or hypoxic (gray columns) conditions were analyzed for 8 cytokine/chemokine content using the MAGPIX® System. Results are expressed as ng/ml and are the mean + SEM of 5 independent experiments. In **(A,C,D)**, the cells were cultured for 20 h in the presence of the indicated stimulating cytokines, in **(B)**, the cells were cultured for 26 h in the presence of IL-2 with the addition of PMA+ionomycin in the latter 6 h. * $p < 0.05$, ** $p < 0.01$.

cells cultured under normoxic or hypoxic conditions for 24, 48, and 96 h were analyzed in classical migration assays using CCL19, CCL21 (CCR7 ligands) and CXCL12 (CXCR4 ligand) as chemoattractants.

Given the peculiar distribution of CCR7 within the PB NK cells, we analyzed whether CCL19 or CCL21 could induce the preferential migration of the $CD56^{bright}CD16^{dim/neg}$ cell subset, resulting in the enrichment of such population within migrated cells. Before performing this analysis, we evaluated by FACS whether the percentage of $CD56^{bright}CD16^{dim/neg}$ cells could be modified over time under hypoxic or normoxic culture conditions. As shown in **Figure 6A** the percentage of $CD56^{bright}$ cells slightly decreased during culture under normoxic conditions, while hypoxia preserved such a population at the 24 and 48 h time points. This observation suggests that hypoxia could contribute to increase the absolute number of migrated $CD56^{bright}$ cells by preserving them over time. In order to selectively evaluate the effect of hypoxia on specific chemotactic properties of the cells, the chemotactic response to chemokines was calculated as ratio of the $CD56^{bright}CD16^{dim/neg}$ cell percentages within cells migrated to specific chemokines or spontaneously. Among NK cells that have been cultured under normoxic conditions for 24 h, $CD56^{bright}$ cells were able to migrate in response to both chemokines and enrich the population of migrated cells. However, this ability progressively disappeared at later culture time points. By contrast, Hy-NK cells gave rise to higher enrichment of $CD56^{bright}$ cells within cells that migrated in response to CCL19/21 and maintained this capability over time (**Figures 6B,C**).

The analysis of CXCR4-dependent chemotaxis indicated that, overall, NK cells exposed to hypoxia were responsive to CXCL12 more than NK cells cultured under normoxic conditions (**Figure 6D**). Remarkably, this difference was more pronounced when considering the $CD56^{bright}$ cell subset. Indeed, "hypoxic" (but not "normoxic") NK cells gave rise to enrichment of the $CD56^{bright}$ cells within cells migrated to CXCL12 (**Figures 6E,F**). These results were in line with the observation that under hypoxia all $CD56^{bright}$ cells expressed CXCR4 at high levels while $CD56^{dim}$ NK cells included a variable fraction of CXCR4neg cells (**Figure 5C**).

Overall, these data demonstrate that hypoxia can sustain CXCR4- and CCR7-dependent chemotactic response of NK cells

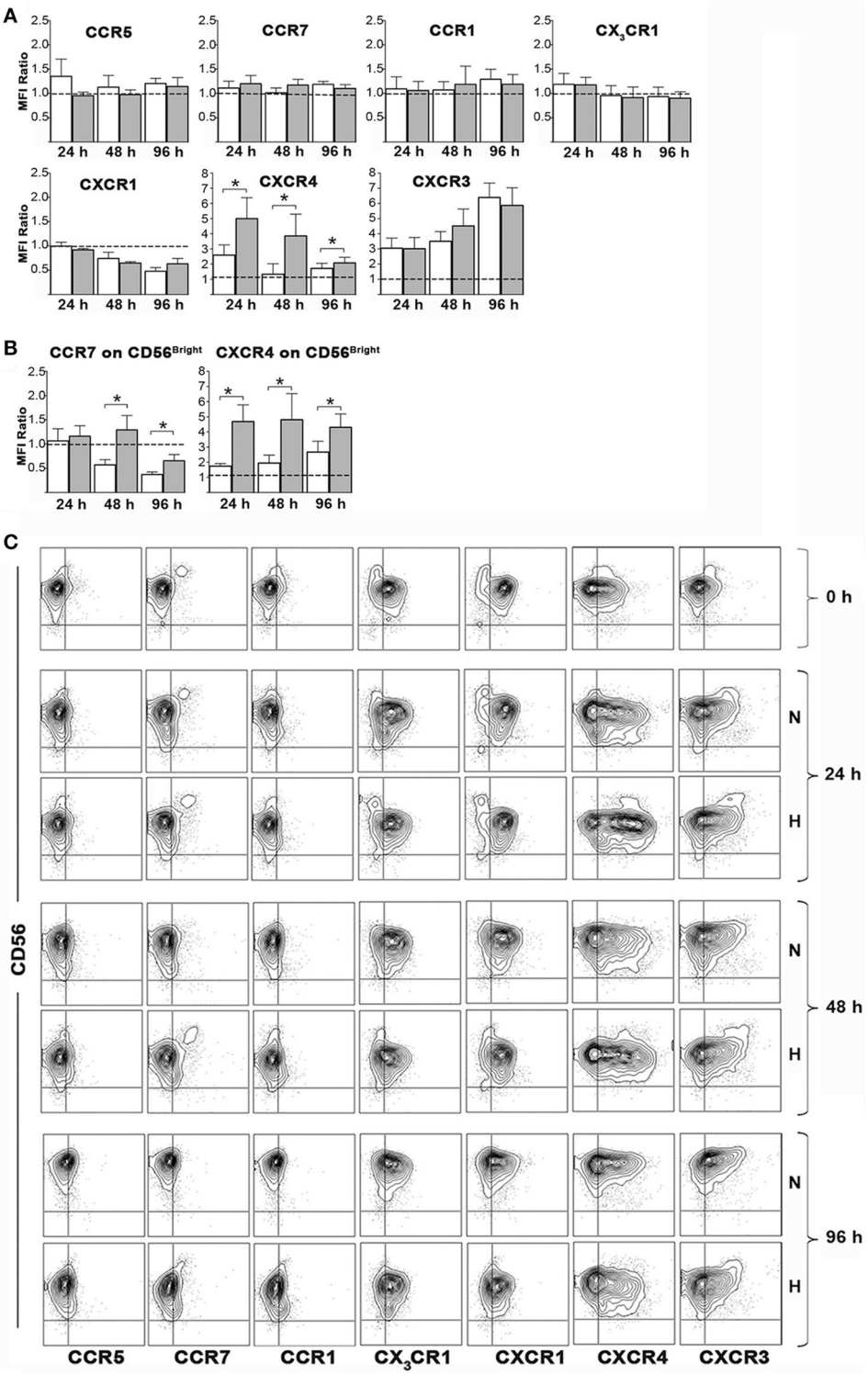

FIGURE 5 | Effects of hypoxia on NK cell chemokine receptor expression**.** Freshly isolated PB-NK cells were cultured in the presence of IL-2 for 24, 48, or 96 h under normoxic or hypoxic conditions and then analyzed by flow cytometry for surface expression of the indicated receptors. **(A,B)** The ratio between the MFI observed at the indicated culture time points and that at t0 (i.e., on freshly isolated cells) is reported for each receptor. Horizontal dotted lines indicate no changes. White and gray bars are referred to NK cells cultured under normoxic or hypoxic conditions, respectively. Data are the mean + SEM of 5 independent experiments. In **(B)** data on $CD56^{bright}$ gated cells are reported for CCR7 and CXCR4 expression. **(C)** FACS profiles of a representative donor are shown. * $p < 0.05$.

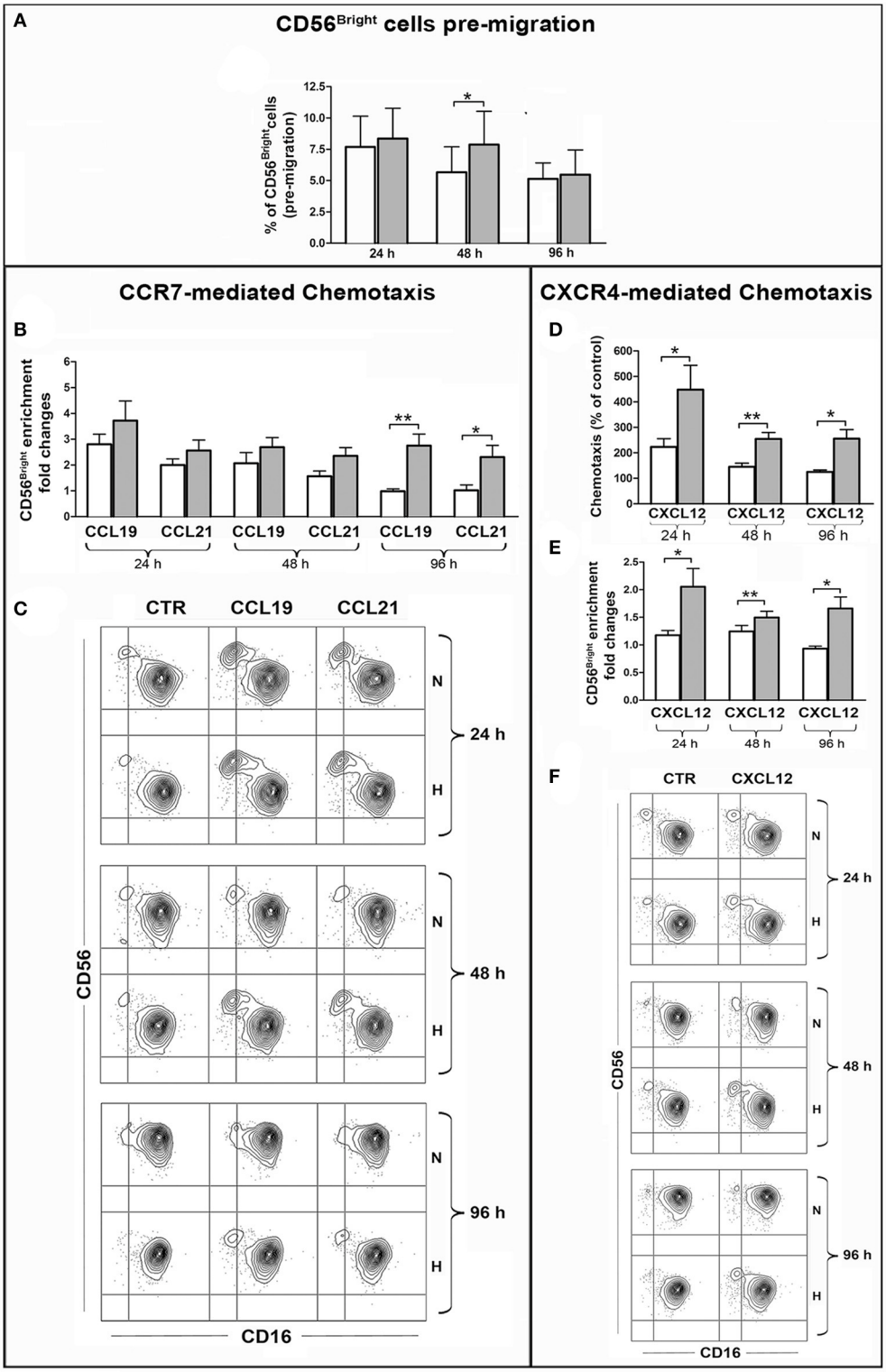

FIGURE 6 | Effect of hypoxia on chemotaxis of PB-NK cells and their $CD56^{bright}/CD56^{dim}$ subset to CXCL12, CCL19, CCL21. PB-NK cells were cultured in the presence of IL-2 for 24, 48, or 96 h under normoxic or hypoxic conditions and analyzed by FACS for the combined expression of CD56 and CD16 markers in order to assess the percentage of the $CD56^{bright}CD16^{dim/neg}$ cells before migration **(A)**. Cells were then assessed for chemotaxis to the indicated chemokines under normoxic conditions for 2 h. Migrated cells were collected from the lower migration chamber compartments and counted or analyzed by FACS for the combined

(Continued)

FIGURE 6 | expression of CD56 and CD16 markers. The specific chemotactic response of $CD56^{bright}CD16^{dim/neg}$ NK cells to CCL19, CCL21 **(B)**, and CXCL12 **(E)** was assessed as enrichment of this cell subset within migrated cells. The enrichment was calculated as fold increase of the $CD56^{bright}CD16^{dim/neg}$ cell percentage within cells migrated to specific chemokines as compared to the $CD56^{bright}CD16^{dim/neg}$ cell percentage within spontaneously migrated cells (see section Materials and Methods for details). **(C,F)** Representative experiments showing the enrichment of $CD56^{bright}CD16^{dim/neg}$ NK cells within cells migrated in response to CCL19, CCL21 **(C)** or CXCL12 **(F)** as compared to cells that spontaneously migrated in the lower compartment in the absence of stimuli (CTR). **(D)** Specific chemotactic response to CXCL12 of the whole PB-NK cell population cultured under normoxic or hypoxic conditions. In **(B,D,E)** white and gray bars indicate data from NK cells cultured under normoxic or hypoxic conditions, respectively and represent the mean ± SEM of 6 independent experiments. * $p < 0.05$, ** $p < 0.01$.

to specific chemokines, such as CXCL12, CCL19, or CCL21, and favor the recruitment of $CD56^{bright}$ cells, suggesting that a hypoxic environment may influence the extent and the nature of the NK cell infiltrate in different types of tumors.

DISCUSSION

In the present study we analyze the global effects of hypoxia on NK cells, which are among the most potent immune effectors available to the host for the control of tumor development and progression (1, 2, 6). We first provide the transcriptional overview of the response of IL-2-primed NK cells to short-term (16 h) and prolonged (96 h) hypoxia, demonstrating that Hy-NK cells are functionally reprogrammed through the differential expression of a large number of genes implicated in various aspects of NK cell biology, including immunoregulation and migration. Then, we document hypoxia influence on the chemotactic properties of specific NK cell subsets and on NK cell ability to release cytokines and chemokines, providing important clues on the effective role of the O_2 tension in determining the composition and the function of the NK cell infiltrate in tumor lesions.

So far, one transcriptional study describing how hypoxia could influence the cytokine-mediated activation of NK cells has been done for IL-15, while no data were available on IL-2, although this factor represents the most known and studied priming cytokine for NK cells. As assessed by GSEA, gene expression changes observed upon NK cell exposure to 1% O_2 conditions follow a consensus hypoxia transcriptional profile. Several hypoxia-related and HIF-1α/HIF-2α target gene sets defined in previous studies are, in fact, significantly enriched in both the 16 and the 96 h Hy-NK transcriptomes. Moreover, we find enrichment of genes involved in glycolysis, gluconeogenesis, glucose transport, non-glycolytic metabolism, and ion transport, which is a common feature of hypoxic cells of different type, origin, and functional state being essential to compensate for the inhibition of oxidative metabolism and the malfunctioning of O_2-dependent enzymes occurring under conditions of reduced oxygenation (32, 34, 47–49).

GO clustering of HMGs in NK cells indicates that hypoxia can modulate several biological processes and suggests that NK cells reaching hypoxic tumor areas may deeply change their mode to respond to stimuli or exert their functions. Processes related to metabolism and biosynthesis, response to stimuli, regulation of apoptosis and response to stress, cell proliferation, and signaling appear to be all affected by hypoxia suggesting that NK cells may modulate a wide range of functions in a hypoxic environment. In particular, the coordinated enrichment of down- or up-regulated genes in specific processes, such as cell cycle, DNA replication and repair, cellular component organization, regulation of gene transcription and expression, indicates that NK cells can moderate their biosynthetic and proliferative capabilities in response to decreased O_2 tension.

Noteworthily, among HMGs we identified a significant cluster of immune-related genes, 43 of which coding for cytokines, chemokines, and their receptors. Several of these cytokine/chemokine-coding genes have not been previously reported to be affected by hypoxia in NK cells, although some of them are known from the literature to be modulated in other immune cells either exposed to short-term hypoxia (typically 8–24 h) (48, 49, 65, 66, 70, 71) or generated under conditions of long-term hypoxia (37, 38, 47, 67–69). On the other hand, some genes appear to be modulated uniquely in NK cells, as they have never been characterized in terms of responsiveness to hypoxia in other immune cells. These findings indicate that hypoxia regulates the expression of genes coding for cytokines/chemokines on different immune cell populations, but it can also activate a distinct transcriptional profile in NK cells.

The data on immune-related HMGs give some hints on how NK cells may be functionally skewed in a hypoxic microenvironment. The early downregulation of genes coding for IFNγ and for several members of the TNF family, such as TNFα, LTA, LTB, TNFSF14, TNFSF10, and TNFSF11, is of particular interest given the role of these molecules in triggering tumor immunogenicity, decreasing tumor proliferation and angiogenesis, and favoring apoptotic tumor cell killing. Likewise, the downregulation of genes coding for TNFRSF18 and IL1RL1 may be crucial, as these molecules act by enhancing IFNγ secretion and potentiating NK cell expansion and response to tumors (62, 63). Consistently, hypoxia also induces the up-regulation of genes coding for important proangiogenic, protumorigenic, prometastatic, and/or immune suppressive factors, namely VEGFA,B, SPP1,2, CXCL8, MIF, TGFβ2, and PDGFD (11, 34, 37, 48, 50–54). Finally, it is remarkable the modulation of genes coding for chemokines and chemokine receptors.

Collectively, our gene expression analysis suggests a major effect of hypoxia on the immunomodulatory functions and the chemotactic properties of NK cells. These aspects are not trivial, as the nature and the function of the NK cell infiltrate at the tumor site, as well as the tissue distribution of specific NK cell subsets, can influence the prognosis of different tumor types (11, 12, 28, 29). For this reason we decided to investigate in detail the effect of hypoxia on these specific functions of NK cells: cytokine/chemokine release and migration to specific stimuli.

Multiplex ELISA analysis of NK cell culture supernatants partly confirmed the indications obtained by the gene chip analysis, showing that, indeed, hypoxia can limit the ability of NK cells to release different factors involved in the host response to the tumor, such as IFNγ, TNFα, GM-CSF, CCL3, and CCL5. These factors are endowed with antitumor activity and/or can induce recruitment, differentiation, proliferation, and activation of APCs, Th1 lymphocytes, and NK cells (9, 11, 72, 73). Hypoxia appears to variably affect cytokine release, depending on the type of NK cell stimulation. Indeed, its inhibitory effect is particularly evident on NK cells exposed to the monokine combination, IL-15 + IL-18, while it does not reach statistical significance in case of IL-12 + IL18 stimulation. We couldn't find any transcriptional modulation of genes coding for IL-12, IL-15, IL-18, or IL-2 receptor subunits, suggesting that the differential effect of hypoxia on the various stimuli may involve other mechanisms such as the interference with specific signaling pathways.

That hypoxia could differentially modulate NK cells under different monokine stimuli is important both because stimulatory monokines can be released by immune cells in inflamed tissues and also in view of the recent lines of research aimed at the definition of effective monokine combinations in NK-based immunotherapy (2, 17, 74). Notably, the hypoxia-related factors CXCL8, VEGF, and MIF were not (or poorly) released by both "normoxic" and "hypoxic" NK cells, although they were induced by hypoxia at the mRNA level. This discrepancy suggests that target-specific translational regulation (49, 67) can shape NK cell response to hypoxia, giving rise to unique functional profiles. The fact that hypoxia fails to induce CXCL8, VEGF, and MIF secretion by NK cells has also been reported in a recent study by Velasquez et al. (41). In that study, however, in contrast to our present data, exposure to hypoxia could induce little, but significant, secretion of CCL3, CCL4, and CCL5. The discrepancy between our and their results may probably be ascribed to the different experimental protocols used, in particular with regard to the priming cytokines (IL-2, IL-12+IL-18, or IL-15+IL-18 in our study vs. IL-15 alone in that of Velasquez) and the time and duration of priming (the whole 20 h culture period in our study vs. the final 6 h culture in the Velasquez study).

Evaluation of chemokine receptor surface expression reveals that hypoxia can significantly increase the expression of CXCR4 receptor on a large fraction of PB-NK cells, suggesting that changes in the levels of O_2 tension within tissues may significantly influence NK cell trafficking (11). The CXCL12-CXCR4 axis represents one of the mechanisms responsible for tumor spread, driven by pro-metastatic CXCR4+ tumor cells. In addition, CXCL12 expressed by Tumor Associated Fibroblasts (TAF) and tumor cells has been demonstrated to play an important role in favoring tumor growth and progression in primary lesions. Thus, sustained CXCR4 expression in NK cells may be important for reaching and infiltrating certain metastatic niches (for example in the bones) and also primary tumors. In this context, in a model of NKp46-targeted HIF1α KO mice, it has been recently shown that NK cells can reach hypoxic tumor tissues, influence angiogenesis, tumor growth, and metastasis spread in a HIF1α-dependent fashion (75). Remarkably, our data indicate that, in humans, hypoxia can differently affect two functionally distinct NK cell subsets. We observed that hypoxia-induced CXCR4 up-regulation involved the whole $CD56^{bright}$ NK cell population, while it affected only a fraction (even if large) of $CD56^{dim}$ cells. Accordingly, hypoxic NK cells that migrated to CXCL12 showed an enrichment of such $CD56^{bright}$ cell subset. Along this line, hypoxia also increased CCR7 expression on $CD56^{bright}$ cells, enhancing their selective migration in response to CCL19 and CCL21. The CCL19/21-CCR7 axis drives metastatic spread to Lymph Nodes but also promotes homing of specific leukocyte subsets. In addition, the CCL21-CCR7 interaction may be effective at the tumor site (76, 77). Overall, our data suggest that hypoxia can intervene in the recruitment of specific NK cell subsets at the site of both primary tumor and metastasis and offer new hints to explain the relative high frequencies of poorly cytotoxic $CD56^{bright}$ cells observed within the NK cell infiltrate of several tumors (29–31). Of course these findings, although suggestive, must be considered within the rich network of factors that regulates lymphocyte trafficking in different tumor sites. As an example, it has been recently described the complex correlation between the chemokine receptor pattern of different T lymphocyte subsets and the control of metastasis in specific sites (78). Also NK cells can respond to multiple chemokines that can be variably released in different tumor sites. In this context, it is worth-noting that NK cells can amplify their recruitment at the tumor site by killing tumor cells and inducing release of chemiotactic HMGB1 (79).

Various escape mechanisms induced by the tumor hypoxic environment have been documented in the last years, most relying on the suppression of different immune cell types, others involving the editing of the tumor cell targets or the tumor microenvironment (23, 33, 38, 47, 80–85). However, only few studies were focused on NK cells. This report represents the first comprehensive transcriptome analysis of human Hy-NK cells, which defines a wide array of hypoxia-modulated immunological genes. Remarkably, our study also describes how hypoxia can influence the type and function of NK cells reaching hypoxic tissues, thus providing new elements useful to design improved NK cell-based immunotherapeutic strategies.

ETHICS STATEMENT

This study was carried out following approved operational procedures of the Ethics Committee of the IRCCS Ospedale Policlinico San Martino (IOH78). Written informed consent was obtained from all the donors in adherence with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MP and FR conducted the experiments, assembled and analyzed data, and participated in MS writing. DC provided statistical analysis of microarray data and performed GSEA analysis. CM, MB, and FB provided experimental support and helped in the analysis of data. AE revised the manuscript. LV, GP, LM, and MM revised the manuscript and provided financial support. MV and

MCB conceived the study, designed experiments, interpreted the data, wrote the manuscript, and provided financial support.

FUNDING

This work was supported by Associazione Italiana Ricerca sul Cancro AIRC under grants: IG 2014 project n. 15428 (MV), IG 2014 project n. 15283 (LM), and IG 2017—Project n. 19920 (LM); PRA 2013 and FRA 2015, DIMES, University of Genoa (GP); 5 × 1000 Min. Sal. 2013 (MV and MM); by the Italian Ministry of Health (MCB); and by Ricerca Corrente 2018 (MCB). MP was recipient of the AIRC fellowship n. 18274, year 2016.

ACKNOWLEDGMENTS

The authors thank Dr. Marzia Ognibene for her valuable support in the carry out of NK cell culture under hypoxic conditions and Dt. Massimo Acquaviva for his support in the statistical analysis of data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02358/full#supplementary-material

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

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frontiers in Immunology

METHODS
published: 30 April 2019
doi: 10.3389/fimmu.2019.00957

Human CAR NK Cells: A New Non-viral Method Allowing High Efficient Transfection and Strong Tumor Cell Killing

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CAR-NK cells may represent a valuable tool, complementary to CAR-T cells, in adoptive immunotherapy of leukemia and solid tumors. However, gene transfer to human NK cells is a challenging task, particularly with non-virus-based techniques. Here, we describe a new procedure allowing efficient electroporation-based transfection of plasmid DNA, including CAR and CCR7 genes, in resting or cytokine-expanded human NK cell populations and NK-92 cell line. This procedure may offer a suitable platform for a safe and effective use of CAR-NK cells in adoptive immunotherapy of cancer.

Keywords: Chimeric Antigen Receptors, chemokine receptors, NK cells, adoptive immunotherapy, electroporation

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Karl-Johan Malmberg, Oslo University Hospital, Norway
Francisco Borrego, BioCruces Health research Institute, Spain

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *03 December 2018*
Accepted: *15 April 2019*
Published: *30 April 2019*

Citation:
Ingegnere T, Mariotti FR, Pelosi A, Quintarelli C, De Angelis B, Tumino N, Besi F, Cantoni C, Locatelli F, Vacca P and Moretta L (2019) Human CAR NK Cells: A New Non-viral Method Allowing High Efficient Transfection and Strong Tumor Cell Killing. Front. Immunol. 10:957. doi: 10.3389/fimmu.2019.00957

INTRODUCTION

Cell-mediated immune responses play a central role in the control of infections and tumor growth. In particular, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are important effectors against solid tumors and leukemias (1, 2). In this context, both T and NK cells are known to play a fundamental role in clearing tumor cells in patients with hematologic malignancies receiving allogeneic hematopoietic stem cell transplantation (allo-HSCT) (3). Another important cell-based immunotherapy is the use of autologous T cells that have been genetically engineered with Chimeric Antigen Receptors (CAR-T cells) specific for tumor antigens. A substantial benefit of this therapeutic approach has been shown by clinical trials, primarily in patients with lymphoid malignancies refractory to chemotherapy or relapsing after allo-HSCT (4–6). However, clinical trials employing CAR-T cell in solid tumors have not been so successful (7) mainly due to the suppressive nature of the tumor microenvironment (8).

Recent studies indicated that also NK cells may be genetically engineered with CAR (9–12). Notably, CAR-NK cells retain the expression of their activating and inhibitory receptors. Thus, different from CAR-T cells, CAR-NK cells can still exert their "natural" anti-leukemia effect (9) in case the tumor antigen targeted by CAR is downregulated (13). In addition, given their different homing properties and the different patterns of cytokines/chemokines released upon activation, CAR-NK cells may be complementary to CAR-T cells in tumor therapy and, possibly, safer with respect to clinical implications, including the cytokine storm syndrome and neurotoxicity (14, 15). Moreover, CAR-NK cells may potentially become an off-the-shelf tool, as they do not seem to require a strict autologous HLA matching as T cells do (14).

A successful CAR-based immunotherapy requires an efficient transfer of the CAR transgene into the immune cells. To this purpose, both viral transduction and non-viral transfection methods in T and NK cells have been attempted. While the use of CAR-NK cells may offer potential advantages, their transfection is considerably less efficient as compared to T cell transfection (16). Recent improvements in viral transduction technology renewed the interest in developing strategies aimed at potentiating NK cell activity through genetic engineering (16–18). However, viral transduction requires dedicated facilities, high costs and a complex preparation. Recently, electroporation of mRNA has been proposed as an alternative method to viral transduction although the short-time expression of the transgene may represent a major limitation (19–21).

In the present study, we developed a new (virus-free) protocol for NK cell electroporation using plasmid DNA that allows a major improvement both in the transfection efficiency and in cell viability. We could successfully transfect different reporter genes (such as EGFP, YFP, Azuride) and functional genes (an anti-CD19 CAR and a chemokine receptor, the CCR7 gene). Our new protocol allows a safer and efficient way to genetically manipulate NK cells, thus offering a novel valuable tool for cancer immunotherapy.

MATERIALS AND METHODS

Human Samples

This study included 18 buffy coats collected from volunteer blood donors admitted to the blood transfusion service of IRCCS Bambino Gesù Pediatric Hospital after obtaining informed consent. The Ethical Committee of IRCCS Bambino Gesù Pediatric Hospital approved the study (825/2014) and conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

Cells Lines and Cell Culture

NK-92 (malignant non-Hodgkin's lymphoma), K562 (chronic myelogenous leukemia, $CD19^-$), Jurkat (acute T cell leukemia, $CD19^-$) Karpas 299 (Human Non-Hodgkin's Ki-positive Large Cell Lymphoma, $CD19^-$), Nalm-8 (Lymphoblastic Leukemia, $CD19^+$) Raji (Burkitt's Lymphoma, $CD19^+$), and DAUDI (Burkitt's Lymphoma, $CD19^+$) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). K562, Karpas 299, Jurkat, Nalm-8, Raji and Daudi cells were cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 1% penicillin-streptomycin-neomycin mixture and 10% heat-inactivated Fetal Calf Serum. For NK-92 20% of FCS and 50 ng/ml of recombinant human IL-2 (Proleukin; Chiron Therapeutics, Emeryville, CA) were added at the culture medium.

Peripheral blood mononuclear cell (PBMC) were obtained from buffy coats after density gradient centrifugation over Ficoll Lympholyte®-H (Cederlane, Burlington, Canada). Highly purified ($\geq$ 98%) NK cells were subsequently obtained by depletion of non-NK cells using the Miltenyi NK cell separation kit (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instruction. To obtain polyclonal activated NK cells, freshly isolated NK cells were cultured on 30 Gy irradiated PBMCs feeder cells in the presence of 600 U/mL recombinant human IL-2 (Proleukin; Chiron Therapeutics, Emeryville, CA) and 1.5 ng/mL phytohemagglutinin (PHA, GIBCO Ltd) for the first week. The culture medium was RPMI 1640 (Euroclone, MI, IT) medium supplemented with 2 mM L-glutamine (Euroclone,MI, IT), 1% penicillin-streptomycin-neomycin mixture (Euroclone,MI, IT), and 10% heat-inactivated Fetal Calf Serum (FCS, Euroclone, MI, IT). Every 3–4 days NK cells were expanded until the right exponential growth phase was reached to perform experiments.

Plasmids

pmaxGFP (3,5 kb; Lonza) was used to setting up the electroporation conditions. The following plasmids were used for the experiments: pEGFP-N1 (BD Biosciences, San Jose, CA) (4,7 kb) and pEGFP-N1-CCR7 (5,5 kb) (22); pLV-Azuride (7,5 kb Addgene plasmid #36086); pCEP4YPet (10,9kb Addgene plasmid #14032). The plasmids carrying the first and second generation CAR were developed by Dr. C. Quintarelli. Both plasmids have been designed to carry the cassette of first or second generation CAR with specificity for the CD19 antigen. Single-chain variable fragment (scFv), derived from a murine antibody of IgG (FMC63) class, was linked to human CD8a hinge-transmembrane domain (CD8aTM) and CD3-ζ to obtain a first generation CAR plasmid or CD8aTM, the costimulatory domains 4-1BB (CD137) and CD3-ζ to obtain a second generation of CAR plasmid. The sequence encoding for a peptide derived from the human phosphoglycoprotein CD34 (ΔCD34) was added as a trackable marker. All plasmids were purified from *Escherichia coli*-transformed cells using EndoFree Plasmid Maxi kit (Quiagen, Hilden, Germany), and re-suspended in endotoxin- free water. Light absorption at 260 nm was used to determine the DNA concentration. Quality of the plasmid purification was assessed by calculating the ratio of light absorption at 260/280 and 260/230 nm.

Cell Electroporation

NK-92 cell line, freshly isolated (resting) or IL-2-activated NK cells were electroporated with the Neon Transfection System (Thermo Fisher Scientific, Waltham, Massachusetts, USA). During the optimization process, a range of conditions for different transfection parameters (DNA concentration, number of cells, pulse settings) was tested. To determine the optimal DNA amount, different plasmid concentrations were tested, ranging from 50 to 200 μg/ml. The Optimal Condition (OC) was 120 μg/ml. For the number of cells, (range from $2*10^7$ to $6*10^7$/ml) the OC was $4*10^7$/ml for both resting and Il-2-expanded NK cells. We also adjusted the electroporation pulse voltage and width. For both the first and the second pulse, a range of voltage (from 1400 to 2300 V for the first pulse and from 500 to 1000 V for the second pulse) was tested and then the three best voltages were used with a range of width (from 10 to 30 ms for the first pulse and from 50 to 300 ms for the second pulse). For the resting NK cells the OC of the first pulse resulted 2050 V and 20 ms followed by a second pulse of 500 V and 100 ms. We found that the OC for the Il-2-expanded NK were a first pulse of 1850 V and 20 ms and a second one of 500 V and 100 ms. For NK-92 cell

line, we used a first pulse of 1650 V and 20 ms and a second one of 500 V and 100 ms. For efficient electroporation of NK-92 and IL-2-expanded NK cells, different electroporation buffers were tested. In particular, different amount of: DMSO (Sigma-Aldrich, St. Louis, USA) (from 0.01 to 10%), sucrose (Sigma-Aldrich, St. Louis, USA) (from 10 to 200 nM), magnesium (Sigma-Aldrich, St. Louis, USA) (from 0.1 mM to 20 mM) and dextran (Sigma-Aldrich, St. Louis, USA) (from 2.5 μg/ml to 10 μg/ml) were diluted in Optimem medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA, henceforth mentioned as buffer O). The components were added individually or in a combination of two or three to buffer O. For the pre-electroporation step, the same buffers were used for a washing step of 5 min at 300 g before electroporation. Buffer O with the 0,1% of buffer CD was the OC. All steps were performed at room temperature. After electroporation, cells were grown on a 96-well round bottom plate in 100 μl of RPMI 1640 medium supplemented with 2 mM l-glutamine, 600 U/mL recombinant human IL-2 and 20% heat-inactivated FCS. Viability and transfection efficiencies were tested 24 h after electroporation. After the electrotransfer, cells were analyzed by flow cytometry on a Cytoflex S (Beckman Coulter, Brea, CA, USA) flow cytometer to evaluate the levels of GFP expression. The number of living cells was evaluated as PI or DAPI (Sigma-Aldrich, St. Louis, USA) negative. The percentage of surviving cells was determined 24 h after electroporation and expressed as a percentage of the number of cells counted in the control. Unless otherwise specified, the percentage of GFP-positive cells reported in the figures was calculated as the percentage of surviving cells expressing GFP.

Migration Assay

Chemotaxis of NK cells was measured by migration through a polycarbonate filter with a 3.0-μm pore size in 24-well transwell chambers (Corning Costar). The assay medium consisted of RPMI 10% FCS. Five hundred microliter of assay medium, containing 250 ng/mL of CCL19 and CCL21 (PeproTech, London, United Kingdom), were added to the lower chamber. Five hundred microliter of assay medium with no addiction was used as a control for spontaneous migration. Then, 5×10^5 NK cells, electroporated with pEGFP-N1-CCR7 or with the empty vector (pEGFP-N1), were added to the upper chamber in a total volume of 350 μL of RPMI 10% FCS. After 3-h incubation at 37°C, NK cells migrated to the bottom chamber were counted by optical microscopy (Leica Microscopy, Wetzlar, Germany) with a 25 × objective. The number of spontaneously migrated cells was subtracted from the total number of migrated cells. Values are given as the chemotactic index compared to the migration of unstimulated NK cells. As further confirmation, migrated cells were also counted by flow cytometry (absolute count) (Cytoflex S, Beckman Coulter, Brea, CA, USA).

Analysis of Cytotoxic Activity

In all experiments NK-cell cytotoxicity was analyzed by incubating of the indicated NK-cell transfectant with the target cell lines at an effector-to-target (E:T) ratio ranging from 10:1 to 0,25:1. Cytotoxicity was assessed using a flow cytometric assay for NK-cell killing developed by McGinnes (23) modified as follow: target cells were stained with 5 μM Cell Tracker Green (CMFDA, Invitrogen, Thermo Fisher Scientific), incubated with NK cells at 37°C for 4 h and then propidium iodide (Sigma-Aldrich, St. Louis, USA) was added. Live target cells were identified as $CMFDA^+$ PI^- whereas dead target cells (Td) were $CMFDA^+$ PI^+. Specific lysis was calculated as Td of target cells cultured with effector cells – Td of target cells cultured without effector cells.

Flow Cytometry Analysis and Measurement of Cell Viability

To verify the purity of NK separation, NK cells were stained with anti-CD56-PC7 (Beckman Coulter, Brea, CA, USA, Clone N901 NKH-1) anti-CD3-ECD (Beckman Coulter, Clone UCHT1) anti-CD14-ECD (Beckman Coulter, Clone RMO52) anti-CD19-ECD (Beckman Coulter, Clone J3-119) antibodies. To study the expression of NK receptors after electroporation, NK cells were stained with antibodies specific for NKG2a PE (Beckman Coulter, Clone Z199); CD158 a/h (Clone EB6B),CD158b1/b2/j (Clone GL183),CD158e1/e2 (Clone Z27.3.7) APC (Beckman Coulter); NKp46 (Clone BAB281), NKp44 (Clone Z231), NKp30 (Clone Z25) PE (Beckman Coulter); NKG2D PE/Dazzle594 (Biolegend, San Diego, CA, Clone 1D11); DNAM1 APC (Biolegend, Clone 11A8); Perforin PE (Biolegend, Clone dG9); CD11a vioblue (Miltenyi Biotech, Bergisch Gladbach, Germany, Clone REA378) NKG2c viobright FITC (Miltenyi Biotech, Clone REA205) CD57 vioblue (Miltenyi Biotech, Clone TB03) CD34-APC APC (R&D Systems, clone QBend10). For detection of surface markers, NK cells were incubated for 20 min at 4°C. For detection of intracellular markers, NK cells were treated with the BD Cytofix/cytoperm kit (BD Biosciences, Erembodegem, Belgium) according to manufacturer's protocols. NK cell samples were acquired using the Beckman Coulter Cytoflex S flow cytometer and analyzed with the CytExpert 2.3 or Kaluza software 2.1 (Beckman Coulter, Brea, CA, USA). For cell sorting, electroporated $CCR7\text{-}GFP^+$ NK cells were sorted to a purity of ≥ 98% with the MoFlo Astrios sorter (Beckman Coulter) using the Summit software (Beckman Coulter).

Intracellular Analysis of IFN-γ Production

IFN-γ expression was analyzed by incubating of the indicated NK-cell transfectant with the K562 cell line in 1:1 ratio for 4 h. Intracellular eFluor 450 anti-human IFN-γ (Invitrogen, Thermo Fisher Scientific Clone 4S.B3.) staining was then performed using a Fix & Perm Cell Permeabilization Kit (Invitrogen) following the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 6.0 (La Jolla, CA, USA) software. Values were expressed as mean ± SD. *P*-values were calculated with Wilcoxon test. For multiple comparison analysis Bonferroni correction was applied. $P < 0.05$ were considered statistically significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

Optimization of the Electroporation Protocol for an Efficient Transfection of NK Cells

A first set of experiments was aimed at increasing the efficiency of NK cell transfection using a plasmid encoding the GFP reporter gene (pmaxGFP). In order to optimize an electroporation protocol for freshly isolated (referred to as "resting") NK cells, we started by modifying classical parameters (**Figure 1A**) including numbers of cells per reaction, voltage, number of pulses applied and concentration of plasmid DNA taking advantage of the Neon™ Transfection System (Thermo Fisher Scientific). **Supplementary Figure 1A** shows the optimal protocols after each optimization step.

Although the quantity of DNA has a small impact on the overall efficiency and no significant effects on cells viability (**Figure 1B** upper panel protocol #2), testing a range of concentrations spanning from 50 to 200 μg/ml, the best results were achieved with 120 μg/ml of DNA. Subsequently, we decided to investigate the effect of cell numbers on NK cells transfection efficiency. Notably, the number of cells used in each reaction was extremely important (**Figure 1B** upper panel, protocol#3) with the optimal condition (OC) being $4*10^7$ cells/ml (in a range from $2*10^7$ to $6*10^7$ cells /ml). Indeed, a lower cell number resulted in a reduced viability, whereas a higher cell number was associated with a decrease in transfection efficiency, even with scaled amount of DNA (data not shown). Notably, the optimization of cell number led to a 10-fold increase of cell viability and to a 3-fold increase of transfection efficiency (**Figure 1B** upper panel, protocol #3).

Pulse voltage and width were the other parameters considered. One of the best approach for difficult-to-electroporate cells is the application of two pulses (24). The first one at high voltage and short width, that induces the openings of the cell membrane pores, and the second one, at low voltage but with long width, that drives the DNA into the cells trough the pores on the cell membrane. For both pulses, we first analyzed a range of voltage (from 1400 to 2300 V for the first one and from 500 to 1000 V for the second one) and then the 3 best voltages were further tested with a range of width (from 10 to 30 ms for the first pulse and from 50 to 300 ms for the second pulse). Our data show that OC for the first pulse was 2050 V for 20 ms followed by a second pulse of 500 V for 100 ms (**Supplementary Figure 1A**).

As summarized in **Table 1**, we were able to define optimal conditions for efficient transfection of resting NK cells (4×10^6 cells/ml and 120 μg/ml DNA, applying a first pulse of 2050 V for 20 ms immediately followed by a second pulse of 500 V for 100 ms). This procedure allows reaching ∼50% of cell viability and ∼50% of transfection efficiency (**Figure 1C** left panel). These results represent a major improvement in NK cell transfection. Indeed, using this protocol we were able to obtain a 5-fold higher efficiency, as compared to the other procedures described so far (17).

In-vitro expanded NK cells are widely used in clinical trials due to their stronger anti-tumor cytolytic activity as compared to resting NK cells.

Thus, we attempted to apply the same optimization steps to improve the transfection of IL-2-expanded NK cells. However, as reported in **Figure 1B** (lower panel), neither cell viability nor transfection efficiency resulted satisfactory (14 and 20%, respectively, protocol #5). Previous reports have shown that the addition of different compounds in the electroporation buffer affected both cell viability and electroporation efficiency (25–27). Therefore, to improve the transfection efficiency of IL-2-expanded NK cells, different buffers were analyzed for electroporation. DMSO (from 0.01 to 10%), sucrose (from 10 to 200 nM), magnesium (From 0.1 to 20 mM) and dextran (From 2.5 to 10 μg/ml) were diluted, at different concentration, in Optimem medium (henceforth mentioned as buffer O). The components were added individually, or in a combination of two or three, to buffer O. The buffer O on its own demonstrated to be beneficial for both transfection efficiency and cell viability (**Figure 1B** lower panel protocol #6), but not in any other combination (data not shown).

In addition, we assessed the effects of these buffers in the washing step that is a critical point for transfection efficiency, as highlighted by the manufacturer. Although Buffer OD (0.01% DMSO in buffer O) improved the efficiency, on the other hand it determined a decrease in cell viability (**Figure 1B** lower panel protocol #7). With Buffer OD we also observed a higher variability in NK cell transfection efficiency among the different donors analyzed. A basis to understanding the high variability among donors is the finding that DMSO stabilizes the pores induced by an electric field preferentially in the presence of a fixed amount of cholesterol in the lipid bilayer (28). In light of this notion, the washing step with 0.1% of saturated cholesterol-DMSO in buffer O (buffer CD) determined an increase in both viability and transfection efficiency (**Figure 1B** lower panel protocol #8 and **Supplementary Figure 1B**). As shown in **Figure 1C** (right panel), the use of these buffers, combined with a first pulse of 1820 V for 20 ms, resulted in a sharp increase in cell viability (up to 58%). Moreover, using these conditions the efficiency of transfection raised up to 51% 24 h after transfection. Notably, the latter protocol proved to be suitable also for electroporation of NK-92, a human NK cell line widely used in clinical trials (16), reaching up to 60% of transfection efficiency (**Figure 1D**). Importantly, this new procedure did not alter the expression of both surface NK receptors and cytoplasmic perforin (**Figure 2A**) in IL-2 activated NK cells. In addition, NK cells maintained the capability of producing interferon-γ (**Figure 2B**).

Plasmids of Different Size Can Be Efficiently Transfected in NK Cells

In the development of our transfection procedure, we used a plasmid DNA of small size (≈3.5 Kb). Since the size of the DNA vectors may influence the transfection efficiency (29), we tested whether the new method was suitable for

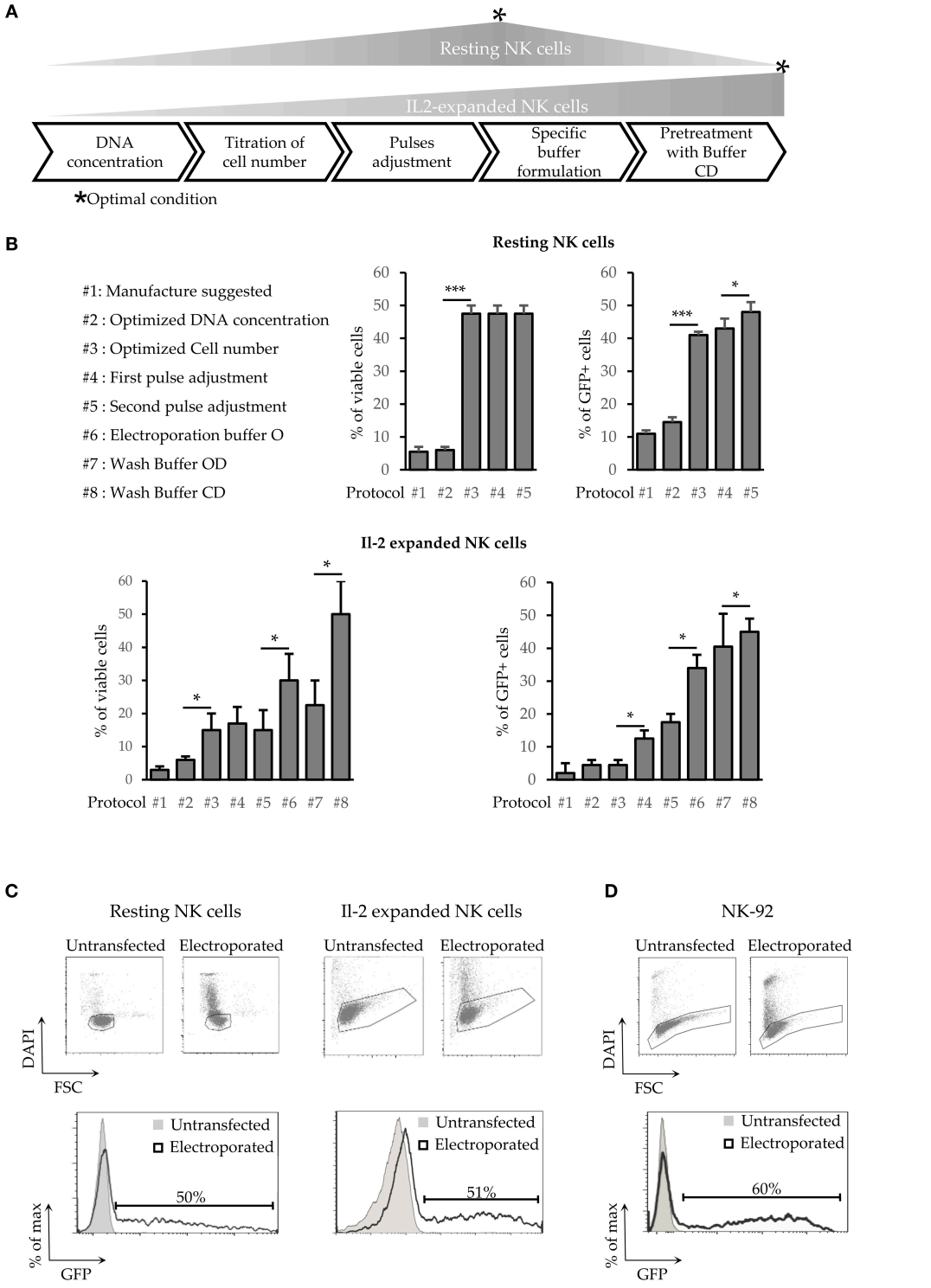

FIGURE 1 | Development of a new transfection method for human NK cells. **(A)** Schematic steps of NK cell electroporation-based transfection methods. The asterisks (*) indicate the step in which we obtained the higher efficiency and viability (Optimal Condition O.C.) of resting or activated NK cells. **(B)** Percentages of cell viability and transfection efficiency obtained for the different protocols (from #1 to #8) applied to improve NK cell electroporation. Error bars indicate Standard Deviation (SD). *P*-values were calculated comparing each protocol with the previous one. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. **(C,D)** GFP expression in electroporated resting NK cells (**C**, left panel), Il-2 activated NK cells (**C**, right panel) and in NK92 cell line **(D)**. One representative experiment out of 4 performed is shown.

TABLE 1 | Table showing the starting electroporation condition (manufacture protocol) compared with the optimal condition determined for resting and IL-2 activated NK cells.

	No. of cells	First pulse	Second pulse	Wash buffer	Electroporation buffer
Manufacture suggested	$2*10^7$ /ml	2100V / 20ms	_	PBS 1X	Buffer R
Resting NK cells O.C.	$4*10^7$ /ml	2050V / 20ms	500V/100ms	PBS 1X	Buffer R
Expanded NK cells O.C.	$4*10^7$ /ml	1820V / 20ms	500V/100ms	Buffer CD	Buffer O

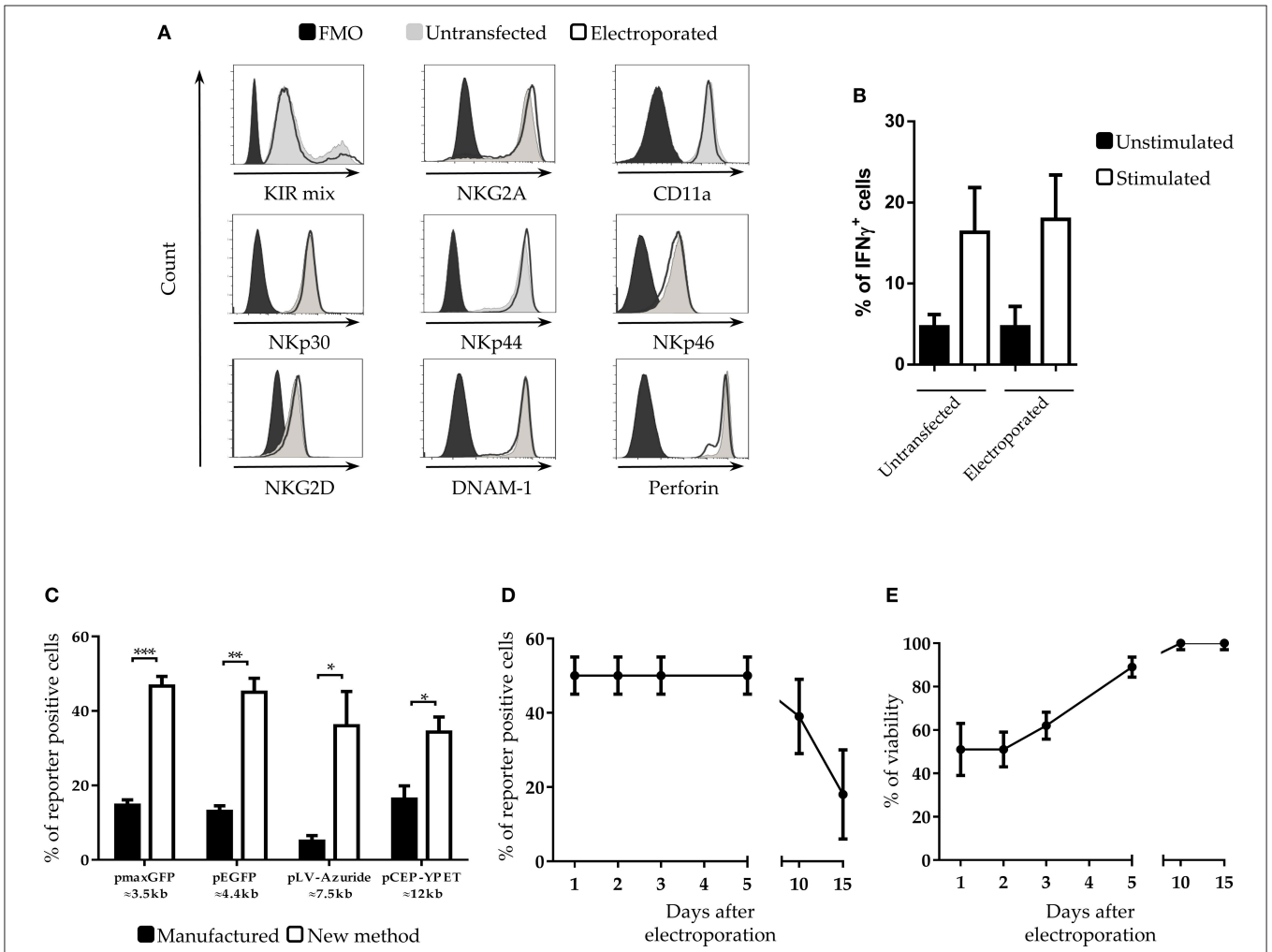

FIGURE 2 | Characterization of NK cells after electroporation **(A)** Cytofluorimetric analysis of NK cell receptors and perforin expression on IL-2-activated NK cells at 5 days after electroporation in FMO control (black filled profiles), untrasfected (gray filled profiles) or with pmaxGFP plasmid (black empty profiles). A representative experiment out of 3 is shown. **(B)** Percentages of interferon-gamma (IFNγ) positive untransfected- and pmaxGFP electroporated-NK cells. Unstimulated (black bars) are compared with K562 stimulated (white bars) NK cells. **(C)** Transfection with different plasmid sizes in NK cells. Percentage of viability and efficiency after electroporation of activated NK cells with plasmids of different size. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. **(D)** Persistence of transfected genes and **(E)** viability of NK cells at different culture intervals after electroporation. Six experiments performed.

the transfection of plasmids of larger size. To this end, IL-2 expanded NK cells were electroporated with four plasmids of different size, ranging from 3.5 to 12 kb. With all the analyzed plasmids we obtained a substantial increase of cell transfection, ranging from 2.5- to 5-fold compared to the standard electroporation protocol (indicated as "manufacture") even though the overall efficiency varied with the different plasmids (**Figure 2C**).

Positive Transfected Cells Can Be Detected Up to 15 Days After Electroporation

We next analyzed the persistence of the transfected genes into the electroporated NK cells. Notably, even after 5 days the percentage of transfected cells did not change (**Figure 2D**) and the overall viability of the culture increased up to 85 % (**Figure 2E**). Remarkably, even after 10 days of culture only a 10% loss of

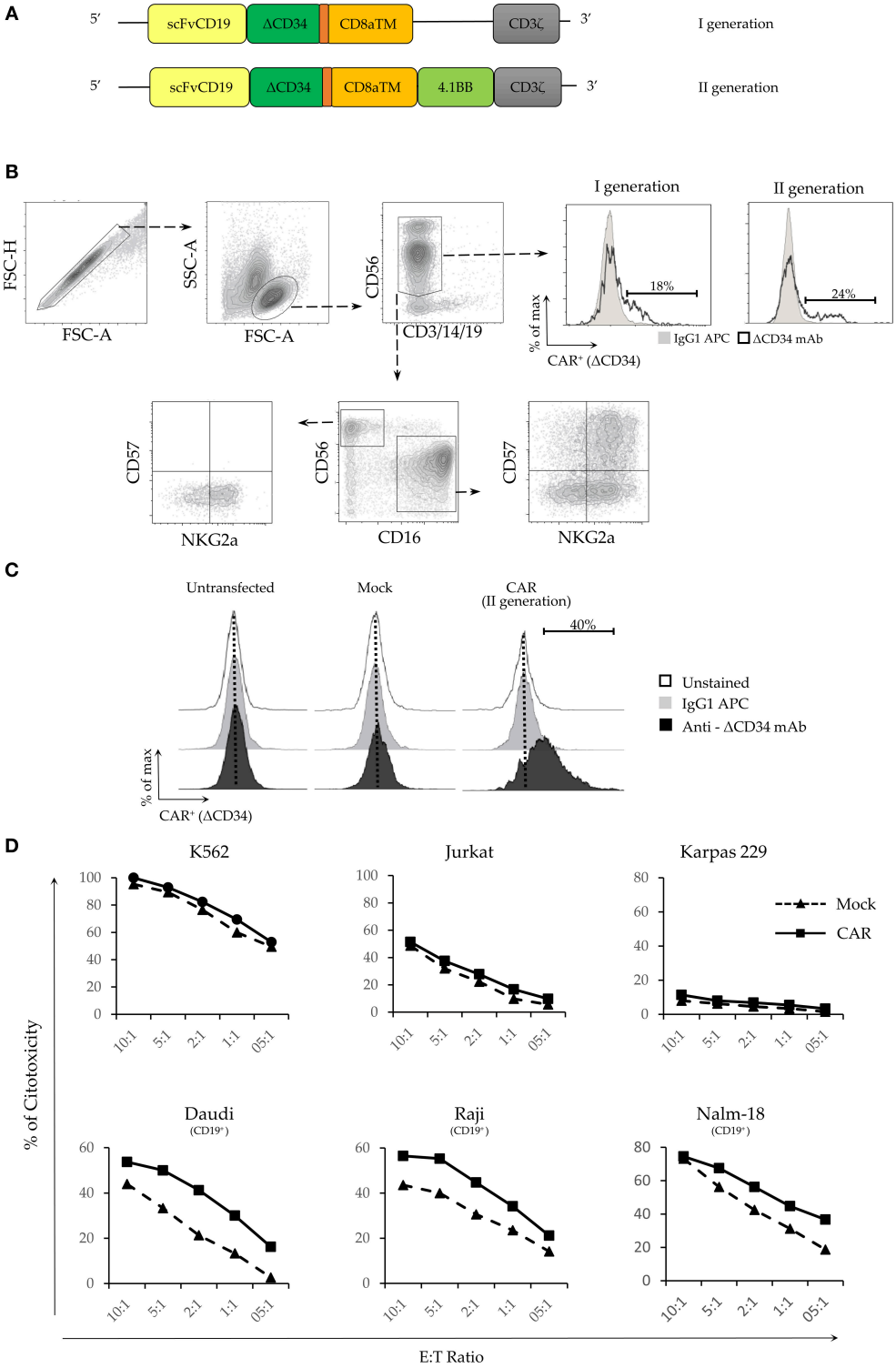

FIGURE 3 | Electroporation of a functional CAR-antiCD19 transgene in NK cells increases their cytolytic activity. **(A)** Schematic representation of the different domains of the two CAR constructs. **(B)** Representative gating strategy used to test the electroporation efficiency of I and II generation CAR constructs in the different NK cell populations. **(C)** Electroporation efficiency of $CD19^{+}$CAR plasmid in untransfected, mock-transfected and CAR-transfected activated NK cells. CAR expression was evaluated on DAPI-negative live cells using the delta (Δ) CD34 marker. Cytometric profiles of unstained (empty profile), isotype stained (gray profile) and anti-ΔCD34 mAb stained (black profile) NK cells are shown. **(D)** Percentage of cytotoxicity was evaluated by flow cytometry on propidium iodide (PI) positive target cells. $CD19^{-}$ cell lines (upper panel) and $CD19^{+}$ (lower panel) were used as target cells. Effector: Target (E:T) ratios are indicated. A representative experiment out of 3 performed is shown.

cells positive for the transfected gene was detected. A relevant reduction of the percentage of positive cells occurred after 15 days of culture.

The Expression of Anti-CD19 CAR in IL-2 Expanded NK Cells Strongly Increases Their Cytolytic Activity Against CD19$^+$ Tumor Targets

We analyzed plasmids encoding for transgenes specific for a first (I) and a second (II) generation CAR recognizing the B cell antigen CD19 (**Figure 3A**). As reported, mRNA electroporation of CARs did not preferentially target different NK cell populations (30). Thus, we tested if with our protocol we could obtain different transfection efficiency for the various NK subpopulations. As illustrated in **Figure 3B**, CAR expression was analyzed in resting NK cells 24 h after electroporation. The efficiency of the two constructs was similar (**Figure 3B** upper right panel). No differences of CAR expression were observed among the NK cells populations analyzed (**Supplementary Figure 2**). Considering that *in vitro* expanded NK cells are those used for cell immunotherapy, we focused our experiment on these cells. IL-2-expanded NK cells were efficiently transfected with the second generation CAR plasmid (9.5 Kb plasmid length). The percentage of transfected cells was ∼40% (**Figure 3C**). Cell viability, measured 24 hours after electroporation, was similar to that obtained in experiments with pmaxGFP (i.e., ∼60%; data not shown). We then assessed the cytolytic activity of CAR-NK cells 5 days after electroporation since, at this time point, the viability of the cell cultures increased, while the percentage of cells expressing the transgene remained stable (**Figures 2D,E**). We tested different target CD19$^+$ cell lines including DAUDI, RAJI and NALM-18. As a negative control, we used different CD19$^-$ cell lines, such as K562, Jurkat and Karpas. On one hand, as shown in **Figure 3D** upper panel, no major changes in the cytolytic activity between Mock- and CAR-electroporated NK cells against all the CD19$^-$ cell lines could be observed. However, NK cells displayed different ability to kill these 3 cell lines, thus K562 showed the maximal susceptibility to lysis, while Karpas, which were virtually resistant to both mock and CAR-electroporated NK cells. On the other hand, CAR-NK cells displayed a higher cytolytic activity against all the CD19$^+$ tumor cell lines tested (**Figure 3D** lower panel). These data clearly indicate that the anti-CD19 CAR transfection confers to NK cells a specific cytolytic activity against CD19$^+$ target cells. Accordingly, these CAR-NK cells may represent suitable candidates for adoptive cell immunotherapy against CD19$^+$ hematologic malignancies.

NK Cells Transfected With the CCR7 Transgene Show Increased Migratory Properties

The surface expression of chemokine receptors is required for cell migration. In this context, NK (or T) cells expressing transgenes encoding for appropriate chemokine receptors may be addressed to tumor sites (31), for example, lymph nodes infiltrated with tumor metastases. Since CCR7$^+$ cells migrate to lymph nodes

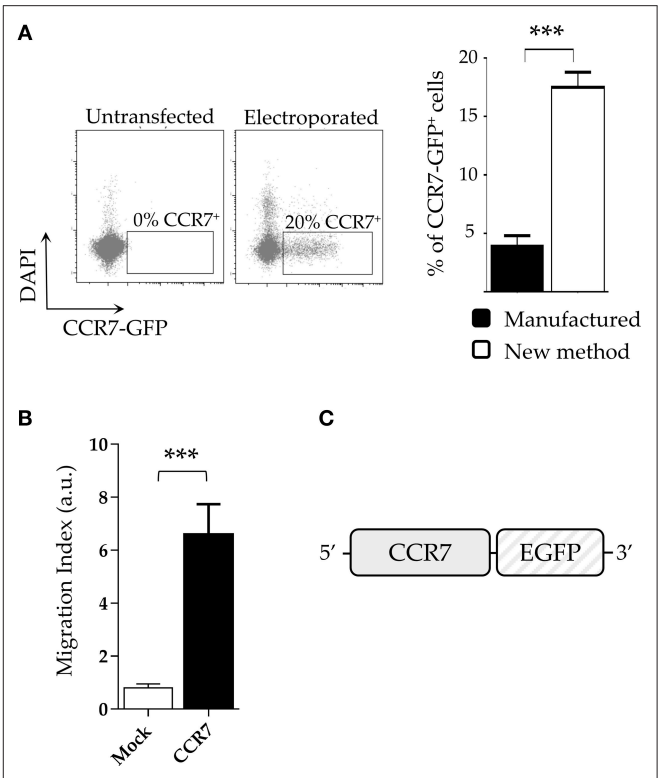

FIGURE 4 | NK cells transfected with CCR7 plasmid acquire migratory capability. **(A)** Comparative expression of CCR7-GFP on DAPI-negative live IL-2-activated NK cells using the new protocol and the manufactured protocol (right panel). A representative experiment (left panel) out of 4 is shown **(B)** Migration index of CCR7$^+$ and mock-electroporated NK cells derived from 5 different donors. ***$p < 0.001$. **(C)** Schematic reppresentation of the CCR7 construct.

in response to CCL19 and CCL21, we investigated whether an expression vector encoding CCR7 fused with GFP (pCCR7-eGFP; **Figure 4C**) could be efficiently transfected in NK cells by applying our new protocol. Although cell viability resulted similar to that of our previous experiments, the transfection efficiency with pCCR7-eGFP was lower compared to pmaxGFP or CAR (∼20%; **Figure 4A** left panel). Notably, even a lower transfection efficiency was obtained for the same plasmid by using a standard ("manufactured") electroporation protocol (∼4%; **Figure 4A** right panel). Thus, also in this case, the transfection efficiency using our protocol resulted ∼5-fold higher than that obtained with the manufacture procedure (**Figure 4A** right panel). It is conceivable that the lower transfection efficiency of pCCR7-eGFP may reflect intrinsic properties of the pCCR7 vector. We next assessed the migration capability of CCR7-transfected NK cells in response to CCL19 and CCL21 chemokines. As shown in **Figure 4B**, purified CCR7$^+$ NK cell transfectants migrate more efficiently than mock-transfected NK cells (CCR7$^-$), displaying a 6 fold increase in migration capacity.

DISCUSSION

Our present study provides a novel important tool for a successful adoptive immunotherapy of cancer using NK

cells transfected with CAR or chemokine receptors, using a non-viral method.

The development of this new methodology allowed a great improvement of the efficiency of NK cell electroporation with plasmids of different size. Importantly, anti-CD19-CAR and CCR7 conferred to NK cells an up to 5 fold increase in killing of $CD19^+$ tumor cells and a 6 fold increase of migratory capability in response to CCL19 or CCL21, respectively.

Although the efficiency of DNA electroporation and viability of transfected cells are lower than those obtained with mRNA electroporation, our method opens new perspectives. Indeed, we show that the transient DNA electroporation leads to a more durable persistence of the transfected genes (up to 15 days). Moreover, while mRNA electroporation could be only transient, the DNA electroporation may allow a stable integrated gene transfer. More generally, our protocol could be applied to improve the efficiency of any electroporation-based transfer of different molecules (DNA, siRNA, mRNA, or proteins) into NK cells. This method was developed using plasmids encoding for reporter genes (i.e., pmaxGFP), and successfully applied to the transfection and functional expression of plasmids encoding transgenes of major relevance for NK cell-mediated tumor cell killing and for NK cell migration. Regarding the migratory capability, the expression of CCR7 is known to promote NK cell migration primarily toward lymph nodes (32) (where CCL19 and CCL21 are primarily produced). However, transfection of other chemokine (or homing) receptors may induce NK cell migration to different tissues and tumor sites (33, 34). An attractive possibility is the development of double transfectants, allowing to address CAR-NK cells where needed. Attempts toward this goal are in progress in our lab. Importantly, the extension of our approach to CAR targeting antigens expressed by solid tumors may provide a valid tool for immuno-therapy of established tumors and prevention of their spreading and metastasis (35). Such acquired functional capabilities are of particular relevance because CAR-NK cells represent suitable candidates for cell-based adoptive therapies to target different solid tumors.

In conclusion, the method of NK cell transfection described in our present study is highly efficient, does not require expensive dedicated structures necessary for viral transduction and avoids possible risks associated with the use of viral vectors. Importantly, it may be applied to NK cells or NK-92 cell line, greatly improving their anti-tumor activity and providing a new NK cell-based platform for new protocols of adoptive immuno-therapy of cancer.

ETHICS STATEMENT

The Ethical Committee of IRCCS Bambino Gesù Pediatric Hospital approved the study (825/2014).

AUTHOR CONTRIBUTIONS

TI designed and performed research, interpreted data, and wrote the article. FM, FB, and NT performed experiments. AP, CC, and FL reviewed the manuscript. CQ and BD developed CAR constructs and revised the manuscript. PV and LM designed research and wrote the paper.

FUNDING

The present study has been supported by the following grants: Associazione Italiana per la Ricerca sul Cancro (AIRC) IG 2014 Id. 15283 (LM), IG 2017 Id. 19920 (LM), Special Project 5X1000 no. 21147 (LM), and Ministero della Salute GR-2013-02356568 (PV). NT is recipient of a fellowship awarded by AIRC.

ACKNOWLEDGMENTS

We thank Ezio Giorda and the Flow Cytometry core facility of IRCCS Bambino Gesù Children's Hospital.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00957/full#supplementary-material

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

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frontiers in Immunology

MINI REVIEW
published: 26 April 2019
doi: 10.3389/fimmu.2019.00910

Innate Lymphoid Cells: Expression of PD-1 and Other Checkpoints in Normal and Pathological Conditions

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Innate lymphoid cells (ILCs) belong to a family of immune cells. Recently, ILCs have been classified into five different groups that mirror the function of adaptive T cell subsets counterparts. In particular, NK cells mirror $CD8^+$ cytotoxic T cells while ILC1, ILC2, ILC3, and Lymphoid tissue inducer (LTi)-like cells reflect the function of $CD4^+$T helper (Th) cells (Th1, Th2, and Th17 respectively). ILCs are involved in innate host defenses against pathogens and tumors, in lymphoid organogenesis, and in tissue remodeling/repair. In recent years, important molecular inducible checkpoints (PD-1, TIM3, and TIGIT) were shown to control/inactivate different immune cell types. The expression of many of these receptors has been detected on NK cells and subsets of tissue-resident ILCs in both physiological and pathological conditions, including cancer. In particular, it has been demonstrated that the interaction between PD-1^+ immune cells and PD-L1/PD-L2+ tumor cells may compromise the anti-tumor effector function leading to tumor immune escape. However, while the effector function of NK cells in tumor is well-established, limited information exists on the other ILC subsets. We will summarize what is known to date on the expression and function of these checkpoint receptors on NK cells and ILCs, with a particular focus on the recent data that reveal an essential contribution of the blockade of PD-1 and TIGIT on NK cells to the immunotherapy of cancer. A better information regarding the presence and the function of different ILCs and of the inhibitory checkpoints in pathological conditions may offer important clues for the development of new immune therapeutic strategies.

Keywords: NK, ILC, PD-1, checkpoint receptors, cancer immunotherapies

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Georg Gasteiger, Julius-Maximilians-Universität, Germany
Rafael Solana, Universidad de Córdoba, Spain

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *03 December 2018*
Accepted: *09 April 2019*
Published: *26 April 2019*

Citation:
Mariotti FR, Quatrini L, Munari E, Vacca P and Moretta L (2019) Innate Lymphoid Cells: Expression of PD-1 and Other Checkpoints in Normal and Pathological Conditions. Front. Immunol. 10:910. doi: 10.3389/fimmu.2019.00910

INTRODUCTION

Innate Lymphoid Cells (ILCs) represent a heterogeneous group of developmentally related lymphocytes (1). However, distinct aspects differentiate T or B lymphocytes from ILCs. Thus, unlike T and B cells, ILCs are characterized by the lack of expression of recombination activating genes (RAG-1 and RAG-2)-dependent rearranged antigen receptors and rely on a set of germ-line encoded receptors to exert their function (2–5). Thus, while T cells responses require longer time intervals due to antigen-mediated clonal selection and expansion, ILCs can exert a prompt response to recognition of conserved molecular patterns from pathogens and infected or injured tissues (6). For this reason and for their tissue-residency ILCs may represent the leading orchestrator of

immune responses. ILCs release effector and regulatory cytokines that play a role in tissue repair and immune defense being also able to coordinate the adaptive immune responses (7–9). Thus, ILCs might also play a primary role in sensing cells that underwent malignant transformation and in initiating anti-tumor immune response, even though, as will be discussed in this review, their actual role in tumor suppression is controversial.

ILC DIFFERENTIATION AND CLASSIFICATION

Recently, five main ILCs groups have been identified according to the transcription factors required for their development and function (6, 10). These groups are represented by natural killer cells (NK), group 1 ILCs (ILC1), group 2 ILCs (ILC2), group 3 ILCs (ILC3), and lymphoid tissue-inducer (LTi) cells. All ILCs derive from common lymphoid progenitors (CLPs) that mainly reside in the bone marrow, even though ILCs progenitors are also found in other tissues, such as fetal liver, tonsils, decidua, and intestinal lamina propria (9, 11–16). ILCs are divided into cytotoxic-ILC and helper-ILC, which resemble cytotoxic and helper T cell subsets (17). In particular, NK cells mirror cytotoxic $CD8^+$ cells while ILC1, ILC2, and ILC3 resemble the T helper cell subsets Th1, Th2, and Th17, respectively. Originally NK and ILC1 represented the group 1 of ILC family because both subsets express the transcription factor T-bet, and secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-α (18, 19) (**Figure 1**). However, it must be noticed that NK cells, apart from T-bet, rely also on Eomes expression for their development (20) (**Figure 1**). NK cells, the first ILC subset to be identified, circulate in the bloodstream where they represent about the 15% of peripheral blood lymphocytes (1, 21). However, tissue-resident NK cells have also been found in liver, uterus and decidua (15, 22–24). In humans, two main PB-NK cell subsets can be distinguished based on the level of CD56 surface expression (25). In particular $CD56^{dim}$, expressing high levels of perforin and granzyme, are characterized by a high cytotoxic activity, while $CD56^{bright}$ cells secrete inflammatory cytokines and are prevalently found in tissues and secondary lymphoid organs (26). NK cells play a major role in innate defenses against viruses and tumors, both by direct cell killing and by promoting the initiation of inflammation (27–30). On the contrary, ILC1 express low level of perforin and are barely found in PB while they mainly reside within tissues, such as intestine, lung, skin and decidua where they are involved in the first line of defense against viruses and bacteria (19, 31–33). ILC2s, which rely on GATA3 expression, are mostly found in lung, intestine, adipose tissue, skin, and gut (34). Upon activation in response to epithelia-derived stimuli (mainly IL-33 and IL-25), they release type-2 cytokines (primarily IL-5, IL-13, and IL-9) and promote defenses against parasites, viral infections, and contribute to metabolic homeostasis (7, 31, 35, 36). In addition, ILC2s produce amphiregulin, an epidermal growth factor family member involved in tissue repair (17). The most heterogeneous ILC subset includes fetal LTi and postnatal ILC3, both depending on the Rorγt transcription factor. They were previously called "group 3 ILCs" but, because of their different developmental trajectories, they are now classified into two distinct subsets (1). LTi cells play a pivotal role in the formation of secondary lymphoid structures, including lymph nodes and Peyer's patches during fetus development (37, 38). After birth, ILC3s can be found in gut, tonsils, and intestine where, through release of IL-22, play an important role in the innate immunity against bacteria and fungi (38, 39). However, LTi and a particular subset of ILC3, namely NCR^+ ILC3, have also been found in decidual tissue during early pregnancy (33).

While the role of NK cells in anti-tumor immunity has been widely studied and well-established, ILCs function in the immune defenses against tumors is still controversial (40). Their preferential localization at the mucosal surfaces may even suggest a negative role, as some of their cytokines exacerbate the development of chronic inflammation and potentially favor tumor growth. Indeed, IFN-γ released by ILC1 in inflamed conditions could have detrimental effects by favoring tumor growth. Similarly, type-2 cytokines produced by ILC2s are associated with poor prognosis in cancer patients and can create a pro-tumorigenic environment through the stimulation of myeloid derived suppressor cells (MDSC) or M2 macrophages (2, 41, 42). Moreover, ILC3s have been associated with tumor growth and metastasis in different type of cancers (10). However, available evidence suggests that ILCs function may depend on the tumor microenvironment (43–48). Indeed, the different cytokines, soluble factors, and cell types that characterize the tumor microenvironment can shape the function of different immune cells, dampening antitumor immunity (7). In this context, ILCs plasticity, that allows them to convert from one subset into another depending on the surrounding stimuli, might have a negative role in immune defenses. Therefore, a deeper understanding of NK and ILCs in protective immunity and how tumor cells and the tumor microenvironment can inhibit their functions is of extreme interest especially for the development of new immunotherapies.

NK/ILC CELL RECEPTORS

NK/ILC are able to discriminate between healthy and virus- or tumor- infected cells through an array of inhibitory and activating receptors that recognize specific ligands induced by virus infection or tumor transformation (49–51). Natural Cytotoxicity Receptors (NCR), which include NKp46, NKp44, and NKp30, represent the major NK cells activating receptors (52–57). NCRs can be also expressed by specific ILCs subsets, with ILC1 expressing NKp46, ILC2 NKp30 and tonsil-derived ILC3 and mucosal NCR^+ ILC3 bearing NKp30 and NKp46, respectively (58). NKG2D and DNAM-1 represent other important activating receptors able to recognize ligands that are *de novo* expressed or upregulated upon cell stress or tumor transformation (59–62). Additionally, NK cells express co-activating receptors, such as NTB-A and 2B4, whose function depends on the simultaneous co-engagement of one or more activating receptors (57, 63–65). The function of activating receptors is counterbalanced by inhibitory receptors that are mainly represented by the killer Ig-like receptors (KIR) and

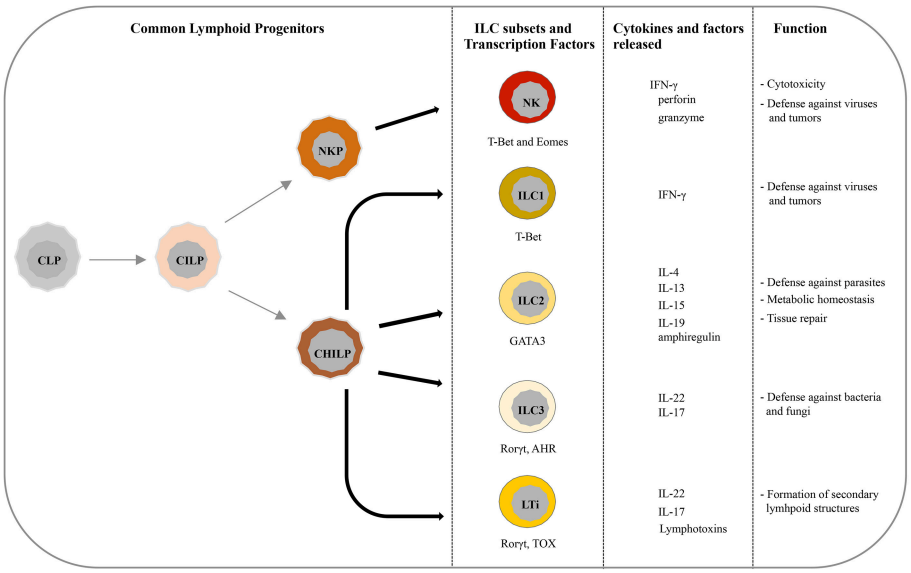

FIGURE 1 | Current view of differentiation and immune function of ILC subsets. ILCs originate from common lymphoid progenitors (CLPs) that give rise to common innate lymphoid progenitors (CILPs). On one side, CILPs can differentiate into NK precursor (NKP) that, through T-bet and Eomes, will originate NK cells, and on the other side into common helper innate lymphoid precursors (CHILP) from which originate both Innate lymphoid precursors (ILP) and lymphoid tissue inducer progenitors (LTiP). ILC1/2/3 subsets and LTi cells differentiate, according to the different transcription factors required, from ILP and LTiP precursors, respectively. As specified in the text, NK/ILC cells release various cytokines and exert different immune functions.

the heterodimer CD94/NKG2A which recognize the main type of HLA class-I molecules and function as true checkpoints in NK cell activation (29, 66–68). Indeed, in normal conditions these inhibitory receptors recognize HLA-I ligands expressed on healthy cells preventing their killing. As a consequence, loss of MHC expression on tumor cells is increasing rather than decreasing their susceptibility to NK cell-mediated killing (69). Recently, additional inhibitory checkpoints (such as PD-1, TIGIT, etc.), which under normal conditions maintain immune cell homeostasis, have been shown to facilitate tumor escape. Indeed, different studies demonstrated that, in these pathological conditions, checkpoint regulators, usually absent on resting NK cells, can be induced *de novo* and contribute to the downregulation of NK cell anti-tumor function upon interaction with their ligands expressed at the tumor cell surface (70).

In the next paragraphs, we will summarize what is known to date about the expression and function of these checkpoint receptors on NK cells and ILCs, with a particular focus on PD-1, TIGIT, and CD96.

PD-1

PD-1, a member of immunoglobulin superfamily, is a cell surface inhibitory receptor, functioning as a major checkpoint of T cell activation. It binds PD-L1 and PD-L2, ligands expressed on many tumors, on infected cells, on antigen-presenting cells in inflammatory foci, and in secondary lymphoid organs. Lack of PD-1 expression results in the suppression of tumor growth and metastasis in mice (71). The efficacy of PD-1 blockade has been mainly correlated with the restoration of a preexisting T cell response. PD-1 expression, initially described on T, B, and myeloid cells, has been recently described also on NK cells (72, 73) (**Figure 2**). In particular, PD-1 expression was shown on NK cells from some healthy individuals and in most cancer patients, including Kaposi sarcoma, ovarian and lung carcinoma and Hodgkin lymphoma, where it can negatively regulate NK cell function (73–78). The contribution of PD-1 blockade on NK cells in immunotherapy has been demonstrated in several mouse models of cancer, where PD-1 engagement by PD-L1$^+$ tumor cells could strongly suppress NK cell–mediated anti-tumor immunity (79). PD-1 expression was found more abundant on NK cells with an activated and more responsive phenotype rather than on NK cells with an exhausted phenotype (79). However, to date the molecular mechanisms regulating the expression of this inhibitory receptor on NK cells are not clear. It has been demonstrated in a mouse model of cytomegalovirus infection (MCMV) that endogenous glucocorticoids integrate the signals from the microenvironment to induce PD-1 expression at the transcriptional level, highlighting the importance of a tissue-specific cooperation of cytokines and the neuroendocrine system in this regulation (80). Regarding the cancer setting, however, recent data suggest that PD-1 is accumulated inside NK cells and translocated on the cell surface rather than induced at the transcriptional level (81). However, the stimuli required for its surface expression are unknown.

Two recent papers described that, in mice, PD-1 expression identifies ILC committed progenitors, capable of generating ILC1s, ILC2s, ILC3s, and a small number of circulating NK cells (82, 83). High expression of PD-1 is lost upon differentiation,

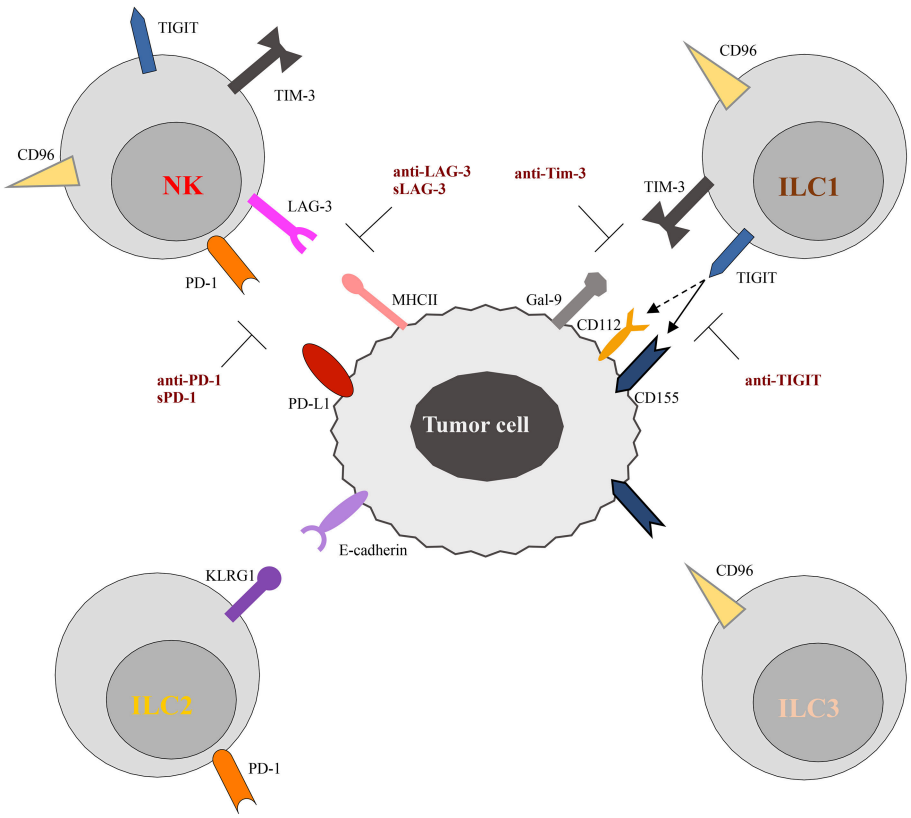

FIGURE 2 | Schematic representation of checkpoint receptors and their ligands expressed by ILC and tumor cells, respectively. NK cells express multiple immune checkpoint receptors, such as PD-1, TIM-3, Lag-3, TIGIT, and CD96. ON the other hand, these checkpoint receptors are instead differentially expressed by ILC subsets. Thus, TIGIT and TIM-3 have been detected only on ILC1 cells, while CD96 is expressed on both ILC1 and ILC2. Surface expression of KLRG1 and PD-1 appears to be restricted to ILC2 cells. The inhibitory ligands expressed by tumor cells, specifically interact with the checkpoint receptors preventing cells activation. However, different therapeutic approaches, aimed to block receptor/ligand interactions, have been demonstrated to restore the anti-tumor activity of immune cells, as illustrated in the Figure. The solid and dotted arrows indicate the strong and weak binding affinity of TIGIT for the different ligands, respectively.

but upregulated on effector tissue resident ILC2s upon lung inflammation (83). In agreement with these findings, it was shown that mouse ILC2s express PD-1 in different percentages depending on their tissue origin and that this expression is enhanced by IL-33 stimulation, reducing their ability to release cytokines (84). This is particularly relevant in type 2 infections, such as helminth infection, but the role of PD-1 expression on ILC2s in the context of cancer remains to be investigated. Nonetheless, the finding that it is possible to modulate ILC2s effector functions by using PD-1 blocking antibodies suggests that targeting this receptor with checkpoint inhibitors could also affect type 2 responses in cancer patients and favor cancer growth by restoring the production of type-2 cytokines. The possible unfavorable effect of this ILC2-mediated response and its contribution to therapy with checkpoint inhibitors should be further explored to further improve the efficacy of cancer treatment.

Recent studies provided the first evidence that also ILC3s can express a functional PD-1 receptor. In particular, PD-1 expression has been detected on both NK cells and ILC3s in malignant pleural effusions of patients with primary and metastatic tumors (85). Moreover, it has been shown that NK cells and ILC3s in human decidua express PD-1 during the first trimester of pregnancy, while the invading trophoblast expresses PD-L1. The PD-1/PD-L1 molecular interaction regulates ILC3 production of cytokines, suggesting that it may play a regulatory role at the feto-maternal interface (16).

TIGIT AND CD96

TIGIT (T cell Ig and ITIM domain) and CD96 are co-inhibitory receptors expressed on subsets of T cells, human NK cells, ILC1s, and ILC3s (**Figure 2**). They belong to a group of immunoglobulin superfamily receptors comprising also the co-stimulatory receptor DNAM-1 (CD226). They recognize nectin and nectin-like ligands, frequently upregulated on tumor cells. CD155 is a ligand shared by the three receptors, with CD96 showing the highest binding affinity (86). These receptors initiate a pathway that is analogous to the CD28/CTLA-4 one. In this pathway, ligands and differential receptor:ligand affinities can fine-tune the immune response. The work of Zhang et al.

(87) recently demonstrated that TIGIT constitutes a previously unappreciated checkpoint in NK cells, and that targeting TIGIT alone or in combination with other checkpoint receptors may represent a promising anti-cancer therapeutic strategy. It has been shown that, in patients with colon cancer, TIGIT expression is increased on tumor associated NK cells. In addition, evidences has been provided that, beyond the targeting of effector and regulatory T cells, the mode of action of TIGIT blockade also involves NK cells (87). In particular, genetic KO or mAb-mediated blockade of TIGIT was able to unleash both NK cell and T cell antitumor activity, leading to a substantial improvement in the control of tumor growth in several preclinical mouse models. Moreover, TIGIT blockade prevented NK cell exhaustion in the absence of adaptive immunity, and elicited a potent T cell–mediated memory response to tumor re-challenge through a not yet identified mechanism (87). Increased TIGIT and CD96 expression and lower levels of DNAM1 were also detected on ILC1s induced by TGF-β, contributing to the impairment of their anti-tumor response (88).

Although the role of TIGIT and CD96 as immune checkpoint receptors are just beginning to be uncovered, accumulating data would support the notion that targeting of these receptors for improving anti-tumor immune responses also involves NK cells and ILCs.

OTHER CHECKPOINTS ON NK CELLS AND ILCs

KLRG1 is another inhibitory receptor expressed by mature NK cells whose expression varies with cell activation. It is a C-type lectin-like receptor containing one ITIM, and it has been used as a marker for distinct NK and T-cell differentiation stages (89). However, KLRG1 knock-out mice showed that it does not play a deterministic role in the generation and functional characteristics of these lymphocyte subsets. KLRG1 is also expressed by mast cells, basophils, eosinophils, and ILC2s, suggesting a role in type 2 immune responses. Experiments in mice showed that *in vivo* administration of IL-25 elicits the expansion of a subset of ILC2s referred to as "inflammatory" ILC2s that are characterized by high expression of KLRG1 and that participate in the control of helminth infection (90). In the tumor context, KLRG1 expression was found on ILC2 associated to the tumor in NSCLC and CRC (91, 92). While the interaction of KLRG1 and its E-cadherin ligand has been shown to inhibit human ILC2s *in vitro*, its function *in vivo* remains to be established (90, 93).

Lag-3 and Tim-3 are inhibitory receptors whose expression has been reported on NK cells and ILC1s (**Figure 2**). Tim-3 is a type 1 glycoprotein expressed by mature NK cells, and its expression is further increased on NK cells in melanoma and lung adenocarcinoma patients, impairing NK cell effector functions (94, 95). More recently, Tim-3 expression has been reported on human decidual NK cells and also on ILC3s. It was demonstrated that Tim-3 is expressed in higher percentages in $CD56^+$ILC3s compared to LTi-like cells, and that its triggering is able to significantly reduce IL-22 production by $CD56^+$ILC3s (16). Lag-3 is a negative costimulatory receptor that is homologous to CD4 and binds MHC-II molecules with very high affinity. Although its role in downregulating T cell proliferation, activation, and homeostasis is clear, its mechanism of action in NK cells remains to be dissected in detail (96).

NKG2A is a HLA-E-specific inhibitory receptor that plays an important regulatory role in NK cell function. Also antigen- or cytokine-stimulated T cells were shown to express a functional NKG2A that may antagonize T cell function (97, 98). It has recently been reported that NKG2A is expressed on NK and T cells in the tumor bed in many human cancers such as squamous cell carcinoma of the head and neck (SCCHN) and colorectal carcinoma (CRC) (99). In addition, its ligand, HLA-E, is frequently overexpressed in these tumors. NKG2A targeting with monalizumab (a humanized anti-NKG2A antibody) has been shown to enhance the anti-tumor immunity mediated by NK and $CD8^+$ T cells when used as a single agent or in combination with other therapeutic antibodies such as durvalumab (blocking PD-L1), or cetuximab (directed against the epidermal growth factor receptor, EGFR) (99, 100).

In mouse tumor models, it has been shown that TGF-β signaling in the microenvironment induces NK cell conversion to ILC1s. These tumor-associated ILC1s express higher levels of inhibitory receptors (NKG2A, KLRG1, CTLA4, LAG3) as compared to NK cells. While NK cells favored tumor immune surveillance in this setting, the higher expression of immunological checkpoint receptors on ILC1s was associated with a lower ability to control local tumor growth and metastasis (88). These evidences suggest that NK cell conversion to ILC1s displaying a functional impairment could represent an additional mechanism by which tumor escapes immune surveillance.

CANCER IMMUNOTHERAPIES

During the past few years, different strategies have been developed to overcome the immunosuppressive tumor environment and restore antitumor immune activity. The use of blocking antibodies against inhibitory receptors or their ligands, in order to restore the T or NK cell function has been demonstrated to be an efficient and safe cancer immunotherapy in the treatment of several tumors (70). Considering the wide expression of PD-1 on immune cells, most therapies have been developed in order to block PD-1/PD-L1/2 interactions. Indeed, some anti-PD-1 mAbs have already been approved by FDA, showing encouraging results in patients with melanoma or lung cancer (101, 102). Currently, there are ongoing phase I and II clinical trials for anti-KIR, -NKG2A, -Tim3, -LAG3, -TIGIT inhibitory receptors (102). Interestingly, considering that checkpoint inhibitors can act in synergy with each other, combinations of mAbs are also under investigation as a new approach for optimal boost of the immune system. In particular, clinical trials are investigating the combination of anti-PD1 therapy with anti-TIM3 or anti-TIGIT blocking antibodies in different tumors (70, 103). Moreover, encouraging results obtained in phase II clinical trial for SCCHN using a combination of monalizumab and cetuximab suggested that,

targeting checkpoint receptors on NK cells, may be an efficient tool to complement first-generation immunotherapies against cancer (99).

Of notice is also the discovery of soluble forms of LAG-3 (sLAG3) and PD-1 (sPD-1) (70, 104). Different studies have been focused on the role of sPD-1 as a putative antitumor agent. Indeed, in mice an increase in anti-tumor activity was observed upon delivery of sPD-1 encoding plasmid at tumor site (105, 106). Moreover, clinical studies have investigated the presence of sPD-1 and its correlation with the overall survival of patients with different cancers (107, 108). It has been shown that sLAG3 is able to induce NK cytokines (IFN-γ and TNF-α) production in *ex vivo* assay (109). Moreover, a phase II clinical trial is investigating the role of sLAG3 in stimulating the immune system in combination with anti-PD-1 therapy (70).

CONCLUSIONS AND FUTURE PERSPECTIVES

It is now evident that NK/ILC family plays a pivotal role in the immune defenses. Recent studies in murine and human settings demonstrated that the expression of several inhibitory checkpoints, that may be detrimental in the tumor context, is not restricted to T lymphocytes, revealing an important, yet poorly appreciated, contribution of their expression on innate immune cells. Thus, in the recent years different immunotherapy approaches, based on the blockade of inhibitory NK cell receptors, have been developed in order to unleash NK cell cytotoxicity. This is particularly important in the context of tumors that downregulate HLA-I expression and become invisible to T cells. However, it must be considered that most inhibitory checkpoints, targeted by mAbs therapies, are shared by T, NK and ILCs. Therefore, further studies are required in order to identify all the receptors regulating NK/ILC cells function for the development of new mAbs-based immunotherapies. In addition, considering the role exerted by the tumor microenvironment on ILCs plasticity and functions, it is necessary to better clarify the role of tumor infiltrating innate immune cells to improve the selectivity of cancer therapies. Therefore, also accurate patient analysis and deeper examinations of tumor biopsies will become key aspects to consider in order to construct personalized protocols. In this context, studies have been performed to determine the exact number of biopsies required to have a more precise PD-L1 expression profile that would more closely resemble to whole tumor section (110–112). Thus, despite the great improvement reached in the last years, further studies are required to investigate the expression of these checkpoints both in NK cells and on the other subsets of ILCs, and their precise role in human pathologies in order to improve the efficacy of immunotherapies thanks to a more personalized approach.

AUTHOR CONTRIBUTIONS

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

FUNDING

The present study has been supported by the following grants: Associazione Italiana per la Ricerca sul Cancro (AIRC) IG 2014 Id. 15283 (LM), IG 2017 Id. 19920 (LM), Special Project 5X1000 no.21147 (LM), Ricerca Corrente OPBG (LM), and Ministero della Salute GR-2013-02356568 (PV).

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frontiers in Immunology

ORIGINAL RESEARCH
published: 11 July 2019
doi: 10.3389/fimmu.2019.01501

Innate-Like Lymphocytes Are Immediate Participants in the Hyper-Acute Immune Response to Trauma and Hemorrhagic Shock

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OPEN ACCESS

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: 17 December 2018
Accepted: 17 June 2019
Published: 11 July 2019

Citation:
Manson J, Hoffman R, Chen S, Ramadan MH and Billiar TR (2019) Innate-Like Lymphocytes Are Immediate Participants in the Hyper-Acute Immune Response to Trauma and Hemorrhagic Shock. Front. Immunol. 10:1501. doi: 10.3389/fimmu.2019.01501

Adverse outcomes following severe traumatic injury are frequently attributed to a state of immunological dysfunction acquired during treatment and recovery. Recent genomic evidence however, suggests that the trajectory toward development of multiple organ dysfunction syndrome (MODS) is already in play at admission (<2 h following injury). Improved understanding of the molecular events during the hyper-acute immunological response to trauma, <2 h following injury, may reveal opportunities to ameliorate organ injury and expedite recovery. Lymphocytes have not previously been considered key participants in this early response; however, two observations in human trauma patients namely, raised populations of circulating NKT and NK cells during the hyper-acute phase and persistent lymphopenia beyond 48 h show association with the development of MODS during recovery. These highlight the need for greater understanding of lymphocyte function in the hyper-acute phase of inflammation. An exploratory study was therefore conducted in a well-established murine model of trauma and hemorrhagic shock (T&HS) to investigate (1) the development of lymphopenia in the murine model and (2) the phenotypic and functional changes of three innate-like lymphocyte subsets, NK1.1+ CD3–, NK1.1+ CD3+, γδTCR+ CD3+ cells, focusing on the first 6 h following injury. Rapid changes in phenotype and function were demonstrated in these cells within blood and spleen, but responses in lung tissue lagged behind. This study describes the immediacy of the innate-like lymphocyte response to trauma in different body compartments and considers new lines for further investigation to develop our understanding of MODS pathogenesis.

Keywords: trauma, major trauma, immune response, MODS, innate immunity, innate-like lymphocytes, trauma immunology

INTRODUCTION

Severe traumatic injury activates an acute inflammatory response, which increases in extent and magnitude during the first 24 h after injury (1, 2). High levels of inflammatory mediators at admission, are associated with the development of infection, Multiple Organ Dysfunction Syndrome (MODS) and death during hospital admission (1–4). The incidence of MODS is ∼30% after severe injury and while advances in trauma resuscitation strategies have reduced

early mortality from hemorrhagic shock, they have also likely increased the complexity of survivors (5–7). Organ support is the mainstay of MODS management, resulting in long critical care admissions, late in-hospital deaths and long-term ill-health in some survivors (6, 8, 9).

These adverse outcomes after trauma have, to date, been attributed to an acquired immunological dysfunction which develops during recovery, recent evidence has however demonstrated that the trajectory toward MODS development is already in-play at admission (1, 5, 10, 11). Patients who go on to develop MODS have high numbers of circulating Natural Killer T (NKT) cells and Natural Killer (NK) cells during the hyper-acute phase suggesting that early immune cell mobilization may influence recovery (10–12). Profound lymphopenia then occurs between 4 and 12 h post injury (12). Historically this has been attributed to widespread lymphocyte apoptosis, although the evidence for this is not strong (13, 14). If persistent after 48 h, lymphopenia is associated with MODS development and increased in-hospital mortality (11, 15). These data suggest that lymphocytes, particularly innate-like lymphocytes, play a significant role in the immunological events immediately following injury; much earlier than previously thought. They also indicate that therapeutic intervention in the pre-hospital phase may abrogate organ injury, if we can improve our understanding of the molecular mechanisms involved (16). Although studies to date have focused on peripheral blood because it is an accessible sample medium, cellular activity in other immunological compartments, such as spleen, bone marrow, liver, and lymph nodes may well differ from those in blood. The events in other compartments and the influence of cell mobilization have not yet been considered in clinical studies.

This exploratory study was conducted using a well-established murine trauma and hemorrhagic shock (T&HS) model. The study focused on NK cells, NK-T cells, and δγ T cells. All are lymphocytes with innate-like properties and we hypothesized that they would be active during the hyper-acute immune response to trauma, <2 h following injury (10). Unlike conventional lymphocytes, they can be directly activated by Danger-Associated-Molecular Patterns (DAMPs), cytokines and adrenergic signals to generate acute inflammation (17–22). They also share some common receptors, interact with one another and are known to orchestrate immune responses in other disease settings (17, 23–28). Our first aim was to determine whether peripheral blood lymphopenia occurred in the murine model and at what time point. We hypothesized that this lymphopenia may not be due to whole body lymphocyte loss from apoptosis, but transient redistribution and so sought evidence of cell death and sequestration into other organs. Our second aim was then to examine NK, NKT, and γδ T cells in blood, bone marrow, spleen and lung to assess cell phenotype and activity status, within these different compartments during the first 24 h following injury.

MATERIALS AND METHODS

This study was conducted at the University of Pittsburgh in accordance with the National Institute of Health guidelines and with the ethical approval of the University of Pittsburgh, Animal Care and Use committee.

Subjects

Male, wild-type C57BL/6 mice aged between 8 and 12 weeks and weighing between 20 and 30 g, were obtained from Charles Rivers Laboratories International (Wilmington, Mass). All were housed in pathogen-free conditions with 12 h light-dark cycles and unlimited access to food and water.

Model Protocol

The tissue trauma and hemorrhagic shock model (T&HS) was performed by experienced operating technicians. The method has been well described in previous literature but shall be detailed in brief (29, 30). A solution of crushed bone fragments was prepared, under sterile conditions, from bilateral femur and tibia harvested from a litter-matched donor. The solution was re-suspended in sterile PBS to achieve a volume of 2 ml. After administration of anesthesia with 70 mg/kg intra-peritoneal (i.p) pentobarbital sodium (Hospira, Inc., Lake Forest, IL, for OVATION Pharmaceuticals, Deerfield, IL), limbs were fixed and bilateral cannulation of femoral arteries performed, via groin incisions, to allow placement of PE-10 catheters. One catheter allowed for real-time BP monitoring (Digi-med BPA 400 Analyzer, Micro-Med Inc, Louisville, KY) and the other enabled blood draw. A bilateral thigh crush was performed using a hemostat for 30 s to induce soft tissue injury, then 0.15 ml of bone solution was injected, via an 18 gauge needle, into each thigh to mimic femur fracture (Pseudofracture, PF). Hemorrhagic shock was induced by withdrawal of blood from the femoral artery cannula to achieve a MAP of 25 mmHg (0 h) which was sustained for 2 h. Time 0 h was defined as the end of the blood draw and the beginning of the shock phase. Animals were resuscitated with Ringer's Lactate solution, at 2 h, using three times the volume of shed blood and given subcutaneous buprenorphine analgesia (0.10 mg/kg, Bedford Laboratories, Bedford, OH). Throughout the experiment, body temperature was maintained by use of a warming plate and controlled ambient room conditions. All subjects had unrestricted access to food and water. Mice were euthanized at 2, 6, or 24 h from the beginning of the hemorrhage induction using excessive isoflurane inhalation (Hospira, Lake Forest, IL, USA). Subjects that were euthanized at the 2 h timepoint did not receive crystalloid resuscitation. Rapid thoracotomy and cardiac puncture were then performed for blood draw in to a heparinized syringe. Intra-cardiac infusion of sterile PBS was performed for systemic vascular flush and then spleen, bone marrow, and lung were harvested.

Preparation of Single Cell Suspensions

For flow cytometry experiments, single cell suspensions of each organ were prepared in accordance with standard protocols. All centrifuge steps are at 350 g for 5 min at 4°C unless otherwise stated.

Blood

Following manual hemocytometer count, blood drawn from the cardiac puncture was centrifuged at 3,400 rpm for 10 min at

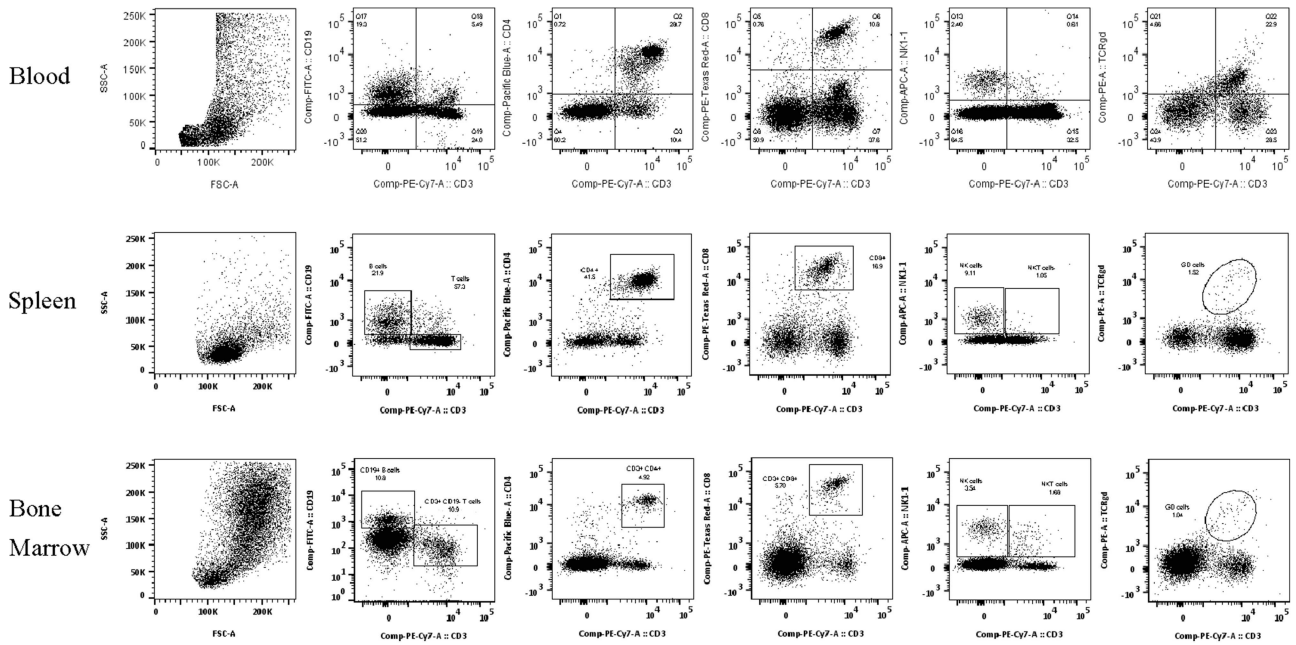

FIGURE 1 | Lymphocyte subset flow cytometry gating strategies. Representative flow cytometry plots demonstrating the gating strategy for the lymphocyte subset identification after removal of doublets and selection of lymphocyte population in **(A)** Blood, **(B)** Spleen, and **(C)** Bone Marrow.

4°C, serum was extracted in aliquots and stored at −80°C. One thousand microliter of warmed (37°C) red cell lysis buffer (prepared in-house: 8.26 g NH4Cl, 1 g KHCO3, and 0.037 g ETDA/L) was added to the eppendorf and the liquid aspirated up and down to loosen the remaining cell pellet. This solution was transferred into a 50 ml Falcon Tube using more lysis buffer to swill out remaining cells. Ten milliliter of warm red cell lysis buffer was then added and the solution incubated for 3 min with periodic vortex. Dilution with 20 ml of cold PBS was followed by centrifuge, removal of supernatant and re-suspension of pellet in 2 ml of PBS. The 2 ml solution was transferred to a flow-cytometry tube, centrifuged and resuspended in 200 µl PBS, ready for antibody application.

Spleen

The harvested spleen was placed in a Petri dish with DMEM (Dulbecco modified Eagle Media) and mechanically fragmented and filtered (70 µm). This solution was centrifuged, decanted and the pellet re-suspended in 5 ml of red cell lysis buffer, vortexed and diluted to 20 ml with cold PBS. The solution was centrifuged, decanted, and re-suspended in 10 ml of DMEM. Manual hemocytometer count was performed.

Bone Marrow

Femur and tibia from both back legs were harvested, cleaned and marrow placed in to a petri-dish. Marrow was fragmented, filtered (70 µm), centrifuged and the pellet re-suspended in 200 µl PBS. Five ml of warmed red cell lysis buffer was added and the solution incubated for 3 min, with periodic vortex. Dilution with 20 ml of cold PBS was followed by centrifuge, supernatant decant and re-suspension in 5 ml of DMEM. Manual hemocytometer count was performed.

Lung

A single cell suspension was produced using a Lung dissociation kit (Miltenyi Biotech 130-095-97). A single lung and the enzyme mixture were placed into the gentle MACS C tube and fragmented using Dissociation program 1 (gentleMACS Dissociator, Miltenyi Biotec #130-093-235). The solution was incubated for 30 min at 37°C, followed by a second dissociation programme and centrifuge. The pellet was re-suspended and the solution filtered through a 70 µm sterile filter. Making the volume up to 5 ml with PBS the solution was centrifuged, supernatant decanted and the pellet re-suspended in 5 ml of DMEM. This was over-laid with 5 ml of Lympholyte-M and centrifuged at 1,500 g for 20 min at room temperature, without the brake. The interphase was then aspirated, washed with PBS and centrifuged. Finally, the solution was centrifuged, the supernatant decanted and the final pellet re-suspended in 1 ml of PBS ready for application of antibodies. Manual hemocytometer count was performed.

Inflammation and Organ Dysfunction

The magnitude of the inflammatory response and end organ damage was quantified using ALT, ALP, and IL-6. These molecules were measured in serum samples drawn at 6 h, which represents the standard end-point for this model. IL-6 was quantified using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN, USA). ALP and ALT were measured using a Dri-Chem 7000 Chemistry Analyzer (Heska Co., Loveland, CO, USA). All were measured in duplicate and

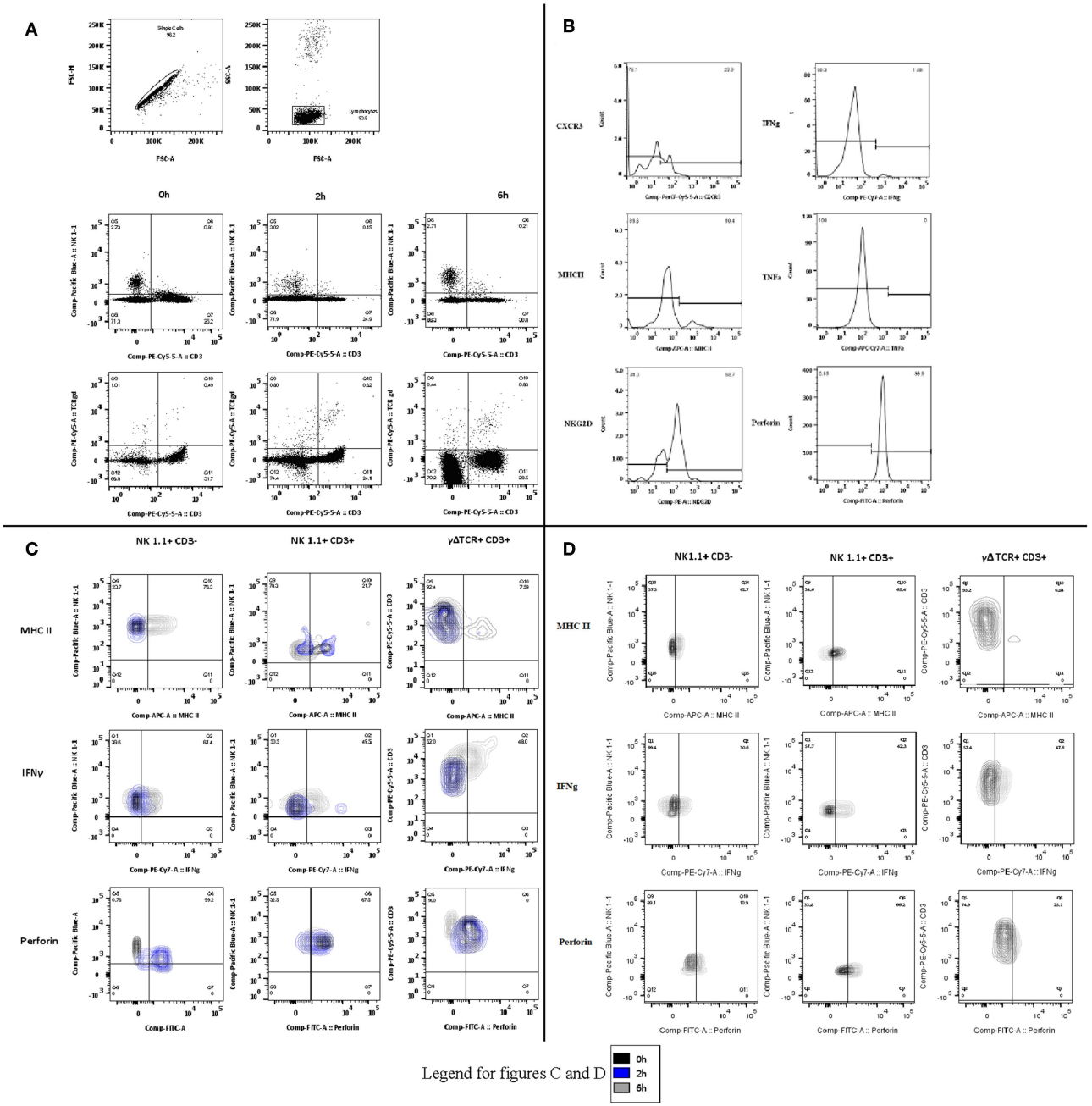

FIGURE 2 | Phenotype and function flow cytometry gating strategies **(A)** Gating strategies for phenotype and function experiments in blood, spleen, and lung. Following identification of single cells, the lymphocyte population was selected and divided by CD3 and either NK1.1 or TCRγδ. Representative plots are displayed for 0, 2, and 6 h. **(B)** Phenotype and function. The phenotype markers (CXCR3, MHCII, and NKG2D) and functional markers (Intracellular IFNγ, TNFα, and perforin) were then examined and recorded as percentage of positive cells for each lymphocyte subset. Representative histograms are displayed. **(C)** Changes in blood over time. Contours plots were overlaid to demonstrate the shifts in phenotype and function within each lymphocyte subset at the different sample points in the protocol. Representative plots of NK1.1 CD3–, NK1.1 CD3+, and CD3 TCRγδ are displayed for blood-borne cells at 0, 2, and 6 h. **(D)** Lung lymphocytes. As in **(C)**, the same process was followed to demonstrate the changes by cell type over time, in this case only Control and 6 h.

average values used for analysis. These end-points were used to ensure comparability between experiments.

Hemocytometer Counts

Standard full blood counts (FBC) were obtained using whole blood and an automated hemocytometer. For flow cytometry experiments leucocyte counts were obtained using a manual hemocytometer. Counts from multiple experiments, were grouped to obtain an average lymphocyte count for each stage ($n = 5$–30). Percentages obtained from the flow cytometry were then multiplied by the average cell count, to calculate absolute cell numbers for data representation and analysis.

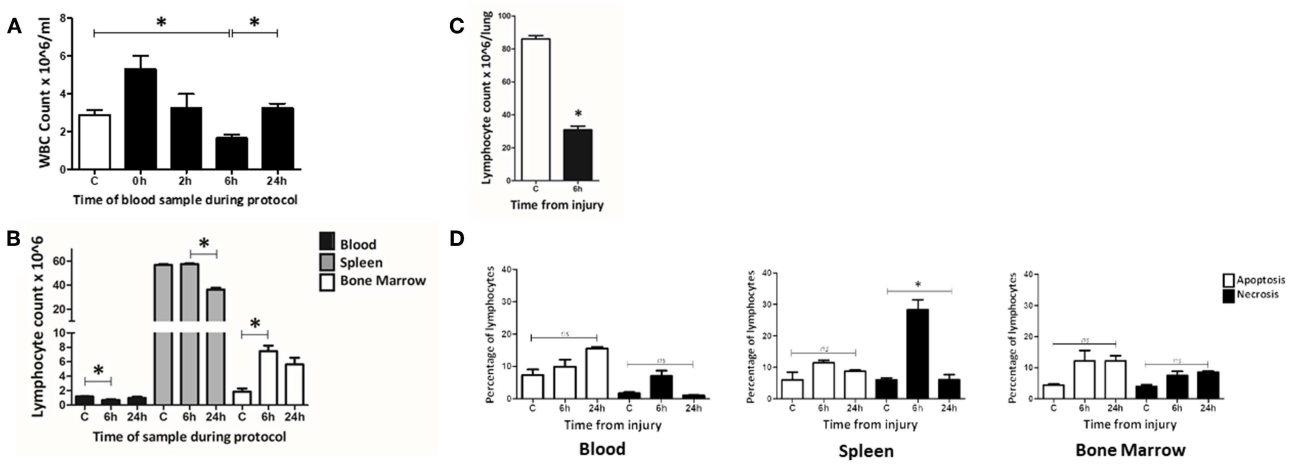

FIGURE 3 | At 6 h following T&HS lymphocyte count was reduced in blood and lung but static or increased in the lymphoid tissues. **(A)** Peripheral blood leucocyte count was determined manually on samples taken at intervals through the T&HS protocol. An initial leukocytosis at the onset of hemorrhagic shock was followed by leucopenia at 6 h. This returned to baseline by 24 h. *C* = 2.9 (2.6–3.1), 0 h = 5.3 (4.6–6.0), 2 h = 3.3 (2.5–4.0), 6 h = 1.7 (1.5–1.8), 24 h = 3.3 (3.0–3.5). Data are presented as mean (SEM) count $\times 10^6$/ml and tested using Kruskal Walis and Dunn's multiple comparison post-test. **(B)** The lymphocyte population in blood, spleen and bone marrow was quantified, at intervals through the protocol, using flow cytometry. The lymphopenia demonstrated in blood at 6 h was not mirrored in the other lymphoid organ compartments. In spleen, lymphocyte count was static at 6 h but reduced by 24 h. In bone marrow, the lymphocyte count had increased by 6 h and remained above baseline at 24 h. Control(C) denotes a naïve subject of the same strain and age. C, 6 h, 24 h = Blood: 1.2 (0.03), 0.7 (0.11), 1.0 (0.18). Spleen: 56.7 (0.71), 57.5 (0.57), 36.3 (1.36). Bone marrow: 1.9 (0.43), 7.5 (0.77), 5.6 (0.93). Data are presented as mean (SEM) absolute cell count $\times 10^6$/ml of blood/spleen/both hind limbs (n = 6–19). Data were tested using Kruskal Walis with Dunn's multiple comparisons test. All had a p-value < 0.01 and *denotes the positive comparisons. **(C)** The lymphocyte count in lung was examined at 6 h using flow cytometry. Lung was selected as a representative, non-lymphoid organ. By 6 h, the absolute count of lymphocytes in the lung tissue had reduced almost threefold C = 86 (84–87), 6 h = 31 (28–32), $p < 0.01$ (n = 6). Data are presented as mean (SEM) and tested with Mann Whitney *U* test, *denotes $p < 0.05$. **(D)** Evidence of widespread lymphocyte apoptosis was sought using a cell death assay on samples of blood, spleen, and bone marrow taken at 6 and 24 h following T&HS. Apoptosis was defined as Annexin V + 7AAD- and necrosis was defined as Annexin V+ 7AAD+. An increase in necrosis was observed in the spleen at 6 h. In all other organs and timepoints, there were no significant changes in apoptosis or cell death over the 24 h protocol. Blood: Apoptosis = 6 (6–9), 7 (7–10), 16 (15–16), $p = 0.15$. Necrosis = 2 (2–2), 7 (4–10), 1 (1–1), $p = 0.08$. Spleen: Apoptosis = 4 (4–7), 12 (10–13), 9 (9–9), $p = 0.10$, Necrosis = 5 (4–6), 29 (10–32), 6 (5–7), $p = 0.02$. Bone Marrow: Apoptosis = 4 (4–5), 12 (9–15), 12 (11–13), $p = 0.10$, Necrosis = 4 (3–5), 7 (6–8), 9 (8–9), $p = 0.06$. C denotes a naïve subject of the same strain and age. Data are presented as median (IQR) and tested with Kruskal Wallis and Dunn's comparison of all columns, *denotes $p < 0.05$.

Flow Cytometry

Using ∼5 million cells per tube, an antibody "master mix" was then added to each tube. The exception was the peripheral blood which had a much lower cell yield (0.5–1.5 million). Concentrations of antibodies were pre-titrated (**Box 1**). The eBioscience staining protocols were used for cell surface and intracellular antibody staining. A viability marker was used in all experiments. Samples were analyzed using the BD LSR Fortessa (BD Biosciences). The innate-like cells were defined as follows: NK cells = CD3– NK1.1+, NKT cells = CD3+ NK1.1+, γδ T cells = CD3+ γδTCR+. The other leucocyte subgroups were defined as: CD3+ CD4+, CD3+ CD8+, CD3– CD19+, and CD3– Gr-1+. The phenotypic markers selected were, CXCR3 a marker of migration potential, MHCII a marker acquired following activation or DC cross-talk (16, 22) and NKG2D an activation marker. The intracellular cytokines were IFNγ, TNFα, and perforin. The apoptosis assay was conducted using a commercially available kit (#559763 BD Biosciences) and analyzed using an LSR Cytometer (**Figures 1**, **2**).

Box 1 | Antibodies used for flow panels.

Viability Dye AmCyan (#65-0866-14)	(NK1.1 APC # 17-5941-82)
Fc Blocker (#553141)	Gr-1 AF700 (#56-5931-82)
CD45 APC-Cy7 (#47-0112-82)	CD183 (CXCR3)
CD3 PE-Cy5.5 (#35-0031-82)	PerCP-Cy5.5 (#45-1831-82)
PE-Cy7 (# 25-5711-82)	MHCII APC (#17-5320-82)
PE (# 12-0033-82)	CD314 (NKG2D) PE (# 12-5882-82)
CD4 e450 (#48-0042-82)	TCRγδ PE-Cy5 (#15-5711-82)
CD8 PE Tex Red (# MCD0817)	IFNγ PE-Cy7 (#25-7311-82)
CD19 FITC (#11-0191-82)	TNFα APC-Cy7 (#502944 Biolegend)
NK1.1 eF450(#MM6628)	Perforin FITC (#11-9392-82)

All antibodies were obtained from eBioscience except Fc Blocker (BD Biosciences) and TNFα (Biolegend)

Statistics and Data Analysis

All data were collected in Excel (Microsoft, USA) and analyzed using Excel or Graph Pad (Prism, USA). The flow cytometry data was analyzed using FlowJo (Treestar, OR, USA). Flow cytometry data are graphically presented as median (min-max) and quoted as median (IQR). These were tested with Kruskal Wallis and Dunns multiple comparison test. All other values are presented as mean (standard error of the mean: SEM) and analyzed using parametric statistics. The statistical applications are detailed in the results.

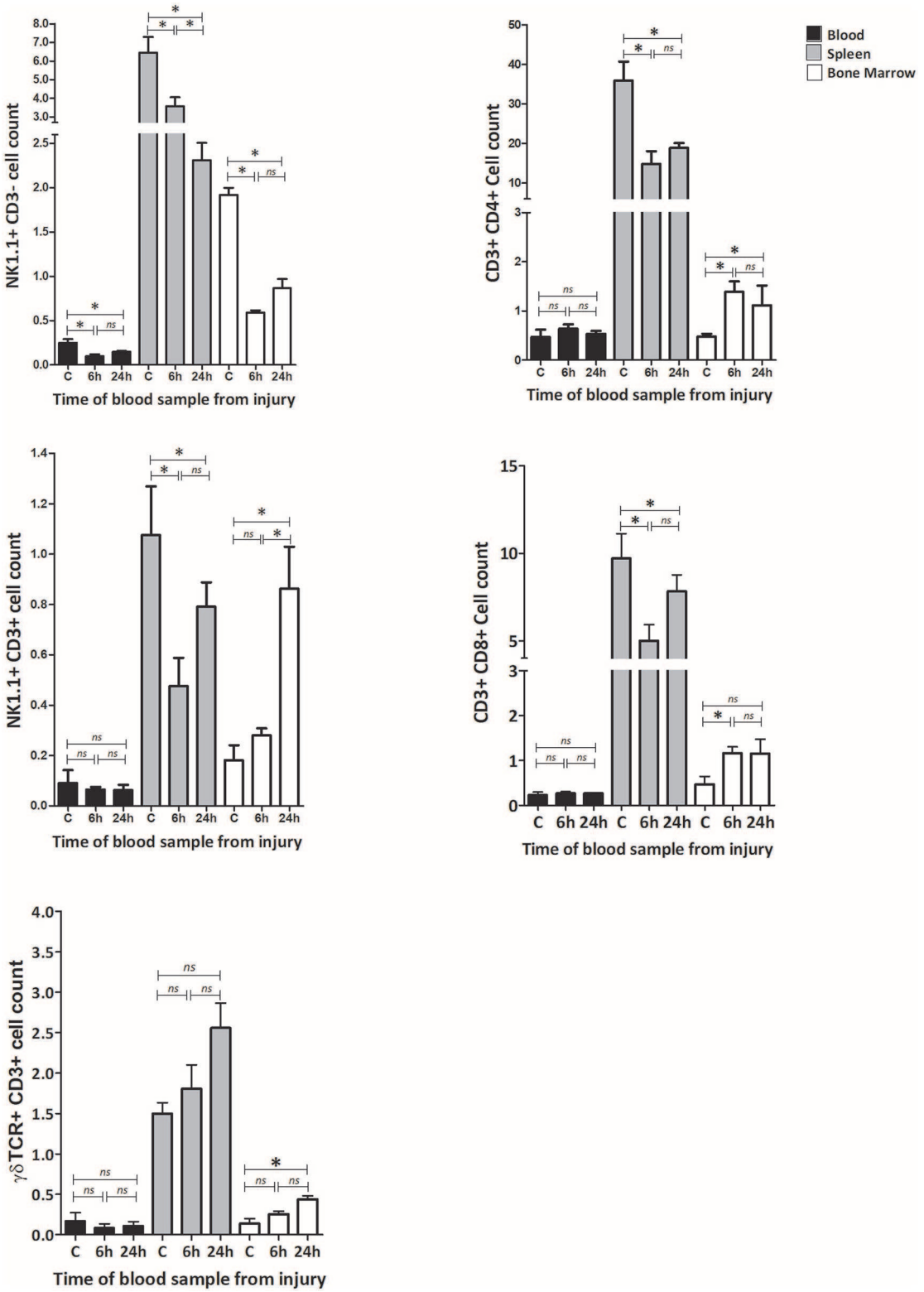

FIGURE 4 | T&HS provokes dynamic changes in lymphocyte subsets which are cell specific, compartment specific and time dependent. Flow cytometry was used to examine the lymphocyte subset populations at 6 and 24 h following injury. Blood, spleen and bone marrow were the compartments selected for examination. Changes in peripheral blood populations of NK 1.1+ CD3– cells were observed at 6 h, while NK1.1+ CD3+, γδTCR+ CD3+ cells, CD3+ CD4+, and CD3+ CD8+

(Continued)

FIGURE 4 | cell populations remained unchanged. In spleen, a decrease in the population of NK 1.1+ CD3–, NK1.1+ CD3+, CD3+ CD4+, and CD3+ CD8+ was observed by 6 h, while γδTCR+ CD3+ cells remained broadly unchanged. In bone marrow, the NK 1.1+ CD3– cell population fell by 6 h while the other cell populations were seen to increase. Examination of lymphocyte sub-set populations revealed dynamic shifts within the immune compartments, principally within the first 6 h. Control (C) denotes a naïve subject of the same strain and age. Data are presented as mean (SEM) absolute cell counts $\times 10^6$ (per ml of blood, per spleen or per both hind limbs) and tested using Kruskal Wallis with Dunn's multiple comparison of columns, *denotes $p < 0.05$.

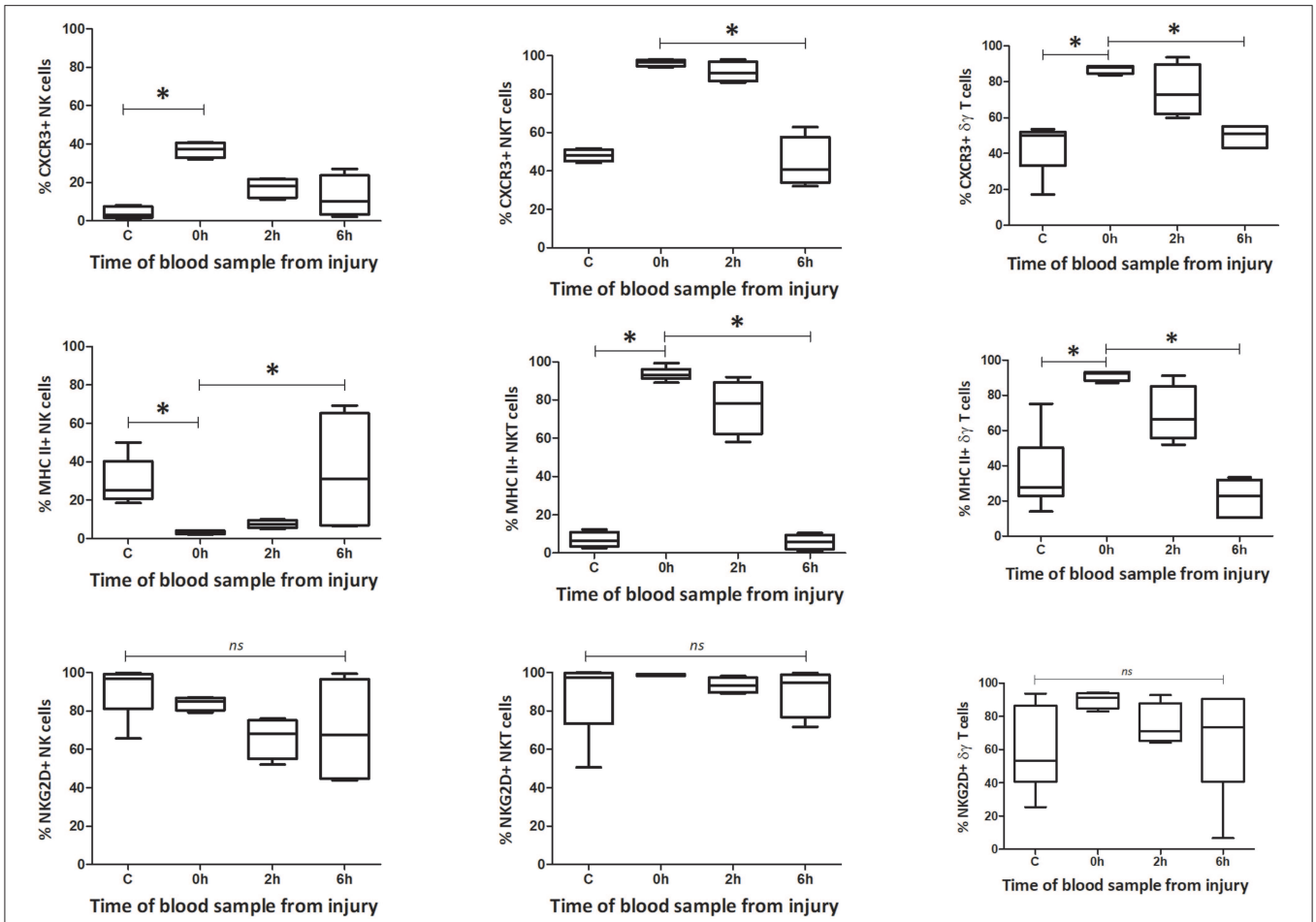

FIGURE 5 | Innate-like cells in blood change their phenotype and function during the first 6 h following T&HS. To determine whether the innate-like cells in this study changed their phenotype after T&HS, flow cytometry was conducted on blood and spleen taken at several sample points during the protocol. This revealed dynamic changes in all three cells, within the first 6 h of injury. Percentage blood phenotype: percentage of CXCR3, MHC II, and NKG2D positive cells. Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

RESULTS

At 6 h Following T&HS Lymphocyte Count Was Reduced in Blood and Lung, Static in Spleen and Increased in Bone Marrow

A manual hemocytometer count of peripheral blood leucocytes was conducted to determine changes at set intervals during a 24 h murine trauma and hemorrhagic shock (T&HS) protocol (**Figure 3A**). At the beginning of the hemorrhagic shock period (0 h) a leucocytosis was observed. This became a leucopenia by 6 h and returned to baseline by 24 h. Flow cytometry was then used to examine changes in lymphocyte count in several different tissue compartments, namely blood, spleen and bone marrow. A significant drop in lymphocyte count was demonstrated at 6 h in peripheral blood but this was not mirrored in spleen or bone marrow (**Figure 3B**). The lymphocyte count in the spleen remained static at 6 h but fell by 24 h. In bone marrow, the lymphocyte count had increased at 6 h and remained elevated at 24 h. An automated full blood count revealed a fall in hemoglobin at 6 and 24 h, as would be expected of this model [$C = 13.3(12.1–14.5)$, $6\,h = 7.6(6.8–8.3)$, $24\,h = 6.2(5.8–6.6) \times 10^3$/ul, $p < 0.01$ C vs. 24 h].

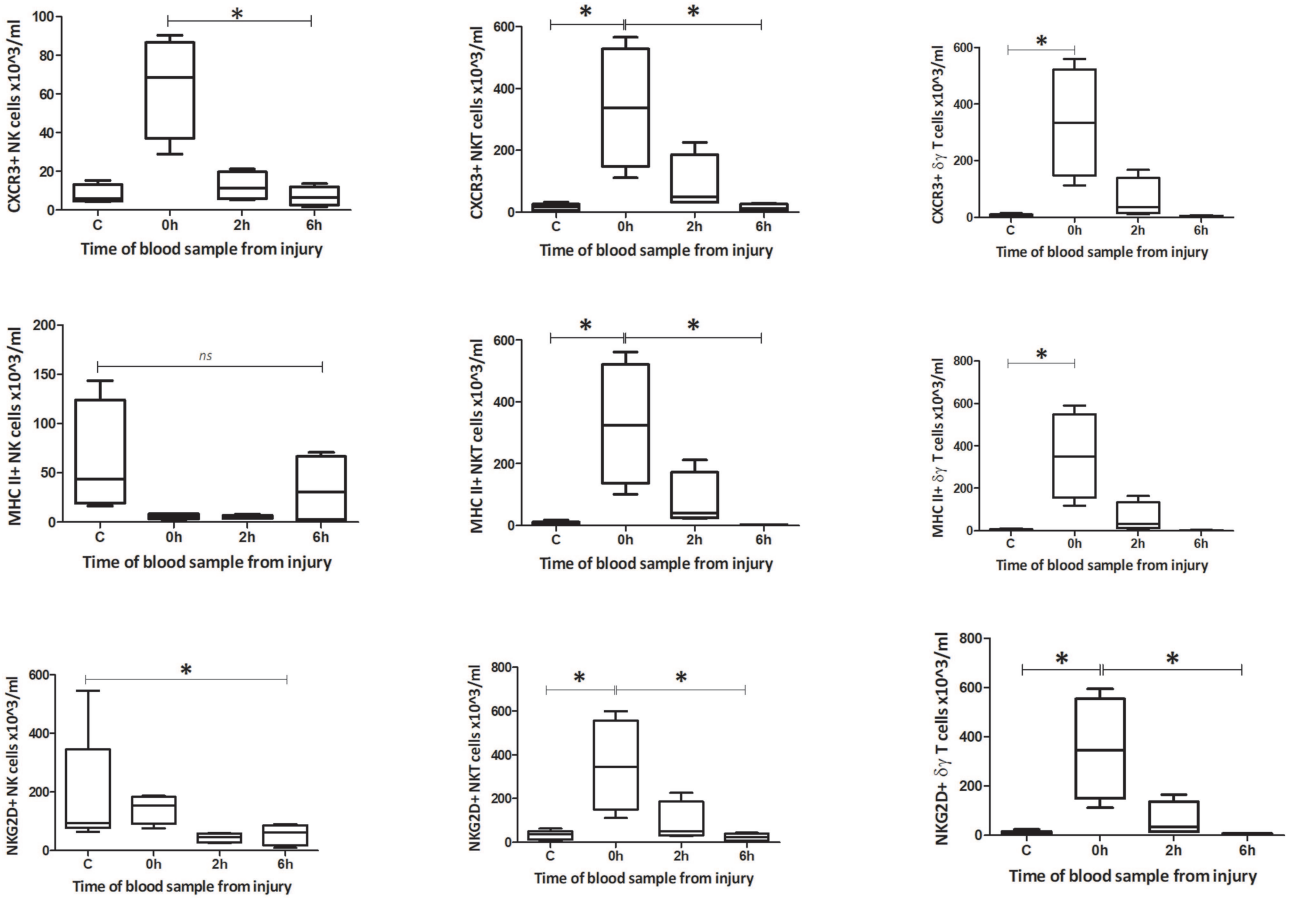

FIGURE 6 | Absolute cell count for blood phenotype: Number of CXCR3, MHC II, and NKG2D positive cells in blood (n = 5–8). Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

To investigate this sudden loss of lymphocytes from circulation, evidence of migration into other "non-lymphoid" tissues was sought. We chose to examine lung as it is commonly involved in human MODS. Lung tissue was examined in Control and 6 h samples using flow cytometry (**Figure 3C**). A marked decrease in the percentage of lymphocytes within lung tissue was observed at 6 h [C = 66% (65–68), 6 h = 39% (36–41), p = 0.02]. This was not unexpected as there is a known influx of granulocytes at this timepoint; however, when calculated, the lymphocyte count also demonstrated an almost 3-fold reduction [C = 86 (84–87), 6 h = 31 (28–32), $p < 0.01$]. Data are presented as mean (SEM) and tested with a Mann Whitney U-test (31).

To determine whether lymphocyte apoptosis could explain the reduction in circulating lymphocytes, a standard cell death assay was used to examine blood, spleen and bone marrow at 6 and 24 h (**Figure 3D**). No organ compartment demonstrated a greater percentage of apoptotic cells (Annexin V + 7AAD-) than the Control; however, an increase in lytic lymphocyte cell death (Annexin V+ 7AAD+) was demonstrated in the spleen at the 6 h timepoint [Annexin V+7AAD+: C =6 (6–7), 6 h = 31 (29–32), 24 h = 6 (5–7), p = 0.02]. Data are reported as median (IQR) and tested with Kruskal Wallis and Dunns comparison of all columns. Lymphocyte loss from circulation was demonstrated at 6 h post injury, although this could not be attributed to widespread apoptosis. Lymphocyte loss from the lungs was also observed. In contrast lymphocyte numbers at 6 h were static in the spleen and increased bone marrow.

T&HS Provokes Dynamic Changes in Lymphocyte Subsets Which Are Cell Specific, Compartment Specific, and Time Dependent

Flow cytometry was used to examine changes in the frequency of lymphocyte subsets. The innate lymphocyte subsets including NK cells (NK1.1 + CD3– cells), NKT cells (NK1.1 + CD3+ cells), and γδTCR cells (γδTCR + CD3 + cells) were examined in three immunological compartments; blood, spleen and bone marrow, at 6 h and 24 h post T&HS. Classical T cell subsets, including CD3+ CD4+ cells and CD3+ CD8+ cells were quantified to provide a comparison (**Figure 4**; **Supplementary Table 1**).

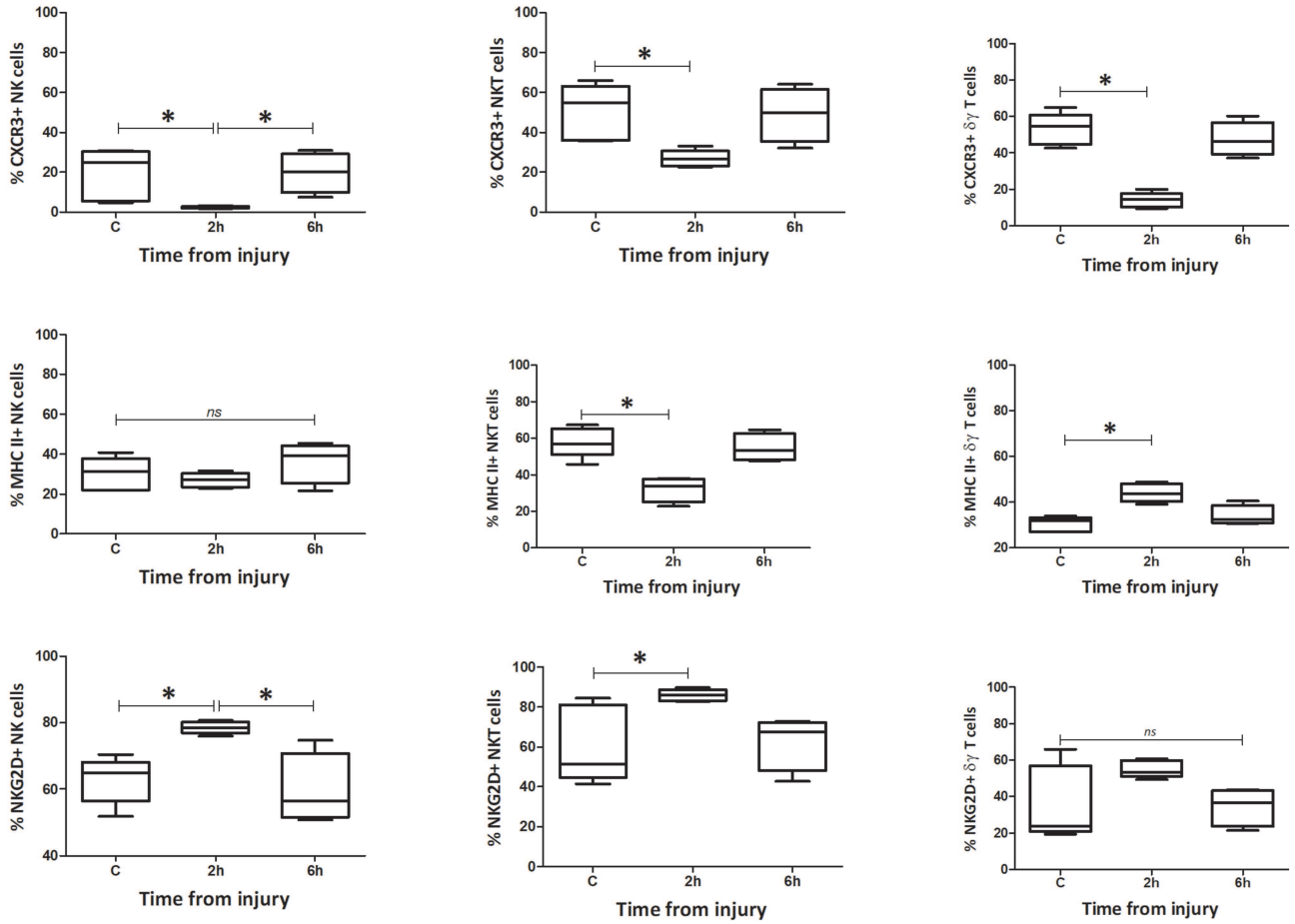

FIGURE 7 | Percentage spleen phenotype: percentage of CXCR3, MHC II, and NKG2D positive cells. Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

Within peripheral blood, the population of NK1.1+ CD3– cells fell by 6 h, while all the other populations remained static. Within spleen, a reduction in cell count was observed at 6 h in NK1.1 + CD3–, NK1.1+ CD3+, CD3+ CD4+ and CD3+ CD8+ cells, while γδTCR+ CD3+ cells remained static. In bone marrow, the NK1.1+ CD3– cell population fell dramatically by 6 h. In contrast, CD3+ CD4+ and CD3+ CD8+ cells increased in cell count while NK1.1+ CD3+ and γδTCR+ CD3+ remained static. At 6 h following injury, the NK1.1+ CD3– cell population had reduced in all three of the examined compartments, CD3+ CD4+ and CD3+ CD8+ cells were down in spleen and up in bone marrow. CD3+ γδTCR+ cells increased in bone marrow by 24 h but displayed no earlier changes. Fluctuations in cell populations at 6 h and 24 h following injury were cell specific, tissue specific, and time dependent.

Innate-Like Cells Change Their Phenotype and Function During the First 6 h Following T & HS

The secondary objective of this study was to investigate changes in phenotype and activity of the innate like cells. As the greatest change in cell numbers occurred within 6 h of injury, we increased our early sampling time-points. Using flow cytometry, the surface markers and intracellular cytokine levels were examined in NK1.1+ CD3–, NK1.1+ CD3+, and γδTCR+ CD3+ cells at 0 h, 2 h, and 6 h following T&HS. Blood and spleen were examined to facilitate comparison between the peripheral circulation and a principal lymphoid organ. The innate-like lymphocytes express many cell surface antigens but we selected, CXCR3, MHC II, and NKG2D for our phenotypic characterization. Similarly, all three cell types produce a range of cytokines but we selected three mutual mediators to indicate functional activity namely: interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα) and perforin.

Phenotype

Within peripheral blood, the percentage and count of CXCR3+ NK1.1+ CD3–, NK1.1+ CD3+, and γδTCR+ CD3+ cells increased immediately following injury (0 h) (**Figures 5–8**). The percentage and number of CXCR3+ cells then returned to baseline, for all three subsets, by 6 h after injury. By contrast, the percentage and number of CXCR3+ cells within the

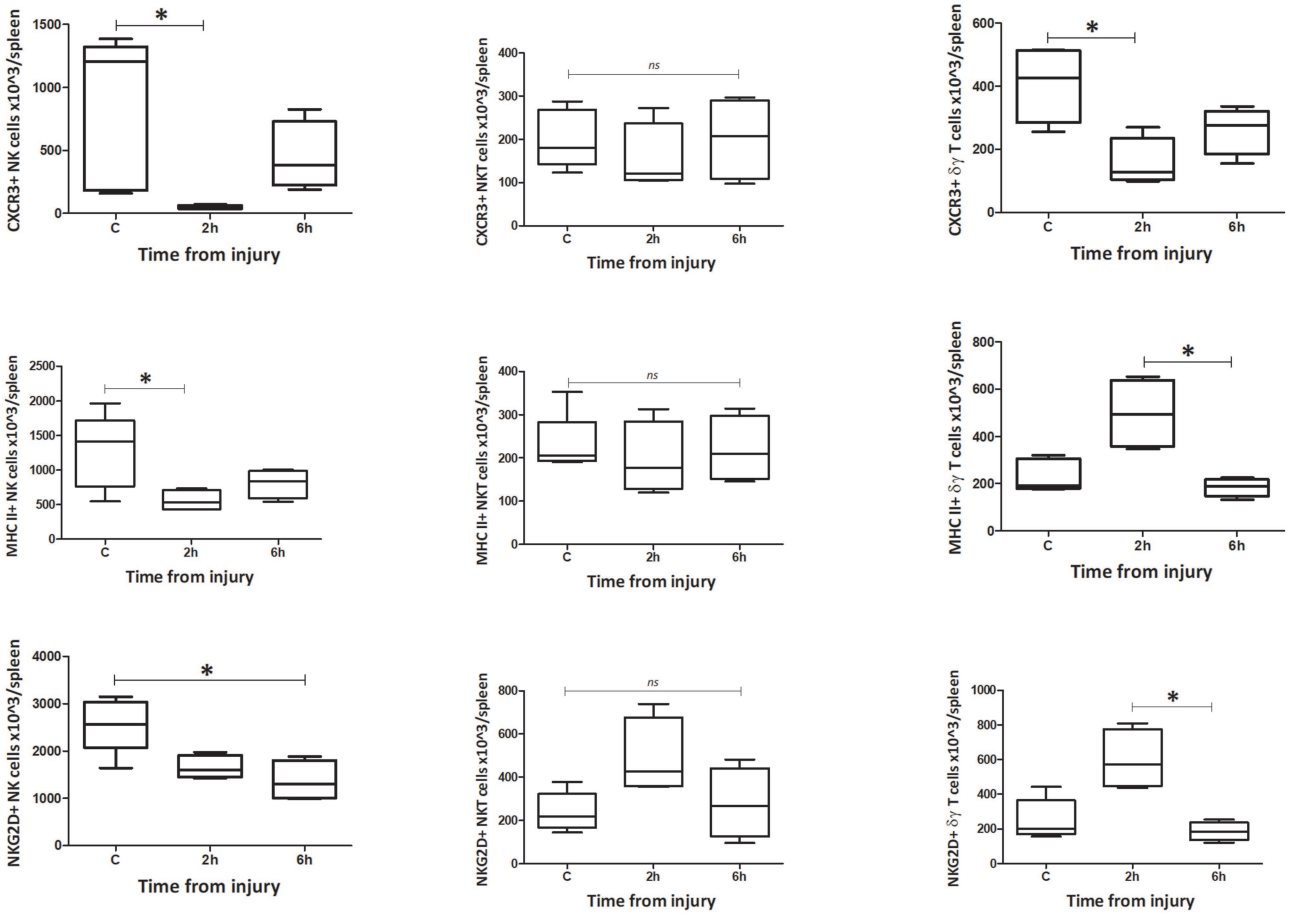

FIGURE 8 | Absolute cell count for spleen phenotype: Number of CXCR3, MHC II, and NKG2D positive cells in spleen ($n = 5$–8). Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

spleen decreased by 2 h for all three subsets but then also returned to baseline by 6 h. The circulating NK1.1+ CD3– population demonstrated a decrease in MHC II+ cells. This contrasted with a dramatic rise in MHCII+ NK1.1+ CD3+, and γδTCR+ CD3+ cells within blood. Changes in the spleen were less clear-cut, but demonstrated a fall in MHCII+ NK1.1+ CD3– and NK1.1+ CD3+ cells, while MHCII+ γδTCR+ CD3+ cells increased. The number of NKG2D+ NK1.1+ CD3– cells gradually decreased over 6 h, while the number of NKG2D+ NK1.1+ CD3+ and γδTCR+ CD3+ cells increased immediately after T&HS (0 h) and then fell back to baseline. Changes in innate-like cell phenotype with respect to CXCR3, MHCII and NKG2D, were observed at 0 h and 2 h following T&HS. All phenotypic changes had returned to baseline by 6 h (**Supplementary Table 2**).

Function

The number of IFNγ+ NK1.1+ CD3– cells within circulating blood fell precipitously by the 0 h time point, but the population was restored by 6 h (**Figures 9–12**). Conversely, NK1.1+ CD3+ and γδTCR+ CD3+ cells demonstrated increased intracellular IFNγ at 0 h, that had returned to baseline levels by 6 h. Within the spleen, a slight increase in IFNγ+ NK1.1+ CD3– cells by 6 h was observed, while IFNγ+ NK1.1+ CD3+ and γδTCR+ CD3+ cells displayed a transient rise at 2 h, that returned to baseline by 6 h. Intracellular TNFα was lower in all three cell types within blood and spleen by 2 h following injury. Intracellular perforin levels within the blood borne cells broadly mirrored IFNγ levels. An immediate fall in Perforin+ NK1.1+ CD3– cells was observed, with a gradual increase back to baseline by 6 h. Whereas, NK1.1+ CD3+ and γδTCR+ CD3+ cells demonstrated a sudden increase at 0 h and a reduction back to baseline by 6 h. Within the spleen, a reduction in perforin+ NK1.1+ CD3– and NK1.1+ CD3+ cells was observed at 2 h. Conversely an increase in perforin positive γδTCR+ CD3+ cells was seen at 2 h. Wave-like fluctuations in intracellular IFNγ, TNFα, and perforin were observed in all three innate-like cell types during the 6 h protocol, predominantly with levels returning to baseline by 6 h. T&HS prompted rapid cytokine production by innate-like lymphocytes within blood and spleen (**Supplementary Table 2**).

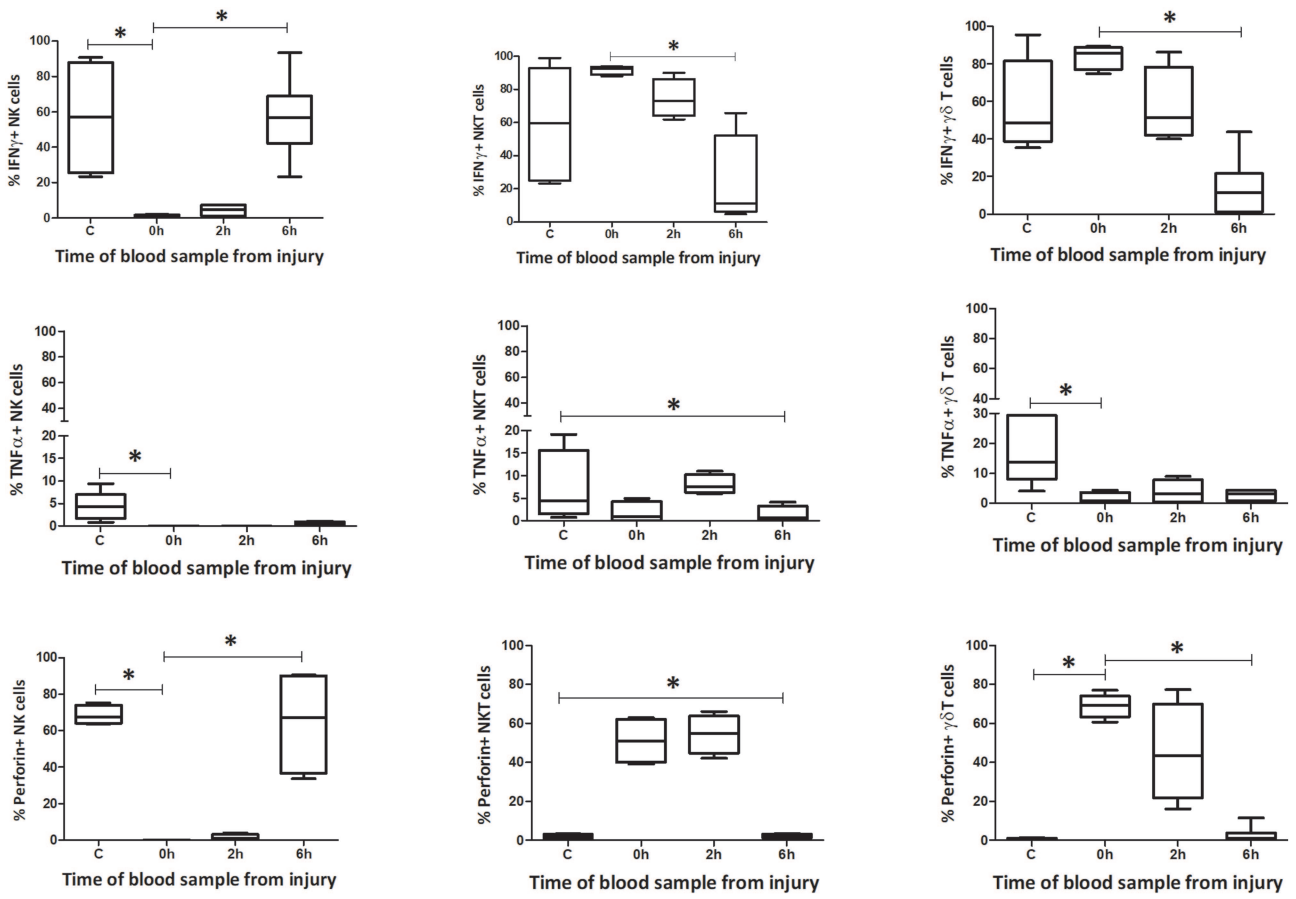

FIGURE 9 | Innate-like cells in the spleen change their phenotype and function during the first 6 h following T&HS. To determine whether the innate-like cells in this study changed their intracellular cytokine levels after T&HS, flow cytometry was conducted on blood and spleen taken at several sample points during the protocol. This revealed extremely dynamic changes in all three cell types, within the first 6 h of injury. Percentage blood: percentage of IFNγ, TNFα, and Perforin positive cells. Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

Changes in Phenotype and Function of Innate-Like Lymphocytes Within Lung, Were Comparatively Delayed

To determine whether the changes in NK1.1+ CD3–, NK1.1+ CD3+, and γδTCR+ CD3+ cells within the lung were consistent with those seen in blood or spleen, we performed the same phenotype and function analyses on the innate-like subsets from lung tissue homogenates. This demonstrated that the populations of NK1.1+ CD3– and NK1.1+ CD3+ cells within lung decreased by 6 h, while the population of γδTCR+ CD3+ cells remained unchanged (**Figure 13A**). NK1.1+ CD3– cells demonstrated a dramatic increase in the percentage of MHC II+ cells at 6 h following trauma, although no change was observed in NK1.1+ CD3+ or γδTCR+ CD3+ cells (C vs. 6h: NK1.1+ CD3– = 12 (2) vs. 52 (9), $p = 0.02$, NK1.1+ CD3+ = 80 (2) vs. 77 (4), $p = 0.56$, γδTCR+ CD3+ = 6 (1) vs. 3 (1), $p = 0.09$) (**Figure 13B**). No difference in NKG2D or CXCR3 expression was identified between the C and 6 h timepoint models, in any of the three cell types. All three subsets increased their percentage of IFNγ+ cells by 6 h following T&HS (**Figure 13C**). No change in TNFα or perforin positive cells was observed [C vs. 6 h: NK1.1+ CD3– = 6 (1) vs. 36 (5), $p = 0.02$, NK1.1+ CD3+ = 6 (1) vs. 22 (6), $p = 0.02$, γδTCR+ CD3+ = 16 (2) vs. 59 (6), $p = 0.02$. $n = 6$–8]. Data are presented as median (IQR) and tested using a Mann-Whitney *U*-test. The responses of the innate like cell subsets in the lung were distinctly different from those seen in blood and spleen. They were characterized by a dramatic rise in MHC II expression on the NK1.1+ CD3– cells and an increase in IFNγ production by all three cell types. In addition, the responses of these lung-based lymphocytes was distinctly delayed when compared to the cells in blood and spleen, where rapid and early changes had predominantly returned to baseline by 6 h.

DISCUSSION

This study used a well-established murine model of T&HS to define the dynamics of lymphopenia and examine innate-like lymphocyte behavior within different organs, at structured intervals following injury. It confirms that lymphopenia occurs

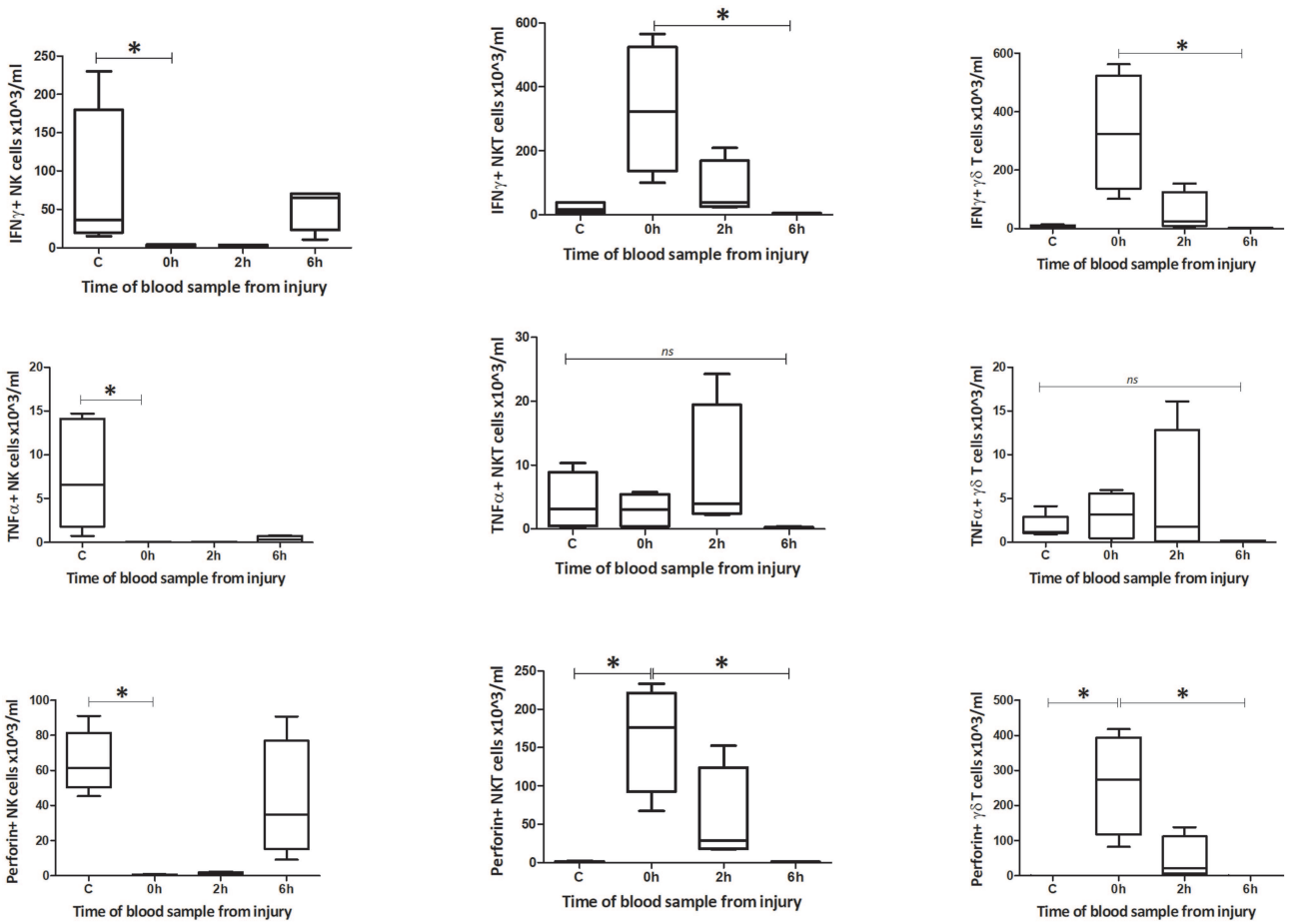

FIGURE 10 | Absolute cell count for blood: number of IFNγ, TNFα, and Perforin positive cells in blood ($n = 5$–8). Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

between 2 and 6 h in this murine model, which supports human observations and suggests murine work is of value to this line of investigation (10, 11, 15). Lymphopenia is frequently attributed to widespread apoptosis, but we were unable to demonstrate this (13, 14, 32). We sought evidence of lymphocyte movement out of circulation and into tissues by examining lung, but this was also not significant. There are many other potential destinations for lymphocyte trafficking and examination of injury sites, liver, lymph nodes and gut may be of value in future. Secondly, this study revealed that T&HS provokes an immediate response from the NK1.1+ CD3–, NK1.1+ CD3+, and γδTCR+ CD3+ lymphocyte subsets, with respect to cell count, phenotypic display, and cytokine production. These changes are cell specific, tissue specific and time-dependent, although the responses of NK1.1+ CD3+ and γδTCR+ CD3+ cells often co-aligned. The findings highlight the need for future study examining each cell type individually, with a focus on the hyper-acute phase (<2 h following injury). The immediacy of the response in blood and lymphoid tissues (spleen and bone marrow) is very clear, as 0 h and 2 h time-points reveal the greatest changes and levels frequently returned to baseline by 6 h. By contrast lung tissue responses were still evolving at 6 h, suggesting that the inflammatory response within non-lymphoid organs may lag behind or follow a different course. This study presents novel information about early immune cell behavior following trauma and suggests that in order to determine the contribution of immune cells to MODS development, we may need to examine cells within the organs themselves.

This study has demonstrated that the hyper-acute response in innate-like lymphocytes is complex and dynamic. Further work is required to fully characterize the behavior but certain observations require particular mention. Bone marrow at 6 h demonstrated an increase in total lymphocyte count, which suggests, cell ingress or increased proliferation. Immature cells have been identified in blood after trauma and bone marrow failure has been demonstrated in humans trauma patients with MODS, therefore formal investigation of the hyper-acute bone marrow response may aid understanding of cell production and trafficking and its implication for MODS development

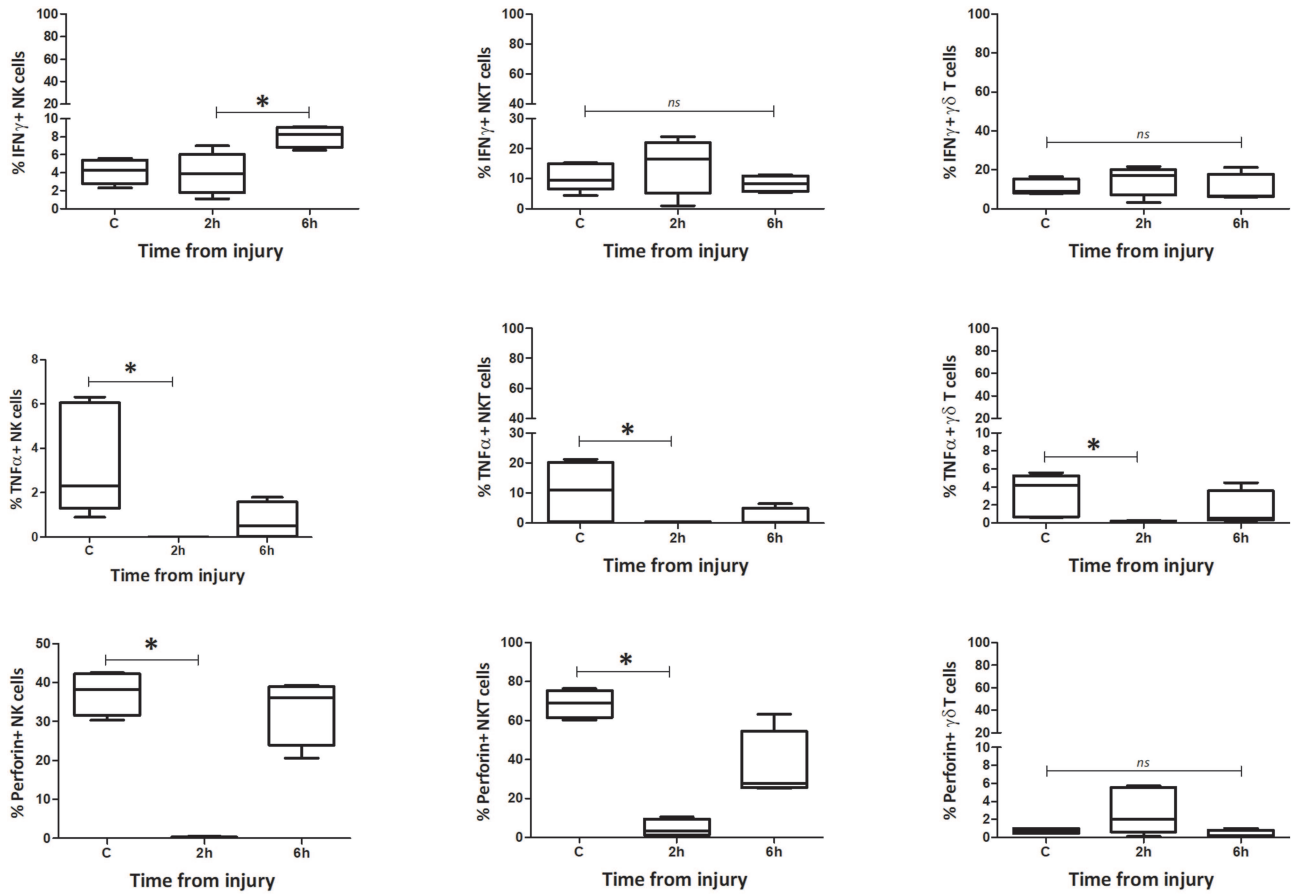

FIGURE 11 | Percentage spleen: percentage of IFNγ, TNFα, and Perforin positive cells. Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test. *denotes $p < 0.05$.

(33, 34). In contrast, NK1.1+ CD3– cells within bone marrow had reduced by 6 h. These cells did not remain in circulation and did not accumulate in lung, therefore their destination and role remain unclear. Bone marrow derived hematopoietic progenitor cells are vital to healing at injury sites and so future investigation could examine areas of injury in more detail (35–38). CXCR3 is a migration marker known to recruit NK and NKT cells to sites of injury. This study revealed an immediate (0 h) increase in CXCR3+ populations of blood-borne NK1.1+ CD3– and NK1.1+ CD3+ cells, which supports a potential for migration (33, 39, 40). MHCII expression was increased on circulating NK1.1+ CD3– cells by 6 h, but increased more quickly on circulating NK1.1+ CD3+ and γδTCR+ CD3+ cells. Within lung, MHCII was substantially increased on NK1.1+ CD3– cells at 6 h, while on NK1.1+ CD3+ and γδTCR+ CD3+ cells it remained unchanged. In other disease settings, NK cells are known to acquire MHCII from activated dendritic cells via trogocytosis (41) Activated γδ T cells are also known to acquire MHCII expression and present antigens, but the relevance for NK1.1+ CD3+ cells is less clear (42, 43) As endogenous alarmin molecules are considered the trigger(s) for the sterile inflammatory response to trauma, the presence of MHCII on these innate-like lymphocyte subsets represents a potential mechanism for organ injury and warrants further investigation (44–46). Finally, IFNγ, TNFα, and perforin represent potentially important cytokine mediators during the hyper-acute phase of inflammation. In the blood and spleen populations, cytokine production appeared sequential and orchestrated, with immediate release from NK1.1+ CD3– cells, followed by production and assumed release from NK1.1+ CD3+ and γδTCR+ CD3+. This wave-like signaling was not only extremely rapid but short-lived as concentrations returned to baseline by 6 h, suggesting that other molecules may then be responsible for downstream propagation. These findings suggest that T&HS activate both the pro-inflammatory and cytoxic functions of NK cells and consistent with this notion, we have previously shown that NK1.1+ CD3– cell populations drive the propagation of monocyte infiltration and injury in the liver in this model (35). These results provide a springboard for future research regarding the role of innate-like lymphocytes in the hyperacute inflammatory response to trauma and the pathogensis of MODS.

There are limitations of this study which we acknowledge, primarily the relatively small group sizes. Confirmation of these

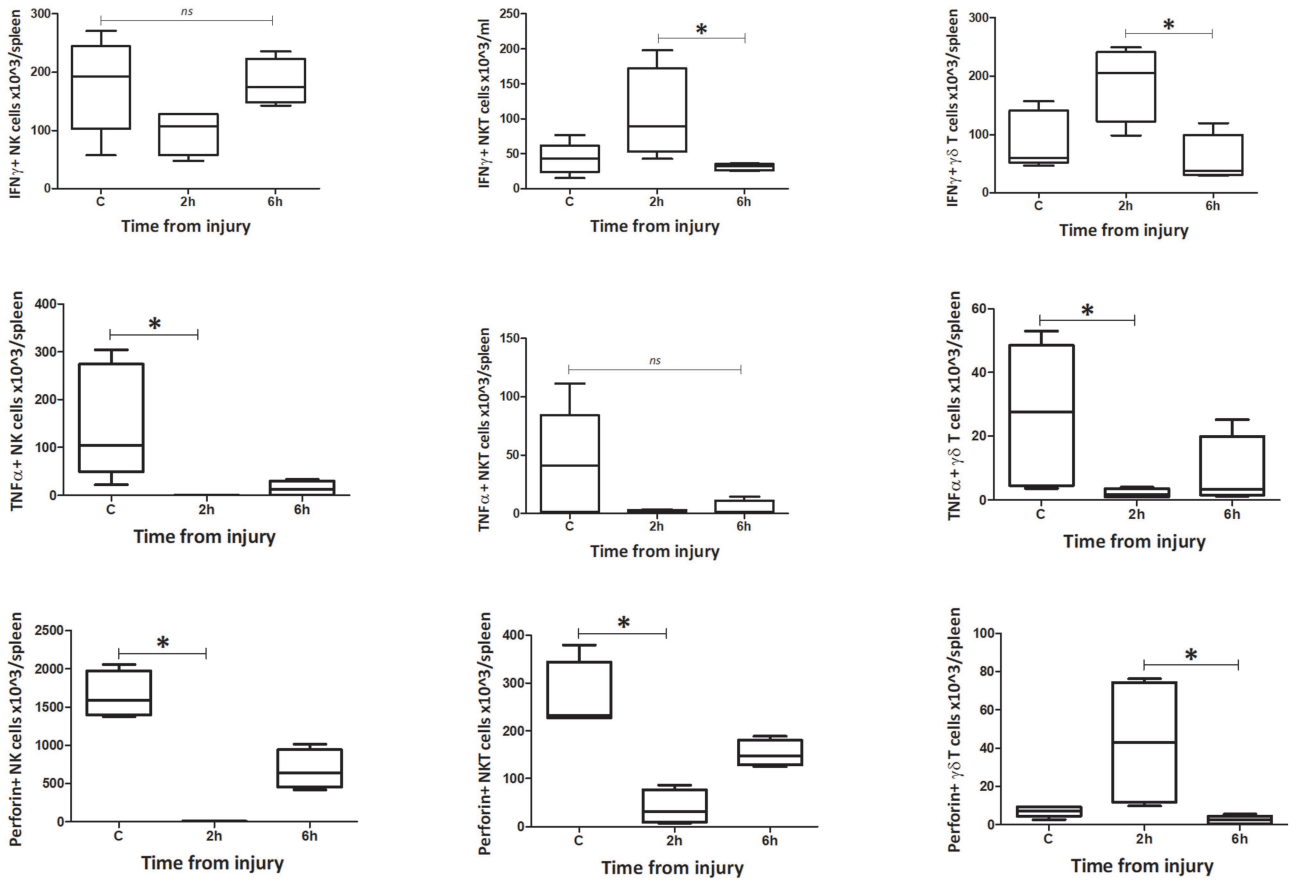

FIGURE 12 | Absolute cell count for spleen: number of IFNγ, TNFα, and Perforin positive cells in spleen ($n = 5$–8). Data are presented as median (min-max) and tested with Kruskal Wallis and Dunns multiple comparison test, *denotes $p < 0.05$.

findings in larger groups and other trauma models would be desirable. More refined NKT cell definition would have been achieved had we included a CD1d marker. Liver necrosis has been demonstrated in this T&HS model which may contribute to the lymphocyte death demonstrated in the spleen at 6 h (47). Further examination of lung tissue at the 0, 2, or 24 h time-points would be required to fully describe the organ events. Investigation of a broader range of immune cells would also be desirable as we have previously demonstrated a key role for innate lymphoid cells in lung injury in this model (48). Lymph node populations and bone marrow cell proliferation were not examined but may be incorporated into future experiments. Cytokine release is assumed but measurement of systemic IFNγ, TNFα, and perforin concentrations at each timepoint would be required for confirmation. Finally, the relevance of these study findings to the development of human MODS is unclear although we hope it adds to the understanding of the hyper-acute inflammatory mechanisms in play following trauma.

In conclusion, this study has demonstrated novel findings regarding the activation and response of three innate-like lymphocyte subsets in the hyper-acute phase of the immunological response to traumatic injury. It demonstrates that the greatest changes in these cells are occurring between 0 and 2 h following injury, reinforcing the importance of early sampling times. It highlights that events within blood are not mirrored by the tissues, therefore study of individual organ tissues will be needed to understand their responses and the pathogenesis of MODS development. These results lend support to the evidence from human studies regarding the potential importance of lymphocytes to the early immune response to injury (10–12, 15). It provides further evidence that the immunological response to trauma is not a dysregulated, dysfunctional reaction, but more likely a consistent, proportional, integrated and complex response (1). Trauma provides a unique population for investigation of these early inflammatory events, as the timing of the initiating stimulus can be precisely determined. Greater understanding of the mechanisms involved in trauma immunology could be extremely valuable, as expediting MODS recovery stands to benefit many other medical specialties.

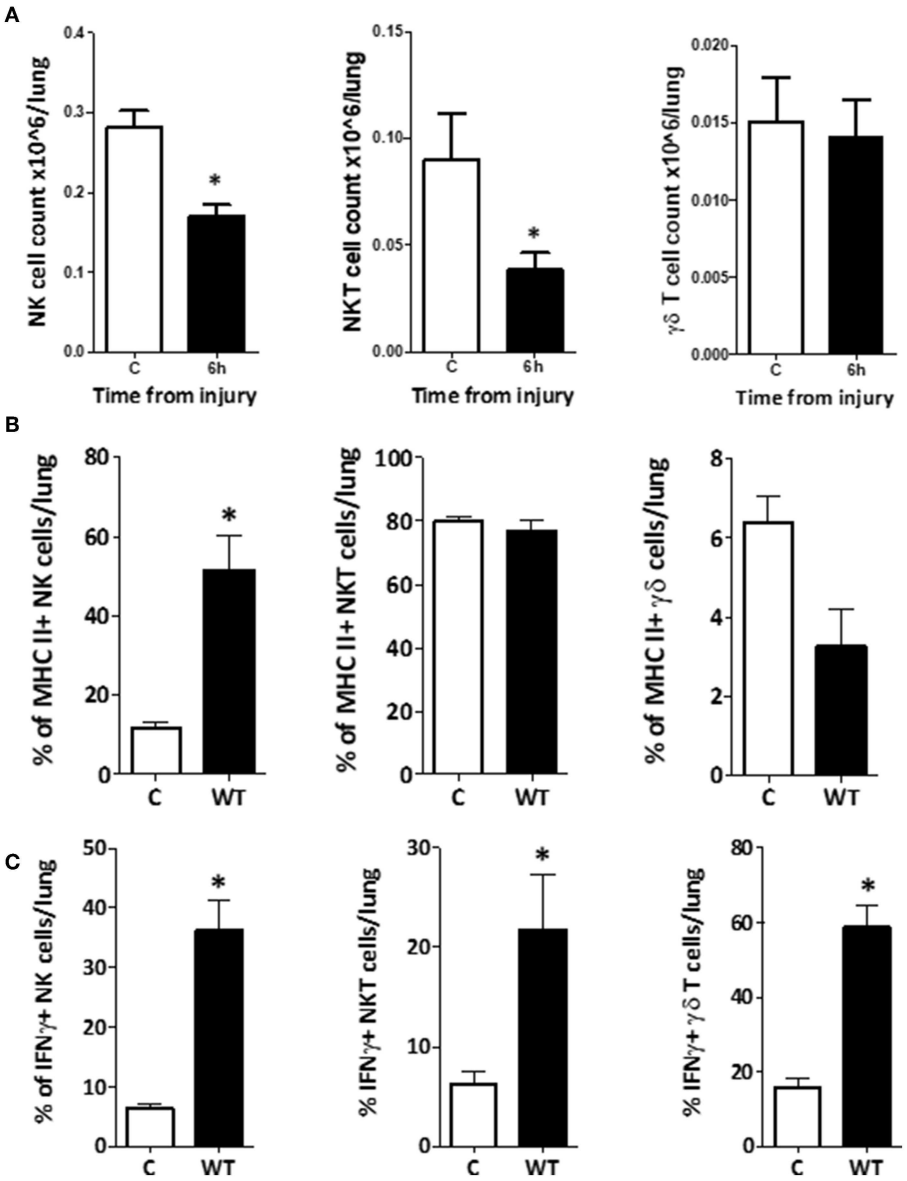

FIGURE 13 | Changes in phenotype and function of innate-like lymphocytes within lung, were comparatively delayed. **(A)** To investigate whether the innate-like lymphocytes within lung also displayed changes in phenotype or function after T&HS, flow cytometry of a single cell suspension of lung tissue was conducted using the same staining protocol. The total number of NK1.1+ CD3– and NK1.1+ CD3+ cells within the lung reduced by 6 h, but no change in γδ TCR+ CD3+ cells was demonstrated. Cell counts ($\times 10^6$/lung): NK1.1+ CD3–: 0.28 (0.26–0.30) vs. 0.17 (0.15–0.18), $p = 0.02$. NK1.1+ CD3+: 0.09 (0.06–0.11) vs. 0.04 (0.03–0.04), $p = 0.04$. γδ TCR+ CD3+: 0.01 (0.01–0.02) vs. 0.02 (0.01–0.02), $p = 0.89$, $n = 6$–8. Control (C) represents a naïve subject of the same strain and age, $n = 4$–6. Data are presented as mean (SEM) and tested with a Mann Whitney test, *denotes $p < 0.05$. **(B)** NK1.1+ CD3– cells in lung tissue upregulated MHCII expression by 6 h following T&HS. Cell surface phenotype markers on NK1.1+ CD3–, NK1.1+ CD3+, and γδ TCR+ CD3+ cell populations were examined in lung tissue, using flow cytometry at 6 h following injury. The NK1.1+ CD3– cell population demonstrated upregulation of MHC II, while NK1.1+ CD3+ MHCII expression remained the same. γδ TCR+ CD3+ cells appeared to downregulate MHCII, although this did not reach significance. NK1.1+ CD3–: C vs. 6h: 12 (2) vs. 52 (9), $p = 0.02$. NK1.1+ CD3+: C = 80 (2) vs. 77 (4), $p = 0.56$. γδ TCR+ CD3+: C = 6 (1) vs. 3 (1), $p = 0.09$. Control (C) denotes a naïve subject of the same strain and age ($n = 4$–6). Data are presented as median (IQR) and tested with a Mann Whitney test, *denotes $p < 0.05$. **(C)** NK1.1+ CD3–, NK1.1+ CD3+, and γδ TCR+ CD3+ cells within lung tissue, all demonstrated an increase in the % of IFNγ+ cells by 6 h following T&HS. To assess activity of innate-like lymphocytes within lung tissue, intracellular IFNγ, TNFα, and perforin were examined using flow cytometry. At 6 h following T&HS more NK1.1+ CD3–, NK1.1+ CD3+, and γδ TCR+ CD3+ cells within lung tissue, were positive for IFNγ compared with their naïve state. No change in TNFα or perforin production was observed. Control (C) denotes a naïve subject of the same strain and age. C vs. 6 h: NK1.1+ CD3– = 6 (1) vs. 36 (5), $p = 0.02$, NK1.1+ CD3+ = 6 (1) vs. 22 (6), $p = 0.02$, γδ TCR+ CD3+ = 16 (2) vs. 59 (6), $p = 0.02$. $n = 6$–8 and data are presented as median (IQR) and tested with a Mann Whitney test, *denotes $p < 0.05$.

AUTHOR CONTRIBUTIONS

JM devised and conducted all the experiments and wrote the manuscript. RH provided technical support and supervision. SC and MR assisted with data collection. The manuscript was edited by all authors. TB stands as guarantor.

FUNDING

The Royal College of Surgeons of England Fulbright Scholars Award—funded JM to conduct the research.

The University of Pittsburgh—hosted JM and funded consumables.

A National Institute of Health Research (NIHR) Clinical Lectureship CL-2014-19-002 funded JM to analyse the data and write the manuscript. This paper presents independent research. The views expressed are those of the authors and not necessarily those of the NHS, NIHR or the Department of Health and Social Care.

FUNDED BY NIHR | National Institute for Health Research

ACKNOWLEDGMENTS

This work was supported by The Royal College of Surgeons of England Fulbright Scholars Award, The University of Pittsburgh and the National Institute of Health Research (NIHR) Clinical Lectureship. Our thanks to Lauryn and Aaron for the technical model work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01501/full#supplementary-material

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

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frontiers in Immunology

CLINICAL TRIAL
published: 20 September 2019
doi: 10.3389/fimmu.2019.02179

Imbalance of Circulating Innate Lymphoid Cell Subpopulations in Patients With Septic Shock

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Background: Septic shock, a major cause of death in critical care, is the clinical translation of a cytokine storm in response to infection. It can be complicated by sepsis-induced immunosuppression, exemplified by blood lymphopenia, an excess of circulating Treg lymphocytes, and decreased HLA-DR expression on circulating monocytes. Such immunosuppression is associated with secondary infections, and higher mortality. The effect of these biological modifications on circulating innate lymphoid cells (ILCs) has been little studied.

Methods: We prospectively enrolled patients with septic shock (Sepsis-3 definition) in the intensive care unit (ICU) of Timone CHU Hospital. ICU controls (trauma, cardiac arrest, neurological dysfunction) were recruited at the same time (NCT03297203). We performed immunophenotyping of adaptive lymphocytes ($CD3^+$ T cells, $CD19^+$ B cells, $CD4^+CD25^+FoxP3^+$ Treg lymphocytes), ILCs ($CD3^-CD56^+$ NK cells and helper ILCs – ILC1, ILC2, and ILC3), and monocytes by flow cytometry on fresh blood samples collected between 24 and 72 h after admission.

Results: We investigated adaptive and innate circulating lymphoid cells in the peripheral blood of 18 patients in septic shock, 15 ICU controls, and 30 healthy subjects. As expected, the peripheral blood lymphocytes of all ICU patients showed lymphopenia, which was not specific to sepsis, whereas those of the healthy volunteers did not. Circulating $CD3^+$ T cells and $CD3^-CD56^+$ NK cells were mainly concerned. There was a tendency toward fewer Treg lymphocytes and lower HLA-DR expression on monocytes in ICU patients with sepsis. Although the ILC1 count was higher in septic patients than healthy subjects, ILC2, and ILC3 counts were lower in both ICU groups. However, ILC3s within the total ILCs were overrepresented in patients with septic shock. The depression of immune responses has been correlated with the occurrence of secondary infections. We did not find any differences in ILC distribution according to this criterion.

Conclusion: All ICU patients exhibit lymphopenia, regardless of the nature (septic or sterile) of the initial medical condition. Specific distribution of circulating ILCs, with an excess of ILC1, and a lack of ILC3, may characterize septic shock during the first 3 days of the disease.

Keywords: sepsis, septic shock, immunosuppression, sepsis-induced immunosuppression, innate lymphoid cells (ILC), NK cells, lymphopenia

OPEN ACCESS

Edited by:
Massimo Vitale,
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Reviewed by:
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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *18 February 2019*
Accepted: *29 August 2019*
Published: *20 September 2019*

Citation:
Carvelli J, Piperoglou C, Bourenne J, Farnarier C, Banzet N, Demerlé C, Gainnier M and Vély F (2019) Imbalance of Circulating Innate Lymphoid Cell Subpopulations in Patients With Septic Shock. Front. Immunol. 10:2179. doi: 10.3389/fimmu.2019.02179

INTRODUCTION

Sepsis and septic shock are major public health concerns. In the absence of therapeutic advances, septic shock is a leading cause of mortality in critically ill patients (1). Septic shock corresponds to an intense and uncontrolled systemic immune response to a bacterial or fungal infection (2). In the first hours, the activation of innate immunity leads to cytokine storm syndrome (CSS), which can lead to multiple organ failure and early death. In some survivors, secondary immune dysfunction can occur. Immunosuppression can lead to secondary infections, such as ventilator-associated pneumonia (VAP), which increases morbidity, and mortality (3). Immunosuppression is not specific to sepsis or septic shock in critical care. It involves both innate and adaptive immunity. The phagocytosis of peripheral blood neutrophils can be altered, leading to VAP (4), as reduced IFN-γ production by natural killer (NK) cells is associated with CMV reactivation (5). Activation markers, such as HLA-DR (MHC class II), on circulating monocytes are underexpressed in septic patients (6), especially in the later stages of the disease (> 72 h) (7), and this is associated with secondary infections, and mortality. Lymphopenia is also common in patients admitted for septic shock, with the loss of splenic $CD4^+$ T cells (autopsy findings), and reduced levels of circulating B lymphocytes (8). Conversely, circulating regulatory T cells (Tregs) are always overrepresented 3 to 7 days after diagnosis of the infection (9), and promote anti-inflammatory responses during the second phase of sepsis (10).

Innate lymphoid cells (ILCs) are innate lymphocytes. ILC subgroups are defined according to their expression of key transcription factors and cytokine production, based on their similarity to T cells, and T helper (T_H) cell subsets. Thus, ILCs can be classified as "cytotoxic" ILCs (bona fide NK cells) or as "helper" ILCs (ILC1, ILC2, and ILC3) (11, 12). Helper ILCs represent 0.1% of all circulating lymphocytes (13). The ILC1, ILC2, and ILC3 subsets are present mainly as sedentary cells in tissues, in which they can be maintained by self-renewal (14). Nevertheless, ILC subsets are detectable in human peripheral blood by flow cytometry by excluding lineage (Lin) positive cells (including $CD3^+$ T cells, $CD19^+$ B cells, $CD94^+$ NK cells, and $CD14^+$ monocyte/myeloid cells), and gating on cells that express the IL-7 receptor (CD127; **Figure 3**). Helper ILC1s require T-bet for development and produce IFN-γ as their main effector cytokine. ILC2s depend on GATA-3 and produce "Th2" cytokines (IL-4, IL-5, IL-9, and IL-13). They express CRTH2, a marker used in the gating strategy. ILC3s depend on RORγt, secrete "Th17" cytokines, such as IL-17, or "Th22" cytokines, such as IL-22, and express CD117 (gating strategy). Many studies have suggested an important role for helper ILCs in immunity, particularly in mouse models. A review on the role of ILCs in inflammatory diseases has been recently published (15). ILC1s are pro-inflammatory cells and may be involved in the pathogenesis of chronic obstructive pulmonary disease (16) and Crohn's disease (17). ILC2s may be involved in atopic diseases (18) and may promote fibrosis (19). ILC3s may be critical effector cells in psoriasis (20). ILC biology has never been well-studied in sepsis, even if they appear to be redundant for protective immunity in humans when T-cell, and B-cell functions are preserved (13). Here, we prospectively evaluated circulating immune cells by flow cytometry, especially circulating ILCs, and their three subsets during the early phase of septic shock, defined as the first 3 days after diagnosis of the infection.

MATERIALS AND METHODS

Patients

Patients with septic shock in the Intensive Care Unit (ICU) of Timone CHU Hospital (Réanimation des Urgences, AP-HM Marseille, France) were prospectively enrolled between June and December 2017. According to the Sepsis-3 definition (21), septic shock was considered to be a bacterial infection responsible for arterial hypotension or hyperlactatemia without hypovolemia. All patients required norepinephrine infusion after adequate fluid resuscitation. Hydrocortisone was systemically added as symptomatic therapeutic support for these patients (200 mg/day) (22, 23). First, we compared septic patients to other patients hospitalized in the ICU for cardiac arrest, trauma, or neurological dysfunction (stroke, status epilepticus). These patients had no sign of infection on the day of inclusion. Exclusion criteria were age < 18 years, life expectancy < 48 h, and bone-marrow aplasia (no circulating lymphocytes). Thirty healthy volunteers were recruited in our laboratory to determine normal values of circulating ILCs. All patient data are shown in **Table 1**. We compared all ICU patients who could have severe tissue injuries vs. all ICU patients with less severe injuries to assess the impact of "aggression intensity" on the depression of immune biomarkers. We used the SOFA (*Sepsis-related Organ Failure Assessment*) score, which adds organ dysfunctions (hemodynamic respiratory, hepatic, renal, neurological, and hematopoietic dysfunctions), to define severe tissue injuries. We arbitrarily defined a SOFA score ≥ 8 as corresponding to severe tissue damage. Patient characteristics according to the SOFA score are shown in **Table 2**. Finally, we compared critically ill patients who had a secondary infection during their stay in the ICU vs. patients who did not (patient data in **Table 3**) to assess the role of "biomarkers depression" in the occurrence of secondary infections. The study protocol was approved by the Committee for the Protection of Persons North-West II—France and the trial was registered online before initiation (NCT03297203). Written informed consent was obtained from each patient.

Immunophenotyping by Flow Cytometry

Biological analyses were performed on fresh blood during the 6 h following blood-sample collection. Blood samples were all collected between 24 and 72 h after ICU admission for all ICU patients. For patients with septic shock, ICU admission corresponded to the diagnosis of infection and the initiation of antibiotics. The first 3 days were considered to be the early phase of the disease based on the clinical and biological systemic inflammatory response (cytokine storm, hemodynamic impairment, multiple organ failure) (24, 25). Lymphocyte populations (total lymphocytes, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD3^-CD56^+$

TABLE 1 | Patient characteristics, ICU-septic patients vs. ICU controls.

	ICU sepsis group *n* = 18	ICU control group *n* = 15	Healthy volunteers *n* = 30	*p*
Age—years	57 [29-77]	50 [21–80]	32 [23–66]	0.12
Gender—F/M	7/11	5/10	19/11	0.74
SOFA	8 [4–15]	6 [2–17]	–	0.10
SAPS II	64 [28–76]	40 [25–85]	–	0.22
Norepinephrine—mg/h	3 [0.3–12]	1 [0–9]	–	**0.01**
RRT—*n* (%)	1 (6%)	2 (13%)	–	0.44
Mechanical ventilation—*n* (%)	12 (67%)	13 (87%)	–	0.18
Mechanical ventilation (days)	3 [0–40]	5 [0–120]	–	0.13
Origin of sepsis				
Pneumonia	6 (33%)	–	–	
Abdominal infections	5 (28%)	–	–	
Urinary tract infections (UTI)	4 (22%)	–	–	
Catheter-related infections (CRI)	2 (11%)	–	–	
Soft tissue infection	1 (6%)	–	–	
Isolated bacteria at time of sepsis diagnosis—*n* (%)	14 (78%)	–	–	–
S. pneumoniae	1			
S. oralis	1			
S. pyogenes	1			
H. influenza	1			
P. aeruginosae	1			
E. coli	4			
K. pneumonia	3			
K. oxytoca	1			
E. cloacae	1			
Secondary infections—*n* (%)	7 (39%)	6 (40%)	–	0.95
–Origin of infection				
Ventilator-associated pneumonia (VAP)	5*	5		
C. difficile colitis	1	0		
Cholecystitis	1	0		
CRI	0	1		
CMV reactivation	1*	0		
–Delay of occurrence since admission—days	7 [5–30]	6 [2–18]		0.38
Mortality day 60—*n* (%)	5 (28%)	6 (40%)	–	0.46
ICU length of stay (days)	6 [2–38]	7 [2–120]	–	0.72

Variables are shown as medians [min-max].

p values result from Mann-Withney U-test (continuous variables) or Chi squared test (categorical variables) and compare the ***SOFA scores*** *(Sequential Organ Failure Assessment score—ranges from 0 to 24 points) of the 2 ICU groups: based on 6 different variables, one each for the respiratory (PaO2/FiO2 ratio and use of mechanical ventilation), cardiovascular (mean arterial pressure and use of vasopressors), hepatic (bilirubin), coagulation (platelets), renal (creatinine and urine output), and neurological (Glasgow coma scale) systems. Significant p values are in bold.*

SAPS II *(Simplified Acute Physiology Score II—ranges from 0 to 163 points): based on age, chronic diseases, type of admission, heart rate, systolic blood pressure, temperature, Glasgow coma scale, PaO2/FiO2 ratio and use of mechanical ventilation, urine output, and biological parameters (urea, sodium, potassium, bicarbonate, bilirubin, and white blood cells). RRT: Renal-Replacement Therapy.*

**Patient with CMV reactivation also had a bacterial VAP.*

NK cells) were quantified using 6-Color BD Multitest and BD Trucount Technologies (Becton Dickinson, Le Pont de Claix, France), according to the manufacturer's instructions. We also determined the HLA-DR expression on circulating monocytes. Whole blood was incubated with BD Quantibrite PE Anti-HLA-DR (clone L243)/Anti-Monocyte Stain (clone MΦP9; Becton Dickinson, San Jose, CA, USA). The analysis was performed on a BD FACSCanto II™ cytometer using BD FACS DIVA Software. The amount of antibody bound per cell (Ab/cell) was calculated by standardizing the HLA-DR geometric mean fluorescence intensity (MFI) of monocytes to BD Quantibrite phycoerythrin (PE) beads (BD CellQuest™ Software). Peripheral blood mononuclear cells (PBMCs) were immediately collected by MSL (Eurobio) density centrifugation. PBMCs were used to identify regulatory T cells, considered to be $CD4^+CD25^+FoxP3^+$ lymphocytes. The following antibodies were used: AmCyan anti-CD3 (Clone SK7), APC-H7 anti-CD4 (clone SK3), PerCP-Cy5.5 anti-CD8 (clone SK1), APC anti-CD25 (clone 2A3), Alexa Fluor 488 anti-FoxP3 (clone 259D/C7), and Alexa Fluor 488 anti-IgG1 (isotype-matched controls) (BD Biosciences). PBMCs were also used to characterize ILCs in peripheral blood. We used a panel of conventional lineage

TABLE 2 | Patient characteristics, SOFA < 8 vs. SOFA ≥ 8.

	SOFA < 8 *n* = 14	SOFA ≥ 8 *n* = 19	*p*
Age—years	56 [21–80]	56 [23–77]	0.91
Gender—F/M	4/10	8/11	0.42
SOFA	4 [2–7]	11 [8–17]	**<0.0001**
SAPS II	35 [26–70]	66 [25–85]	**0.0004**
Type of admission			
Sepsis	5	13	**0.03**
Cardiac arrest	2	4	0.6
Trauma	4	1	**0.04**
Stroke	1	1	0.8
Status epilepticus	1	0	0.2
Voluntary overdose	1	0	0.2
Norepinephrine—mg/h	0.2 [0–4]	3 [0.5–12]	**<0.0001**
RRT—*n* (%)	0	3 (16%)	0.12
Mechanical ventilation—*n* (%)	9 (64%)	16 (84%)	0.19
Mechanical ventilation—days	2 [0–120]	5 [0–40]	0.19
Secondary infections—*n* (%)	6 (42%)	7 (37%)	0.73
–Origin of infection			
Ventilator-associated pneumonia (VAP)	5	5*	
C. difficile colitis	1	0	
Cholecystitis	0	1	
CRI	0	1	
CMV recurrence	0	1*	
–Time of occurrence since admission—days	7 [2–30]	6 [5–13]	0.91
Mortality day 60—*n* (%)	2 (14%)	9 (47%)	**0.046**
ICU length of stay—days	4 [2–120]	7 [3–38]	0.14

Variables are shown as medians [min-max].
p values result from Mann-Withney U-test (continuous variables) or Chi squared test (categorical variables). Significant p values are in bold.
***SOFA score** (Sequential Organ Failure Assessment score—ranges from 0 to 24 points).*
***SAPS II** (Simplified Acute Physiology Score II—ranges from 0 to 163 points).*
**Patient with CMV recurrence had also a bacterial VAP. RRT: Renal-Replacement Therapy.*

TABLE 3 | Patient characteristics, secondarily infected vs. others.

	Secondarily infected patients *n* = 13	Others *n* = 20	*p*
Age—years	57 [23–80]	56 [21–73]	0.26
Gender—F/M	5/8	7/13	0.84
SOFA	8 [4–17]	8 [2–16]	0.59
SAPS II	65 [28–77]	48 [25–85]	0.51
Type of admission			
Sepsis	7	11	0.95
Cardiac arrest	2	4	0.74
Trauma	3	2	0.30
Stroke	0	2	0.24
Status epilepticus	1	0	0.22
Voluntary overdose	0	1	0.41
Norepinephrine—mg/h	1.5 [0–12]	1.5 [0–8]	0.89
RRT—*n* (%)	2 (15%)	1 (5%)	0.31
Mechanical Ventilation—*n* (%)	11 (85%)	13 (65%)	0.22
Mechanical Ventilation—days	14 [0–40]	2 [0–120]	**0.004**
Mortality day 60—*n* (%)	5 (38%)	6 (30%)	0.61
ICU length of stay—days	18 [2–60]	4 [2–120]	**0.001**

Variables are shown as medians [min-max].
p-values from the Mann-Whitney U-test (continuous variables) or Chi squared test (categorical variables). Significant p values are in bold.
***SOFA score** (Sequential Organ Failure Assessment score—ranges from 0 to 24 points).*
***SAPS II** (Simplified Acute Physiology Score II—ranges from 0 to 163 points). RRT: Renal-Replacement Therapy.*

markers (Lin: CD3, CD19, CD14, TCRαβ, TCRγδ, CD94, CD16, FcεRI, CD34, CD123, and CD303) and cell-surface expression of CD127, CD117, and CRTH2 to identify the ILC1 subset as Lin^- $CD127^+$ $CD117^-$ $CRTH2^-$ cells, the ILC2 subset as Lin^- $CD127^+$ $CRTH2^+$ cells, and the ILC3 subset as Lin^- $CD127^+$ $CD117^+$ $CRTH2^-$ cells, among the circulating lymphocytes. The following antibodies were used: FITC anti-CD3 (clone UCTH1), FITC anti-CD19 (clone HIB19), FITC anti-CD14 (clone M5E2), FITC anti-TCRαβ (clone T10B9), FITC anti-TCRγδ (clone B1), FITC anti-CD94 (clone HP-3D9), FITC anti-CD16 (clone 3G8), FITC anti-CD34 (clone 581/CD34), FITC anti-CD123 (clone 7G3) (BD Pharmigen), FITC anti-CD303 (clone AC144) (Miltenyi Biotec), FITC anti-FcεRI (AER-37) (Ebioscience), PE-C7 anti-CD127 (HIL-7R-M21) (BD Pharmigen), Alexa Fluor 647 anti-CD294/CRTH2 (clone BM16) (BD Horizon), and PE-Cy5 anti-CD117 (clone YB5.B8) (BD Pharmigen). Data was acquired using a BD LSRFortessa™ cytometer and data analysis was performed using FlowJo 10.2 Software.

Statistical Analysis

The results are presented (text, tables, and figures) as the median [min, max]. For continuous variables, multiple group comparisons were analyzed using the Mann-Withney *U*-test for two groups and the Kruskal-Wallis test for more than two groups. For categorical variables, the Chi squared test was used. Statistical analyses were performed with Prism 6 (GraphPad Software, San Diego, CA, USA). Results were considered significant for a $p < 0.05$.

RESULTS

Patient Characteristics (Tables 1–3)

Eighteen patients with septic shock were included (*ICU Sepsis Group*). The sources of infection and bacterial strains are detailed in **Table 1**. The ICU control group contained 15 patients (*ICU Control Group*): six cardiac arrests, five severe trauma, two strokes, one status epilepticus, and one voluntary overdose with benzodiazepine.

Patients with sepsis were slightly, but not significantly, older (57 years [29–77]) than the controls (50 years [21–80]; $p = 0.12$). Most patients were male (21/33), with no difference between the two groups ($p = 0.74$). The median SOFA score was higher for the *Sepsis Group* (8 [4–15]) than the ICU controls (6 [2–17]; $p = 0.10$). The same was true for the SAPSII (64 [28–76]

TABLE 4 | Immunological analysis.

	ICU sepsis $n = 18$	ICU controls $n = 15$	Healthy controls $n = 30$	Secondary infections $n = 13$	No secondary infection $n = 20$	p	SOFA < 8 $n = 14$	SOFA ≥ 8 $n = 19$	p
Lymphocytes (/mm^3)	992 [298–2,487]	856 [298–2,246]	2,042 [708–3,606]	710 [319–1,517]	1,056 [298–2,487]	0.14	1,495 [298–2,487]	658 [298–1,987]	0.02
CD4$^+$ T Cells	386 [124–1,510]	405 [100–1,265]	858 [366–1,731]	320 [124–845]	545 [100–1,510]	0.13	729 [100–1,510]	309 [124–1,265]	0.02
CD8$^+$ T Cells	183 [32–914]	160 [23–687]	448 [166–1,181]	195 [23–687]	165 [32–914]	0.64	236 [23–914]	129 [32–820]	0.12
CD19$^+$ B Cells	131 [20–380]	163 [37–599]	196 [72–713	150 [37–325]	141 [20–599]	0.54	173 [37–599]	121 [20–410]	0.31
CD3$^-$CD56$^+$ NK Cells	73 [18–320]	68 [38–247]	245 [57–787]	71 [18–198]	75 [31–320]	0.28	103 [38–247]	62 [18–320]	0.03
Monocytes HLA-DR expression									
MFI	2649 [959–8,752]	4882 [1,314–16,298]		3,702 [959–12,941]	3533 [1,048–16,298]	0.52	6176 [1,314–16,298]	3,479 [959–11,183]	0.08
Ab/Cell	5286 [2,089–16,832]	7,882 [2,760–24,756]		6,044 [2,089–24,756]	6,959 [2,257–23,846]	0.55	11,958 [2,760–24,756]	6856 [2,089–15,804]	0.05
Regulatory T Cells (CD4$^+$CD25$^+$FoxP3$^+$)									
Absolute count (/mm^3 of whole blood)	9 [1–80]	17 [5–64]		11 [3–38]	14 [1–80]	0.67	17 [1–80]	11 [3–34]	0.04
ILC									
Total ILC (/mL of whole blood)	1,293 [483–4,468]	1,242 [326–4,085]	1,632 [505–5,846]	1,120 [326–4,085]	1,277 [440–4,468]	0.7	1,031 [326–4,468]	1,264 [483–3,638]	0.98
ILC1									
Absolute count (/mL)	931 [282–3,478]	512 [133–2,978]	468 [178–1,980]	514 [209–2,978]	931 [133–3,478]	0.67	505 [133–3,127]	883 [282–3,478]	0.57
Percentage across ILCs	66 [32–96]	64 [27–83]	30 [13–71]	61 [27–77]	69 [29–96]	0.25	66 [27–82]	68 [32–96]	0.28
ILC2									
Absolute count (/mL)	239 [29–1,592]	147 [39–791]	584 [117–1,597]	294 [45–791]	161 [29–1,592]	0.3	211 [29–1,592]	147 [29–518]	0.60
Percentage across ILCs	20 [3–58]	14 [3–43]	28 [14–66]	21 [7–39]	13 [3–58]	0.14	20 [6–44]	14 [3–58]	0.20
ILC3									
Absolute count (/mL)	162 [41–469]	258 [71–1,460]	513 [83–2,424]	261 [41–800]	188 [41–1,460]	0.74	180 [59–1,460]	182 [41–644]	0.41
Percentage across ILCs	13 [2–31]	25 [8–49]	32 [12–55]	17 [5–49]	14 [2–48]	0.64	16 [3–49]	14 [3–43]	0.44

Variables are shown as median [min-max].

vs. 40 [25–76], $p = 0.22$). According to the protocol for septic shock, all patients with sepsis received norepinephrine infusion with a median dosage of 3 mg/h [0.3–12], higher than for the controls, for whom norepinephrine infusion was from 1 mg/h [0–9] ($p = 0.01$). Only one patient in the *ICU Sepsis Group* required renal replacement therapy (RRT) vs. two in the control group ($p = 0.44$). Three quarters of the patients were mechanically ventilated (more in the sepsis group, 12 vs. 13, $p = 0.18$), with a median duration of mechanical ventilation of 3 days [0–40] in the sepsis group and 5 days [0–120] in the ICU control group ($p = 0.13$). Secondary infections in the ICU concerned seven patients with anterior septic shock and six ICU controls ($p = 0.95$). Secondary infections corresponded to 10 cases of VAP, one CRI, one case of cholecystitis, and one of *C. difficile* colitis. One patient with VAP also had *CMV* reactivation. These secondary infections occurred between day 2 and day 30 (median of seven days). Overall mortality at day 180 was 11/33 (33%) and was higher in the controls (6/15 vs. 5/18 deaths in the sepsis group, $p = 0.46$). The median length of stay in the ICU was 7 days and was nearly the same for the two groups (6 [2–38] for the sepsis group vs. 7 [2–20] for the control group, $p = 0.72$).

Immunological Analysis (Table 4)

Conventional Blood Lymphocyte Immunophenotyping

Most patients with septic shock develop lymphopenia (8, 26). Patients of the ICU groups developed lymphopenia, with no difference between the two groups ($p = 0.90$), whereas the lymphocyte counts of the healthy volunteers remained normal. The median lymphocyte count was 2,042/mm^3 [708–3,606] for the healthy subjects vs. 992/mm^3 [298–2,487] for the septic patients and 856/mm^3 [298–2,246] for the ICU controls ($p < 0.0001$). Lymphopenia concerned all lymphocyte subsets, above all CD3$^+$ T cells (TCD4$^+$ and TCD8$^+$), and CD3$^-$CD56$^+$ NK cells (**Figure 1A**). There was no statistical difference for the CD19$^+$ B cell counts between the three groups. Patients with a secondary infection tended to have fewer circulating lymphocytes than those without, with no statistical difference (**Table 4**). The more extensive the tissue damage was (SOFA ≥ 8), the more pronounced was the deficit of circulating lymphocytes, with a correlation between a higher SOFA score, and lower lymphocyte counts (Rho (Spearman): −0.446 [IC 95%: −0.684, −0.121], $p < 0.001$). These results were only significant concerning CD4$^+$ T cells and CD3$^-$CD56$^+$ NK cells (**Figure 1B**).

Circulating Treg Lymphocytes

Treg lymphocytes are expected to be overrepresented in patients with septic shock and could promote the post-aggression anti-inflammatory response (9, 27). Here, the Treg counts were lower in patients with septic shock than the ICU controls (9/mm^3 [1–80] vs. 17/mm^3 [5–64], $p = 0.04$) (**Figure 2A,** top panel). There was no difference in the number of Treg cells in patients who developed secondary infections and those who did not (11/mm^3 [3–38] and 14/mm^3 [1–80], respectively, $p = 0.67$; **Table 4**). ICU patients who were more severely ill (SOFA ≥ 8) had fewer Treg

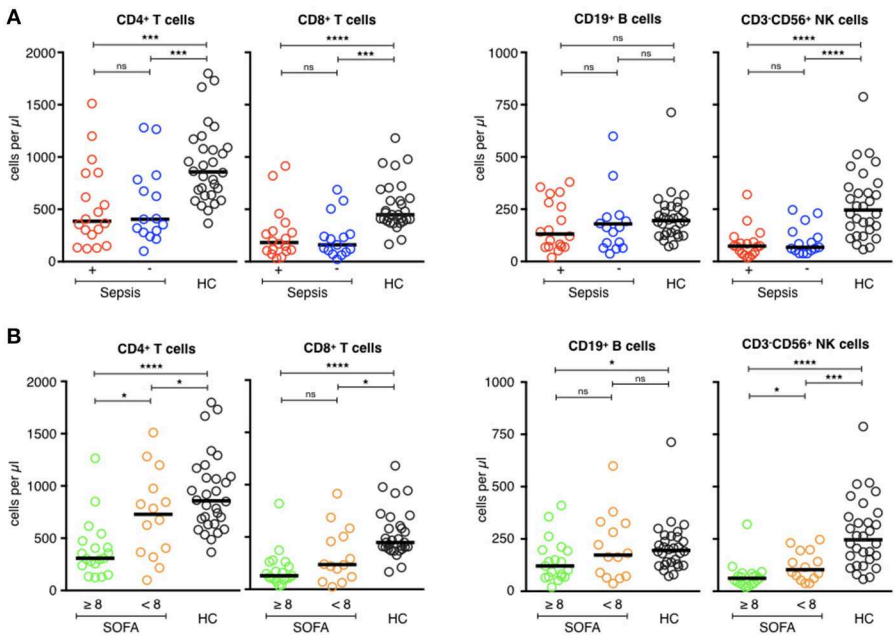

FIGURE 1 | Phenotypic analysis of circulating lymphocyte subsets in ICU patients. **(A)** Comparison of helper T-cell, cytotoxic T-cell, B-cell, and NK-cell counts (cells/µl) of septic patients (red circles) with those of healthy controls (dark circles), and ICU patients without sepsis (blue circles). **(B)** Comparison of helper T-cell, cytotoxic T-cell, B-cell, and NK cell counts (cells/µl) of patients with severe tissue injuries (green circles) with those of healthy controls (dark circles), and patients with less severe lesions (orange circles). The bars show the median. Statistical analyses were performed using the Mann Whitney *U*-test. Differences were considered significant when $P < 0.05$: $^*P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ns: not significant.

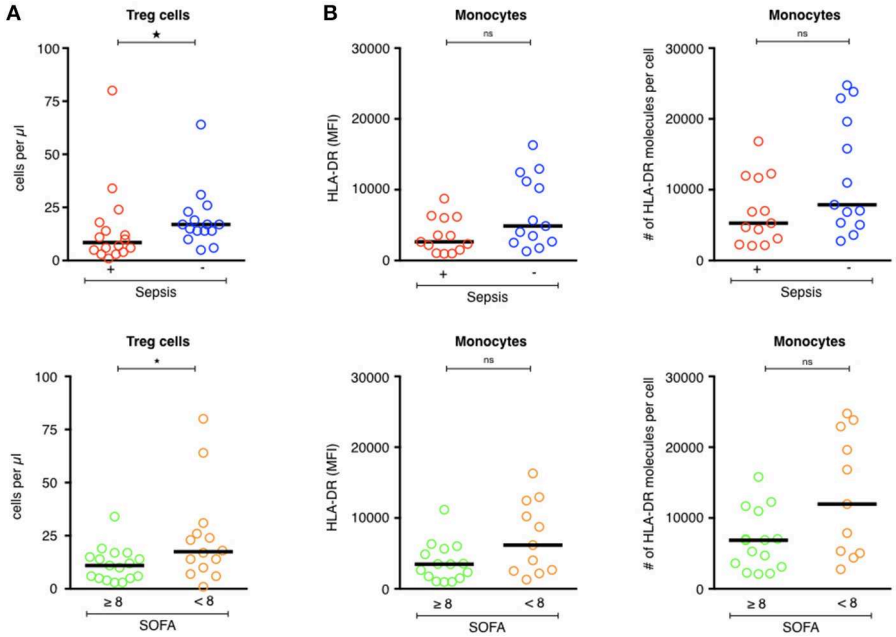

FIGURE 2 | Phenotypic analysis of circulating Tregs and monocytes in ICU patients. (**A**, upper) Comparison of Treg cell counts (cells/µl) of septic patients (red circles) with those of ICU patients without sepsis (blue circles). (**A**, lower) Comparison of Treg cell counts (cells/µl) of patients with severe tissue injuries (green circles) with those of patients with less severe lesions (orange circles). (**B**, upper) Comparison of the MFI of HLA-DR expression and the number of HLA-DR molecules on monocytes of septic patients (red circles) with those of ICU patients without sepsis (blue circles). (**B**, lower) Comparison of the MFI of HLA-DR expression and the number of HLA-DR molecules on monocytes of patients with severe tissue injuries (green circles) with those of patients with less severe lesions (orange circles). The bars show the median. Statistical analyses were performed using the Mann Whitney *U*-test. Differences were considered significant when $P < 0.05$: $^*P < 0.05$. ns: not significant.

lymphocytes than those who were less ill (SOFA < 8; 11/mm^3 [3–34] vs. 17/mm^3 [1–80], $p = 0.04$; **Figure 2A**, bottom panel).

HLA-DR Expression by Circulating Monocytes

HLA-DR expression on circulating monocytes is frequently used as a marker for the monitoring of immune alterations in critically ill patients, especially those with septic shock (6, 7, 28–31). As expected, HLA-DR expression was lower on circulating monocytes of patients with septic shock (MFI = 2,649 [959–8,752]—HLA-DR Ab/cell = 5,286 [2,089–16,832]) than those of the ICU controls (MFI = 4,882 [1,314–16,298]—HLA-DR Ab/cell = 7,882 [2,760–24,756]; $p = 0.08$; **Figure 2B**, top panel). There was no difference in HLA-DR expression between patients with secondary infections and those who were uninfected (MFI = 3,702 [959–12,941]—Ab/cell = 6,044 [2,089–24,756] for secondarily infected patients vs. MFI = 3,533 [1,048–16,298]—Ab/cell = 6,959 [2,257–23,846] for patients without secondary infection, $p = 0.5$; **Table 4**). Less ill patients (SOFA < 8) showed higher HLA-DR expression on their circulating monocytes than those who were more ill (SOFA > 8; **Figure 2B**, bottom panel), without statistical significance.

Circulating ILCs

Circulating ILCs have never been studied in patients with septic shock. As tissue-resident cells, they represent one of the first immune gates encountered by the infection and participate in the innate immune response through the production and release of pro-inflammatory cytokines (32–39). Total ILCs are defined as lineage-negative CD127$^+$ lymphocytes (**Figure 3**). Among total ILCs, ILC subsets can be discriminated according to the expression of CD294 (CRTH2) and CD117 (cKit): ILC2s are Lin$^-$CD127$^+$CRTH2$^+$ cells, ILC3 are Lin$^-$CD127$^+$CRTH2$^-$CD117$^+$ cells, and ILC1 are Lin$^-$CD127$^+$CRTH2$^-$CD117$^-$ cells. ILCs in critically ill patients are not spared by the global deficit in circulating lymphocytes. Total ILC counts were slightly lower in both ICU groups than in healthy controls (1,293/mL [483–4,468], 1,242/mL [326–4,085], and 1,632/mL [505–5,846], respectively).

However, the ILC distribution was not the same in the two ICU groups (**Figure 4A**). Septic patients had more circulating ILC1s (931/mL [282–3,478]) than the ICU controls (512/mL [133–2,978]) and, above all, than the healthy volunteers (468/mL [178–1,980], $p = 0.04$). The proportion of ILC1s within total ILCs followed the same pattern: 66% [32–96] of circulating ILC1s in the sepsis group, 64% [27–83] in the ICU controls, and 30% [13-71] in the healthy controls ($p < 0.0001$). Balancing the excess of ILC1s, the ILC2, and ILC3 counts and percentages were significantly lower in both ICU groups than the healthy volunteers. The number of ILC2s in septic patients was 239/mL [29–1,592] (20% [3–58] of total ILC) vs. 147/mL [39–791] (14% [3–43] of total ILCs) in ICU controls and 584/mL [117–1,597] (28% [14–66] of total ILCs) in healthy subjects. The most significant finding for patients with septic shock was a severe deficit in the ILC3 subset (162/mL [41-469] or 13% [2–31])

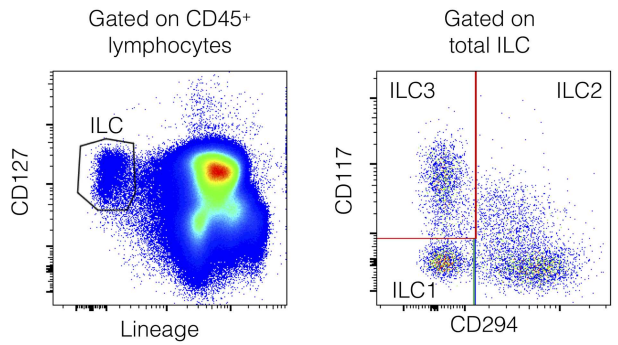

FIGURE 3 | Flow cytometry gating strategy for the identification of human peripheral blood ILCs. ILCs are defined as Lin$^-$CD127$^+$ cells with a lineage cocktail containing antibodies directed against CD3, CD19, CD14, TCRαβ, TCRγδ, CD94, CD16, FcεRI, CD34, CD123, and CD303 **(Left)**. Within the ILC gate, ILC2s are CD294$^+$ cells, whereas ILC1s are CD294$^-$CD117$^-$ cells and ILC3s are CD294$^-$CD117$^+$ cells **(Right)**.

relative to both the healthy subjects (513/mL [83–2,424], $p < 0.0001$ or 32% [12–55], $p < 0.0001$), and critically ill controls (258/mL [71–1,460], $p = 0.2$ or 25% [8–49] $p = 0.04$). Finally, we compared ILCs and ILC subsets between patients who had a secondary infection and those who did not (**Table 4**). There was no statistical difference between the two groups based on this criterion. Similarly, there was no difference according to the severity of tissue damage (SOFA ≥ 8 or SOFA < 8) (**Figure 4B**).

DISCUSSION

Lymphopenia is common in critically ill patients, especially when the reason for admission is septic shock. The immune deficit following sepsis is called sepsis-induced immunosuppression (25). Other pathological situations, such as trauma (40), or extensive burns (41), can induce the same biological modifications. These situations can all be complicated by "opportunistic" or, more precisely, secondary infections. Here, we confirmed that acute injuries (sepsis, trauma, cardiac arrest) are associated with circulating lymphopenia, which affected all lymphocyte subsets, above all circulating T cells, NK cells, and helper ILCs. Treg lymphocytes were also affected. This appears to contradict previous reports (9), but an examination of the results of Venet et al. show the absolute number of Treg lymphocytes to be lower in septic-ICU patients than in healthy subjects. No ICU controls were considered. Only the percentage of Treg lymphocytes among all CD4$^+$ T cells was higher in patients with septic shock, although we did not find this result. The method to identify Treg cells was also not the same as in our study (reagents and samples, whole blood in their study vs. PBMCs in ours). In ICU patients, the deficit in ILCs (as Treg lymphocytes) could be explained by the global lymphopenia.

We also confirmed that lymphopenia is not specific to sepsis. Ischemia-reperfusion (after a cardiac arrest for example), traumatic injuries, or major surgical procedures (42–44) led to the same biological states. Our results are however limited by the small number of patients and our monocentric recruitment. Although "ICU immunosuppression" is not specific to sepsis, it appears to correlate with the severity of the tissue injuries. Comparison of patients with more severe tissue damage (arbitrarily defined by a SOFA score ≥ 8) with those with less severe lesions (arbitrarily defined by a SOFA score < 8) showed that the most ill patients showed a lower count of circulating lymphocytes and lower HLA-DR expression on circulating monocytes. However, there were no major differences in terms of circulating ILCs and their three subsets. It is possible that choosing a SOFA score ≥ 8 to define the severity of tissue injuries may have been somewhat arbitrary. However, the SOFA score adds all organ dysfunction and their intensity. Moreover, the prognosis of these two groups of patients was very different in terms of mortality (2/14 (14%) vs. 9/19 (47%), $p = 0.046$; **Table 2**). After a severe injury, lymphocyte apoptosis must occur to control inflammation. The initial cytokine storm is subsequent to overstimulation of the innate immune system (45). Pathogen-associated molecular patterns (PAMPs—microbial patterns) and damage-associated molecular patterns (DAMPs—"sterile" patterns) (46) bind to their Toll-like receptors (TLRs) to start an effective immune response to aggression. The resolution of inflammation requires negative feedback involving lymphocyte apoptosis and "tolerization" (47). Lymphocyte apoptosis is the main mechanism leading to lymphopenia in the ICU (40, 48, 49). The more severe the organ damages are, the greater is the deficit in lymphocytes and the resulting so-called "secondary immunosuppression."

We also observed the absence of a correlation between the depression of biological immune markers (for example, circulating lymphocytes) and the occurrence of secondary infections. This observation contradicts published reports (50, 51). These contradictory findings can be explained by the timing of the blood sample collection. Our biological samples were collected relatively early after the onset of critical illness (24 to 72 h), whereas previous studies used samples taken later (>72 h after the initial infection) (7, 9). It is likely that the risk of secondary infection correlates more with the persistence of an immune deficit than the immune deficit itself, especially in the first hours of care. Even though immunosuppression may be a risk factor (52), the mechanism behind secondary infections appears to be more complex than a simple deficit of several biomarkers (53). In the ICU, comorbidities, length of stay, and the retention of invasive materials can lead to nosocomial infections. Mechanical ventilation can promote VAP and the retention of a central venous catheter can promote catheter-related infections (CRI). For example, in our study, the median length of stay was 18 days [2–60] for secondarily infected patients vs. 4 days [2–120] for uninfected subjects ($p = 0.001$). The length of stay may have been the consequence of secondary infections but an extended length of stay may have also promoted it.

Among all immune modifications, the distribution of circulating ILCs and their subsets appear to show some specificity for the critically ill patients with septic shock. These patients showed a greater proportion of ILC1s balanced by a lower proportion of ILC3s. This result for the ILC1 subset contradicts the recent findings of Cruz-Zárate et al. (49). However, there were differences between their study and ours. Our patients were

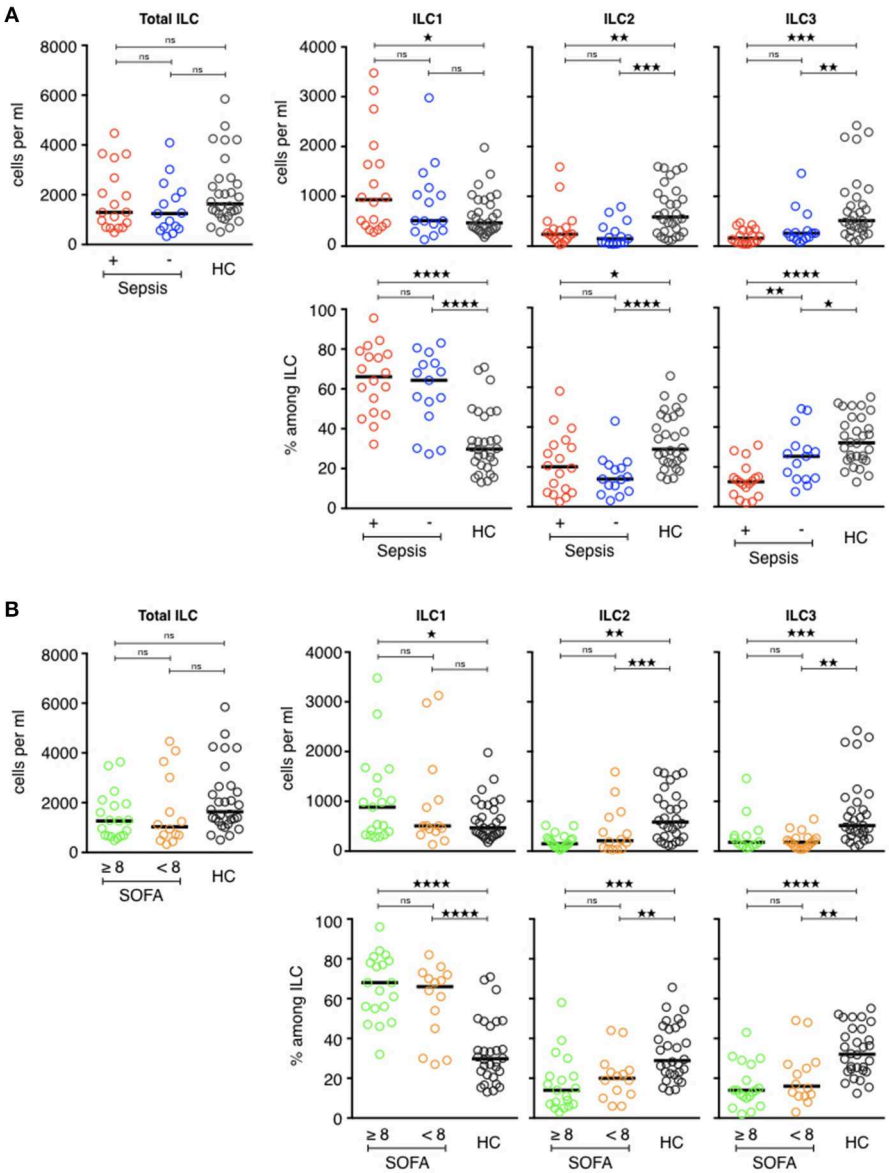

FIGURE 4 | Phenotypic analysis of circulating ILCs in ICU patients. (**A**, upper) Comparison of total ILC, ILC1, ILC2, and ILC3 counts (cells/ml) of septic patients (red circles) with those of healthy controls (dark circles) and ICU patients without sepsis (blue circles). (**A**, lower) Distribution of each ILC subset among total ILCs. (**B**, upper) Comparison of total ILC, ILC1, ILC2, and ILC3 counts (cells/ml) of patients with severe tissue injuries (green circles) with those of healthy controls (dark circles) and patients with less severe lesions (orange circles). (**B**, lower) Distribution of each ILC subset among total ILCs. The bars show the median. Statistical analyses were performed using the Mann Whitney *U*-test. Differences were considered significant when $P < 0.05$: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ns: not significant.

all in septic shock (infusion of norepinephrine), whereas theirs were mainly in sepsis. Moreover, the distribution of ILCs in their healthy controls is surprising. Although an equal distribution between the ILC subsets has been reported (13, 54), ∼85% of the ILCs in their study belonged to the ILC1 subset.

ILC1 is characterized by its ability to produce IFN- γ, which plays a role in the fight against bacterial infections. The ILC1 subset has already been shown to play a role in microbial infections, such as *C. difficile* or *rodentium* (33, 35) colitis and *T gondii* invasion (34) in mouse models. The lack of the ILC3 subset in sepsis suggests that these cells may represent a pool of future and effective ILC1s (ex-ILC3). Lim et al. recently showed (55) that cultured human peripheral blood $CD7^{+}CD127^{+}CD117^{+}$ cells (ILC3) can give rise to both mature cytotoxic NK and helper ILC subsets, showing an important role for ILC3 in ILC-poiesis (56). ILCs are highly plastic cells (57, 58), which can change phenotype and function depending on their microenvironment. In animals, ILC2s and

ILC3s exposed to IL-12 loose the expression of GATA-3 and RORγt, respectively, and acquire features of ILC1s, including T-bet expression and IFN-γ production (59). In the early phase of septic shock, the increase in the pool of ILC1s, originating from ILC3s (ILC1 precursors), may promote the pro-inflammatory response to eliminate the pathogen. Concerning the risk of secondary infections due to the lack of circulating ILCs, our study revealed no significant differences between secondarily infected and uninfected patients, although there was a trend toward a lower proportion of ILC1s and higher proportion of ILC2s in secondarily infected patients (**Table 3**). ILC2s have been shown to play a role in the resolution of inflammation in ischemic-reperfusion (60) and central nervous system inflammation (61) models. However, they are not "infection models." In a mouse model of *E. faecalis* infection after burn injury (62), ILC2s had a detrimental role in sepsis and the use of an inhibitor of ILC2 development improved survival. In another study, ILC2s were protective against acute lung injury in a sepsis-induced acute lung inflammation model (63). Shifting the balance in favor of ILC2s (anti-inflammatory) vs. ILC1s (pro-inflammatory) would bear the risk of promoting "immunosuppression" and secondary infections in the ICU.

The study of ILCs in humans is made difficult because of their mostly being located in mucosal tissues (64). Circulating ILCs are considered to be immature relative to tissue-resident ILCs. In humans, the mechanisms by which ILCs circulate between peripheral blood and tissues are still unknown; it is unknown whether a deficit in circulating ILCs is associated with an equal deficiency in tissues. Nevertheless, peripheral blood is the main biological compartment available in humans to analyze the immune response and we show, for the first time, a disequilibrium in the distribution of ILC subsets in patients with septic shock, in which ILCs could participate in the pro-inflammatory immune response and may account for certain immunological post-injury modifications.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

This study protocol was approved by the Committee for the protection of persons West-North II - France - and the trial was registered online before initiation (NCT03297203).

AUTHOR CONTRIBUTIONS

JC and FV devised and supervised the study, designed the research, and wrote the manuscript, with the help of the other co-authors. JC, CP, NB, CF, and CD designed the research, performed experiments, and analyzed the data. JB and MG provided key expertise and samples.

FUNDING

This work was supported by APHM, MSDAvenir, and institutional grants to the CIML (INSERM, CNRS, and Aix- Marseille University) and to Marseille Immunopole.

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

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frontiers in Immunology

REVIEW
published: 07 May 2019
doi: 10.3389/fimmu.2019.00909

The Natural Cytotoxicity Receptors in Health and Disease

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The Natural Cytotoxicity Receptors (NCRs), NKp46, NKp44, and NKp30, were some of the first human activating Natural Killer (NK) cell receptors involved in the non-MHC-restricted recognition of tumor cells to be cloned over 20 years ago. Since this time many host- and pathogen-encoded ligands have been proposed to bind the NCRs and regulate the cytotoxic and cytokine-secreting functions of tissue NK cells. This diverse set of NCR ligands can manifest on the surface of tumor or virus-infected cells or can be secreted extracellularly, suggesting a remarkable NCR polyfunctionality that regulates the activity of NK cells in different tissue compartments during steady state or inflammation. Moreover, the NCRs can also be expressed by other innate and adaptive immune cell subsets under certain tissue conditions potentially conferring NK recognition programs to these cells. Here we review NCR biology in health and disease with particular reference to how this important class of receptors regulates the functions of tissue NK cells as well as confer NK cell recognition patterns to other innate and adaptive lymphocyte subsets. Finally, we highlight how NCR biology is being harnessed for novel therapeutic interventions particularly for enhanced tumor surveillance.

Keywords: receptors, natural killer cell, immunoregulation, disease association, tissue homeostasis

OPEN ACCESS

Edited by:
Michael A. Caligiuri, City of Hope National Medical Center, United States

Reviewed by:
Vincent Vieillard, Centre National de la Recherche Scientifique (CNRS), France
Frank Momburg, German Cancer Research Center (DKFZ), Germany

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: *06 February 2019*
Accepted: *09 April 2019*
Published: *07 May 2019*

Citation:
Barrow AD, Martin CJ and Colonna M (2019) The Natural Cytotoxicity Receptors in Health and Disease. Front. Immunol. 10:909. doi: 10.3389/fimmu.2019.00909

INTRODUCTION

Natural Killer (NK) cells constitute a population of large, granular lymphocytes that are located in the blood, lymphoid organs, such as the thymus and spleen, and non-lymphoid organs, such as the liver and uterus, as well as tissues, such as skin (1–4). NK cells were originally identified based on their ability to lyse certain tumor and virally infected cells (5, 6). In contrast, normal healthy cells that express sufficient levels of MHC class I molecules are spared from NK cell attack (7). Subsequently, the process whereby the effector functions of developing NK cells are adapted to the levels of MHC class I expressed by a host, termed NK cell "education", was described (8, 9). It was during this exciting era of discovery that the functional activity of NK cells was shown to be exquisitely controlled by inhibitory receptors specific for MHC class I molecules, such as the Ly49 receptors in mice (10), and the Killer immunoglobulin-like receptors (KIR) in humans (11–14). Fully educated NK cells efficiently lyse target cells lacking MHC class I molecules, implying the existence of a set of activating NK cell receptors for non-MHC class I ligands expressed on target cells that are either not present or expressed at much lower levels on healthy cells, although formal molecular evidence for this was lacking during this period.

In contrast to T and B lymphocytes, NK cells lack the expression of rearranging cell-surface antigen receptors and so it remained unclear how NK cells might be aroused by surface ligands expressed by tumor or virus-infected cells. Even though NK cells lack expression of the T cell

receptor (TCR), they still retain expression of the ζ chain from the CD3 signaling complex. These data suggested that NK cells express cell-surface receptors that might share similar downstream signaling mechanisms to the TCR but are regulated by different ligands. For example, whereas T cells recognize short antigenic peptides in the context of MHC class I, NK cells can be inhibited by the expression of MHC class I molecules. Thus, NK cells express activating receptors that may recognize ligands expressed by tumor and virus-infected cells in a non-MHC-restricted fashion. Whilst the function and downstream signaling events of the low affinity Fc receptor, CD16, that mediates antibody-dependent cellular cytotoxicity (ADCC) were well-defined at this time (15–17), the identity of other activating receptors and their cognate ligands involved in the natural cytotoxicity of NK-susceptible targets cells were largely unknown, which stimulated further research into the receptors that trigger NK cell cytotoxicity.

The Natural cytotoxicity receptors (NCRs) were originally identified by the Moretta group almost 20 years ago in a series of elegant redirected lysis experiments using human NK cells (18–20). The NCR family are comprised of three type I transmembrane (TM) receptors, termed NKp46, NKp44, and NKp30, which are encoded by the genes, *NCR1*, *NCR2*, and *NCR3*, respectively. Even though the NCRs were discovered based on their ability to induce NK cell cytotoxicity of monoclonal antibody (mAb)-coated tumor cell targets, the blocking of individual NCR activity using soluble mAbs had only a mild effect on NK cell cytotoxicity and different tumor cells varied in their susceptibility. Combinations of soluble mAbs to the NCRs were found to have a much stronger blocking effect for selected tumor cell-lines indicating that the NCRs can cooperate with each other to mediate NK cell cytotoxicity of certain tumor cell-types (18, 20, 21). These results suggest that the coordinated expression of NCR ligands by different tumor cell-types as well as the level of NCR expression by different NK cell clones governs NK cell cytotoxicity, which is counterbalanced by the level of MHC class I expressed for a given tumor cell type.

Recent success in identifying the various ligands for the NCRs is now beginning to illuminate the biological roles that this important class of receptors plays in NK cell surveillance of malignant or pathogen-infected cells and in different tissue microenvironments. One striking aspect that has become apparent is that each NCR may interact with several different pathogen- and host-encoded molecules that can either be expressed on cell-surfaces, secreted or shed extracellularly, or incorporated into the extracellular matrix (ECM). Moreover, cytokines expressed in the local tissue microenvironment can influence which particular NCR isoform is expressed. These different NCR isoforms have now been shown to deliver either activating or inhibitory signaling functions depending on their interaction with ligand. Here we review NCR biology in the different tissue NK cell populations as well as other innate and adaptive immune subsets, their functional interactions with a diverse set of cellular- and pathogen-encoded ligands, and the potential for these interactions to be harnessed for tumor immunotherapy.

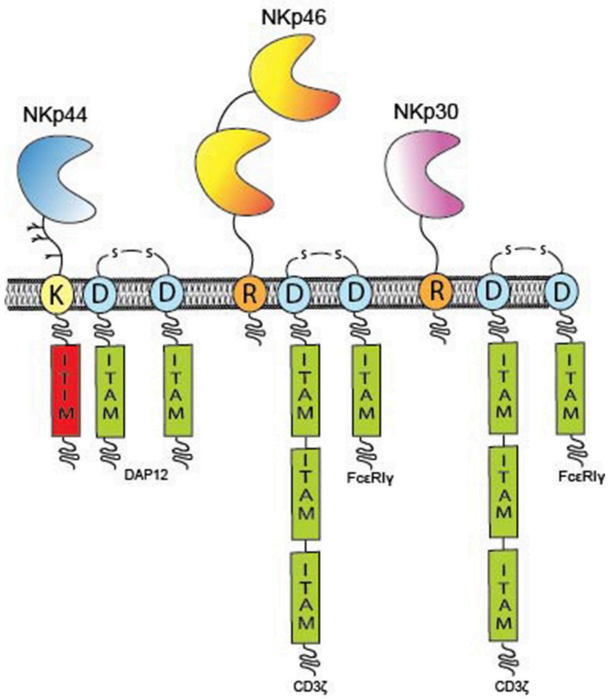

FIGURE 1 | Overview of individual NCR domain structures. The domain architecture of the NCRs and TM signaling adaptors encoding ITAM residues (green boxes) are displayed. The NCRs are type I TM proteins expressed on the plasma membrane of immune cells. NKp46 (yellow) has two Ig-like domains, whereas NKp30 (pink) and NKp44 (blue) possess only one Ig-like domain. All NCRs contain either a positively charge arginine (R) or lysine (K) residue in their hydrophobic TM domains that can form a salt bridge with a corresponding aspartate (D) residue in the TM domains of the ITAM adaptors; CD3, FcR, or DAP12, respectively. The cytoplasmic domains of the NCRs do not encode any inherent signaling capacity with the exception of NKp44 that contains a putative ITIM sequence (red) in its cytoplasmic tail and thus maintains potential for inhibitory signaling.

NKp46

Molecular cloning of the cDNA for NKp46 (also known as natural cytotoxicity receptor 1, NCR1) revealed an open reading frame (ORF) encoding a 46 kDa type I TM protein belonging to the immunoglobulin (Ig) superfamily characterized by two extracellular C2-type Ig-like domains followed by a stalk region (**Figure 1**) (21). The two Ig-like domains of NKp46 are arranged in a V-shaped conformation positioned at an angle of 85° to each other similarly to the D1D2 domains of KIRs and Leukocyte Ig-like receptors [LILR, also known as ILTs (22)] that share a distant ancestral evolutionary relationship with NKp46 (23, 24). The cytoplasmic domain of NKp46 lacks an Immunoreceptor Tyrosine-based Activation Motif (ITAM), instead the TM domain contains a positively charged arginine residue that mediates association with the negatively charged aspartate residue in the TM domain of the ITAM signaling adaptors, CD3ζ or the Fc receptor common γ (FcRγ) (**Figure 1**). The gene encoding NKp46, *NCR1*, is located in the Leukocyte Receptor Complex (LRC) on chromosome 19q13.4 (25). A murine *NCR1*

ortholog has also been cloned and maps to mouse chromosome 7, the syntenic region of human chromosome 19 (21).

The expression of NKp46 on NK cells is conserved across all mammalian species (26). In humans, NKp46 is expressed by all $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ human NK cells irrespective of their activation status (19). Cross-linking with anti-NKp46 mAb results in calcium release and the secretion of IFN-γ and TNF-α by NK cells and blocking NKp46 signaling with specific mAbs can result in reduced NK cell cytotoxicity of certain tumor cell-lines, although the most potent blocking activity is observed in combination with mAbs to other NCRs (19, 21). Subsequent studies have now shown that NKp46 is also expressed by innate lymphoid cells (ILCs) of group 1 (ILC1) and a subset of group 3 ILCs (NCR^{+} ILC3) (27, 28), γδ T cells (29, 30), a population of oligoclonally expanded intraepithelial (IEL) cytotoxic T lymphocytes (CTL) (31) and a population of IL-15-dependent innate-like IEL lacking surface TCR expression (32) in celiac disease patients, and umbilical cord blood (UCB) T cells cultured in IL-15 (33). NKp46 is also expressed by malignant NK, NKT, and T cell lymphomas (32, 34–36) (**Table 1**).

NKp44

The functional activity of NK cells against tumor cells deficient in the expression of MHC class I molecules is greatly enhanced by culture in IL-2, suggesting that NK cells upregulate activating receptors for additional non-MHC ligands. Whereas, NKp30 and NKp46 are constitutively expressed by resting NK cells obtained from peripheral blood, the expression of NKp44, also known as natural cytotoxicity receptor 2 (NCR2), is upregulated on NK cells stimulated by IL-2, IL-15 or IL-1β, particularly on the $CD56^{bright}$ subset (20, 37–39). NKp44 is a 44 kDa protein comprised of a single extracellular V-type Ig (IgV) domain followed by a long stalk region and a hydrophobic TM domain containing a charged lysine residue that mediates association with the ITAM adaptor, DAP12 (also known as KARAP and TYROBP) (**Figure 1**) (40, 41). Structural studies have shown that the IgV domain of NKp44 forms a saddle-shaped dimer with a positively charged groove on one side of the protein (42).

The gene for NKp44 (*NCR2*) is encoded in the human TREM receptor locus at 6p21.1, which is centromeric of the MHC (20, 43) and is also found in several primates species but not in mice (44). Three major transcripts are expressed from *NCR2* (NKp44-1,−2, and−3) that have been investigated in detail. Whereas, NKp44-2 and NKp44-3 are predicted to encode proteins with short cytoplasmic domains with no inherent signaling capacity, NKp44-1 is predicted to encode a protein with a long cytoplasmic tail containing the amino acid sequence "ILYHTV" that conforms to the sequence of an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM). The NKp44-1 isoform thus has the potential for inhibitory as well as activating signaling since this isoform also retains the capacity to associated with DAP12. A molecular characterization of the NKp44-1 ITIM showed that it has the potential to be phosphorylated in the NK92 NK cell-line but could not recruit the phosphotyrosine phosphatases, SHP-1 or SHP-2, or the 5′-inositol phosphatase, SHIP, in order to mediate cellular inhibition (45). Thus, it was concluded that the activating function of NKp44-1 is not influenced by the presence of the cytoplasmic ITIM. Nevertheless, NKp44-1 has the potential for dual signaling functions through the cytoplasmic ITIM and association with the ITAM adaptor, DAP12.

Like the other NCRs, NKp44 has also been shown to be expressed by ILC1 (46, 47), ILC3 (48, 49), γδ T cells (30, 50), oligoclonally expanded IEL CTL in celiac disease patients (31), and UCB T cells cultured in IL-15 (33), in addition to plasmacytoid dendritic cells where NKp44 may be involved in the regulation of type I interferon secretion (51) (**Table 1**).

NKp30

NKp30, also known as natural cytotoxicity receptor 3 (NCR3), was identified as a 30 kDa protein that, similarly to NKp46, is expressed on all mature resting and activated NK cells (18). Molecular cloning of the NKp30 cDNA revealed an open reading frame predicted to encode one extracellular IgV domain and a hydrophobic TM domain with a charged arginine residue capable of associating with the ITAM adaptors, CD3ζ and/or FcRγ (**Figure 1**) (18, 88). The crystal structure of NKp30 reveals some structural similarity to CTLA-4 and PD-1 and NKp30 is thus considered a member of the CD28 family of receptors (83, 89). Both NKp30 and NKp46 have reduced surface expression on adaptive memory NK cells most likely due to the downregulated expression of the FcRγ signaling chain required for the surface expression of these receptors (90, 91).

In humans, *NCR3* is encoded in the class III region of the Major Histocompatibility Complex (MHC) but was found to be a pseudogene in 13 strains of mice with the exception of *Mus caroli* (92). Six alternatively spliced transcripts are transcribed from the NKp30 gene, termed NKp30a-f. Three NKp30 isoforms, NKp30a, NKp30b, and NKp30c, which differ in their cytoplasmic tails due to alternative splicing in exon 4, have been studied in detail. Whilst NKp30a and NKp30b evoke NK cell activation, the NKp30c isoform was shown to elicit secretion of the immunosuppressive cytokine, IL-10, from NK cells (93). NKp30 has also been shown to be expressed by γδ T cells (30), $CD8^{+}$ T cells (94), and UCB T cells cultured in IL-15 (33).

NCRs AND THEIR LIGANDS IN CANCER

The NCRs were first characterized based on their ability to evoke the cytotoxic and cytokine-secreting functions of NK cells toward tumor cell-lines *in vitro*. Several studies have now provided evidence that the NCRs are involved in tumor surveillance *in vivo*. For example, genetic deficiency of NKp46 in mice results in the impaired clearance of subcutaneous T lymphoma (95) and melanoma (96) tumors and melanoma lung metastases (97, 98). Moreover, transgenic overexpression of NKp46 resulted in the enhanced clearance of melanoma lung metastases (99). Intriguingly, enhanced NKp46 signaling elicited IFN-γ secretion and increased tumor deposition of fibronectin, which altered the solid tumor architecture and resulted in the decreased formation of melanoma metastases (100). Importantly, the T lymphoma and melanoma cell-lines used in these studies all expressed cell-surface ligands for NKp46. Moreover, in human patients with melanoma, normal melanocytes had negligible expression of NKp46 ligands in comparison to malignant melanocytes deep

TABLE 1 | Expression of Natural cytotoxicity receptors and their ligands.

Receptor	Gene	Cellular expression	Ligands	Ligand-induced signal	References
NKp46	*NCR1*	NK cells, ILC1, NCR$^+$ ILC3, Small intestine TCRαβ$^+$ CD8$^+$ IEL, Small intestine TCRαβ$^-$ innate-like IEL, TCRγδ^{low}CD3$^-$, Expanded peripheral blood γδ T cells (Vδ1$^+$), NK, NKT and T lymphomas, Cord blood T cells cultured in IL-15	Heparan sulfate (HS) gylcosaminogylcans (GAGs) HA (hemagglutinin) of influenza virus HA of human vaccinia virus HN of avian Newcastle disease virus, Sendai virus and human parainfluenza virus (DBL)-1 α domain of *Plasmodium falciparum* erythrocyte membrane protein (PfEMP1) Vimentin expressed on cells infected with *Mycobacterium tuberculosis* Unidentified ligand expressed by *Fusobacterium nucleatum* Unidentified ligand expressed by pancreatic β-cells *C. glabrata* Epa proteins Complement Factor P (properdin)	Activation Activation Activation Activation Activation Activation Activation Activation Activation Activation Activation	(52) (53) (54) (53, 55–57) (58) (59, 60) (61) (62) (63) (64)
NKp44	*NCR2*	NK cells, ILC1, ILC3, Plasmacytoid dendritic cells, Small intestine TCRαβ$^+$ CD8$^+$ IEL, Expanded peripheral blood γδ T cells (Vδ1$^+$), Cord blood T cells cultured in IL-15	HS GAGs Syndecan-4 (*in cis*) HA of Influenza virus HN of avian Newcastle disease virus, Sendai virus and human parainfluenza virus PDGF-DD Nidogen-1 PCNA NKp44L expressed on tumor cells, bystander CD4$^+$ T cell during HIV infection, or cartilage-derived chondrocytes Domain III envelope protein from West Nile and Dengue viruses Unknown ligand(s) on *Mycobacterium tuberculosis, M. bovis, Nocardia farcinica and Pseudomonas aeruginosa*	Activation Inhibition Activation Activation Activation Inhibition Inhibition Activation Activation Unclear	(52, 65) (65) (66) (55–57) (67) (68) (69, 70) (71–73) (74) (75, 76)
NKp30	*NCR3*	NK cells, CD8$^+$ T cells, Expanded peripheral blood γδ T cells (Vδ1$^+$), ILC2 Cord blood T cells cultured in IL-15	HS GAGs HA of human vaccinia virus pp65, main tegument protein of human cytomegalovirus (DBL)-1 α domain of Plasmodium falciparum erythrocyte membrane protein (PfEMP1) BAT3/BAG6 B7-H6 Galectin-3 β-1,3 glucan	Activation Inhibition Inhibition Activation Activation Activation Inhibition Activation	(52, 77, 78) (54) (79) (58) (80–82) (83, 84) (85) (86, 87)

within melanoma lesions that stained strongly for NKp46-Fc showing that NKp46 ligands can be upregulated on malignantly transformed cells but are not expressed by healthy cells (101). Several cellular ligands for the NCRs have now been proposed (**Table 1**) and we discuss these here in the context of anti-tumor responses (**Figure 2**).

Heparan Sulfate Glycosaminoglycans

Heparan sulfate (HS) glycosaminoglycans (GAGs) are found on cell surfaces and within the ECM and consist of long, branched, anionic polysaccharides that are incorporated into proteins to form HS proteoglycans (HSPGs), such as the syndecans and glypicans, that form an integral and dynamic component of normal tissue architecture (102, 103). HSPGs also play vital roles in tumor progression allowing cancer cells to proliferate, elude immunosurveillance, invade neighboring tissues, and metastasize to distal tissue sites from the primary tumor. The negative charge of HSPGs can also provide docking sites for the basic domains of various secreted factors, such as chemokines and growth factors like fibroblast growth factor (104).

All three NCRs have been reported to bind to different HS sequences. HS GAGs are heterogenous in structure with a preference for highly sulfated HS structures and with each NCR possessing a distinct HS binding specificity. NKp30 and NKp46 binding to HS is similar although the binding varies significantly. In contrast, NKp44 displays a very different binding pattern to NKp30 and NKp46 (52, 77). A cis interaction of NKp44 with the cell-associated HSPG, syndecan-4, has been reported. The interaction between syndecan-4 and NKp44 was shown to regulate the membrane distribution of NKp44, and constitutively

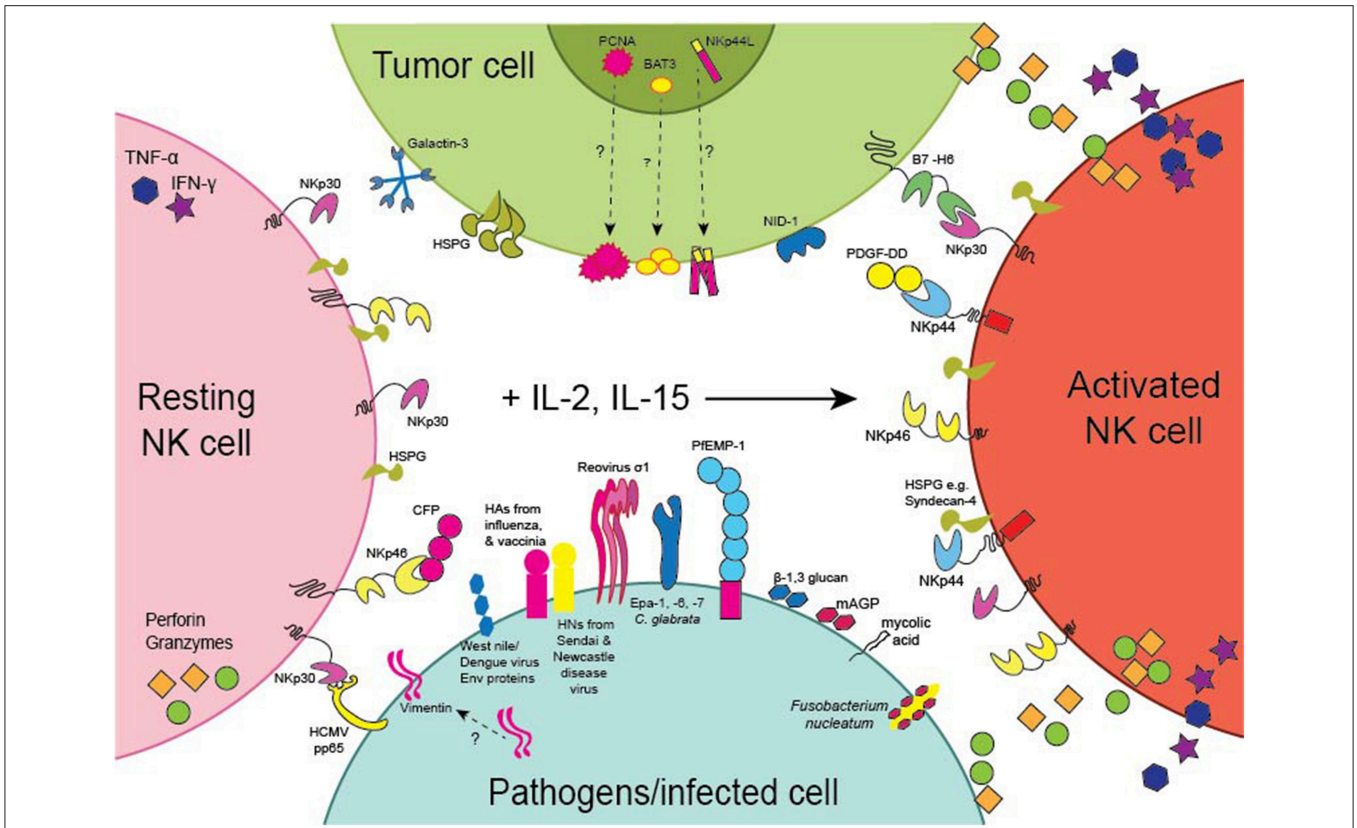

FIGURE 2 | Expression of the NCRs and their Ligands. Schematic representation of the expression of the NCRs on NK cells and the NCR ligands by tumors (green) or pathogens or cells infected with pathogens (light blue). NKp30 and NKp46 are expressed by resting (pink) as well as activated (orange) NK cells, whereas NKp44 is only expressed by NK cells activated with IL-2 or IL-15 (arrow). PCNA, BAT3 and NKp44L are nuclear proteins but the pathways whereby they become expressed on the surface of tumor cells remain to be identified. NKp44L is a splice variant of the Mixed-lineage leukemia 5 (MLL5) protein encoding a C-terminal modification reported to mediate cell-surface expression of NKp44L on tumor cells. Similarly, the pathway whereby vimentin becomes expressed on the surface of *M. tuberculosis* infected cells also remains to be described.

dampen NKp44 activity. Treatment of NK cells with soluble HS was proposed to disrupt the cis association with syndecan-4 and potentiate NKp44 signaling (65).

It is an attractive hypothesis to speculate that NK cells could utilize the NCRs to sense changes in HSPGs in the tumor microenvironment or possibly even during infection to activate NK cell cytotoxicity and IFN-γ secretion, particularly since several cancers exhibit aberrant regulation of key HS biosynthetic enzymes, such as 3-*O*- and 6-*O*-Sulfotransferases, and catabolic enzymes, such as heparanase and the HS endosulfatases, SULF1 and SULF2 (105). Conversely, pathogens or tumor cells could modify HSPGs to evade NK cell surveillance. Indeed, the over-expression of telomere repeats binding factor 2 (TRF2), a key factor in telomere protection, can result in the upregulation of heparan sulfate-glucosamine 3-*O*-sulfotransferase 4 (HS3ST4) in cancer cells by binding to the interstitial telomeric repeat located within the HS3ST4 intron (106). Silencing of HS3ST4 expression using short hairpin RNAs in cancer cells resulted in increased tumor infiltration of activated NK cells. Thus, aberrant sulfation of cell-surface HSPGs can modify the capacity for tumor surveillance by NK cells. However, the precise role of the NCRs and HS GAGs in NK cell tumor surveillance remains unclear since NKp30-dependent NK cell cytotoxicity was unaffected by GAG-deficiency or heparanase treatment of tumor cell targets (78). It is possible that HS GAGs may also serve as "co-receptors" facilitating interactions with other NCR ligands, such as growth factors like PDGF-DD (67).

B7-H6

K562 cells are highly susceptible to lysis by human NK cells, which is mediated by NKp30-mediated recognition of a tumor cell-surface ligand. A proteomics approach designed to trap ligands bound to an NKp30 Fc-fusion protein by chemical cross-linking resulted in the co-immunoprecipitation of B7-H6 from K562 cell lysates (84). B7-H6 is type I TM protein possessing two extracellular Ig-like domains and is a member of the B7 family of costimulatory molecules. Like other members of the B7 family, the extracellular domain of B7-H6 is composed of a membrane distal IgV domain and a membrane proximal IgC domain (83). NKp30 was found to use the front and back β-sheets of its IgV domain to engage the side and face of the corresponding β-sandwich of B7-H6, whereas CTLA-4 and PD-1

use only the front β-sheet of their Ig-like domains to contact their ligands. B7-H6 contacts NKp30 through the complementarity-determining region (CDR)-like loops of its IgV domain, thus resembling the binding interaction of antibodies with antigen, which is not observed for CTLA-4 binding to B7.1/B7.2 or PD-1 binding to PD-L1/PD1-L2 (83).

B7-H6 expression was found to be negligible on normal cells but was highly expressed by a wide range of tumor cells, showing that cellular transformation serves as a mode of immunosurveillance in the innate immune system (84). In support of this, the expression of B7-H6 is upregulated by the proto-oncogene Myc (107) and is associated with greater overall survival of patients with oral squamous carcinoma (108). In contrast, some tumors are thought to escape NKp30 recognition by metalloprotease-mediated shedding of B7-H6 from the cell-surface (109). The soluble form of B7-H6 is detected in the serum of patients with hepatocellular carcinoma (110), metastatic gastrointestinal tumors (GIST) (111), neuroblastoma (112, 113), and peritoneal fluid from ovarian cancer patients (114), and is associated with impaired NKp30 expression and NK cell dysfunction as well as poor overall patient survival.

Differential expression of transcripts encoding the different NKp30 isoforms have also been associated with cancer prognosis. The preferential expression of the immunosuppressive NKp30c isoform over the activating NKp30a and NKp30b isoforms was associated with the reduced overall survival of GIST patients treated with imatinib mesylate (93, 111). The NKp30c isoform was reported to trigger secretion of the immunosuppressive cytokine IL-10 from NK cells and was associated with NK cell dysfunction characterized by defective NK cell degranulation and secretion of IFN-γ and TNF-α. Interestingly, polymorphism in the 3′ untranslated region of *NCR3* can result in the preferential expression of the immunosuppressive NKp30c isoform by NK cells and predicted the clinical outcome for GIST patients independently from KIT mutation (93). In pediatric neuroblastoma, expression of the immunostimulatory (NKp30a/b) versus immunosuppressive (NKp30c) isoforms is also associated with a higher risk of relapse and elevated serum levels of soluble B7-H6 that inhibit NK function were associated with the bone marrow metastasis and chemoresistance of neuroblastoma cells (112, 113). Expression of B7-H6 is also detected on monocytes and neutrophils treated with TLR ligands and pro-inflammatory cytokines. A soluble form of B7-H6 was also detected under these conditions and in sepsis patients suggesting B7-H6 may also regulate NK cell activity via NKp30 isoforms during inflammation (115).

Nuclear Proteins

NKp44 has been reported to bind to several intracellular ligands that have been proposed to be aberrantly expressed on the cell-surface of tumor or virus-infected cells. An NKp44 ligand was reported to be expressed on the surface of tumor or $CD4^+$ T cell from HIV patients (71) that was dependent on expression of HIV gp41. Moreover, a peptide sequence derived from HIV gp41 was shown to upregulate the expression of this NKp44 ligand on $CD4^+$ T cells (71). A yeast two-hybrid screen using a cDNA library from the Jurkat T cell-line was employed to find interacting partners for NKp44 (72) and a cDNA encoding a splice variant of the mixed-lineage leukemia protein-5 (MLL5) was subsequently cloned. MLL5 is normally a nuclear antigen but the cDNA sequence revealed a new exon resulting in a transcript encoding an MLL5 variant protein with an alternative C-terminal amino-acid sequence. This new protein termed NKp44L was reported to be expressed on the cell-surface and in the cytoplasm of various tumor cell-lines but not in normal tissues (72). However, the mechanism driving NKp44L cell-surface expression remains obscure. NKp44L expression has also been reported on human articular chondrocytes (73).

NKp44 was also reported to interact with Proliferating cell nuclear antigen (PCNA). PCNA is found in the nucleus and functions as a co-factor for DNA polymerase δ that helps leading strand synthesis during DNA replication (116). PCNA was identified as an NKp44 ligand following a screen of a yeast surface display library using an NKp44-Fc fusion protein (69). Cell-surface expression of PCNA was reported following transfection into HeLa cells, which reduced NK cell cytotoxicity and IFN-γ secretion. PCNA was found to localize at the plasma membrane where it was shown to form an immunological synapse with NKp44 to mediate NK cell inhibition. The inhibition of NK cell signaling was reported to occur via the NKp44-1 splice variant that potentially encodes a cytoplasmic ITIM (69). The interaction of PCNA with NKp44 was proposed to form a unique interaction capable of transducing an inhibitory rather than an activating signal (69, 117). PCNA has also been shown to associate with Human Leukocyte Antigen (HLA) class I molecules on the cell-surface of tumor cells to form an inhibitory ligand for NKp44 and suppression of NK cell cytotoxicity (70). The authors suggested that NCR ligands could act as Damage-Associated Molecular Patterns (DAMPs) through association with HLA class I molecules (118). Interestingly, preferential expression of the transcript encoding the ITIM-bearing NKp44-1 isoform is associated with poor survival in acute myeloid leukemia and was proposed to be a novel target for checkpoint blockade on NK cells (119).

HLA-B-associated transcript 3 (BAT3), also known as Bcl2-associated anthogene 6 (BAG6), is another intracellular protein that was identified as an NKp30 ligand using a yeast two-hybrid screening approach (80). BAT3 is a multifunctional molecular chaperone that contributes to several cell processes including apoptosis, gene regulation, protein synthesis, protein quality control and protein degradation. For example, BAT3 can function by preventing the aggregation of misfolded and hydrophobic-patch containing proteins ensuring their correct delivery to the endoplasmic reticulum or the sorting of proteins that have mislocalized to the cytoplasm for proteosomal degradation (120). BAT3 also accumulates in the nucleus where it is involved in regulating apoptosis following DNA damage (121). BAT3 was proposed to be released from tumor cells to engage NKp30 and trigger NK cell activation (80, 81). Exosomal release of BAT3 was also reported to promote NK:DC cross-talk (82). In contrast, the release of a soluble form of BAT3 found in the plasma of CLL patients was found to suppress NK cell activation through competition for the exosomal form of BAT3 and other tumor ligands (122, 123).

Platelet-Derived Growth Factor-DD

Growth factors (GFs) are important for guiding various cellular and developmental processes and GF pathways are often dysregulated in cancer. The platelet-derived growth factor (PDGF) family are comprised of four polypeptides that can assemble into at least five dimeric isoforms, PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD, and which engage with the receptor tyrosine kinases, PDGF receptor (PDGFR)-α and PDGFR-β (124). Cancer cells frequently express PDGFs which can trigger autocrine and paracrine PDGFR signaling to promote tumor growth, proliferation, stromal recruitment, angiogenesis, epithelial to mesenchymal cell transition, and metastasis (125).

PDGF-DD was identified as a ligand for NKp44 by screening a library of secreted proteins with NKp44 GFP reporter cells (67). PDGF-DD induced NK cell degranulation and the secretion of IFN-γ and TNF-α from NK cells and ILC1 as well as TNF-α secretion from ILC3 in agreement with a previous study (67, 126). IFN-γ and TNF-α secreted by NK cells stimulated with PDGF-DD could induce cell cycle arrest in melanoma, ovarian, and breast tumor cell-lines. Thus, innate immune cells have the capacity to sense the expression of GFs by tumor or tumor-associated stroma cells and potentially even infected or developing tissues to evoke NK cell responses. Remarkably, the upregulation of PDGF-DD-induced NK cell cytokines and chemokines and the downregulation of tumor cell cycle genes correlated with *NCR2* expression and was associated with greater survival in a cohort of glioblastoma patients. Moreover, transgenic expression of NKp44 in mouse NK cells resulted in greater control of tumors expressing PDGF-DD showing that whilst PDGF-DD can support tumor growth it also exposes tumor cells to NK cell immunosurveillance via NKp44. The ability of NK cells and ILCs to engage in GF surveillance via NKp44 is a new immunological paradigm that remains to be fully explored (67). Interestingly, a polymorphism in *PDGFD* is associated with serum IFN-γ levels in humans (127), suggesting the PDGF-DD/NKp44 interaction may play wider biological roles beyond cancer (**Table 2**).

Nidogen-1

The ECM protein, Nidogen-1 (NID1, also known as Entactin) was identified as an NKp44 ligand, in another screen to identify soluble ligands that might regulate NCR activity and NK cell function (68). NID1 is an essential component of the basement membrane (BM) where it plays a role in BM assembly and stabilization, as well as adhesion between cells and the ECM (128). Soluble NID1 can be detected in the serum from patients with ovarian and lung cancer that may be derived from proteolytic degradation by cathepsin-S (CatS) (129, 130). Thus, it is conceivable that the release of NID1 into extracellular fluids may regulate the activity of activated NK cells in the blood or at specific tissue sites. Indeed, CatS-degradation and release of NID1 was proposed to reflect the loss of BM integrity, which is frequently associated with invasive tumors. Soluble NID1 was able to inhibit cytokine secretion mediated by PDGF-DD or cross-linking with anti-NKp44 mAbs. Consequently, the release of soluble NID1 was proposed to be a novel immunosuppressive mechanism exploited by tumor cells to evade NK cell surveillance (68).

Galectin-3

Galectin-3 is a β-galactoside-binding lectin that has been reported to be a critical immune regulator in the tumor microenvironment (131). Galectin-3 can be expressed in the cytoplasm, nucleus, cell-surface, or extracellularly depending on the cell type and proliferative status. For example, extracellular galectin-3 can facilitate metastasis by promoting cell adhesion, invasiveness and immune evasion.

The genetic manipulation of galectin-3 expression levels in tumor cells showed that downregulation of galectin-3 lead to tumor growth inhibition, whereas the upregulation of galectin-3 led to enhanced tumor growth in human cervical and breast cancer models. Moreover, soluble galectin-3 released from tumor cells was found to bind specifically to NKp30 thereby inhibiting NKp30-mediated cytotoxicity. Thus, it was proposed that the secretion of galectin-3 by tumors represents a novel pathway to escape NKp30-mediated NK cell immunosurveillance (85).

NCR-MEDIATED CONTROL OF TUMORICIDAL PATHWAYS

The NCRs have been proposed to bind to many cellular ligands which are implicated in NK cell surveillance of tumor cells. Many of these interactions have been shown to evoke the cytotoxic and cytokine-secreting functions of NK cells. However, it is also possible that the NCRs may regulate other anti-tumor pathways. For melanoma metastases, NK cells were attributed to play a major role (99). However, ILC1 are phenotypically closely related to conventional NK cells as both these cell-types express T-bet and secrete IFN-γ (132, 133). ILC1 are characterized by higher TRAIL expression than NK cells due to tissue-imprinting by TGF-β (134). TRAIL (TNFSF10) can bind to TRAIL receptors (TRAIL-Rs), such as TRAIL-R1 in humans and TRAIL-R2 in humans and mice, which carry death domains that can induce caspase-8-mediated apoptosis of TRAIL-R^+ tumor cells. TRAIL expression can be induced on NK cells activated by various cytokines and interferons (135–137) and several reports have described NK cell-mediated clearance of TRAIL-sensitive transplantable tumors (135, 138), chemical-induced fibrosarcoma (139), liver metastases (135, 139, 140), and hematological malignancies (141).

NKp46 was shown to regulate TRAIL surface expression resulting in the diminished cytotoxicity of TRAIL-R^+ target cells by NKp46-deficient NK cells and ILC1 (142, 143). The NKp46-mediated pathway that regulates surface TRAIL expression remains to be fully characterized but appears to involve a post-transcriptional mechanism (142, 143). Whether NKp46 can regulate surface TRAIL expression when expressed by other immune cell subsets, such as γδ T cells and $CD8^+$ T cells that have been expanded with IL-15, remains to be determined. These data show that NKp46 can endow NK cells or ILC1 with the ability to lyse TRAIL-sensitive tumors as well as contribute to

TABLE 2 | Disease and biological trait association of the NCRs and their ligands.

NCR gene	Gene location	Disease association/reported trait	Reference (PubMed ID)	NCR Ligand gene	Gene location	NCR ligand disease association	Reference (PubMed ID)
NCR1	19q13.42	Blood protein levels	29875488 30072576	*KMT2E* (parental gene for NKp44L)	7q22.3	Intelligence (MTAG)	29326435
						Educational attainment (MTAG)	30038396
						Educational attainment (years of education)	30038396
						Cognitive performance (MTAG)	30038396
				PCNA	20p12.3	No data available	
				VMAC (vimentin)	19p13.3	Post bronchodilator FEV1/FVC ratio	26634245
				CFP	Xp11.23	No data available	
NCR2	6p21.1	Alzheimer's disease biomarkers (p-tau measurement)	23562540	*PDGFD*	11q22.3	Interferon gamma levels	27989323
						Corticosteroid-induced adrenal suppression (peak cortisol < 350 nmol/L)	29551627
		Total ventricular volume (brain measurement)	21116278			Corticosteroid-induced adrenal suppression (peak cortisol < 500 nmol/L)	29551627
						LDL cholesterol	21347282
		Telomere length	29151059			Blood protein levels	29875488 30072576
						3-hydroxypropylmercapturic acid levels in smokers	26053186
				SDC4 (Syndecan-4)	20q13.12	Bacterial meningitis	28928442
						Psoriasis	20953189
						Velopharyngeal dysfunction	29855589
						Lung function (FEV1/FVC)	30595370
				NID1	1q42.3	Urate levels in overweight individuals	25811787
						Cutaneous nevi	21478494
						Blood protein levels	30072576
						Waist-hip ratio	30595370
						Facial morphology (factor 20)	28441456
NCR3	6p21.33	C3 and C4 complement levels	23028341	*BAG6*	6p21.33	Small cell lung carcinoma	28604730
						Diastolic blood pressure	28739976 21909115
		Susceptibility to shingles	28928442			Systolic blood pressure	28739976 27618447
		Blood protein levels	29875488			Feeling worry	29500382
		Mosquito bite reaction, itch intensity measuremet	28199695			Hypertension	21909115
						Neuroticism measurement	29500382
						Psoriatic arthritis	30552173
		Neutrophil collagenase measurement	28240269			Urinary tract infection frequency	28928442
		Allergic disease (asthma, hay fever or eczema)	29083406	*LGALS3* (Galactin-3)	14q22.3	Blood protein levels	30072576
						Protein biomarker	23056639
						No data available	
		Menopause (age at onset)	29773799	*NCR3LG1* (*B7-H6*)	11p15.1		
		Laryngeal squamous cell carcinoma	25194280				
		Crohn's disease	22936669				
		Pulmonary function	23284291 28166213 21946350				
		IgG glycosylation	23382691				

(Continued)

TABLE 2 | Continued

NCR gene	Gene location	Disease association/reported trait	Reference (PubMed ID)	NCR Ligand gene	Gene location	NCR ligand disease association	Reference (PubMed ID)
		Height	23563607				
		Type 1 diabetes and autoimmune thyroid diseases	25936594				
		Weight	19079260				
		Diastolic blood pressure	28135244				
		Cold sores	28928442				
		Pneumonia	28928442				
		Strep throat	28928442				
		Tonsillectomy	28928442				
		Educational attainment (MTAG)	30038396				
		Self-reported mathematical ability	30038396				
		Highest math class taken (MTAG)	30038396				
		Heel bone mineral density	30598549				

TRAIL-mediated immunoregulation of infectious diseases, tissue inflammation (144–146), and potentially autoimmunity. It will also be interesting to see which NKp46 ligands can induce surface TRAIL expression in NK cells and ILC1 and the functional consequence for TRAIL-sensitive tumors *in vivo*.

Control of Malignancy by Unconventional NCR$^+$ Lymphocyte Subsets

Until recently NCR expression was considered to be restricted to NK cells. The NCRs have now been shown to be expressed by CD8$^+$ T cell populations and γδ T cells expanded with IL-15, in addition to ILC1 and subsets of ILC3. Moreover, antibody-mediated stimulation of NKp44 and NKp46 on "NK-like" IL-15-expanded IEL CTL elicits the secretion of IFN-γ showing that these NCRs can function independently of TCR stimulation in T lymphocytes (31). Moreover, a population of NKp30$^+$ CD8$^+$ T cells with anti-tumor potential was shown to be induced by IL-15 (94). These NKp30$^+$ CD8$^+$ T cells exhibited high "NK-like" anti-tumor activity and NKp30 synergized with TCR signaling to control tumor growth in a preclinical xenograft mouse model.

Murine γδ T cells do not express NKp46, however, all three NCRs are expressed on human γδ T cells following continuous stimulation with TCR agonists or mitogens in the presence of IL-2 or IL-15 (30). Interestingly, NCR induction was mostly restricted to the Vδ1$^+$ T cell subset and not Vδ2$^+$ T cells. Like NK cells, the NCR$^+$ Vδ1$^+$ T cells were highly cytolytic against primary leukemia cells and tumor cell-lines in redirected cytotoxicity assays and NCR triggering also enhanced the expression of IFN-γ. Based on these observations, IL-15 expanded populations of "NK-like" CD8$^+$ or Vδ1$^+$ T cells that express NCRs have tremendous potential to be used clinically for adoptive cancer immunotherapy (147). The acquisition of NCRs by Vδ1$^+$ T cells and CD8$^+$ T cells is thought to require strong TCR activation, which is consistent with the oligoclonal expansion of gut IEL CTL in celiac disease that express a highly restricted TCR repertoire (31).

Although unconventional NCR$^+$ lymphocyte subsets have been implicated in tumor surveillance they may also promote immunosuppression and facilitate tumor immune evasion. For example, a unique CD3$^-$CD56$^+$ ILC population was recently described that inhibits tumor-infiltrating lymphocytes (TILs) from high-grade serous ovarian carcinomas (148). The CD3$^-$CD56$^+$ ILC population was associated with a reduced TIL expansion and altered TIL cytokine production. This novel population of CD3$^-$CD56$^+$ ILCs exhibited low cytotoxic potential, secreted IL-22, and exhibited a transcriptional profile overlapping that of NK cells and other ILCs. NKp46 was highly expressed by the regulatory CD3$^-$CD56$^+$ ILC population and NKp46 signaling promoted the ability to suppress TIL expansion in co-culture (148).

Group 2 ILCs (ILC2s) secrete large amounts of type 2 cytokines, such as IL-5, IL-9, and IL-13, which promote alternative macrophage activation, eosinophilia, and goblet cell hyperplasia to limit parasite infection (149, 150). Increased levels of hyperactivated ILC2s expressing NKp30 and Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) are found in patients with peripheral acute promyelocytic leukemia (APL). APL blasts were found to express high levels of surface B7-H6 and prostaglandin D2 (PGD2) that bind to NKp30 and CRTH2 (151) on ILC2s, respectively, to drive potent IL-13 secretion and activation of IL-13R$^+$ myeloid-derived suppressor cells (152). Disruption of this tumor immunosuppressive axis by specifically blocking PGD2, IL-13, and NKp30 signaling partially normalized ILC2 and MDSC levels resulting in enhanced survival in leukemic mice (152).

NCRs AND THEIR LIGANDS IN INFECTIOUS DISEASE

The central role of the NCRs in resistance to infectious disease is exemplified by the susceptibility of NKp46-deficient mice to infection with different microorganisms. For example,

human metapneumovirus (HMPV) causes acute respiratory tract infections in infants and children worldwide that can be fatal in immunosuppressed hosts. HMPV-infected cells express an unidentified ligand for NKp46 and NKp46-deficient mice are more susceptible to HMPV infection (153). The NCRs have also been shown to contribute to humoral immune responses and the generation of protective pathogen-specific antibodies. For example, deficiency in NKp46 resulted in the impaired maturation, function, and migration of NK cells to regional lymph nodes of mice infected with murine cytomegalovirus (MCMV). This was accompanied by a reduction in $CD4^+$ T cell activation and follicular helper T cell generation leading to diminished B cell maturation in germinal centers and reduced titers of MCMV-specific antibodies (154).

Given the central role of NCRs in resistance to infectious disease, it is unsurprising that pathogens have evolved mechanisms to avoid NCR recognition. Herpesviruses are DNA viruses that have evolved various strategies of immune evasion as exemplified by their ability to establish latent infection with limited transcription of viral genes. Cells infected with human cytomegalovirus (HCMV) and human herpesvirus 6B (HHV6B) can express the NKp30 ligand, B7-H6. Both HMCV and HHV6B have evolved strategies to downregulate B7-H6 and ligands for other activating NK cell receptors, such as NKG2D, from the cell-surface, thus impairing NK cell recognition of virus-infected cells (155, 156). In the case of HCMV infection, HCMV expressed two gene products US18 and US20, that interfere with B7-H6 surface expression by promoting the lysosomal degradation of B7-H6 (155).

Many of the genes for the NCRs and their ligands are associated with infectious diseases highlighting the importance of the NCRs for pathogen recognition (**Table 2**). For example, *NCR2* is associated with chronic periodontitis in pregnant women and *NCR3* is associated with susceptibility to cold sores and shingles (**Table 2**). Here we document various NCR ligands that have been reported to play a role in pathogen surveillance by NK cells and other NCR^+ immune cell subsets (**Figure 2**).

Hemagglutinins and Hemagglutinin Neuraminidases

Even though the NCRs were first identified based on the ability to lyse certain tumor cell-lines, some of the first NCR ligands identified were viral proteins. Hemagglutinins (HA) from influenza (53, 66) and hemagglutinin-neuraminidases (HN) from parainfluenza, Sendai, and Newcastle disease viruses (53, 55) expressed on infected cells were shown to bind to NKp46 and NKp44. HAs from poxviruses, such as vaccinia and ectromelia, were also identified as ligands for NKp30 and NKp46 (54). However, the NCRs do not bind to HA from measles virus, showing that whilst the NCRs are capable of recognizing a broad range of viral HA and HNs there is some selectivity, which is predominantly dependent on α-2,3- and α-2,6-sialylated *O*-glycans on NCRs (53, 55, 56).

The interaction of viral-encoded HA and HNs on the surface of infected cells results in NK cell activation via NKp46 and NKp44 (53, 55, 56) and binding of the HN of Newcastle disease virus to NKp46 results in activation of downstream signaling molecules in the ITAM pathway e.g., Syk and surface upregulation of TRAIL on murine NK cells (157). In support of these observations, mice deficient in NKp46 expression are more susceptible to infection with influenza A virus (158, 159). In contrast, the interaction of NKp30 with HA expressed on the cell-surface or shed from vaccinia-infected cells counteracted activation by NKp46 resulting in the inhibition of NK cell lysis (54), suggesting a novel pathway of viral immune escape via NKp30. Moreover, exposure of NK cells to influenza virions or soluble HA can result in lysosomal degradation of the CD3ζ signaling chain and a reduction of NK cell cytotoxicity mediated by NKp46 and NKp30 (160). In contrast, the cleavage of sialic acid residues from NKp46 by the influenza virus neuraminidase (NA) was proposed as a mechanism of immune evasion by disrupting NKp46-binding to the viral HA and inhibitors specific for the influenza NA enhanced NKp46 recognition and virus clearance (161).

Human parainfluenza virus type 3 (HPIV3) is a virus that causes various respiratory illnesses, such as pneumonia, croup, and bronchiolitis, during infancy and childhood. Even though reinfection with HPIV3 throughout life is common there is no development of immunological memory and currently no effective vaccine or anti-viral therapies available. Poor T cell proliferation is observed following HPIV3 infection, which may be responsible for the lack of memory associated with the virus. NK cells have been shown to induce T cell cycle arrest in a contact-dependent manner, which was shown to be dependent on NKp44 and NKp46 but not NKp30 recognition of the HPIV3 HN on HPIV3-infected monocytes co-cultured with allogeneic-mixed lymphocytes (57). Consequently, the authors proposed that the success of future vaccines to HPIV3 may require the generation of modified HN proteins that retain immunogenicity but do not interact with NKp44 or NKp46.

Human Cytomegalovirus Tegument Protein pp65

Intracellular staining of human cytomegalovirus (HCMV) infected fibroblasts revealed an unidentified protein that interacted with NKp30-Fc fusion protein. Purified virions from HCMV-infected fibroblasts also bound to NKp30-Fc but not to control Fc-fusion proteins. These data suggested the unidentified viral protein that interacted with NKp30 may be incorporated into HCMV virus particles. The NKp30 Fc fusion was used to immunoprecipitate proteins from HCMV-infected fibroblasts and the HCMV-encoded tegument protein pp65 was subsequently identified as a ligand for NKp30 using mass spectrometry (79). HCMV pp65 was shown to bind directly to NKp30 and induce the dissociation of the signaling adaptor CD3ζ. HCMV-infected fibroblasts were found to be less susceptible to NK cell cytotoxicity compared to fibroblasts infected with a pp65-deficient HCMV or in the presence of blocking antibodies to NKp30. Thus, pp65 furnishes HCMV with a novel immune escape pathway by disrupting the surveillance of virus-infected cells by NKp30.

Flavivirus Proteins

NKp44 Fc-fusion proteins have also been shown to interact with purified envelope protein from the flaviviruses, dengue virus (DV), and West Nile Virus (WNV) and WNV virus-like particles. NKp44 was shown to specifically bind to domain III of the envelope protein from WNV, which also bound to NK cells expressing high levels of NKp44 and the binding of infectious WNV and WNV-infected cells stimulated NK cell degranulation and IFN-γ secretion in an NKp44-dependent manner (74). Unlike the interaction with viral hemagglutinins, NKp44 binding to domain III of the DV and WNV envelope proteins was independent of glycan-linked sialic acid residues on NKp44.

Bacterial NCR Ligands

In contrast to viral ligands, the NCRs have also been shown to bind directly to molecules encoded by bacteria and parasites in addition to host molecules exposed on the surface of cells infected with bacteria. For example, an Fc-fusion protein of NKp44 (NKp44-Fc), but not NKp30 or NKp46, was shown to bind to *Mycobacterium bovis* (*M. bovis*), *M. tuberculosis, Nocardia farcinica*, and *Pseudomonas aeruginosa* (75, 76). NKp44 was specifically shown to bind to cell wall components, such as arabinogalactan-peptidoglycan (mAGP) as well as to mycolic acids and arabinogalactan and derivatives, from *M. tuberculosis*. Whilst a direct role in NK cell cytotoxicity could not be demonstrated, anti-NKp44 antibodies inhibited mAGP and BCG-induced CD69 expression on IL-2-activated NK cells, suggesting NKp44 recognition of bacterial wall components may sustain NK cell activation during infection with *M. tuberculosis* (76). In contrast to NKp44, NKp46 was reported to play a dominant role in the lysis of mononuclear phagocytes infected with *M. tuberculosis* (59). NKp46 was subsequently shown to recognize vimentin expressed on the surface of cells infected with *M. tuberculosis* (60). Antibodies to vimentin inhibited NK cell lysis of monocytes infected with *M. tuberculosis*, whereas transfection of vimentin enhanced NK cell lysis of Chinese hamster ovary cells (60).

Fusobacterium nucleatum and *Porphyromonus gingivalis* are involved in the pathogenesis of periodontitis. The severity of periodontal disease is determined by the host's immune response. Alveolar bone loss occurred in wild-type mice but was minimal in NKp46-deficient mice in an oral infection model using *Fusobacterium nucleatum* or *Porphyromonus gingivalis* (61). Expression of an NKp46 ligand by *F. nucleatum* was demonstrated by the binding of a NKp46-Fc and the activation of NKp46 reporter cells as well as the secretion of TNF-α from wild-type but not NKp46-deficient NK cells (61). Interestingly, *NCR2*, but not *NCR1*, is associated with chronic periodontitis in humans (**Table 2**) (162), which could reflect cross-talk in NCR signaling (163).

Parasite NCR Ligands

Parasite infection has also been shown to induce the expression of NCR ligands. Fc fusion proteins of NKp30 and NKp46 were shown to specifically bind to the Duffy binding-like (DBL)-1 α domain of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1), which is expressed on the cell-surface of parasitized erythrocytes (58). NK cells lysed erythrocytes infected with *P. falciparum*, which was blocked by antibodies to NKp30 and NKp46 with the greatest inhibition observed when both antibodies were used in combination.

Fungal NCR Ligands

NKp46 and NKp30 have been shown to interact with fungal ligands. Both mouse and human NKp46 were shown to bind to the emerging fungal pathogen, *Candida glabrata* but not to *C. albicans, C. parapsilosis*, *C. krusei*, or *Cryptococcus gattii* (63). Fc fusion proteins from human and mouse NKp46 were specifically shown to bind to the *C. glabrata* adhesins Epa1, Epa6, and Epa7. The Epa proteins are lectins that bind to glycans to facilitate the *C. glabrata* infection of mammalian cells and mutation of *O*-linked glycosylation of Threonine 225 of mouse and human NKp46 abolished binding to *C. glabrata* Epa proteins (63). Moreover, the clearance of systemic *C. glabrata* infection was impaired in NKp46-deficient mice.

NKp30 mediates the recognition of *Cryptococcus neoformans* and *C. albicans* resulting in the formation NK cell-fungal conjugates and direct fungal killing (164). NKp30 was specifically shown to bind to the fungal cell wall component β-1,3 glucan to stimulate the polarization of cytotoxic granules in NK cell-fungal conjugates, which enhanced the release of perforin required for fungal cytotoxicity (86). Rather than blocking the interaction between NK cells and fungi, soluble β-1,3 glucan enhanced fungal killing (86). Fungal infections are the leading cause of death in AIDS patients and NK cells from HIV-infected patients had reduced expression of NKp30. IL-12 and β-1,3 glucan restored NK cell expression of NKp30 and fungal killing by NK cells (86, 164). The activation of src family kinases by NKp30 synergized with β1 integrin signaling and the activation of integrin-linked kinase (ILK) and Rac1, which converged into a central PI3 kinase signaling pathway that was required for fungal killing by NK cells (87).

Complement Factor P (properdin)

The complement system is an evolutionary ancient component of the innate immune response. Complement factor P (CFP, also known as properdin) is a plasma glycoprotein that binds to microbial surfaces and is the only known positive regulator of the alternative pathway of complement (165). CFP stabilizes the C3- and C5-convertase enzyme complexes that ultimately leads to the formation of the membrane attack complex and target cell lysis. NKp46 was shown to bind to CFP. Patients deficient in CFP are more susceptible to lethal *Neisseria meningitidis* due to an inability to activate the alternative pathway of complement (166).

CFP was shown to bind to a recombinant NKp46 Fc-fusion protein and to activate NKp46 reporter cells but could not induce classical NK cell activation characterized by degranulation and IFN-γ secretion (64). Instead CFP induced the upregulation of genes encoding the leucine zipper protein FosB and the chemokine XCL1 (also known as lymphotactin). Thus, CFP binding to NKp46 was proposed to activate a non-canonical pathway of gene expression in NK cells. In agreement with patients deficient in CFP, NKp46 and ILC1 were required for mice to survive infection with *N. meningitidis*. Moreover, the beneficial

effects of CFP supplementation for *N. meningitidis* infection were dependent on NKp46 and ILC1 (64).

Unconventional Roles for NCRs in the Control of Infection

NKp30 is also expressed on γδ T cells (30) bearing TCR Vδ1 but not TCR Vδ2. Circulating γδ T cells are normally devoid of NCR expression. However, all three NCRs were induced on γδ T cells following TCR engagement in conjunction with cytokines, such as IL-2 and IL-15 (30). Moreover, mAb cross-linking of NKp30 on NCR^+ Vδ1 T cells, and to a lesser extend NKp44 and NKp46, induced the chemokines CCL1, CCL2 and CCL3, which suppressed HIV infection by binding the HIV co-receptor, CCR5 (167).

NCRs AND THEIR LIGANDS IN DEVELOPMENT

In steady state, human uterine NK (uNK) cells maintain endometrial arteries (168). However, during pregnancy, uterine NK cells maintain key developmental processes, such as trophoblast invasion, remodeling uterine vasculature, and the promotion of fetal growth (169). The tissue origins of uNK remain obscure and are thought to constitute a heterogenous population derived from local endometrial NK cells as well as conventional NK cells recruited from the circulation (169, 170). Uterine NK cells possess limited cytotoxicity and actively secrete cytokines, chemokines, GFs, and angiogenic factors required for the correct remodeling of maternal spiral arteries and likely function by promoting the recruitment of invading trophoblast cells and promoting angiogenesis during pregnancy (171).

The NCRs have been reported to be expressed by uNK (171–173) and the ligands for NKp30 and NKp44 are also expressed by trophoblast cells (171). Consequently, trophoblast cells have the potential to directly stimulate uNK functions via the NCRs and ligation of the NCRs with specific antibodies resulted in the secretion of cytokines, chemokines, GFs, and angiogenic factors by uNK (171–173). Thus, NCR expression endows uNK with the capacity to orchestrate key developmental processes at the fetal-maternal interface following interactions with trophoblast cells or other NCR ligands expressed in decidua. Uterine NK and peripheral blood NK (pNK) operate in different tissue microenvironments and also differ in NCR expression. For example, resting pNK do not express NKp44 unlike uNK. The decidual stroma expresses the cytokines IL-15, IL-18, and TGF-β, that are required for successful pregnancy. Uterine NK were found to express distinct NKp30 and NKp44 splice variants compared to pNK, characterized by predominant expression of the inhibitory NKp30c and NKp44-1 isoforms and lower amounts of the activating NKp30a/b and NKp44-2/3 isoforms (174). A combination of IL-15, IL-18, and TGF-β shifted the pNK NCR splice variant expression profile toward that of uNK suggesting the decidual tissue microenvironment can confer a decidual-like uNK phenotype on pNK (174).

NCRs AND THEIR LIGANDS IN ALLERGY

Allergic disease is caused by inappropriate immune responses to harmless antigens driven by type 2 cytokines. Very few papers have documented a role for NK cells or the NCRs in allergic disease. However, recent studies have suggested that NK cells and the NCRs may be important in allergy due to the immunoregulatory activity of NK cells on other immune cell subsets (175) and NK cells have also been shown to drive allergic responses independently of T and B cells (176, 177). NKp46-deficient mice were shown to have reduced skin and airway hypersensitivity (178). In a delayed type IV hypersensitivity model of airway inflammation, the number of IFN-γ producing NK cells and the proliferation of OVA-specific T cells was reduced following intranasal OVA challenge in NKp46-deficient mice, suggesting that differences in the stimulation of T cells resulting in an altered cytokine profile upon challenge with ovalbumin (OVA) (178). These results suggested that NKp46 signaling enhances allergic responses. In contrast, a separate study using a delayed type I hypersensitivity model, showed that NKp46 signaling suppressed the development of airway eosinophilia following intranasal OVA challenge (179). It will be interesting to see which NKp46-expressing cell types, such as NK cells, ILC1 or ILC3, might be responsible for either suppressing (179) or augmenting (178) airway inflammation in models of type I and type IV hypersensitivity, respectively.

NCR expression by other lymphocyte subsets may also regulate allergic response. For example, whilst ILC2s secrete type 2 cytokines to limit parasite infection they are also important contributors to allergic inflammation (149, 150). Activated human ILC2s can express NKp30 and stimulation of ILC2s with B7-H6 induced the rapid production of type 2 cytokines, such as IL-13, which was blocked by anti-NKp30 blocking mAb and galectin-3 (180). Higher expression of B7-H6 is observed in skin lesion biopsies from patients with atopic dermatitis and stimulation of keratinocytes with proinflammatory as well as type 2 cytokines upregulated B7-H6 expression leading to enhanced ILC2 production of type 2 cytokines. Thus, the NKp30-B7-H6 interaction is a novel cell contact-dependent interaction that can mediate ILC2 activation and is a potential target for the development of novel therapeutics for atopic dermatitis and possibly other atopic diseases (180).

NCRs AND THEIR LIGANDS IN AUTOIMMUNITY

NK cell have also been implicated in the development of autoimmune diseases, such as type 1 diabetes (181–184). NKp46-deficient mice were reported to be resistant to the development diabetes induced by streptozotocin and a soluble NKp46-Fc protein blocked the development of diabetes in mice (62). NKp46 was suggested to play a dominant role in NK cell cytotoxicity of human pancreatic β-cells through the recognition of an as yet unidentified NKp46 ligand expressed in the β-cell insulin granules (97). Anti-NKp46 mAbs that can induce the downregulation and lysosomal degradation of mouse and human

NKp46 have now been developed, which may be effective in the treatment of diabetes (185, 186).

Primary Sjogren's syndrome (pSS) is a chronic autoimmune disease characterized by lymphocytic exocrinopathy that can affect up to 0.1 to 0.6% of the general population. An excess accumulation of NK cells in minor salivary glands of pSS patients is correlated with the severity of exocrinopathy. B7-H6, the ligand for NKp30, is expressed in minor salivary glands and a higher cell-surface expression of NKp30 on circulating NK cells is observed in pSS patients compared to healthy controls (187). A case control study of genetic polymorphisms revealed that a single nucleotide polymorphism (SNP) rs11575837 ($G > A$) in the promoter region of *NCR3* was associated with reduced transcription and protection from pSS. These findings suggested that NK cells may promote NKp30-dependent inflammation in salivary glands and blockade of the NKp30-B7-H6 interaction may represent a novel clinical target for pSS (187).

NCR-BASED CANCER IMMUNOTHERAPIES

In addition to the development of therapeutic antibodies to the NCRs for potential use in the treatment of autoimmune diseases, such as diabetes (see above), several reports are now beginning to provide evidence that the NCRs and their ligands can be successfully targeted for cancer immunotherapy. Moreover, these systems have shown to be effective in various pre-clinical mouse tumor models highlighting the tremendous potential of NCR-based strategies for tumor immunotherapy.

Virotherapy

Reovirus is a double-stranded RNA virus that infects much of the population during childhood causing mild or subclinical infections. Interestingly, reovirus efficiently infects tumor cells and is currently be tested in human clinical trials as a potential cancer virotherapy although the mechanism whereby reovirus infected tumor cells are recognized and eliminated by the immune system is not well-understood. The reovirus σ1 protein forms an elongate trimer that serves as a viral attachment protein (188). Human and mouse NKp46 were shown to bind to reovirus σ1 protein in a sialic acid-dependent manner that led to NK cell activation *in vitro* (189) (**Figure 2**). NK cells and NKp46 were shown to be essential for the clearance of reovirus infection from the lungs of infected mice and for the success of reovirus-based tumor therapy (189).

Bi-Specific T Cell Engagers (BiTE)

The NKp30/B7-H6 interaction has been targeted using various therapeutic strategies. One approach has been to target T cells to B7-H6 expressing tumors using a BiTE that incorporated a single-chain F_V (ScFV) to B7-H6 fused to an anti-CD3ε ScFV. The B7-H6-specific BiTE promoted T cell cytotoxicity and IFN-γ secretion against B7-H6$^+$ tumor cells, which enhanced the survival of lymphoma-bearing mice and decreased the tumor burden of melanoma- and ovarian cancer-bearing mice (190). Similar conceptual approaches, have involved fusing the B7-H6 ectodomain (with the aim of engaging NKp30 on NK cells) to either an anti-Human Epidermal Growth factor 2 (HER2) scFv to enhance NK cell killing of HER2$^+$ breast cancers (191) or an anti-CD20 scFv to target lymphomas (192, 193). Such reagents were reported to be effective at nM concentrations and to enhance ADCC mediated by therapeutic antibodies, such as trastuzumab, cetuximab, and rituximab (191–193).

NCR-Based Chimeric Antigen Receptors (CARs)

Yet another therapeutic strategy has been to engineer chimeric antigen receptor (CAR) constructs that incorporate either the NKp30 ectodomain (NKp30-CAR) or an anti-B7-H6 scFv (B7H6-specific CAR) with TM domains fused to the CD3ζ or and/or CD28 cytoplasmic signaling domains for cell-surface expression in T or NK cells intended for use in adoptive cancer immunotherapy (194–196). NKp30-CAR and B7-H6-specific CAR T cells elicited robust cellular cytotoxicity and IFN-γ secretion when co-cultured with B7-H6$^+$ tumor cells. Dendritic cells (DCs) also enhanced IFN-γ secretion from NKp30-CAR expressing T cells, whereas B7-H6-specific CAR T cells exhibited little self-reactivity to DCs and monocytes expressing B7-H6 ligands. The adoptive transfer of T cells expressing NKp30-CAR or B7-H6-specific CAR T cells enhanced the survival of mice bearing RMA lymphoma cells transduced with B7-H6. Moreover, mice that remained tumor-free, were resistant to subsequent rechallenge with B7-H6-deficient parental RMA tumor cells (194, 195). NKp44- and NKp46-derived CARs have also been developed and were shown to elicit clinical efficacy for various solid tumors that express ligands for these receptors (197, 198).

DISCUSSION

The NCRs were originally identified as activating receptors expressed on NK cells. However, it is now apparent the NCRs are expressed by ILCs in addition to adaptive Vδ1$^+$ and CD8$^+$ T cells. The NCRs have been shown to synergize with TCR signaling as well as function independently on T cells. Thus, like NK cells, Vδ1$^+$ and CD8$^+$ T cells expressing NCRs hold potential for adoptive cancer immunotherapy (147, 199). It will be interesting to see how NCR$^+$ Vδ1 T cells and CD8$^+$ T cell functions are regulated by the range of NCR ligands now reported in the literature.

The NCRs can interact with soluble as well as membrane bound ligands. Such polyfunctionality is not uncommon in the immune system (25). For example, the activating immunoreceptor OSCAR signals via FcRγ and possesses two Ig-like domains similar to NKp46 and genes for both proteins are encoded in the Leukocyte Receptor Complex (LRC), suggesting a common evolutionary origin (200). OSCAR interacts with at least three different types of ECM collagens to costimulate osteoclastogenesis (201–203) and also binds to the secreted collagen-like lectin, Surfactant Protein D (SP-D) (204). The LRC-encoded immunoreceptors, LAIR-1 and LAIR-2, also bind to multiple ECM collagens as well as SP-D (205–207). Moreover, NKG2D, a member of the C-type lectin family of activating NK cell receptors, is highly promiscuous and binds to a number of

cell-surface and shed MHC class I-like ligands to regulate NK cell functions (199, 208–211). However, in contrast to OSCAR, LAIR-1 and−2, and NKG2D, which all bind structurally similar surface or soluble ligands, respectively, the NCRs have been reported to bind a structurally diverse set of ligands. Thus, many open questions regarding NCR ligand binding still remain, such as how can a given NCR bind to such a broad range of structurally diverse ligands? Conversely, how can the structurally diverse NCRs all bind the same classes of ligands, such as viral HAs and cellular HS GAGs? Structural studies are clearly required to resolve many of these outstanding questions and may reveal fascinating insights into the polyfunctionality of the NCRs as well as intriguing comparisons for other immunoreceptor families.

Such a structurally diverse set of soluble and surface bound NCR ligands might also be expected to induce different strengths and duration of activating or inhibitory NCR signaling that may regulate NK cell activity in different tissues and inflammatory settings. Recent data have raised the importance of secreted ligands in the "alternative activation" of NK cells and the NCRs are no exception (64, 67, 210). For example, PDGF-DD has been reported to bind to NKp44 to induce the secretion of IFN-γ and TNF-α and chemokines, such as CCL1, CCL3, CCL4, XCL1 and XCL2 (67), whereas CFP binding to NKp46 only elicits the expression of XCL1 (64). Firstly, these data challenge the view that stimulation with soluble or shed ligands invariably leads to the desensitization of activating receptor pathways and NK cell inhibition. Secondly, the range of different response to soluble ligands suggests the NCR interaction with each ligand may be tailored to the tissue microenvironment for a specific function or to avoid immunopathology. For example, CFP binding to NKp46, is critical for resistance to *N. meningitidis* infection, which causes septicaemia and meningitis (64). It is conceivable that the reduced NK cell activation induced by the CFP/NKp46 interaction may be desirable in the meninges given the proximity of the inflammatory response to the brain. Further research into the molecular basis and cell biology of NCR signaling for surface-bound and soluble ligands is warranted to fully understand how soluble ligands, such as PDGF-DD and CFP, as well as shed ligands, such as MULT1, (210), evoke appropriate NK cell activation in different organs and tissues and within the tumor microenvironment (212). Such approaches will inform methods to enhance NK cell targeting and to stimulate or inhibit their functions *in vivo* and will lead to the development of novel clinical interventions (212, 213). For example, CARs have already been engineered for soluble growth factors, such as TGF-β, that function in T cells and promote anti-tumor responses *in vivo* (67, 214, 215).

Many of the proposed NCR ligands are normally localized to the nucleus or cytoplasmic proteins, such as BAT3, NKp44L, and PCNA. However, the cellular pathways responsible for the cell-surface localization of these ligands and their selective upregulation in malignant vs. non-malignant cells still remain to be characterized in molecular detail. These questions are critical for understanding how the NCR ligands regulate the activity of NK cells and other NCR^+ immune cell subsets in different tissues and pathological processes. Interestingly, the NCRs and their ligands are associated with a diverse range of diseases and biological traits (**Table 2**). It might be expected that many of these disease associations are independent of the NCRs and result from changes to the primary biological function of the NCR ligand in question. However, it is also feasible that the NCRs and their ligands could play a pathogenetic role for some of these aforementioned diseases. Further research into the role of the NCRs and their ligands that are genetically associated with disease should be encouraged and will provide novel insights into the role of tissue NK cells for diseases that may have an as yet unrecognized immunopathological component.

AUTHOR CONTRIBUTIONS

AB, CM, and MC authored and edited the manuscript. CM contributed figures and tables.

ACKNOWLEDGMENTS

CM was the recipient of a Susan Keyes-Pearce Microbiology and Immunology laboratory experience award.

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

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frontiers in Immunology

MINI REVIEW
published: 09 April 2019
doi: 10.3389/fimmu.2019.00719

NKp44-NKp44 Ligand Interactions in the Regulation of Natural Killer Cells and Other Innate Lymphoid Cells in Humans

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Specialty section:
This article was submitted to NK and Innate Lymphoid Cell Biology, a section of the journal Frontiers in Immunology

Received: 07 February 2019
Accepted: 18 March 2019
Published: 09 April 2019

Citation:
Parodi M, Favoreel H, Candiano G, Gaggero S, Sivori S, Mingari MC, Moretta L, Vitale M and Cantoni C (2019) NKp44-NKp44 Ligand Interactions in the Regulation of Natural Killer Cells and Other Innate Lymphoid Cells in Humans. Front. Immunol. 10:719. doi: 10.3389/fimmu.2019.00719

Natural Killer (NK) cells are potent cytotoxic cells belonging to the family of Innate Lymphoid Cells (ILCs). Their most characterized effector functions are directed to the control of aberrant cells in the body, including both transformed and virus-infected cells. NK cell-mediated recognition of abnormal cells primarily occurs through receptor-ligand interactions, involving an array of inhibitory and activating NK receptors and different types of ligands expressed on target cells. While most of the receptors have become known over many years, their respective ligands were only defined later and their impressive complexity has only recently become evident. NKp44, a member of Natural Cytotoxicity Receptors (NCRs), is an activating receptor playing a crucial role in most functions exerted by activated NK cells and also by other NKp44$^+$ immune cells. The large and heterogeneous panel of NKp44 ligands (NKp44L) now includes surface expressed glycoproteins and proteoglycans, nuclear proteins that can be exposed outside the cell, and molecules that can be either released in the extracellular space or carried in extracellular vesicles. Recent findings have extended our knowledge on the nature of NKp44L to soluble plasma glycoproteins, such as secreted growth factors or extracellular matrix (ECM)-derived glycoproteins. NKp44L are induced upon tumor transformation or viral infection but may also be expressed in normal cells and tissues. In addition, NKp44-NKp44L interactions are involved in the crosstalk between NK cells and different innate and adaptive immune cell types. NKp44 expression in different ILCs located in tissues further extends the potential role of NKp44-NKp44L interactions.

Keywords: natural kiiler cells, innate lymphoid cells (ILC), natural cytotoxicity receptors (NCR), NKp44, ligands, tumor immunology, viral infections

INTRODUCTION

NK cells are cytotoxic Innate Lymphoid Cells (ILCs) that patrol the body and play a crucial role in the defense against viruses and tumors by circulating in peripheral blood (PB) and extravasating into tissues, in particular at the sites of injury (1–8). In addition to recirculating NK cells, specific NK cell subsets reside in different tissues and organs fulfilling unique regulatory functions. Depending on the organ and the local microenvironment, tissue-resident NK (trNK) cells release proangiogenic factors, regulatory cytokines or specific chemokines, and interact with different cell types. Recirculating NK cells, besides killing target cells, can promote inflammation by cytokine and chemokine release and interaction with dendritic cells (DC), monocytes/macrophages, granulocytes, and T cells (9–14).

NKp44, together with the other Natural Cytotoxicity Receptors (NCRs), NKp46 and NKp30 (14–16) can play an important role in most functions exerted by NK cells and also by other immune cell types (17). Indeed, NKp44 expression is wider than initially thought, and includes activated PB-NK cells (15), interferon-producing intraepithelial ILC1 in tonsils (18, 19), a subset of ILC3 in the decidua (20), and in mucosal-associated lymphoid tissue (MALT) (21), a small subset of decidual trNK cells (22), and plasmacytoid dendritic cells (pDC) (23, 24). Typically, NKp44 is implicated in the killing of virus-infected or tumor cells; however, the increasing panel of NKp44$^+$ cells and the identification of new NKp44-ligands (NKp44L), possibly expressed in different tissues or released in the circulation, supports an important role for this receptor in tissue homeostasis and immune regulation (25–29).

NKp44 is a transmembrane glycoprotein characterized by a single extracellular V-type Ig-like domain and a cytoplasmic tail containing an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) and no known activating signaling motifs (16). The crystal structure of the NKp44 Ig-V domain reveals the presence of a prominent positively charged groove, likely involved in recognition of anionic patterns shared by different ligands (30). The transmembrane domain contains a charged amino acid (Lys) that allows the association with the Immunoreceptor Tyrosine-based Activating Motif (ITAM)-containing KARAP/DAP12 adaptor protein, a strong transducer of activating signals (16, 31–33). Even if the NKp44 ITIM sequence was originally shown to be non-functional (34), in certain contexts NKp44 can also transduce inhibitory signals. Indeed, NKp44 expressed on pDC inhibits IFN-α release induced upon TLR stimulation (23). More recently, three NKp44 mRNA splice variants (NKp44-1,−2,−3) have been demonstrated to display different signaling capabilities based on the presence (NKp44-1) or absence (NKp44-2 and−3) of the ITIM in their cytoplasmic tail. Local physiologic or pathologic cytokine milieus could influence NKp44 splicing, determining the relative expression levels of these variants and, accordingly, affecting the functional features of NK cells in different locations (see below) (35–37).

The local microenvironment can also determine the function and role of NKp44 by orchestrating development, recruitment, and modulation of specific regulatory, tissue-remodeling, or cytotoxic NKp44$^+$ cell subsets (either NK cells or ILCs). Thus, for example, lungs contain a large majority of CD56^{dim} NK cells recirculating from blood; spleen and liver also include CD56^{bright} trNK cells; lymphoid tissues associated to epithelia include NKp44$^+$ ILCs; uterus comprises NKp44$^+$ NK cells and NKp44$^+$ ILC3 with peculiar regulatory and tissue-remodeling functions (2, 4, 5, 18, 19, 38, 39).

Pathologic conditions, and in particular tumors, can result in altered patterns of NKp44$^+$ cells. Tumor tissues can produce specific cytokines and chemokines that can drive recruitment of cytokine-producing or cytotoxic NK cell subsets (i.e., CD56^{bright} or CD56^{dim} cells) (40, 41) and induce NKp44 expression. In addition, NKp44$^+$ ILC3 have also been described in lung tumors and have been found to be associated with a better clinical outcome (39). On the other hand, in the tumor microenvironment, hypoxic conditions (42), or soluble mediators such as PGE_2 (43–45) and TGF-β (46) can induce NK cells to down-regulate expression and/or function of major activating NK receptors including NKp44. Similar effects can also be induced by tumor cells and tumor-associated fibroblasts (45, 47).

Thus, depending on the type of available NKp44L, NKp44-expressing cells, tissues, or environmental conditions, the role of NKp44 greatly varies and extends to novel functional contexts, beyond the classical NK-mediated target cell killing.

NKP44-NKP44L INTERACTIONS INVOLVED IN RECOGNITION OF TUMOR AND VIRUS-INFECTED CELLS

NKp44-Mediated Recognition of Tumor Cells

Although NKp44L have been elusive for a long time since the discovery of NKp44, experimental evidence suggested that NKp44-NKp44L interactions occurring in the context of tumor cell recognition could play an important role in potentiating NK-mediated cytotoxicity against tumor cells (15, 16, 48, 49). **Figure 1A** summarizes information on the presently known NKp44-L and their role in the NK-tumor cell interaction.

The first evidence of an activating cellular NKp44L was provided in 2005, when Vielliard et al. reported that an NKp44L is induced on CD4$^+$ T lymphocytes from HIV-1-infected individuals (see below) (50). Later on, such NKp44L was identified as a peculiar isoform of mixed-lineage leukemia protein-5 (MLL5), termed 21spe-MLL5, encoded by a splice variant lacking the last five exons of MLL5 and containing a unique C-terminal exon, required for both localization at the cell surface and interaction with NKp44 (51). MLL5 (or lysine methyltransferase 2E), is primarily a nuclear protein with a wide expression pattern in healthy tissues and plays a role in regulating cell cycle progression and hematopoietic differentiation; on the other hand, 21spe-MLL5 is mainly found in the cytoplasm and at the cell surface, is barely expressed in healthy tissues but is frequently detected at high levels in hematopoietic and non-hematopoietic tumor cells. The mechanism allowing the expression of this ligand on the surface of cancer cells is still to

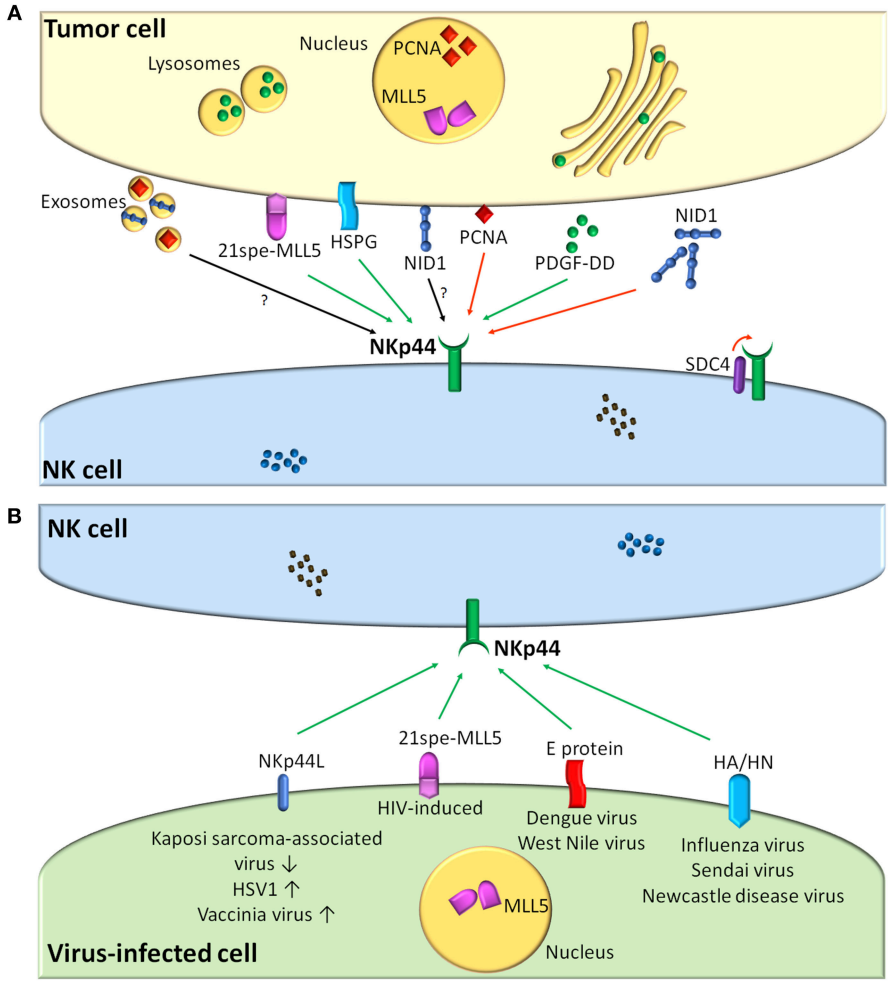

FIGURE 1 | NKp44-NKp44L interactions and their role in the recognition of tumor **(A)** or virus-infected cells **(B)** by human NK cells. **(A)** Shows cellular and cell-released NKp44 ligands expressed by tumor cells. **(B)** Shows viral and viral-regulated NKp44 ligands. Viruses up-regulating/down-regulating NKp44L expression on infected cells are indicated with up and down arrows. Green arrows indicate interactions resulting in NK cell activation, while red arrows indicate inhibition. Black arrows refer to still undefined interactions. MLL5, mixed-lineage leukemia protein-5; PCNA, Proliferating Cell Nuclear Antigen; HSPG, heparan sulfate proteoglycans; NID1, Nidogen-1; PDGF, Platelet-Derived Growth Factor; SDC4, syndecan-4.

be defined, but 21spe-MLL5 surface expression on tumor cells triggers NKp44-mediated cytotoxicity (51).

Another cellular NKp44L is represented by Proliferating Cell Nuclear Antigen (PCNA) (52), a ubiquitously expressed nuclear protein involved in the regulation of DNA replication, DNA repair, and cell cycle progression. Interestingly, it may be overexpressed in cancer cells where it contributes to tumor survival and enhanced malignancy (53). PCNA can be recruited to the plasma membrane of tumor cells, probably through the formation of a PCNA/HLA-I complex (54) or shuttling via exosomes (52). NKp44 engagement by PCNA results in the inhibition of NK-mediated cytotoxicity and IFN-γ secretion by NK cells; this inhibitory effect is mediated by the ITIM in NKp44 cytoplasmic tail and is observed only following an interaction with the ITIM-containing isoform NKp44-1, whose expression has been associated with poor survival in AML patients (36). In view of these findings, PCNA has been proposed as an immune evasion mechanism, enhancing tumor cell survival by promoting a paradoxical NKp44-mediated inhibition of tumor cell killing by NK cells. Recently, the NKp44 binding site for PCNA was identified and an NKp44-derived peptide has been shown to target intracellular PCNA, resulting in the apoptosis of cancer cell lines *in vitro* and tumor growth arrest *in vivo* (55).

Cell surface-associated heparan sulfate (HS) proteoglycans (HSPGs) represent a peculiar category of NCR ligands (56). The three NCRs display a distinct pattern of HS/heparin recognition, based on the heterogeneity and structural complexity of these macromolecules (57). NKp44 recognizes highly sulfated HS/heparin-type structures by binding to negatively charged stretches of HS. Mutations of basic residues in the positively charged NKp44 groove resulted in a decreased binding to HS/heparin. In the context of tumor cell recognition, NKp44 may

bind to HS expressed on different cancer cell lines. Moreover, HS was able to enhance NKp44-induced IFN-γ secretion, while the role of HS in the induction of NK-mediated cytotoxicity is less clear (58). Although membrane-associated HSPGs are present on all cells, their expression is heterogeneous in different tissues and can be altered in tumor cells (59). Modified levels of HS in cancer cells may result in altered recognition, in their association with other ligands, or in their structural alterations by tumor-induced modifying enzymes. In this context, HS moieties of HSPGs may be considered as self "modified" ligands for NCRs and may serve as co-ligands, cooperating with other ligands to influence NK cell functions.

NKp44 has also been shown to interact in *cis* with syndecan-4 (SDC4), one of the HSPGs expressed on the surface of NK cells, thereby constitutively dampening NKp44-mediated activation by preventing the receptor binding to other ligands expressed on target cells (60).

More recently, the search for glycolipid ligands by microarray screening led to the identification of Globo-A (GalNAcα1,3(Fucα1,2) Galβ1,3GalNAcβ1,3Galα1,4Galβ1,4Galβ1-Cer) as NKp44L (61). This glycolipid, which was originally isolated from human kidney, displays a globo-series structure and includes a terminal part similar to that of blood group A antigen (62). At present, its functional relevance in the regulation of NK cell function has not been demonstrated yet.

NKp44-Mediated Recognition of Virus-Infected Cells

Concerning the role of NKp44 in the context of virus recognition, roughly three types of viral interactions have been described: viral NKp44L, virus-induced up-regulation of cellular NKp44L, and virus-mediated inhibition of NKp44 recognition (**Figure 1B**).

In 2001, Mandelboim et al. reported that the hemagglutinin (HA) of the orthomyxovirus H1N1 influenza virus and the hemagglutinin-neuraminidase (HN) of the paramyxovirus Sendai virus, both expressed on the surface of infected cells, are recognized by NKp46, and thereby trigger the lysis of infected cells by NK cells (63). Shortly thereafter, these viral proteins were also found to serve as NKp44L, but not NKp30L, and the interaction with NKp44 could contribute to the killing activity of certain NK cell clones (64). NKp44 not only recognizes the influenza virus HA of H1 strains but also of H5 strains (65). In addition, HN of other paramyxoviruses, avian Newcastle disease virus and human parainfluenza virus 3 (HPIV3), also appear to serve as NKp44L and trigger NK cell activity (66, 67).

The recognition of both HA and HN depends on sialylation of NKp44, similar to that reported for NKp46 (63–65). Remarkably, the E envelope glycoproteins of two flaviviruses, West Nile and Dengue viruses, also bind to NKp44 thereby increasing NK cell activity, but in a sialylation-independent manner (68).

As mentioned above, in 2005, Vieillard et al. reported that a fraction of $CD4^+$ T cells from HIV-infected patients, but not from healthy subjects, showed an increased expression of NKp44L (50). The percentage of $NKp44L^+$ $CD4^+$ T cells was inversely correlated with the $CD4^+$ T cell count and correlated with the viral load, suggesting that NKp44L expression may be implicated in T cell depletion and disease course. In addition, the HIV gp41 envelope protein (and its precursor gp160) was found to trigger NKp44L expression and NK-mediated lysis. In particular, a highly conserved peptide in gp41 (NH2-SWSNKS-COOH or 3S) triggers NKp44L expression (50). Another study suggested that a role for virus-induced NKp44L expression in $CD4^+$ T cell depletion during HIV infection may be virus strain-dependent (69).

Members of two large DNA virus families, the poxviruses and the herpesviruses, also up-regulate the expression of NKp44L early after infection of host cells. The immediate early ICP0 protein of herpes simplex virus 1 and an unidentified early protein of the poxvirus vaccinia virus up-regulate expression of NKp30L, NKp44L, and NKp46L, thereby triggering NCR-mediated lysis of infected human fibroblast cells (70, 71).

Viral interference with the NKp44-mediated killing of infected cells typically consists in the down-regulation of cellular or viral NKp44L from the cell surface. Remarkably, the same HIV gp41 S3 peptide that triggers NKp44L expression in $CD4^+$ T cells, suppresses NKp44L expression in astrocytes. This led the authors to hypothesize that HIV may use this strategy to protect astrocytes from NK cell-mediated killing during HIV infection of the CNS (72). How the same viral peptide exerts such opposing effects on NKp44L expression and NK cell-mediated cytotoxicity in two different cell types is currently unclear.

The viral ORF54-encoded protein of Kaposi's sarcoma-associated herpesvirus, but not that of other human herpesviruses, suppresses the expression of NKp44L during lytic infection. Although this viral protein is a dUTPase, its enzymatic activity is neither necessary nor sufficient to down-regulate NKp44L (73).

Finally, although the influenza virus HA binds NKp44 and NKp46, thereby contributing to NK-mediated lysis of infected cells, the virus can suppress this effect via expression of its NA protein (74, 75). NA displays sialidase activity which may remove the sialic acids from NKp44 and NKp46, thereby interfering with their binding to HA (74, 75). Although the HA-NA-NKp44 interplay may appear paradoxical at first sight, perhaps this should be looked upon from an evolutionary point of view. The highly conserved function of influenza HA is to interact with sialic acids on the cell surface as an essential initial step to enter host cells. It has been hypothesized that NK cells utilize this general HA sialic acid-binding property to recognize the infected cell via NKp46 and NKp44 (75). The NA protein may then serve as a viral countermeasure against these and other unwanted side-effects of the essential sialic acid-binding activity of HA.

REGULATORY AND HOMEOSTATIC FUNCTIONS OF NKP44-NKP44L INTERACTIONS

NKp44 is also expressed on helper ILCs, which typically reside at the epithelial/mucosal surfaces, becoming involved in tissue remodeling, inflammation, and maintenance of barrier integrity (76). Notably, NKp44 expression allows to discriminate two

ILC3 subsets characterized by unique cytokine patterns: NKp44$^+$ ILC3 are able to produce IL-22 (21) and are dependent on the Aryl Hydrocarbon Receptor, whereas human LTi and NKp44^{neg} ILC3 produce IL-17A (77). Remarkably, in NKp44$^+$ ILC3, IL-22 expression is preferentially induced by cytokine stimulation, whereas NKp44 triggering selectively activates a coordinated pro-inflammatory program via TNF-α (78). In this context, NKp44 has been suggested to play a potential role in the pathogenesis of immune-mediated diseases, including psoriasis. Indeed, more than 50% of circulating NKp44$^+$ ILC3 in the blood of psoriasis patients express cutaneous lymphocyte-associated antigen, suggesting their role in skin homing. Moreover, NKp44$^+$ ILC3 frequency in non-lesional psoriatic skin is significantly increased when compared to that in normal skin (79).

NKp44$^+$ ILC3 are also present in secondary lymphoid organs (SLO). Differently from ILC3 in inflamed tonsils, ILC3 in non-inflamed lymph nodes and spleen, irrespective of NKp44 expression, lack the transcription of cytokines typically mediating ILC3 function. However, both NKp44^{neg} and NKp44$^+$ resting ILC3 can produce IL-22 in response to inflammatory stimuli. According to these data, SLO-residing ILC3 may represent a pool of resting cells that can be rapidly activated by inflammatory signals present in the local microenvironment (80).

NKp44$^+$ ILC3 can also infiltrate tumor tissues. In particular, in non-small cell lung cancer, NKp44 can interact with tumor cells and synergize with IL-1β and IL-23 for IL-22 production (39). In addition, NKp44-mediated recognition of tumor associated fibroblasts can lead to the release of high amounts of TNF-α, thus influencing the vascular permeability and inducing pro-inflammatory responses in the tumor microenvironment (39).

In the uterus, the NKp44$^+$ pool is composed of ILC3, LTi-like cells, IFN-γ-producing ILC1-like cells, and NK cells (20, 22). These cell populations are variably involved in tissue remodeling related to decidua development and vascularization during pregnancy, the induction of maternal-fetal tolerance, and the control of viral infections (i.e., CMV) (81). For some of these functions the role of NKp44 has been suggested by its ability to induce IP10, IL-8, and VEGF release (82). It has also been recently shown that the decidual cytokine milieu can favor the expression of the ITIM-bearing NKp44-1 inhibitory isoform (37), which indeed can induce cytokine release but inhibits cytotoxicity. In this context, the overexpression of the NKp44-L PCNA in decidual throphoblasts in the first trimester of gestation suggests that inhibitory signaling of NKp44 might contribute to the maintenance of immune tolerance of maternal NK cells during pregnancy (83).

As mentioned in the Introduction, the functional role of NKp44 also depends on the nature and body distribution of its ligands. Very recently, the discovery of Platelet-Derived Growth Factor (PDGF)-DD as a novel NKp44L represented a major breakthrough, since for the first time NKp44 was demonstrated to recognize a soluble molecule (28, 84). PDGF-DD belongs to the PDGF family and represents the active processed form of PDGF-D (85, 86). Noteworthy, PDGF-DD-NKp44 interaction does not trigger NK cell-mediated cytotoxicity, rather, it induces potent release of TNF-α and IFN-γ by IL-2-activated NK cells. Transcriptomic analysis clearly indicated that stimulation of activated NK cells by PDGF-DD induced genes encoding pro-inflammatory cytokines and chemokines, cell surface activation markers, and transcription factors involved in ITAM signaling, cellular activation, and proliferation. Notably, the induction of an NKp44-dependent pro-inflammatory program upon PDGF-DD-NKp44 interaction has been demonstrated not only in activated NK cells but also in other NKp44$^+$ ILCs, namely ILC3 and intraepithelial ILC1. In particular, PDGF-DD induced IFN-γ and TNF-α production in ILC1, while it stimulated TNF-α but not IL-22 secretion in ILC3 (28).

PDGF-DD is involved in embryonic development, placenta formation, angiogenesis, and wound healing, and has been implicated in various pathological conditions, including vascular diseases, mesangioproliferative glomerulonephritis, and fibrosis (85, 87). Thus, PDGF-DD interaction with NKp44$^+$ cells present in various tissues (including decidua) may play diverse biological roles. Nevertheless, PDGF-DD can be secreted by different tumors and promotes cellular proliferation, stromal cell recruitment, angiogenesis, epithelial-mesenchymal transition, and metastasis through autocrine and paracrine PDGFRβ signaling (88, 89). In this context, PDGF-DD, as NKp44L, can also induce NK-, ILC1- and ILC3-mediated release of cytokines with anti-tumor activity. Indeed, supernatants of PDGF-DD-activated NK cells inhibit tumor cell growth *in vitro*, while *in vivo* experiments indicated that the introduction of the NCR2 transgene limited the spread of PDGF-DD-expressing tumor cells in mice (28).

The example of PDGF-DD as an extracellular ligand is not unique among NCRs, since NKp46 can also bind to a soluble molecule, namely the plasma glycoprotein Complement Factor P (CFP or properdin) (90). Both CFP and PDGF-DD activate NK cell functions. However, soluble ligands of activating receptors generally represent mechanisms to modulate NK cell functions (91–97). In this context, another peculiar NKp44L was recently described (29). Nidogen-1 (NID1) glycoprotein is an essential component of ECM and basement membrane (BM) (98, 99) able to interact with NKp44. When released as soluble molecule, NID1 modulates NK cell function by reducing NKp44-induced IFN-γ release or cytotoxicity. Notably, it also regulates IFN-γ production induced by PDGF-DD following NKp44 engagement. Thus, the release of NID1 in extracellular fluids may act as regulatory mechanism for NKp44$^+$ NK cells in the blood stream or in tissues. Interestingly, NID1 release has been observed in different cancer types (100–102), and may represent a suppressive mechanism exploited by tumors to avoid NK-mediated attack. Actually, the functional outcome of the NKp44-NID1 interaction may be rather complex, as NID1 can associate with other ECM components, including laminin, collagen type IV, and perlecan (98, 99), or be modified by extracellular proteases secreted in the tumor microenvironment (103, 104). In addition, NID1 can also be exposed at the cell surface of different NID1-releasing tumor cell lines (29).

As shown by the examples of PDGF-DD and NID1, NKp44L can be expressed not only on neoplastic or virus-infected cells, but also on normal cells. Thus, NKp44L expression was observed on the surface of articular chondrocytes (105), although the

TABLE 1 | Expression of NKp44 ligands in human tissues.

Ligand (inhib/act NKp44)*	Tissues
PCNA[a,b] (inhibition through ITIM)	***Nervous system***: cerebral and prefrontal cortex, spinal cord, cerebellum
	Endocrine tissues: adrenal, thyroid, parathyroid, and salivary glands, pancreas
	BM[e] ***and immune system***: B, T, and NK cells, monocytes, BM stromal cells, LN[e], tonsil, spleen, appendix
	Muscle tissues: heart, smooth and skeletal muscle
	Respiratory system: lung, nasopharynx, bronchus
	Skeletal system: bone
	Digestive system: colon and ileum epithelial cells, colon, esophagus, gallbladder, gut, cardia, liver, oral epithelium, stomach, rectum, duodenum, small intestine
	Urinary system: kidney, bladder
	Male tissues: testis, seminal vesicle, prostate gland, epididymis
	Female tissues: breast, amniocytes, fallopian tube, myometrium, ovary, placenta, uterus, uterine cervix, vagina, endometrium
	Adipose and soft tissues: adipocytes
	Integumentary system: hair follicle, skin
21spe-MLL5[c] (activation)	***BM and immune system***: $CD4^+$ T cells from HIV-infected patients
	Skeletal system: articular chondrocytes
Syndecan-4[a,b] (inhibition through receptor masking)	***Nervous system***: cerebral and prefrontal cortex, spinal cord, cerebellum, hippocampus, caudate
	Endocrine tissues: adrenal, thyroid, parathyroid, and salivary glands, pancreas
	BM and immune system: platelets, LN, tonsil, spleen, appendix
	Muscle tissues: heart, smooth and skeletal muscle
	Respiratory system: lung, nasopharynx, bronchus
	Skeletal system: bone, chondrocytes
	Digestive system: colon, gallbladder, liver, oral epithelium, rectum, small intestine, duodenum, stomach, esophagus
	Urinary system: kidney, bladder
	Male tissues: testis, seminal vesicle, prostate gland, epididymis
	Female tissues: breast, amniocytes, fallopian tube, myometrium, ovary, placenta, uterine cervix, vagina, endometrium
	Adipose and soft tissues: adipocytes, fibroblasts
	Integumentary system: skin
PDGF-DD[b,d] (activation)	***Nervous system***: cerebral cortex
	Endocrine tissues: adrenal and thyroid gland
	BM and immune system: LN, tonsil, spleen, appendix
	Muscle tissues: heart, smooth muscle
	Respiratory system: lung
	Digestive system: colon, oral epithelium
	Male tissues: seminal vesicle
	Female tissues: breast, ovary
	Integumentary system: skin
NID1[a,b] (inhibition through receptor masking)	***Nervous system***: arachnoid cyst, cerebral cortex, prefrontal cortex, spinal cord
	Endocrine tissues: adrenal, thyroid, and salivary glands, pancreas
	BM and immune system: platelets, mesenchymal stem cells, NK cells, LN, tonsil, spleen
	Muscle tissues: colon muscle, heart
	Respiratory system: lung, nasopharynx
	Digestive system: colon, esophagus, gallbladder, gut, cardia, liver, oral epithelium, stomach, rectum;
	Urinary system: kidney, bladder
	Male tissues: testis, seminal vesicle, prostate gland, epididymis
	Female tissues: breast, amniocyte, myometrium, ovary, placenta, uterus, uterine cervix
	Adipose and soft tissues: adipocytes
	Integumentary system: skin

**NKp44-NKp44L interactions can result in a different outcome depending on the ligand: activation or inhibition of NK cell functions.*
[a]*Data obtained from ProteomicsDB.*
[b]*data obtained from The Human Protein Atlas.*
[c]*data obtained from refs (50, 51, 105).*
[d]*data obtained from ref (107).*
[e]*BM, bone marrow; LN, lymphnodes.*

nature of this ligand is not well-defined. Since NK cells can kill human chondrocytes, it has been suggested that the NKp44-NKp44L interaction may play a role in cartilage destruction occurring in chronic inflammatory joint disorders. Interestingly, human NK cells were previously shown to kill porcine chondrocytes mainly through NKp44-NKp44L interaction (106).

More recently, it has been demonstrated that human astrocytes express an NKp44L whose interaction with NKp44 contributes to NK-mediated cytotoxicity against astrocytes and to IFN-γ production (72).

CONCLUDING REMARKS

The finding that NKp44 is expressed by different immune cell types and tissues (**Table 1**) and can recognize multiple ligands (soluble or associated to the cell surface or ECM) strongly suggests that this receptor is used to fulfill various functions, adapted to different body compartments or even to temporary micro-environmental changes.

The expanding knowledge on the multifaceted role of NK cell subsets and ILCs, residing in the different tissues, suggests that NKp44-NKp44L interactions may be involved in the activation/regulation of several biological/immunological processes. A deeper understanding of these issues will be beneficial for the design of NK-based immunotherapeutic approaches in different pathologic conditions, including but not limited to tumors and infections.

AUTHOR CONTRIBUTIONS

MP, HF, SS, MV, and CC wrote the manuscript. GC, SG, MCM, and LM reviewed the manuscript and provided critical input.

FUNDING

This work was supported by the following grants: AIRC 5X1000, 2018 Project Code 21147 (LM and SS); AIRC IG 2017, Project Code 19920 (LM); V806A 5X1000 Ministero della Salute 2015 (MCM); AIRC IG 2017 Id. 20312 (SS); AIRC IG 2014 project no. 15428 (MV); Funds from Renal Child Foundation (GC); MP was a recipient of the AIRC fellowship no. 18274, year 2016.

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