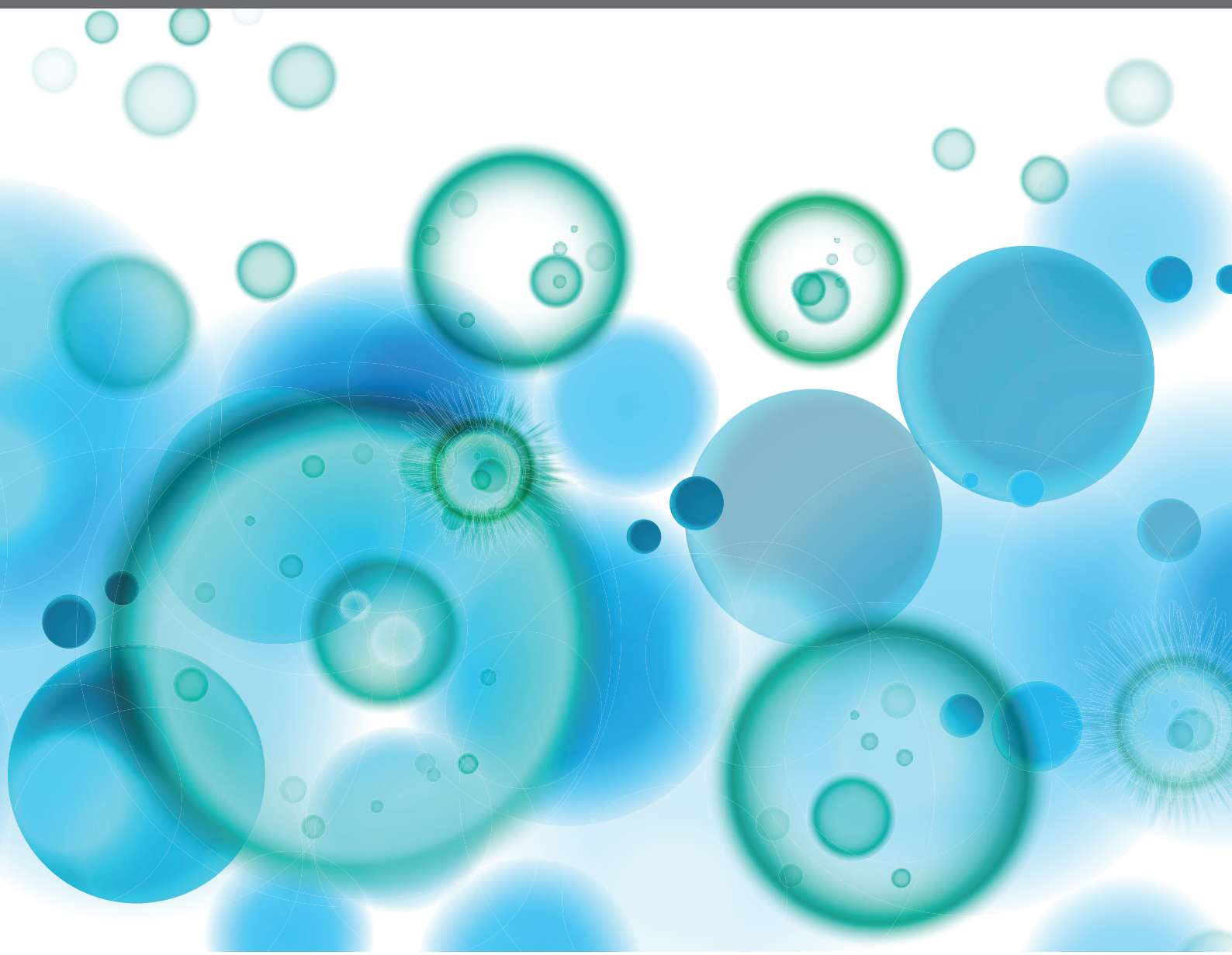
MOLECULAR AND CELLULAR PATHWAYS IN NK CELL DEVELOPMENT

EDITED BY: Ewa Sitnicka, Yenan Bryceson, Aharon Freud and Emily Mace
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-977-9
DOI 10.3389/978-2-88963-977-9

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MOLECULAR AND CELLULAR PATHWAYS IN NK CELL DEVELOPMENT

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Citation: Sitnicka, E., Bryceson, Y., Freud, A., Mace, E., eds. (2020). Molecular and Cellular Pathways in NK Cell Development. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-977-9

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frontiers in Immunology

EDITORIAL
published: 14 July 2020
doi: 10.3389/fimmu.2020.01448

Editorial: Molecular and Cellular Pathways in NK Cell Development

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Keywords: NK cells, developmental and maturation stages, regulatory pathways, disease-induced defects, clinical applications

Editorial on the Research Topic

Molecular and Cellular Pathways in NK Cell Development

We are delighted to present this Research Topic for *Frontiers in Immunology*, focusing on "Molecular and Cellular Pathways in NK Cell Development."

This collection comprises five primary research articles, seven reviews of the current literature, and one opinion piece by experts in the field. Natural killer (NK) cells have immense therapeutic potential. Understanding how to acquire large numbers of functional cells and how to guide their activity is a focus of basic research with potential clinical application.

Papers included in this collection highlight recent advances in our understanding of NK cell origins, their cellular developmental stages and regulatory networks during normal hematopoiesis. These manuscripts also address molecular mechanisms responsible for NK cell defects found in patients with hematological malignancies and the degree to which NK cell impairments contribute to disease progression.

Despite having been discovered more than 40 years ago and used in the clinic for immunotherapy, several aspects of NK cell biology remain unexplored and are still being debated. In contrast to the mouse hematopoietic hierarchy, the development of human blood lineages is less characterized. Although the production and maintenance of NK cells are sustained by the pool of hematopoietic stem cells, the sites of NK cell development and the sequential intermediate differentiation stages are poorly defined. Cichocki et al. discuss two potential hierarchical models of human NK cell development: (1) a linear model where the lineage commitment occurs stepwise from hematopoietic stem cells, through the lymphoid–primed multilineage progenitors, the common-lymphoid progenitors, to NK cell-restricted progenitors and $CD56^{dim}$ NK cells; and (2) a branched model where different NK cell populations, $CD56^{dim}$, $CD56^{bright}$, and adaptive NK cells, are generated from both early lymphoid and myeloid progenitors.

NK cells represent the founding member of a family of innate lymphoid cells (ILCs) and are placed within group 1. The ILC family consists of four subsets: NK cells/ILC1, ILC2, ILC3, and lymphoid tissue inducer cells. Stokic-Trtica et al. review the function, properties, diversity, and developmental relationship between NK cells and the other members of the ILC family. The authors summarize similarities and differences between NK cells and other ILCs, and discuss different potential therapeutic strategies to activate and harness anti-tumor immunity mediated by NK cells.

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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: *27 May 2020*
Accepted: *04 June 2020*
Published: *14 July 2020*

Citation:
Sitnicka E, Bryceson Y, Freud AG and Mace EM (2020) Editorial: Molecular and Cellular Pathways in NK Cell Development. Front. Immunol. 11:1448. doi: 10.3389/fimmu.2020.01448

Among several transcription factors critical for NK cell development and maturation, Eomes represents a candidate that drives NK cell lineage-specification. O'Sullivan discusses heterogeneity within ILC1 cells in mice, where in addition to Eomes-dependent NK cells, there is a unique population of Eomes-independent ILC1s. This Eomes-independent ILC1 population represents a distinct lineage of group 1 ILCs rather than a developmental or functional stage of NK cells.

NK cells are heterogeneous in terms of their tissue location, phenotype, and function. In addition to the most abundant and the best studied conventional NK cells found in the blood and spleen, there are distinct subsets of tissue resident NK cells and helper ILC1s that have been identified in multiple organs and tissues including the liver, uterus, thymus, skin, and adipose tissue among others. Whereas, the development and regulation of bone marrow dependent conventional NK cells is well-characterized, the origin and regulation of recently described unique tissue-specific and tissue resident NK cells is less understood. Valero-Pacheco and Beaulieu provide a comprehensive overview of transcriptional regulatory pathways controlling and driving the development of tissue resident NK cells and helper ILC1s in mice.

To better characterize diverse populations of human NK cells, Filipovic et al. developed a 29-paremeter analysis panel to investigate NK cell subsets across three different tissues: liver, peripheral blood, and tonsil. This novel approach allows high dimensional profiling of NK cells in different tissues and can be applied as a potential diagnostic tool.

Adaptive NK cells represent a distinct long-lived population of NK cells that emerges after cytomegalovirus (CMV) infection providing the evidence for virus-specific NK cell immunological memory. Since NK cells are critical anti-viral effectors, these memory NK cells represent important potential therapeutic targets. In their studies, Gyurova et al. investigated changes in phenotype and function of NK cells from healthy individuals after treatment with CMV vaccine. Lack of changes in $NKG2C^{+}$ NK cells was consistent with the absence of CMV infection, whereas the other NK cell subsets showed dynamic changes over time.

The origin and regulation of adaptive NK cells is not well-understood. Truitt et al. investigated CMV driven expansion of adaptive NK cells in rhesus macaques using lentivirally-barcoded autologous hematopoietic stem and progenitor transplantation that enabled tracking of $CD56^{-}CD16^{+}$ and $CD56^{+}CD16^{-}$ NK cell generation at the clonal level. The authors used this model to test the impact of infection on NK cell clonal dynamics and demonstrate long lasting clonal expansion in response to RhCMV, providing evidence for a clonal adaptive response and immunological memory within the NK cell compartment.

NK cell development and maturation have been driven and controlled by a network of cytokines (including: IL-2, IL-7, IL-12, IL-15, IL-21, IL-27, and interferons) that signal via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Gotthardt et al. review the current understanding of cytokine requirements and the downstream signaling involved in development and maturation of NK cells and ILC1s. The Authors also discuss the role of negative regulators of JAK/STAT signaling—the family of proteins called suppressor of cytokine signaling (SOCS) and their potential application as immunotherapeutic strategy. Scarno et al. previously applied next generation sequencing technology (NGS) to explore how JAK/STAT pathway regulate NK cells at different states of differentiation and function. The authors review how different STAT pathways are required in resting, effector and adaptive NK cells to control their expansion, differentiation, and function. Studies by Vian et al. further support the differential impact of cytokine signaling in NK cells and ICL1s, by demonstrating a high level of *Bcl2* expression in ILC1s after JAK inhibition compared to NK cells.

IL-15 role plays a central and unique role in NK cell biology. Pfefferle et al. review new insights into regulation of NK cell maturation and homeostasis, and discuss metabolic requirements, intra lineage NK cell plasticity, and transcriptional reprogramming of NK cells during differentiation and homeostatic proliferation in response to IL-15.

NK cells undergo phenotypic and functional changes in the presence of cytokines, and IL-2 has a crucial role in NK cell activation. Ranganath et al. have demonstrated that blocking IL-2 signaling by daclizumab beta used as a treatment for multiple sclerosis leads to the expansion of $CD56^{bright}$ NK cells with enhanced ability to kill autoreactive T cells.

Ample data support a role of NK cells in tumor immune-surveillance and elimination of malignant transformed cells. There is clinical evidence supporting potent NK cell anti-tumor activity in the settings of chronic myeloid leukemia, acute myeloid leukemia, and myelodysplastic syndromes. However, disease-associated mechanisms induce NK cell defects and impairment in their cytotoxic function. Carlsten and Järås provide an overview of the mechanisms involved in disease-induced NK cell dysfunctions and discuss potential therapeutic approaches to restore NK cell function in patients with myeloid malignancies. They also discuss novel strategies to unleash NK cells against leukemic cells.

Together, the papers in this collection add new knowledge on the complex map of NK cell development, while also suggesting potential novel therapeutic strategies to modulate NK cell development and activity. These papers also lend new insights into how to endow NK cells with potent activity to control hematopoietic and non-hematopoietic malignancies.

We would like to take this opportunity to thank all the reviewers for their time and input. We also thank the authors for their valuable contributions to this Research Topic.

AUTHOR CONTRIBUTIONS

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

FUNDING

ES was supported by funding provided by the Swedish Research Council including the Stem Therapy Program,

the Swedish Foundation for Cancer Research and the Lund University Medical Faculty. YB was supported by the Swedish Research Council, the Swedish Foundation for Cancer Research, the Swedish Foundation for Childhood Cancer, and the Center for Innovative Medicine. AF was supported by funding from the USA National Institutes of Health/National Cancer Institute (CA199447 and CA208353). EM was supported by NIH-NIAID R01AI137073 and R0AI137275.

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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REVIEW
published: 29 August 2019
doi: 10.3389/fimmu.2019.02078

Human NK Cell Development: One Road or Many?

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$CD3^{-}CD56^{+}$ NK cells develop from $CD34^{+}$ hematopoietic progenitors (HPCs) *in vivo*, and this process can be recapitulated *in vitro*. The prevailing model is that human NK cell development occurs along a continuum whereby common lymphocyte progenitors (CLPs) gradually downregulate CD34 and upregulate CD56. Acquisition of CD94 marks commitment to the $CD56^{bright}$ stage, and $CD56^{bright}$ NK cells subsequently differentiate into $CD56^{dim}$ NK cells that upregulate CD16 and killer immunoglobulin-like receptors (KIR). Support for this linear model comes from analyses of cell populations in secondary lymphoid tissues and *in vitro* studies of NK cell development from HPCs. However, several lines of evidence challenge this linear model and suggest a more branched model whereby different precursor populations may independently develop into distinct subsets of mature NK cells. A more definitive understanding of human NK cell development is needed to inform *in vitro* differentiation strategies designed to generate NK cells for immunotherapy. In this review, we summarize current evidence supporting the linear and branched models of human NK cell development and the challenges associated with reaching definitive conclusions.

Keywords: NK cell, development, precursor, innate, adaptive, progenitor, immune, differentiation

OPEN ACCESS

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

Received: *21 June 2019*
Accepted: *16 August 2019*
Published: *29 August 2019*

Citation:
Cichocki F, Grzywacz B and Miller JS (2019) Human NK Cell Development: One Road or Many? Front. Immunol. 10:2078. doi: 10.3389/fimmu.2019.02078

THE PLASTICITY OF EARLY HUMAN HEMATOPOIESIS

The population of cells comprising human blood is organized as a cellular hierarchy derived from multipotent stem cells. The first *in vivo* experiments demonstrating reconstitution of the hematopoietic system from stem cells were based on rescue of lethal irradiation by bone marrow transplant in mice (1). Subsequent bone marrow transplant experiments in mice provided estimates of the minimal number of hematopoietic stem cells (HSCs) that could reconstitute hematopoiesis (2) and revealed *in vivo* proof for the multipotent nature of stem cells (3). The advent of flow cytometry and cell sorting allowed for purification of hematopoietic stem cells and demonstration that a small number of these cells could reconstitute all blood cell types in lethally irradiated mice (4).

Throughout the past two decades there have been numerous studies characterizing hematopoietic stem cells and determinants of self-renewal or differentiation. In early models of the hematopoietic differentiation tree, the first branch point segregated common lymphoid progenitor cells (CLPs) from common myeloid progenitors (CMPs). Subsequent modifications to the tree have been made based on work showing that the HSC pool is very heterogeneous in terms of self-renewal and differentiation properties. One landmark discovery that challenged the standard branched tree paradigm of human hematopoiesis was the identification of a population of multi-lymphoid progenitor cells (MLPs) that could generate all lymphoid cell types, as well as

monocytes, macrophages, and dendritic cells (DCs). MLPs were characterized as a distinct Thy-1$^{neg-low}$CD45RA^{+} population within the CD34^{+}CD38^{-} HSC pool of both cord blood and bone marrow. When cultured on the MS-5 murine stromal cell line, MLPs differentiated into myeloid cells, B cells, and NK cells at a nearly 1:1:1 ratio. A large fraction of MLPs could also differentiate into T cells when cultured on OP9 murine stromal cells transduced with the Notch ligand DL1 (5). This work, along with other studies showing macrophage potential in thymic progenitors, CLPs, and B cell progenitors call into question the lymphoid-restricted state of the presumed CLP (6–10) and led to a model whereby multipotential progenitors (MPPs) initially differentiate into lymphoid-primed multipotential progenitors (LMPP) (11–14) in route to definitive myeloid and lymphoid commitment (15, 16).

Several important conclusions can be drawn from these studies. First, there exists considerable heterogeneity and plasticity with regards to hematopoiesis and lineage potential of precursors. Second, precursors with some degree of B and T cell lineage restriction appear to retain NK cell and myeloid potential. From an evolutionary perspective, the innate myeloid and NK cell lineage pathways may represent ancestral programs that are retained in progenitors. Adaptive immunity, when it arose, may have been layered onto the ancestral programs, resulting in further hematopoietic lineage diversification. Third, signals within the microenvironment in which a progenitor resides provide instructive signals that strongly influence the developmental path of a given progenitor.

NK CELL PRECURSORS AND ONTOGENY

One of the first reports aimed at defining the precursor origin of NK cells was performed by Kumar and colleagues in the mid 1980's. The authors transplanted syngeneic bone marrow cells into lethally irradiated mice that were also depleted of NK cells by injection of an anti-asialo GM1 antibody. Using this system, the authors demonstrated that an intact bone marrow microenvironment was necessary for the development of mature, lytic NK cells, and that NK cell precursors lack expression of several surface antigens that define mature NK cells (17). Subsequently, an early foray into human NK cell ontogeny was undertaken by Lanier et al. who characterized freshly isolated NK cells from fetal tissue. The most striking finding from this study was that fetal NK cells, in contrast to adult peripheral blood NK cells, expressed intracellular (but not surface) CD3δ and CD3ε. This led to the hypothesis that NK cells and T cells may share a common precursor that splits to the T or NK cell lineage depending on environmental cues (18). Contemporaneously, Reinherz and colleagues identified a dominant fetal thymocyte population in mice lacking expression of CD4 and CD8 but expressing Fc gamma RII/III prior to TCR acquisition *in vivo*. If maintained in a thymic environment, these precursors exhibited stepwise differentiation into canonical CD8^{+} T cells. If removed from the thymus, these precursors developed into canonical NK cells with cytotoxic function (19). Subsequently, *in vitro* fetal thymic organ culture experiments using mouse fetal thymocytes demonstrated that a T/NK-committed progenitor defined as NK1.1^{+}CD117^{+}CD44^{+}CD25^{-} could efficiently develop into T cells if cultured in a thymic microenvironment, whereas co-culture with bone marrow-derived stromal cells resulted in the generation of mature NK cells (20). Support for a developmental relationship between NK cells and T cells also comes from whole-genome microarray analyses of murine splenic leukocyte populations. At the transcriptome level, NK cells and T cells cluster within a complex that is distinct from those formed by subsets of B cells, DCs, and macrophages by principal components analysis (21).

Compelling evidence exists for the idea that T cell-determining factors are needed to enforce the development of precursor cells into the T cell lineage, and the NK cell lineage becomes the default pathway in the absence of these factors. Several murine studies have shown that one of the earliest checkpoints in T cell development is dependent on the zinc-finger transcription factor Bcl11b. Bcl11b-deficient mice exhibit impaired thymocyte development between the DN3 to immature SP stage because of an inability to rearrange the TCR V_{β} to D_{β} gene segments (22). Genetic deletion of Bcl11b in conditional knockout mice results in a loss of T cell identity in developing DN3 thymocytes and reprograming to a morphological and transcriptional state resembling that of NK cells (23–25). One interpretation of these results is that early progenitor cells with intrinsic T cell potential but low or absent Bcl11b expression differentiate into NK cells, providing support for the existence of a common T/NK progenitor. Another interpretation is that Bcl11b expression is necessary to enforce T cell identity during development by overriding a more ancestral NK-like program, and there is no actual NK/T lineage split determined by Bcl11b. The latter interpretation seems more likely based on a report of a patient that contained a mutant *BCL11B* variant causing dysregulated binding of BCL11B to promoter targets. The patient exhibited a "leaky" form of severe combined immunodeficiency (SCID) and very low T cell counts. However, NK cell counts were within the normal range (26).

There has been a continuous evolution regarding our understanding of the earliest stages of progenitor cell commitment to the NK cell lineage. An updated model of human lymphopoiesis has been put forth postulating that lymphoid development stems from distinct populations of CD127^{-} and CD127^{+} early lymphoid progenitors (ELPs). Evidence for this model is supported by experiments where CD34^{+} HSCs were engrafted into immunodeficient mice and subsequently phenotyped for surface expression of various lineage markers. Representation of flow cytometry data using tree-plots suggested that lymphoid cells differentiated along two pathways, distinguished by expression of CD127, that originates from CD34^{high}CD45RA^{+} progenitors. A series of *in vitro* differentiation assays showed that CD127^{-} ELPs could generate T cells, marginal zone B cells, NK cells, and innate lymphoid cells (ILCs), while CD127^{+} ELPs could generate marginal zone B cells, NK cells, and ILCs. Molecular characterization of *in vitro*-generated NK cells identified substantial differences according to whether cells originated from CD127^{-} or CD127^{+} NKIPs. NK cells derived from CD127^{-} NKIPs expressed higher

levels of *GZMB*, *IFNG,* and *GZMK* and secreted higher levels of IFN-γ and TNF-α when stimulated with PMA. NK cells derived from $CD127^+$ NKIPs expressed higher levels of genes encoding several transcription factors including *RUNX1*, *TCF4*, *NFIL3*, *MYC*, *LEF1*, *EOMES*, *ETS1*, *TCF12,* and *BCL11B* and exhibited marginally higher degranulation in response to K562 stimulation (27). Further dissection of the relative contribution of $CD127^-$ and $CD127^+$ NKIPs to the mature peripheral blood NK cell pool and NK cell subsets in various tissues will be of interest.

Because of the complexity and plasticity of early hematopoiesis and lineage commitment, it has been challenging to define lineage-restricted NK cell progenitors. A foundational study by Chen and colleagues showed that a subpopulation of $CD34^+Lin^-CD45RA^+$ cells expressing CD10 could give rise to T cells, B cells, NK cells, and DCs under supportive culture conditions (28). Similar results were reported by Miller et al. who demonstrated that 2% of bone marrow cells with a $CD34^+Lin^-CD38^-$ phenotype could give rise to at least three lineages (NK cells, B cells, and myeloid cells) under the same culture conditions (29). Subsequent work comparing lymphoid potential of $CD34^+Lin^-CD45RA^+$ cells isolated from cord blood concluded that $CD34^+Lin^-CD45RA^+CD10^+$ progenitors predominantly exhibited B cell potential, while $CD34^+Lin^-CD45RA^+CD7^+$ progenitors skewed more toward the T cell and NK cell lineages when differentiated *in vitro* (30). Support for CD7 expression by the putative NK cell progenitor came from experiments showing a high cloning efficiency of $CD3^-CD56^+$ NK cells from $CD34^+CD7^{bright}$ bone marrow progenitors (31). Another study of lymphoid and myeloid lineage commitment using precursors from cord blood described B cell and NK cell potential from $CD34^+CD38^-CD10^+CD7^+$ progenitors with barely detectable expansion of these cells in myeloid stromal cultures (32). An important step forward in identifying a lineage-restricted NK cell progenitor was made about a decade later with the identification of a very rare population of cord blood and bone marrow progenitors with a $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$ phenotype that gave rise exclusively to NK cells when co-cultured at limiting dilutions with supporting stroma and after transplantation into newborn immune-deficient mice. These NK cell precursors were shown to be "downstream" of CLP-like cells with a $Lin^-CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^+$ phenotype (33). It would be of considerable interest to revisit the place of these lineage-restricted NK cell precursors in the hematopoietic hierarchy in the context of the "two-family" model, which posits that $CD127^-$ and $CD127^+$ ELPs differentiate independently and can each give rise to NK cells (27).

Another population of lineage-restricted progenitor cells with NK and T cell potential characterized as $Lin^-CD34^+DNAM\text{-}1^{bright}CXCR4^+$ has been described. These cells were bone-marrow-resident, but increased markedly in circulation in individuals with chronic infections. *Ex vivo* culture of these cells with cytokines (FLT3, SCF, IL-7, IL-15) led to the development of NK cells and $TCR\alpha/\beta^+$ T cells, but not myeloid cells. In contrast, cord blood-derived $CD34^+DNAM^-CXCR4^-$ progenitor cells in the same culture conditions gave rise to NK and myeloid cells (34). Where these $Lin^-CD34^+DNAM\text{-}1^{bright}CXCR4^+$ progenitor cells fit within the developmental hierarchy of NK cells is unclear. It will be of interest to determine whether cytotoxic lymphocytes that arise from these unique progenitors play an important role in the anti-viral immune response.

THE LINEAR MODEL OF NK CELL DEVELOPMENT

$CD3^-CD56^+$ NK cells with cytotoxic function can be generated *in vitro* after long-term culture of $CD34^+$ cells isolated from cord blood, bone marrow, fetal liver, thymus, or secondary lymphoid tissue with IL-2 or IL-15 (31, 35–38). Based on the anatomical locations of progenitors and their capacity to develop into NK cells under supportive conditions, a stepwise model for development and maturation of human NK cells has been put forth by Freud and Caliguiri. In this model, HSCs give rise to "Stage 1" progenitors that retain CD34 expression and acquire CD45RA and CD10. These cells give rise to "Stage 2" progenitors marked by loss of CD10 expression and acquisition of CD117. "Stage 3" is marked by downregulation of CD34 and acquisition of LFA-1. These cells are presumed to be restricted to the NK cell lineage given their inability to differentiate into T cells or DCs *in vitro* and their capacity for efficient differentiation into bona fide NK cells in response to IL-15. "Stage 4" is marked by acquisition of CD94, and these cells represent the $CD56^{bright}$ NK cell subset. The precursor population for $CD56^{bright}$ cells has been identified as exhibiting a $CD34^{dim}CD45RA^+$integrin $\alpha_4\beta_7$ phenotype (38). Further differentiation into "Stage 5" cells is marked by downregulation of CD94 and acquisition of CD16 and killer immunoglobulin-like receptors (KIR). These cells represent the $CD56^{dim}$ NK cell subset (39).

The presumed developmental transition from a $CD56^{bright}$ to a $CD56^{dim}$ phenotype is perhaps the most controversial step in this developmental model. A good case can be made for this developmental pathway. $CD56^{bright}$ NK cells are the predominant population early after hematopoietic cell transplant. Their frequency decreases by 3 months post-transplant, concomitant with an increase in the percentage of $CD56^{dim}$ NK cells (40). While this pattern of NK cell reconstitution could reflect a developmental relationship, an alternative hypothesis is that the abundance of $CD56^{bright}$ NK cells early post-transplant is due to high levels of homeostatic expansion of this subset in the setting of lymphopenia induced by transplant conditioning. Additional support for a developmental relationship between $CD56^{bright}$ and $CD56^{dim}$ NK cells comes from the identification of a functionally and phenotypically intermediate population of $CD56^{dim}CD94^{high}$ NK cells that have been described as a transitional population between $CD56^{bright}$ and $CD56^{dim}CD94^{low}$ NK cells (41). However, whether human NK cells differentiate from $CD56^{bright}$ to $CD56^{dim}CD94^{high}$ to $CD56^{dim}CD94^{low}$ has not been definitively established. Interestingly, CD94 has also been used as a marker to define phenotypically and functionally distinct NK cell subsets in mice. Murine $CD94^{high}$ NK cells share phenotypic and functional

properties with human $CD56^{dim}CD94^{high}$ NK cells. When $CD94^{high}$ and $CD94^{low}$ NK cells were purified and adoptively transferred into congenic mice, $CD94^{low}$ NK cells became $CD94^{high}$ but not vice versa (42).

Perhaps the strongest evidence in support of the idea that $CD56^{bright}$ NK cells differentiate into $CD56^{dim}$ NK cells comes from studies where $CD56^{bright}$ NK cells were sorted and stimulated *in vitro*. In a study by Chan et al., a fraction of sorted $CD56^{bright}$ NK cells co-cultured with synovial fibroblasts exhibited CD56 downregulation and had a phenotype consistent with $CD56^{dim}$ NK cells. The apparent differentiation of $CD56^{bright}$ NK cells to $CD56^{dim}$ NK cells could be inhibited by the addition of an antibody that blocks fibroblast growth factor receptor 1 (FGFR1). Of note, stimulation of $CD56^{bright}$ NK cells with a combination of IL-2, IL-15, and 721.221 cells did not induce differentiation to a $CD56^{dim}$ phenotype. $CD56^{bright}$ NK cells that were adoptively transferred into NOD-SCID mice were almost uniformly $CD56^{dim}CD16^{+}$ when analyzed 10 days later in the blood, spleen, and lymph nodes. Furthermore, $CD56^{bright}$ NK cells were shown to have longer telomere repeat lengths relative to $CD56^{dim}$ NK cells, suggesting that they are more naïve (43). A contemporaneous study by Romagnani et al. reported on the acquisition of $CD56^{dim}$ NK cell features such as KIR and CD16 upregulation after stimulation of sorted $CD56^{bright}$ NK cells with IL-2 or IL-15 and confirmed the existence of longer telomere repeats in $CD56^{bright}$ NK cells (44). The discrepancies between these two studies with respect to the role of cytokines in driving maturation of $CD56^{bright}$ NK cells may be due to experimental techniques, but additional studies are needed to gain a more definitive understanding of this stage of NK cell maturation. Indeed, it's somewhat surprising that no follow up studies looking deeper into the FGF signaling pathway and its role in driving NK cell maturation have been published.

The $CD56^{dim}$ NK cell subset in peripheral blood is heterogeneous mix of cells with respect to the expression of KIR, CD94, NKG2A, CD62L, and CD57. Relative surface expression levels of these molecules are indicative of maturation status. The current model based on analysis of peripheral blood NK cells from healthy donors and NK cell reconstitution after hematopoietic cell transplantation suggests that as $CD56^{dim}$ NK cell mature, they downregulate NKG2A and CD62L and subsequently acquire KIR and CD57. Sequential maturation is associated with a gradual decline in proliferative capacity in response to IL-2 or IL-15 (45, 46). The acquisition of inhibitory KIR and NKG2A after lineage commitment has been studied using *in vitro* models of human NK cell development from $CD34^{+}$ precursors (47, 48). However, late stage NK cell differentiation and maturation is difficult to study using current culture conditions. NK cell development from CD34 precursors *in vitro* is a slow process that takes ∼4 weeks, and $CD3^{-}CD56^{+}$ NK cells generally exhibit low-to-absent expression of KIR, CD16, and CD57. Additionally, signaling through the common γ-chain cytokines IL-2 and IL-15 drives high expression of CD56, NKG2A, and cytotoxic granule components in cultured NK cells. Thus, innovative new approaches need to be developed in order to study the paths of late stage NK cell maturation and the mechanisms that influence NK cell heterogeneity.

EVIDENCE FOR A NON-LINEAR MODEL OF NK CELL DEVELOPMENT

The linear model of human NK cell development is a useful construction. Within it lie some fundamental truths, such as the concept that multipotent progenitor cells become lineage restricted and further mature. However, we may need to go beyond this model to understand NK cell heterogeneity. NK cells were once thought to be a relatively homogenous lymphocyte population, particularly in comparison to T and B cells that can generate remarkable receptor diversity through somatic DNA recombination. This view has changed with the advent of more sophisticated technologies for cellular analysis and computing power. Using mass cytometry with a panel of 28 NK cell receptors, Horowitz et al. phenotyped peripheral blood NK cells from five sets of monozygotic twins and 12 unrelated donors with defined KIR and HLA genotypes. Using a Boolean gating strategy to analyze the mass cytometry data, they estimated 6,000–30,000 phenotypic populations within an individual and more than 100,000 phenotypes in the entire donor panel. Interestingly, no single phenotype accounted for more than 7% of the total NK cells, and subsets comprising the top 50 phenotypes accounted for an average of only 15% of a given individual's NK cells. Hierarchical clustering of NK cell populations on the basis of surface receptors showed that the major distinguishing receptors were CD94, NKG2A, CD16, and CD57. Two separate clusters emerged: a less mature $CD94^{+}NKG2A^{+}$ cluster and a mature $CD16^{+}CD57^{+}$ cluster (49). With the existence of these new technologies and sophisticated methods of analysis, it will be exciting to find out how population frequencies shift in the context of aging and disease. It will also be of interest to know whether less mature populations such as the $CD94^{+}NKG2A^{+}$ population continually mature and alter their phenotype or whether they are more static and fixed at their stage of differentiation. It is currently unknown whether the astounding diversity found within the peripheral blood NK cell population is largely a reflection of a spectrum of maturational states and stochastic receptor expression influenced by the environment or whether clonal diversity within the precursor pool dictates NK cell phenotypes. In this section we review evidence for the hypothesis that NK cell diversity could be determined at the precursor level.

The idea that NK cells develop exclusively from CLPs was challenged by experiments showing that CMPs and granulocytic-monocytic precursors (GMPs) isolated from cord blood could efficiently differentiate into NK cells when cultured in the presence of NK-supporting cytokines and stroma. Additionally, NK cells derived from myeloid precursors variably expressed colony-stimulating factor receptor (CSFR) during culture. Both $CSFR^{-}$ and $CSFR^{+}$ progenitors gave rise to functional $CD56^{+}$ NK cells if cultured in NK-supporting conditions, and addition of colony-stimulating factor (CSF) to NK cell cultures skewed development toward the monocyte lineage in a dose-dependent manner. Interestingly, NK cells derived from $CSFR^{+}$ myeloid precursors exhibited significantly higher killer immunoglobulin-like receptor (KIR) expression (50). More KIR acquisition on NK cells derived from myeloid precursors could be related

to CSFR, which signals through the transcription factor Myc (51). Upstream distal *KIR* promoters have binding sites for Myc, and Myc overexpression drives *KIR* gene transcription (52). Importantly, a fraction of NK cells with a more mature $NKG2A^{-}KIR^{+}$ phenotype was identified in cultures where NK cells were derived from $CSFR^{+}$ progenitors, and this population was absent in cultures where NK cells were derived from $CSFR^{-}$ progenitors (50). Supporting evidence for human NK cell differentiation from myeloid progenitors was reported in a more recent study of NK cell reconstitution in humanized mice. In this model, 80% of $CD56^{+}$ cells in the bone marrow co-expressed myeloid markers such as CD33 or CD36. These cells lacked expression of conventional NK cell markers including NKG2D and NKp46 and were hypofunctional with regards to IFN-γ production and cytotoxicity. However, $CD56^{+}CD36^{+}$ NK cells sorted from the bone marrow of these mice and cultured in differentiation media containing stem cell factor (SCF), IL-15, and FLT-3 ligand exhibited maturation toward the conventional NK cell lineage as evidenced by loss of CD36 expression and acquisition of NKp46 and NKG2D. Similar observations were reported using $CD56^{+}CD36^{+}$ cells isolated from human cord blood. Finally, the authors demonstrated that when purified $CD4^{+}CD38^{+}CD123^{low}CD45RA^{+}$ cells with a GMP phenotype were cultured in conditions supporting NK cell development, transient CD36 expression was observed followed by significant upregulation of CD56 (53).

It could be argued that NK cell development from myeloid progenitors is an artifact of the culture systems used and that it does not occur *in vivo*. Indeed, further studies need to be done *in vivo* to substantiate *in vitro* results. Nonetheless, given the plasticity of hematopoiesis described above, we believe that it's likely that some fraction of lineage-committed NK cells in humans derive from myeloid precursors. This notion is supported by other studies showing that under certain circumstances NK cells can share properties with DCs, such as MHC class II upregulation and antigen-presentation (54, 55). Conversely, there are conditions under which DCs acquire cytotoxicity characteristic of NK cells (56).

While much of the above discussion has highlighted hematopoietic plasticity and the multi-lineage potential of progenitor cells, results from a recent study by Dunbar and colleagues utilizing autologous transplantation of rhesus macaques with barcode-labeled $CD34^{+}$ cells suggest that the NK cell lineage is ontologically distinct. This contention was based on analysis of peripheral blood from macaques between 3- and 6.5-months post-transplant. Within this window, many shared clones were contributing to the granulocyte, monocyte, T cell and B cell lineages, while the clonal composition of NK cells was distinct. Additionally, distinct clonal patterns were observed for the more abundant $CD16^{+}CD56^{-}$ NK cell subset compared to the less abundant $CD16^{-}CD56^{+}$ NK cell subset (57).

In a follow up study, the same group reported on NK cell reconstitution from the same rhesus macaques out to 4 years post-transplant. In this subsequent analysis, the differences in clonal contributions to the $CD16^{+}CD56^{-}$ and $CD16^{-}CD56^{+}$ NK cell populations were still evident, and the $CD56^{-}CD16^{+}$ NK cell subset exhibited low clonal diversity. Despite technical challenges related to limited reagents to phenotype macaque NK cells, the authors also showed that reconstituted NK cells segregated by expression of KIR also exhibited clonal segregation. Furthermore, these clonal patterns were maintained after short term *in vivo* depletion with an anti-CD16 antibody. This finding suggests persistence and self-renewal of oligoclonal NK cell populations (58). If it can be assumed that (a) reconstitution of hematopoiesis after adoptive transfer of transduced $CD34^{+}$ progenitors accurately recapitulates NK cell ontogeny, (b) macaque and human NK cell development are reasonably equivalent, and (c) macaque $CD16^{+}CD56^{-}$ and $CD16^{-}CD56^{+}$ NK cells are analogous to $CD56^{dim}$ and $CD56^{bright}$ NK cells, the results from this study suggest that $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells are distinct lineages. This has obvious implications for the current model of human NK cell development where $CD56^{bright}$ NK cells are assumed to be precursors of $CD56^{dim}$ NK cells. While the debate over whether $CD56^{bright}$ NK cells are precursors of $CD56^{dim}$ NK cells or an independent lineage may seem somewhat trivial, it has important implications for generating NK cells for immunotherapy. It is possible that current culture systems which predominantly generate cells with a $CD56^{bright}$ phenotype favor the expansion/differentiation of a particular subset of precursor clones at the expense of other clones that differentiate into $CD56^{dim}$ NK cells.

CONSIDERATIONS OF NK CELL DEVELOPMENT IN RELATION TO ILCS

In recent years, much knowledge has been gained by studying NK cell development in parallel with the closely related ILCs and lymphoid tissue inducers (LTi). In one report describing committed ILC precursors, the immune systems of $PLZF^{GFPcre+/-}$ mice carrying the ROSA26-floxstop-yellow fluorescent protein fate (YFP)-mapping allele were analyzed in detail. GFP marked cells actively expressing promyelocytic leukemia zinc finger (PLZF), and YFP marked cells that had previously expressed PLZF at some point during their development. PLZF is a transcription factor that plays an important role in the effector differentiation of NKT cells (59, 60). Hematopoietic reconstitution experiments using progenitor cells from $PLZF^{GFPcre+/-}$ mice demonstrated that the vast majority of NKT cells expressed YFP, whereas CLPs, B cells, and T cells were unlabeled. ILC1, ILC2, and ILC3 cells were YFP-labeled to varying extents. Interestingly, non-recirculating $DX5^{-}CD49a^{+}CD3\varepsilon^{-}NK1.1^{+}$ NK cells in the liver were heavily labeled, whereas classical recirculating $DX5^{+}CD49a^{-}$ NK cells were mostly negative. In a search for the PLZF-expressing ILC precursor, the authors identified a rare subset of $PLZF^{high}$ cells in fetal liver and adult bone marrow with a $Lin^{-}IL\text{-}7R\alpha^{+}cKit^{+}\alpha4\beta7^{high}$ phenotype that demonstrated ILC1, ILC2, and ILC3 potential at the clonal level. This potential excluded classical LTi and NK cells, but included non-recirculating $DX5^{-}CD49a^{+}CD3\varepsilon^{-}NK1.1^{+}$ NK cells. The results of this study suggest that liver-resident NK cells share a common progenitor with ILCs and that a distinct $PLZF^{-}$ progenitor gives rise to

circulating NK cells (61). Whether PLZF expression is associated with the divergence of canonical NK cells and ILCs in humans has not yet been determined, and there may be important differences in PLZF expression patterns between species that limit the application of knowledge gained from these mouse experiments to human biology. In contrast to mice, recirculating canonical NK cells in humans are PLZF$^+$, and PLZF downregulation through promoter DNA methylation is a hallmark of adaptive NK cells that arise in response to human cytomegalovirus (HCMV) (62).

Mice with an inhibitor of DNA binding 2 (Id2) reporter allele (*Id2*$^{Gfp/+}$) have also been employed to track ILC progenitors (63). Id2 is a transcriptional regulator and inhibitor of E proteins (64). Genetic deletion of *Id2* in mice abrogates the development of all ILC lineages, including NK cells (65, 66). In *Id2*$^{Gfp/+}$-reporter mice, a Lin$^-$Id2$^+$IL-7Rα^+CD25$^-\alpha_4\beta_7^+$ cell population representing a common progenitor to the ILC1, ILC2, and ILC3 lineages was identified. This progenitor population was termed the common progenitor to all helper-like ILCs (CHILP). CHILP cells did not give rise to conventional NK cells in adoptive transfer experiments, indicating early divergence of the ILC and NK cell lineages (63). However, this interpretation has been recently challenged by DiSanto and colleagues who studied ILC and NK cell development using Id2^{RFP}-reporter mice. The genomes of these mice contain an internal ribosome entry site monomeric red fluorescent protein (IRES-mRFP) cassette within exon 2 of the *Id2* gene. In these mice, RFP was highly expressed in all ILC subsets and in splenic and liver NK cells (67). In the current model of ILC development, Lin$^-$CD117$^+$CD135$^-\alpha_4\beta_7^+$CD25$^-$ ILC progenitors (ILCP) are considered the earliest precursor population giving rise to ILCs downstream of CLPs (68, 69). Analyses of immune cell reconstitution 5 weeks after adoptive transfer of bone marrow-derived Id2^{RFP} ILCPs into sub-lethally irradiated immunodeficient mice showed that all ILC subsets as well as conventional NK cells were present in these mice. Additionally, when Id2^{RFP} ILCPs were sorted and cultured on stroma with cytokines, single-cell cultures gave rise to both single and mixed colonies of ILC1s, ILC2s, ILC3s, and NK cells. To assess PLZF as a distinguishing factor of ILC progenitors, Id2^{RFP} mice were crossed with *Zbtb16*^{GFPcre} mice to generate double-reporter mice. *Zbtb16* is the gene encoding PLZF. *Id2*$^+$*Zbtb16*$^-$ and *Id2*$^+$*Zbtb16*$^+$ ILCPs were purified from double-reporter mice and adoptively transferred into immunodeficient mice. Both populations gave rise exclusively to ILC subsets and NK cells with no detection of B cells, T cells or myeloid cells. Sorted *Id2*$^+$*Zbtb16*$^+$ ILCPs could also give rise to all ILC subsets as well as NK cells in single-cell cultures. Results from these experiments performed with a more sensitive reporter system suggest that conventional NK cells and ILCs are derived from a common early precursor and that neither Id2 nor PLZF distinguishes progenitors with differing lineage potential (67).

Not surprisingly, human ILC development is less well-characterized. A lineage-committed CD34$^+$ ILC3 precursor expressing the transcription factor RORγt has been found in tonsil and intestinal lamina propria tissues but not in the peripheral blood, bone marrow or thymus (70). Freud and colleagues also identified a Lin$^-$CD34$^+$CD45RA$^+$CD117$^+$IL-1R1$^+$RORγt$^+$ progenitor population that expressed *ID2* and could differentiate into all ILC types, including conventional CD56^{bright} NK cells, *in vitro*. This progenitor was found in several different secondary lymphoid tissues (SLT) but not in hematopoietic tissues or thymus. Intriguingly, *RORC1* and *RORC2* (encoding RORγ) transcripts were present in all mature ILC subsets and CD56^{bright} NK cells but not CD56^{dim} NK cells (71). This finding contrasts with fate-mapping studies in mice where RORγt expression was found to be restricted to ILCs, and a RORγt$^+$ progenitor gave rise to subsets of ILCs but not NK cells (72, 73). The observation of *RORC2* expression in CD56^{bright} but not CD56^{dim} NK cells raises questions about the developmental relationship between these two subsets. It's possible that *RORC2* expression is downregulated during the presumed developmental transition of CD56^{bright} NK cells into CD56^{dim} NK cells. Alternatively, *RORC2* expression could be a lineage-defining factor that marks two distinct lineages (71).

While the two studies referenced above describe ILCs located in SLT, a recent report has extensively characterized human ILCPs that circulate in peripheral blood. These cells are found at a low frequency in blood and are CD45$^+$CD7$^-$CD56$^-$CD25$^+$CD127$^+$CD117$^+$IL1R1$^+$CD69$^-$. Analysis of progeny from single ILCP cell cultures showed that all ILC subsets as well as NK cells developed from ILCPs. ILCPs as defined in this study represented a heterogeneous population comprised of unipotent and multipotent progenitors, and some ILCPs exhibited the potential to generate both NK cells and ILCs at the single-cell level. In addition to peripheral blood, human ILCPs were identified in cord blood, SLT, fetal liver, and adult lung. Results from this work support the idea that circulating ILCPs can seed various tissues, and that environmental factors within the tissue can "instruct" further differentiation toward the ILC1, ILC2, ILC2, and NK lineages. Some of this instruction is likely given by the presence or absence of Notch ligands and the cytokine milieu (74). Collectively, these studies provide strong evidence that a precursor population exists in humans that has the potential to differentiate into ILCs and conventional NK cells. To what extent the total NK cell pool in humans is derived from an ILC/NK-restricted precursor is unknown and is a challenging question to address. It is possible that many tissue-restricted NK cell populations could arise from an ILC/NK-restricted precursor, while circulating peripheral blood NK cells arise from other CLP, CMP, or GMP populations. Because fate-mapping experiments cannot be carried out in humans for obvious ethical reasons, the continued refinement of humanized mouse models for analysis of human NK cell development might be the best approach for advancing our understanding.

ADAPTIVE NK CELL DEVELOPMENT

Over the past decade there has been considerable interest in the concept of NK cell memory. The idea that NK cells may possess attributes of immunological memory began with the discovery that mouse cytomegalovirus (MCMV) encodes an MHC-like protein (m157) that engages the activating receptor

Ly49H on NK cells. This interaction was shown to be important for host protection against the virus (75, 76). Further analysis of the Ly49H$^+$ NK cell population in MCMV-infected mice revealed that these cells expanded robustly in the liver and spleen after infection. Following a contraction phase, the remaining Ly49H$^+$ cells remained in lymphoid and non-lymphoid organs for several months. Adoptive transfer experiments showed that these "memory" cells could undergo secondary expansion in response to viral challenge and conferred protective immunity (77). An analogous population of NK cells expressing the activating receptor NKG2C expands specifically in response to HCMV (78). While the Ly49H/m157 interaction is crucial for host protection against the virus, the same is not true for the NKG2C/HLA-E interaction. Approximately 4% of humans carry a homozygous deletion of *KLRC2*, the gene that encodes NKG2C. Because of built-in redundancy in the human response to HCMV, NK cells from NKG2C$^{-/-}$ individuals can still mount a response against the virus through other activating receptors (78, 79). This redundancy is reflected in the epigenetically regulated diversification of NK cell signaling and function that has been reported in HCMV seropositive individuals (62, 80). Another more general form of NK cell memory for haptens or viruses has also been described in mice. These NK cells are hepatic and express the chemokine receptor CXCR6 (81). This work has been extended to humans where it has been shown that a population of NK cells expressing tissue residency markers (CD69, CD62L, CXCR6) exhibit recall responses to varicella-zoster virus (VZV) and appear to be very long lived (82).

Little is known regarding the developmental origin or adaptive or memory NK cells. There is circumstantial evidence to suggest that the liver may be a site for NK cell memory acquisition. Two recent studies have characterized liver-resident NK cells from biopsied human tissue. These cells express the liver-specific adhesion molecules CXCR6 and CD49a. High frequencies of these cells also express NKG2C and KIR (83, 84). It is possible that the liver is the primary extramedullary site for the development of adaptive NK cells. These cells could then traffic to sites of infection, expand upon activation and traffic through peripheral blood.

The developmental path from CD34$^+$ hematopoietic progenitor cell to adaptive NK cell has not yet been elucidated. It may be that the same lineage-restricted

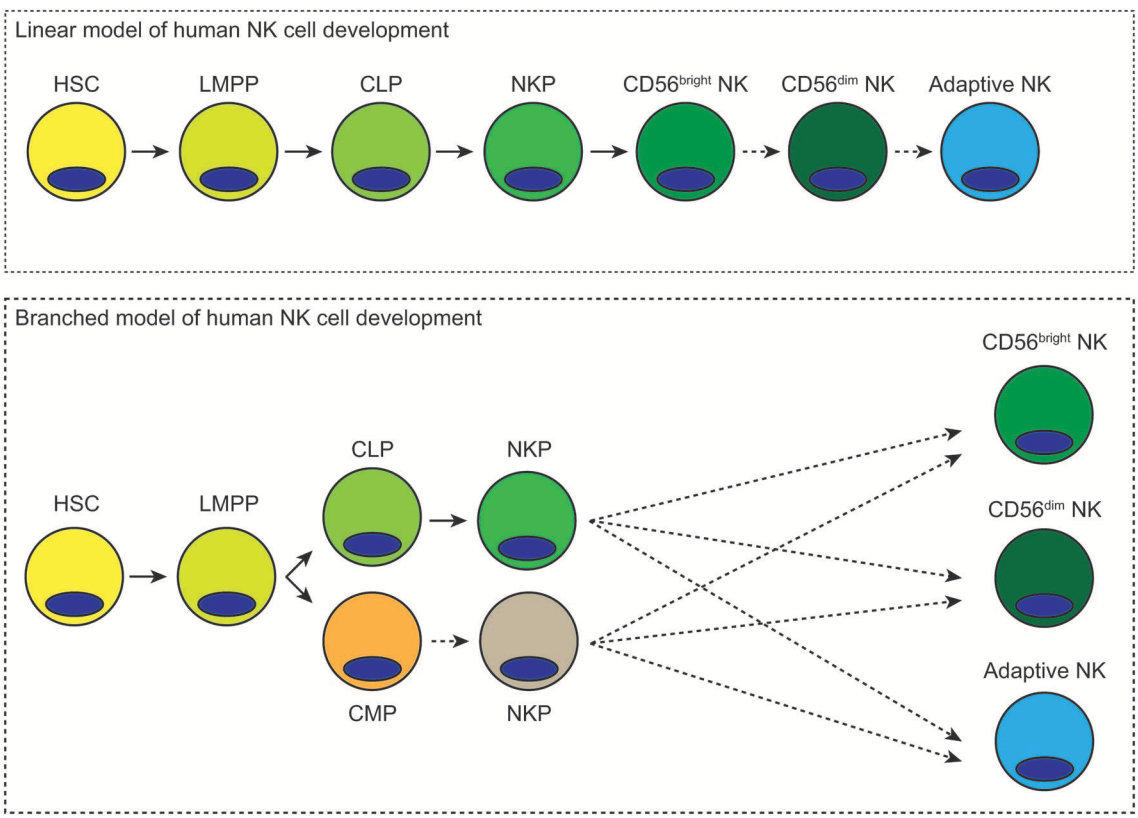

FIGURE 1 | Possible linear and branched models of human NK cell development. In the linear model of human NK cell development, hematopoietic stem cells differentiate into lymphoid-primed multipotential progenitors, which then become common lymphoid progenitors. Lineage commitment occurs at the NK precursor stage. These cells then mature first into CD56^{bright} NK cells and then CD56^{dim} NK cells. Differentiation into adaptive NK cells could subsequently occur in response to viral infection. In the branched model of human NK cell development, hematopoietic stem cells differentiate into lymphoid-primed multipotent progenitors, which then differentiate toward common lymphoid or myeloid progenitors. Either of these progenitors could give rise to NK cell progenitors. These NK cell progenitors could then differentiate into CD56^{bright}, CD56^{dim}, or adaptive NK cells. Dashed arrows represent hypothetical routes of development/differentiation.

$Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ NK cell precursor that has previous been described (33) can differentiate into adaptive NK cells under supportive conditions. Alternatively, there could exist a unique precursor cell that gives rise to an adaptive NK cell lineage. New experimental systems and approaches will likely be needed to understand the ontological relationship between adaptive and canonical NK cells. Currently, there are no studies that have reported on the ability to take canonical NK cells from HCMV seronegative donors and induce and adaptive NK cell state *ex vivo*.

DISCUSSION

Our understanding of hematopoiesis in general and NK cell development in particular has advanced considerably over the past several decades. There is now increased awareness of the plasticity of hematopoietic progenitor cells and their capacity for differentiating toward multiple lineages. One major unresolved question is whether human NK cells arise from a distinct set of clonal precursors or whether they arise from multi-potent progenitors that also split off into the T cell, B cell or myeloid lineages. If NK cells have a particularly unique ontogeny, at what stage of hematopoiesis do they diverge? Another major question that remains to be resolved is whether $CD56^{bright}$ NK cells represent a distinct lineage or whether they are precursors of $CD56^{dim}$ NK cells. A third major question is the developmental origin of adaptive NK cells and whether they represent a lineage distinct from canonical NK cells. To what degree is NK cell development a linear path from hematopoietic stem cell to terminally mature NK cell, and to what degree is it a branched process where different progenitor cell populations give rise to distinct NK cell lineages (**Figure 1**)? More in depth investigation and the development of new approaches and technologies should shed more light on these difficult questions and provide more definitive answers. It is also important to keep in mind the myriad differences between mice and humans with regards to hematopoiesis and immune cell development.

While these questions are interesting from an academic perspective, advancements in our understanding of human NK cell development will be critical for the development of new immunotherapies. One major challenge is how to successfully treat patients with solid tumors with an NK cell therapy. We know that NK cells exist within peripheral tissues where tumors can arise and can infiltrate the tumor microenvironment. However, we do not know the precise developmental pathway these NK cells take, which precursors they differentiate from or what environmental cues instruct their maturation. With this knowledge, we could potentially guide the differentiation of either a subset of $CD34^{+}$ progenitors or induced pluripotent stem cells (iPSCs) *in vitro* to generate NK cells for adoptive immunotherapy that will home to specific tissues and persist. Similarly, we know that certain subsets of NK cells respond specifically to HCMV, VSV, and EBV infections. There is potential to develop an NK cell-based immunotherapy to treat patients who have complications from these infections. For this approach to become a reality, we need a better understanding of the developmental origins of these virus-specific NK cell subsets.

AUTHOR CONTRIBUTIONS

FC, BG, and JM wrote the manuscript.

FUNDING

This work was supported by NIH grants K99/R00 HL123638 (to FC), P01 CA111412 (to JM), P01 CA65493 (to JM), and R35 CA197292 (to JM).

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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 SS and handling editor declared their shared affiliation at the time of review.

frontiers in Immunology

OPINION
published: 20 September 2019
doi: 10.3389/fimmu.2019.02235

Dazed and Confused: NK Cells

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Keywords: NK cells, group 1 ILCs, development, heterogeneity, ILC1

INTRODUCTION

Innate lymphoid cells (ILCs) are rapid producers of both proinflammatory and regulatory cytokines in response to local injury, inflammation, pathogen infection, or commensal microbiota perturbation (1). Because most ILCs have been shown to be tissue-resident during homeostasis (with the exception of circulating NK cells) in almost all organs analyzed, their ability to quickly respond to tissue stress and inflammation underpins their critical role in regulating tissue homeostasis and repair during infection or injury (2–4). Recent evidence has suggested that mature ILCs can be further classified into group 1, 2, and 3 ILCs based on different expression of transcription factors, cell surface markers, and effector cytokines (1). Mouse group 1 ILCs, which include natural killer (NK) cells and ILC1, were initially distinguished from other ILCs based on their constitutive expression of the transcription factor *Tbx21* (T-bet), co-expression of activating receptors NKp46 and NK1.1, and production of interferon (IFN)-γ following activation (5). In humans, group 1 ILCs are harder to definitively differentiate from other ILCs due to the lack of lineage defining markers and reported functional plasticity amongst group 2 and group 3 ILCs (6).

ILC1 are recently discovered tissue-resident sentinels that function to protect the host from bacterial and viral pathogens at initial sites of infection (2, 7, 8). ILC1 rapidly produce IFN-γ following local dendritic cell activation and interleukin (IL)-12 production to limit viral replication and promote host survival before the recruitment of circulating lymphocytes into infected tissue (2). Unlike ILC1, NK cells can be recruited from the circulation into the parenchyma of infected or cancerous tissues where they display potent perforin-dependent cytotoxicity in addition to rapid IFN-γ production (9, 10). However, persistent inflammatory signals can also lead to unrestrained activation of group 1 ILCs during obesity and inflammatory bowel disease (IBD) (3, 11–14). While these studies suggest important roles for group 1 ILCs during host protection and pathology, gaps in evidence have inhibited the ability of recent studies to definitively distinguish between the roles of ILC1 and NK cells in these contexts.

GROUP 1 ILC PHENOTYPIC AND FUNCTIONAL HETEROGENEITY

NK cells, the founding member of ILCs, were initially defined based on the cell surface expression of NK1.1 in mouse or CD56 in human with the absence of cell surface expression of other lineage (Lin) defining markers including CD3, CD14, CD19, and TCR proteins (15). In subsequent mouse studies over the last 30 years, $Lin^{-}NK1.1^{+}$ cells were found to be heterogeneous for the expression of activating and inhibitory Ly49 receptors, cell surface integrins [α1β1 (CD49a), α2β1 (CD49b), αEβ7 (CD103)], cell surface proteins (TRAIL, CD69, CD27, CD11b), transcription factors (Eomes), chemokine receptors (CXCR6), and cytokine receptors (IL-7Rα) in various organs (1, 16). Similarly, human $Lin^{-}CD56^{+}$ cells have been reported to be heterogeneous for the expression of transcriptions factors (EOMES and T-BET), cell surface markers (CD49a, CD56, CD16, NKp80, CXCR6, IL-7Rα, CD94, CD69, NKp44), and cytotoxic molecules (Perforin) (1, 16).

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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 July 2019*
Accepted: *04 September 2019*
Published: *20 September 2019*

Citation:
O'Sullivan TE (2019) Dazed and Confused: NK Cells. Front. Immunol. 10:2235. doi: 10.3389/fimmu.2019.02235

Early studies concluded that cells with an alternative cell surface or transcription factor phenotype from putative mature NK cells (mouse: $Lin^-NK1.1^+T\text{-}bet^+Eomes^+CD49b^+$; human: Lin^- IL-$7R\alpha^-CD56^{dim}CD16^+$) in peripheral organs and blood likely represented immature NK (iNK) cells (17–21). This hypothesis is supported by studies demonstrating that subsets of developing mouse NK cells can be distinguished based on CD27 and CD11b expression (22, 23). Similarly, previous studies have suggested that $CD56^{bright}CD16^-$ human NK cells in the blood may be immature precursors to $CD56^{dim}CD16^+$ mature NK cells (18, 19). However, whether other phenotypic differences observed in mouse and human group 1 ILCs are due to tissue-specific microenvironments, distinct lineages of cells, or developmental/activation states of NK cells is still under considerable debate and investigation.

Insight into these questions came shortly after the identification of Lin^-IL-$7R\alpha^+$ "helper" ILCs. Specifically, genetic evidence suggested that *Tbx21*-dependent IL-$7R\alpha^+Tbet^+Eomes^-NK1.1^+NKp46^+$ "ILC1" in the small intestine did not require Eomes for their development, whereas NK cells did require Eomes (7). A recent study further supported these initial data by using *Eomes*-GFP reporter mice to generate core transcriptional signatures of $Eomes^-$ ILC1 and $Eomes^+$ NK cells from 4 independent tissues. The identified core ILC1 signature led to the discovery of the inhibitory receptor CD200r1 as a stable marker expressed by ILC1 but not NK cells during homeostasis and inflammation (2). Additional lineage tracing experiments suggested that $CD200r1^+Eomes^-CD49b^-$ group 1 ILCs constituted a stable lineage during homeostasis, distinct from CD200r1-$Eomes^+CD49b^+$ mature NK (mNK) cells (2, 7, 24). Functional evidence suggestive of distinct group 1 ILCs in peripheral organs was supported by the findings that $T\text{-}bet^+Eomes^-CD49b^-$ group 1 ILCs (in addition to ILC2 and ILC3) were long-term tissue-resident cells, whereas $Eomes^+CD49b^+$ mNK cells were derived from the circulation in almost all organs tested in mouse parabiosis experiments (2, 4). Similarly, in one human study a subset of donor liver $CXCR6^+$ group 1 ILCs was found to be maintained up to 13 years post-liver transplant while donor $CXCR6^-$ NK cells were absent, suggesting that a subset of long-term tissue-resident $CXCR6^+$ group 1 ILCs are conserved in mammals (25). Furthermore, $CD49b^-Eomes^-$ group 1 ILCs with a phenotype consistent with ILC1 in the liver express higher levels of TRAIL than mNK cells at steady state, and these ILC1 can produce higher levels of tumor necrosis factor (TNF)-α and IFN-γ following activation *ex vivo* (2, 17, 20, 24). While ILC1 in the small intestine were observed to have poor cytotoxicity and liver group 1 ILCs with a phenotype consistent with ILC1 express lower levels of granzymes A/B and perforin at steady state compared to NK cells (7, 24), peripheral ILC1 express higher transcript levels of granzyme C in addition to TRAIL and may kill target cells through alternative mechanisms (2, 24, 26–28). However, it will be important for future studies to determine whether perforin-independent killing mechanisms can be used as definitive criteria to functionally separate ILC1 from NK cells across all mouse and human tissues. Thus, significant phenotypic and functional heterogeneity has been demonstrated in group 1 ILCs; however, it is still unclear to what extent these individual pieces of evidence can be used in isolation to define group 1 ILC subsets.

DEVELOPMENTAL AND ACTIVATION STATES OF GROUP 1 ILCs

Collective reports have demonstrated that iNK cells in mouse bone marrow and periphery can express Ly49 receptors, CD49a, CD90, TRAIL, CD69, and Eomes, and lack CD49b expression (3, 21, 29–31). Upon adoptive transfer into lymphopenic mice, iNK cells can induce CD49b expression and retain Eomes expression (3). During activation, mNK cells can induce expression of CD49a, CD69, TRAIL, and CD90 while also decreasing Eomes expression (2, 17, 29, 32), suggesting that iNK and mNK cell phenotypes can overlap with other reported group 1 ILC phenotypes based on these markers. Consistent with these findings, NK cells can repress Eomes expression and induce CD49a, TRAIL, and CD103 in response to TGFβ and IL-2 stimulation *ex vivo* (33, 34). These key findings make the current dogma of utilizing CD49a, CD49b, and Eomes expression in $Lin^-T\text{-}bet^+NK1.1^+NKp46^+$ cells insufficient to distinguish between group 1 ILC subsets and activation or developmental states of NK cells. Furthermore, adipose and small intestine iNK cells have also been found to be short-term (1 month), but not long term (4 months) tissue-resident in mouse parabiosis experiments (3), suggesting that short-term parabiosis (2 weeks-1 month) experiments are not sufficient to distinguish iNK cells from ILC1 without additional evidence. Thus, there is currently insufficient evidence to conclude that $T\text{-}bet^+$ group 1 ILCs with the phenotype of $CD49a^+CD49b^+Eomes^+NK1.1^+$ are either tissue-resident NK (trNK) cells or transitional states of group 1 ILCs, because these cells may be activated NK cells in the tissue parenchyma following recruitment from circulation. Furthermore, $CD49a^+CD49b^-Eomes^+NK1.1^+$ cells may not represent a transitional subset of group 1 ILC, but instead may represent iNK cells in peripheral tissues, although further lineage tracing experiments will be necessary to clarify these issues in the field.

In the healthy state, mature human group 1 ILCs have been described to be heterogeneous for cell surface expression of CD56, CD16, and NKp80 in peripheral tissues (35). However, CD56 can be expressed on ILC progenitor populations and ILC3 in the tonsil (36), and may be downregulated during activation in a similar manner to CD16 and NKp80 (37–39). Thus, to date there are no known stable cell surface markers that can unequivocally distinguish between human mNK cells (or their developmental intermediates, which may be tissue-resident) and other proposed group 1 ILCs in inflamed human tissues, because activated mNK cells can lose expression of these cell surface markers during inflammation.

Mouse Group 1 ILC Development

Recent unbiased chromatin accessibility studies in mice suggest that NK cells can be defined epigenetically as a distinct ILC lineage through the enrichment of accessible T-bet and Eomes binding sites compared to other leukocytes (40). Similarly, mNK

and iNK cells require Eomes for their development (2, 20, 41), suggesting that Eomes may be the master transcription factor that defines NK cell lineage identity in mice during homeostasis. In support of this hypothesis, mNK cells in the peritoneum, liver, spleen, salivary gland, and adipose tissue were all found to have a cell-intrinsic developmental requirement for Eomes and T-bet (2), arguing against tissue-specific transcription factor developmental requirements for mNK cells. While certain studies have observed that mNK cell numbers are normal in the absence of T-bet (7, 8, 42), it has been demonstrated previously that *Tbx21*$^{-/-}$ NK cells display an immature phenotype and are functionally deficient (3, 43–45). Therefore, because *Tbx21* is required for optimal mature ILC1 and mNK development (2, 3, 46), *Rag2*$^{-/-}$ × *Tbx21*$^{-/-}$ mice are not a suitable model to test for the contributions of mature group 1 ILCs *in vivo*.

The transcription factors *Id2* and *Nfil3* have also been shown to be required for mature mouse ILC1 and NK cell development (47, 48). Certain studies have identified "tissue-resident NK cells," "salivary gland ILCs," and "type 1 ILCs" based on their development in the absence of *Nfil3* (27, 33, 49). However, similar subsets have been also found to be *Nfil3*-dependent in a cell-intrinsic manner in other studies (2, 50). Because mNK cells can develop in an *Nfil3*-independent manner during virus-induced inflammation and aging (33, 51), analysis of *Nfil3*$^{-/-}$ mice is likely not sufficient to define group 1 ILC subsets due to these caveats. Previous studies have also utilized Zbtb16 fate-mapping studies and Id2 reporter mice to identify a common helper ILC precursor population that gives rise to all tissue-resident ILCs, but not mNK cells, to argue that ILC1 comprise a developmental lineage distinct from NK cells (7, 52, 53). However, a recent study using dual *Zbtb16* and *Id2* reporter mice demonstrated that both NK cells and ILC1 can develop from a *Id2*$^{+}$*Zbtb16*$^{+}$ shared precursor, suggesting that these transcription factors alone cannot be used to identify different group 1 ILC subsets during ontogeny (54). Instead, several studies have identified the transcription factor *Zfp683* (Hobit) as highly expressed in peripheral ILC1 compared to mNK cells (2, 55, 56). *Zfp683*$^{-/-}$ mice display a loss of liver ILC1 but not other ILC populations (including ILC1 in other tissues) (2, 55), suggesting that mature liver ILC1 have a unique developmental pathway from other mouse ILCs. While developmental dependence on Eomes expression can be used to identify NK lineage cells in peripheral organs of mice, there is still no definitive evidence that a single transcription factor can define the development of other group 1 ILC subsets across all mouse tissues.

DISCUSSION

While collective evidence supports the hypothesis that mouse group 1 ILCs are composed of *Eomes*-dependent iNK and mNK cells, their activation or developmental states may be mistaken for novel subsets of group 1 ILCs. *Eomes*-independent ILC1 have been shown through single- cell sequencing, parabiosis, lineage tracing, and transcription factor deficient mouse experiments to be a distinct lineage of group 1 ILCs, and not a developmental or activation state of NK cells. In human tissues, there is currently no definitive evidence that can distinguish between developmental or activation states of group 1 ILCs during inflammation. Single cell sequencing studies will be needed to determine the extent of group 1 ILC heterogeneity in various peripheral tissues, and to identify stable markers that can distinguish between stable subsets of group 1 ILCs through lineage tracing in humanized mouse models.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by the National Institutes of Health (P30 DK063491 and AI145997 to TO'S).

ACKNOWLEDGMENTS

With acknowledgments to *Pennied Days* by Night Moves.

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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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 09 October 2019
doi: 10.3389/fimmu.2019.02381

Impact of CMV Infection on Natural Killer Cell Clonal Repertoire in CMV-Naïve Rhesus Macaques

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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: *30 July 2019*
Accepted: *23 September 2019*
Published: *09 October 2019*

Citation:
Truitt LL, Yang D, Espinoza DA, Fan X, Ram DR, Morström MJ, Tran D, Sprehe LM, Reeves RK, Donahue RE, Kaur A, Dunbar CE and Wu C (2019) Impact of CMV Infection on Natural Killer Cell Clonal Repertoire in CMV-Naïve Rhesus Macaques. Front. Immunol. 10:2381. doi: 10.3389/fimmu.2019.02381

Recent functional, gene expression, and epigenetic studies have suggested the presence of a subset of mature natural killer (NK) cells responsible for maintaining NK cell memory. The lack of endogenous clonal markers in NK cells impedes understanding the genesis of these cell populations. In humans, primates, and mice, this phenotype and memory or adaptive functions have been strongly linked to cytomegalovirus or related herpes virus infections. We have used transplantation of lentivirally-barcoded autologous hematopoietic stem and progenitor cells (HSPC) to track clonal hematopoiesis in rhesus macaques and previously reported striking oligoclonal expansions of NK-biased barcoded clones within the $CD56^{-}CD16^{+}$ NK cell subpopulation, clonally distinct from ongoing output of myeloid, B cell, T cell, and $CD56^{+}16^{-}$ NK cells from HSPC. These $CD56^{-}CD16^{+}$ NK cell clones segregate by expression of specific KIR surface receptors, suggesting clonal expansion in reaction to specific environmental stimuli. We have now used this model to investigate the impact of rhesus CMV(RhCMV) infection on NK clonal dynamics. Following transplantation, $RhCMV^{neg}$ rhesus macaques display less dominant and oligoclonal $CD16^{+}$ NK cells biased clones compared to $RhCMV^{pos}$ animals, however these populations of cells are still clearly present. Upon RhCMV infection, $CD16^{+}$ NK cells proliferate, followed by appearance of new groups of expanded NK clones and disappearance of clones present prior to RhCMV infection. A second superinfection with RhCMV resulted in rapid viral clearance without major change in the mature NK cell clonal landscape. Our findings suggest that RhCMV is not the sole driver of clonal expansion and peripheral maintenance of mature NK cells; however, infection of macaques with this herpesvirus does result in selective expansion and persistence of specific NK cell clones, providing further information relevant to adaptive NK cells and the development of NK cell therapies.

Keywords: NK cells, cytomegalovirus, adaptive memory, barcoding, clonality

INTRODUCTION

Natural killer (NK) cells are classically-defined as circulating and tissue-resident immune effectors responsible for production of regulatory and supportive cytokines as well as the killing of infected and malignant cells. NK cells have been historically considered innate effector cells, lacking both the rearranged diverse antigen receptors present in B and T cells conferring specificity and the self-renewal and/or longevity of reactive clones necessary to confer immune memory. However, there is increasing direct and correlative evidence for properties of NK cells providing adaptive memory in mice, non-human primates, and humans in response to viral infection, immunization, or cytokine stimulations (1–8). Functional, gene expression, and epigenetic studies have defined subsets of natural killer cells potentially responsible for these adaptive properties (1, 6, 9–11).

Clonal expansions are integral in understanding T and B cell memory; yet, the lack of endogenous clonal markers in NK cells has impeded understanding of the genesis and maintenance of putative memory or adaptive NK cell populations. Efforts to elucidate mechanisms underlying NK memory have focused on analyzing expression patterns and "pseudo-clonal" expansions of NK cells with specific patterns of surface receptors known to interact with MHC molecules or viral targets, specifically Ly49 receptors in mice (9) or killer immunoglobulin-like receptor (KIR) in humans. The pathways resulting in heterogeneity of NK cell functions are complex and not completely understood, with the character of responses to the environment appearing to depend on the timing and amalgamation of expression of activating and inhibitory cell surface receptors.

Mouse NK cells are phenotypically and functionally distinct from human NK cells, limiting extrapolation from this model organism. The Ly49 family of receptors in the mouse has been shown to have some analogous functions to human KIRs; however in terms of their structure, these molecules are highly dissimilar (12). In contrast to murine models, non-human primates, specifically rhesus macaques (RMs), are phylogenetically closely related to humans, and their NK cells share many phenotypic and functional properties with human NK cells (13–15).

We have recently utilized genetic barcoding of transplanted autologous RM hematopoietic stem and progenitor cells (HSPCs) to track hematopoiesis at a clonal level *in vivo* (16, 17). Previously, we observed striking expansions of circulating mature $CD56^{-}CD16^{+}$ NK cell clones, clonally distinct from myeloid, B cell, T cell, and $CD56^{+}16^{-}$ NK cells implying an independent differentiation and maintenance pathway distinct from ongoing production from HSPC, perhaps due to peripheral self-renewal (18). Groups of peripheral expanded clones appeared rapidly following transplantation and showed variable degrees of waxing and waning over time, as if in response to environmental stimuli, similarly to peripheral mature effector T cell clonal dynamics. Strikingly, these expanded NK clones segregated by KIR expression long-term, with specific clones either expressing or not expressing specific KIRs, for the first-time linking expression of specific interacting receptors with clonal expansions and suggesting a potential explanation for maintenance of NK memory. The concept of NK memory was further strengthened by a study showing evidence for antigen-specific NK cell memory following SIV/HIV vaccination in RM indicating the existence of functional memory NK cells (19).

In humans, recent studies have demonstrated populations of mature adaptive NK cells with a distinctive signaling, functional, and transcription factor profiles along with epigenetic characteristics similar to T effector cells that closely correlated with seropositivity for the herpesvirus cytomegalovirus (CMV) (10, 11). Expansions of "pseudoclonal" KIR-segregated NK cells expressing maturation markers such as CD57 and the activating receptor NKG2C have been linked to CMV reactivation post-allogeneic transplantation (20). In the context of reactivation of CMV post-transplant, increases in the NKG2C+ population persisted over time (21, 22). Further, NKG2C gene copy number variation has been shown to play a role in the human NK cell response to CMV infection (23, 24).

Rhesus CMV (RhCMV) has been considered an emerging animal model for studying human CMV due to close phylogenetic relationship, immunogenicity, and identical life cycles, including latency and reactivation following immunosuppression (25). Virtually 100% of RM in the wild or reared in standard captive breeding populations become RhCMV positive by 1 year after birth (26). The RMs previously studied in our barcoded transplantation model were all RhCMV seropositive. We hypothesized that the massive clonal expansions arising post-transplantation may have arisen wholly or in part in response to RhCMV reactivation. We have now used this model to investigate the impact of RhCMV infection on NK cell clonal dynamics and phenotypic subsets by transplanting two RhCMV naïve monkeys with autologous barcoded HSPCs and tracking NK clonal dynamics post-transplantation in comparison to historical barcoded $RhCMV^{pos}$ recipients. To then directly test the relationship between RhCMV infection and NK clonal dynamics, we infected these $RhCMV^{neg}$ animals with RhCMV 9 months post transplantation. Our results provide new insights into NK adaptive features and clonal dynamics related to RhCMV infection and details the phenotype of a model relevant to the human clinic.

MATERIALS AND METHODS

Rhesus Macaque Autologous HSPC Transplantation

Animal studies were carried out on protocols approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee. Indian-origin $RhCMV^{neg}$ RMs ($n = 3$) were obtained from the expanded specific-pathogen free colony maintained at the Tulane National Primate Research Center and confirmed to be RhCMV-seronegative by whole virion ELISA screening for RhCMV-specific IgG antibodies. These animals were housed in isolation from $RhCMV^{pos}$ RMs and special precautions were taken to maintain their $RhCMV^{neg}$ status before and after transplantation and before RhCMV inoculation, including use of one $RhCMV^{neg}$ animal as a blood donor for the

two transplanted RhCMV^{neg} macaques following conditioning radiation and before engraftment.

Peripheral blood CD34+ HSPCs were mobilized, collected via apheresis, immunoselected, and transduced with diverse barcoded lentiviral libraries as described (16–18, 27). Following transduction, CD34+ HSPC were infused into autologous recipients conditioned with 10 Gy total body irradiation.

RhCMV Infection and Monitoring

RhCMV strain 180.92 (28) was used to infect animals in this study. The virus stock of RhCMV 180.92 used for experimental infection of RhCMV-seronegative rhesus macaques was derived after transfection of virion DNA purified from infected cells into primary rhesus macaque fibroblast lines as previously described (29) (2×10^6 TCID50 was slow thawed on ice, reconstituted with RPMI (Thermo Fisher, cat# 11875119) to a final volume of 1 ml, and given as a slow IV push. Immediately following inoculation, infected animals were housed in a regular specific pathogen free (SPF) room and separated from remaining RhCMV^{neg} animals.

RhCMV DNA copy numbers were determined via real-time qPCR as described. DNA was extracted from 200 μL plasma, urine, or saliva using Qiagen QIAamp DNA Mini kit (Qiagen # 51306) and eluted into 50 μL buffer and three replicates for each sample were amplified using TaqMan Universal PCR MasterMix (Thermo Fisher Cat#4304337). Primers and probes were custom designed for the glycoprotein B gene (UL55) of RhCMV (**Table S2**). Absolute quantification of RhCMV copy number was calculated based on a standard curve of plasmid containing the target region. RhCMV DNA copy numbers were expressed as copies per ml of plasma or copies per microgram of input DNA in saliva or urine.

RhCMV-specific IgG was measured as previously described (30, 31) by whole virion ELISA, which uses a 96-well plate coated with purified virion preparation of filtered, fibroblast-passaged RhCMV strain 180.92 (32) at 1:3000 dilution. Plasma (1:50 dilution) was incubated in duplicate wells and RhCMV-binding IgG was detected using 1:500 dilution of an HRP-conjugated goat anti-monkey IgG Ab (Santa Cruz Biotechnology, sc-2458) and substrate incubation. The magnitude of the RhCMV specific IgG binding responses is reported as optical density (OD) at 450 nm.

T Cell Depletion

The recombinant immunotoxin (termed "A-dmDT390-scfbDb(C207)", referred to as FN18) was produced by fusion of the affinity-matured form of the anti-macaque CD3 monoclonal antibody C207 expressed as a fold-back single chain Fv diabody to a truncated diphtheria toxin (DT390) and produced in yeast (33, 34). This immunotoxin was obtained from the Massachusetts General Hospital-Dana Farber Cancer Center Recombinant Protein Expression and Purification Core Facility, supported by the NIAID/NIH Non-human primate reagent resource program (https://www.nhpreagents.org/NHP/default.aspx). 0.25 ug/kg FN18 was administered via IP push twice daily for 4 days.

Cell Lineage Purification

Blood samples were processed using Lymphocyte Separation Medium (GE Healthcare, cat# 17144002) to obtain a PB mononuclear cell (PBMC) layer, followed by red blood cell lysis with ACK lysis buffer (Quality Biological, cat# 118156101). PBMCs were stained with a panel of antibodies (**Table S1**), and specific subsets (**Figure S1E**) were sorted via fluorescence-activated cell sorting to high purity on a BD FACSAria II instrument. Intracellular staining was performed using the FoxP3/Transcription Factor Staining Buffer (Thermo Fisher Cat#00552300). Subsequent analyses were performed using FlowJo V10 (FlowJo, LLC).

Clonal Tracking via Barcode Retrieval

Each integrated lentiviral provirus includes a marker copGFP transgene, a 6bp library ID and a 35 or 27 random bp barcode sequence (35). Use of these documented high diversity barcode libraries ensures that each barcode uniquely marks individual engrafting HSPC, as detailed and validated in prior publications (16, 17, 35). By targeting relatively low transduction of HSPC, the majority of HSPC contain a single barcode. The barcode is passed onto each daughter cell and serves as a clonal tag.

DNA was extracted from cell samples using the DNAeasy kit (Qiagen, cat##69506) and 200 ng DNA was amplified via low-cycle PCR with primers bracketing the library ID and barcode (**Table S2**) with Phusion high fidelity DNA polymerase (Thermo Fisher, Cat #F530L). PCR products were gel purified (Qiagen, cat#28706) and sequenced using the Illumina HiSeq2500 or HiSeq3000 system. Barcode retrieval from the sequencing output was processed to retrieve valid barcodes and analyzed as described, using custom Python and R code which can be accessed at https://github.com/dunbarlabNIH/CMV (16, 17). Only barcodes contributing above a threshold taking into account sequencing errors and sampling constraints were included in analyses (17).

RNA-Flow Discrimination of Rhesus NKG2C vs. NKG2A

Analysis of NKG2C vs.NKG2A expression on RM NK cells was carried out using RNA probe-based staining and flow cytometry (PrimeFlow # 88-18005-204, Affmetrix) as described (36). Briefly, KLRC1(NKG2A)-Alexa-647 and KLRC2(NKG2C)-Alexa-488 probe sets complementary to unique sequences in RM KLRC1 and KLRC2 mRNAs were purchased from Thermo Fisher (KLRC1 Assay ID VF1-20995-PF, KLRC2 Assay ID VF4-4221856-PF). Frozen PBMCs were thawed and rested in RPMI1640 with 10% FBS (Sigma, #F2442) at 37°C and 5% CO_2 for ∼12 h before staining. Surface marker antibody staining was performed followed by cell fixation and permeabilization for intracellular antibody and probe staining, using antibodies listed in **Table S1**. After staining and hybridization to probe sets, the cells were analyzed on the BD LSRFortessa II instrument.

Computational and Statistical Analyses

R was used to realize the data and preform statistical analysis. Code can be found at https://github.com/dunbarlabNIH/CMV.

RESULTS

Autologous Transplantation With Barcoded HSPC in RhCMV^{neg} Macaques

We utilized genetic barcoding of RM HSPC to study the impact of RhCMV infection on the clonal dynamics of NK and T cells following myeloablative autologous transplantation (**Figure 1A**). In this model, a high diversity library of barcodes is introduced into RM CD34$^+$ HSPC, followed by total body irradiation (TBI) and autologous transplantation. Each individual barcode uniquely marks a HSPC and its progeny, and can be quantitatively retrieved to track the dynamics of thousands of barcoded clones (16, 18). RMs in our prior studies ($n = 5$) (16, 18) were all RhCMV seropositive, indicating prior infection and the presence of latent virus. Although serum and saliva were negative for detectable RhCMV DNA before myeloablative and highly immunosuppressive conditioning with high dose TBI, samples collected immediately following TBI and autologous transplantation showed evidence for RhCMV reactivation, before clearing 3–8 weeks later (**Figure 1B**). Three RhCMV^{neg} RM were obtained and two (JD76, JM82) underwent barcoded HSPC transplantation (**Figure 1A**, **Figure S1A**), with the third macaque (JC95) retained as a blood donor to support the other two animals following myeloablation until engraftment. As expected, RhCMV DNA was not detectable in either serum or saliva collected before or post-transplantation (**Figure 1B**, **Figure S1B**). RhCMV IgG remained negative post-transplantation. Both RhCMV^{neg} animals recovered neutrophil, red blood cell and platelet counts in the expected time frame (**Figure S1C**). Successful barcoded lentiviral vector transduction of engrafted HSPC was documented by detection of expression of the marker CopGFP gene at appreciable levels in engrafted circulating myeloid and lymphoid cells (**Figures S1D,E**).

To assess whether RhCMV status affects post-transplantation cellular immune reconstitution, we analyzed circulating numbers of T cells, B cells, CD56$^+$CD16$^-$ NK cells (analogous to human CD56^{bright} immature NK cells) and CD56$^-$CD16$^+$ NK cells (analogous to human CD56^{dim} mature NK cells) (13). RM NK cells were defined by expression of NKG2 using the anti-human NKG2A antibody which stains both NKG2A and NKG2C on RM NK cells (13, 38–40). There was no discernible difference in the pace or degree of recovery of these cell types post transplantation comparing RhCMV^{neg} to RhCMV^{pos} animals, including the mature CD56$^-$CD16$^+$ NK cells of most interest (**Figure 1C**). The distribution and staining pattern of CD56$^+$CD16$^-$ and CD56$^-$CD16$^+$ NK cells were also similar between RhCMV^{neg} and RhCMV^{pos} animals (**Figure 1D**).

Clonal Dynamics in RhCMV^{pos} vs. RhCMV^{neg} Animals Post-transplantation

As previously shown in our clonal tracking studies (16, 18), the CD56$^-$CD16$^+$ subset of NK cells is dominated by a limited number of very large barcoded clones highly biased in contributions toward only this NK subset, in comparison with polyclonal contributions from stable multilineage HSPC to all other circulating cell types appearing by 2–3 months post-transplant, as shown for RhCMV^{pos} ZJ31 in **Figure 2** and for other RhCMV^{pos} animals in **Figure S2**. Individual clonal contributions can be visualized using heatmaps mapping the fractional contributions of individual barcodes, each corresponding to an individual clone derived from the same precursor (**Figure 2A**, **Figure S2**), and overall clonal diversity and richness for the entire population of clones can be represented via Shannon index plots (**Figure 2A**, **Figure S2**). In RhCMV^{pos} animals, mature circulating CD56$^-$CD16$^+$ NK cells are of much lower diversity than other lineages, and are primarily composed of expanded NK-biased clones that can wax and wane over time. Our previous work has demonstrated that these mature NK cell clones express specific KIR and likely self-renew and proliferate independent of ongoing production from HSPC (16, 18).

As shown in **Figures 2A,B**, CD56$^-$CD16$^+$ NK-biased clones arose in both RhCMV^{neg} macaques (JD76 and JM82) after engraftment. However, in comparison to RhCMV^{pos} animals, we observed that the CD56$^-$CD16$^+$ NK-biased clones were relatively smaller and in aggregate dropped to 10% or less of all clonal contributions in this cell type, in contrast to persistent contributions at levels of 10–50% from NK-biased clones in the RhCMV^{pos} animals (**Figures 2B,C**, **Figure S2**). In addition, the clonal diversity of CD56$^-$CD16$^+$ NK cells in the two RhCMV^{neg} recipients became similar to that of other lineages over time, in contrast to decreasing and generally markedly lower diversity in this cell population in RhCMV^{pos} animals (**Figures 2D,E**, **Figures S2**, **S3**). Taken together, the results indicate that biased peripherally-expanding NK clones are still generated post-transplantation in RhCMV^{neg} macaques, however they are less prominent than in RhCMV^{pos} animals, suggesting that RhCMV may be a major but not the only driver of mature NK clonal dynamics.

Impact of RhCMV Infection on NK Cell Clonal Dynamics in RhCMV^{neg} Macaques

To directly analyze the NK response to RhCMV, we infected RhCMV^{neg} barcoded macaques JD76 and JM82 with RhCMV strain 180.92 (28) 9–9.5 m post-transplantation. In addition, we also infected the non-transplanted RhCMV^{neg} macaque JC95 (**Figure 3A**). In JM82, CD3$^+$ T cells were depleted following RhCMV administration in an attempt to reduce competition for proliferative cytokines by T cells. In all three macaques, productive infection was detected by 7–10 days after administration (**Figure 3B**, **Figure S4A**). After RhCMV DNA disappeared from the plasma around 60 days post infection, it remained undetectable, however small amounts of RhCMV DNA could found intermittently over time in saliva and urine, consistent with the normal pattern of virus shedding (**Figure S4A**). All three animals seroconverted between days 7 and 21(**Figure 3C**).

Following RhCMV administration, we enumerated circulating CD4$^+$ and CD8$^+$ T cells and CD56$^+$CD16$^-$ and CD56$^-$CD16$^+$ NK cells (**Figure 3D**), B cells (**Figure S4B**), and monocytes (**Figure S4B**), and the fraction of proliferation in each cell type by Ki67 staining (**Figure 3E**). In JD76, the numbers of CD8$^+$ T cells and both NK cell subsets increased

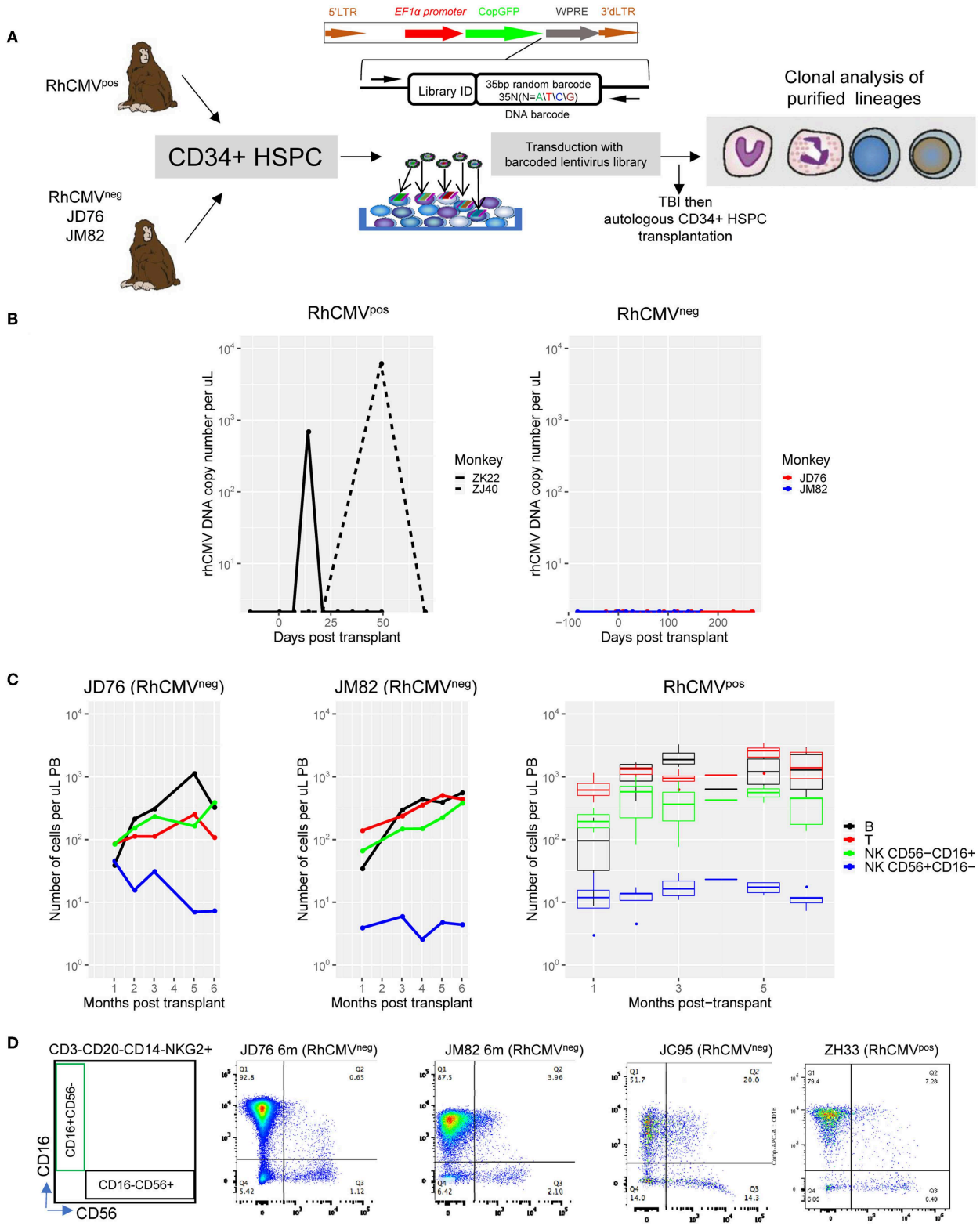

FIGURE 1 | Experimental design and post-transplant reconstitution. **(A)** Pictorial representation of the experimental plan. The study includes five RhCMV^{pos} RM and two RhCMV^{neg} RM (JD76 and JM82) that were autologously transplanted with barcoded CD34^{+} HSPCs. One RhCMV^{neg} RM(JC95) was left untransplanted as normal blood donor. **(B)** RhCMV DNA in plasma of RhCMV^{pos} (ZK22 and ZJ40) and RhCMV^{neg} (JD76 and JM82) RMs before and post transplantations. The cell

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FIGURE 1 | counts for B cells (black line), T cells (red), and CD56^{-}CD16^{+} NK cells (green) and CD56^{+}CD16^{-} NK cells (blue) over the first 6 months post-transplant for **(C)** RhCMV^{neg} and RhCMV^{pos} monkeys. The median and the 25–75% percentile range of cells count from 5 RhCMV^{pos}(ZH33, ZG66, ZH19, ZJ31, and ZK22) for each lineage are shown. **(D)** FACS plots showing the CD16 and CD56 expression of CD3^{-}CD20^{-}CD14^{-}NKG2^{+} NK cells for RhCMV^{neg} (JD76, JM82, and JC95) and a representative RhCMV^{pos} monkey (ZH33). The schematic of gating for sorts is shown on the left.

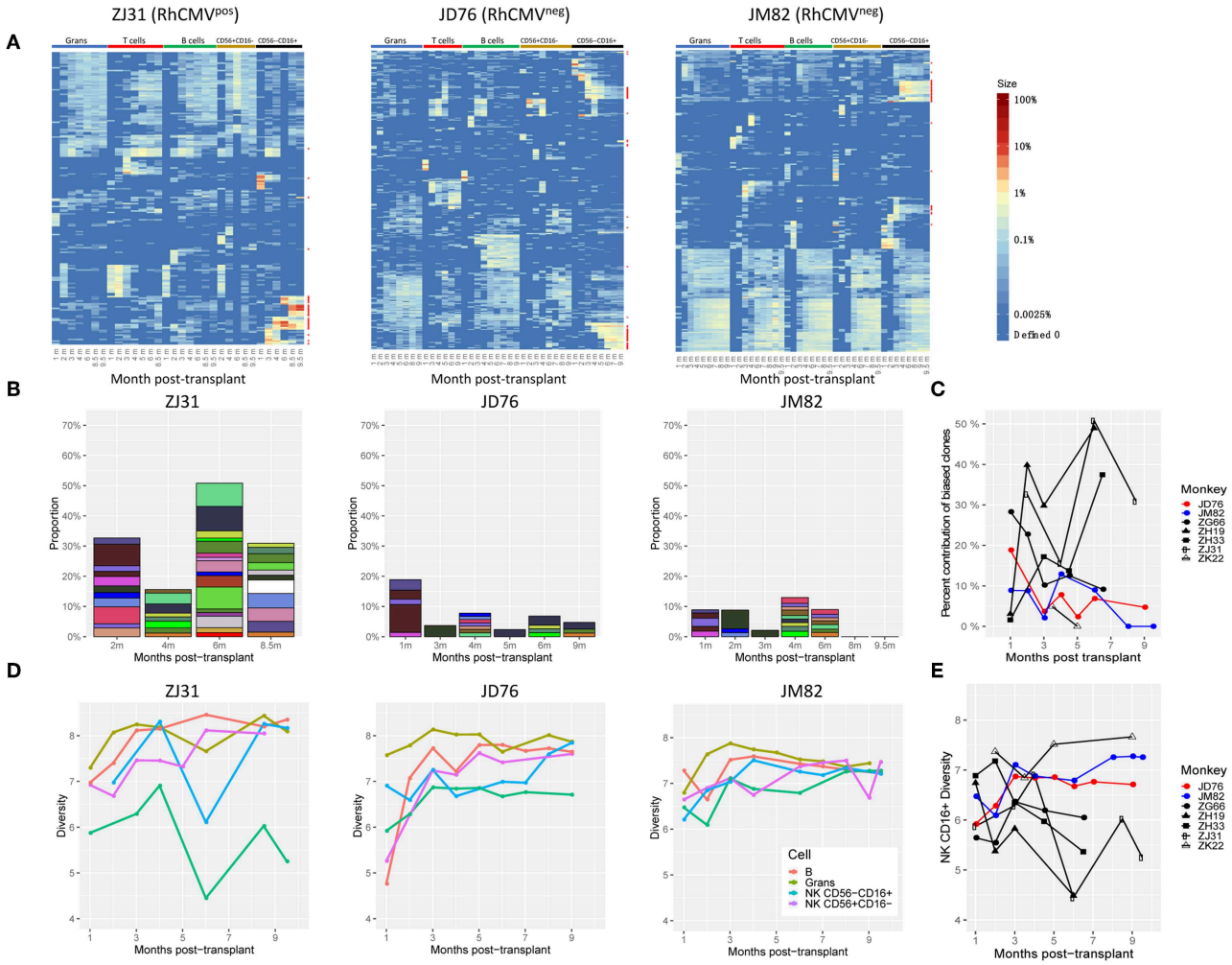

FIGURE 2 | Clonal characterization of NK cells post-transplantation. **(A)** The upper panels show heatmaps of the top 10 barcoded clones chosen by rank order relative contributions to each sample for RhCMV^{pos} monkey ZJ31 and RhCMV^{neg} monkeys JD76 and JM82 over time post-transplantation. Each column shows a single sample and each row represents an individual barcode (clone). Contributions from the top 10 clones for each sample are plotted over all samples included in the analysis. Colors represent the relative percent contribution of the barcoded clone in that sample (column) as shown in the color bar on the right. Clones (rows) with at least 10-fold greater contribution to CD56^{-}CD16^{+} NK cells (termed biased clones) than to any other lineage, including T cells, B cells, granulocytes and CD56^{+}CD16^{-} NK cells are designated with red stars. **(B)** The stacked bar plots showing the fractional contribution of the biased CD56^{-}CD16^{+} NK cell clones over time for RhCMV^{pos} monkey ZJ31 and RhCMV^{neg} monkeys JD76 and JM82 post transplantation. Each colored box represents a barcode clone. **(C)** The total percent contribution of CD56-CD16+ NK-biased clones for RhCMV^{pos} (black lines) and RhCMV^{neg} (red and blue lines) RM over time. **(D)** Shannon diversity plots of each lineage [T, B, Granulocytes(Grans), CD56^{-}CD16^{+} NK and CD56^{+}CD16^{-} NK] over time for RhCMV^{pos} ZJ31 and RhCMV^{neg} JD76 and JM82 post transplantation. **(E)** Shannon diversity of CD56^{-}CD16^{+} NK cell lineage in various RM (black: RhCMV^{pos} monkeys, red and blue: RhCMV^{neg} monkeys).

markedly after infection, coincident with increased proliferation. In JM82, administration of the anti-CD3 immunotoxin delayed the initial increase in CD8^{+} T cells, accompanied, as expected, by more marked initial proliferation of NK cells. In untransplanted JC95, T cells and CD56^{-}CD16^{+} NK cells increased following infection. Overall, there was a rapid and marked immune response to RhCMV infection in all compartments. Of note, we also observed a second increase in cell numbers and proliferation of T and NK subsets at 4-5m post RhCMV infection in all three animals (**Figures 3D,E**).

We followed the clonal patterns over time in each lineage before and after RhCMV infection. As shown in **Figure 4A**, JD76

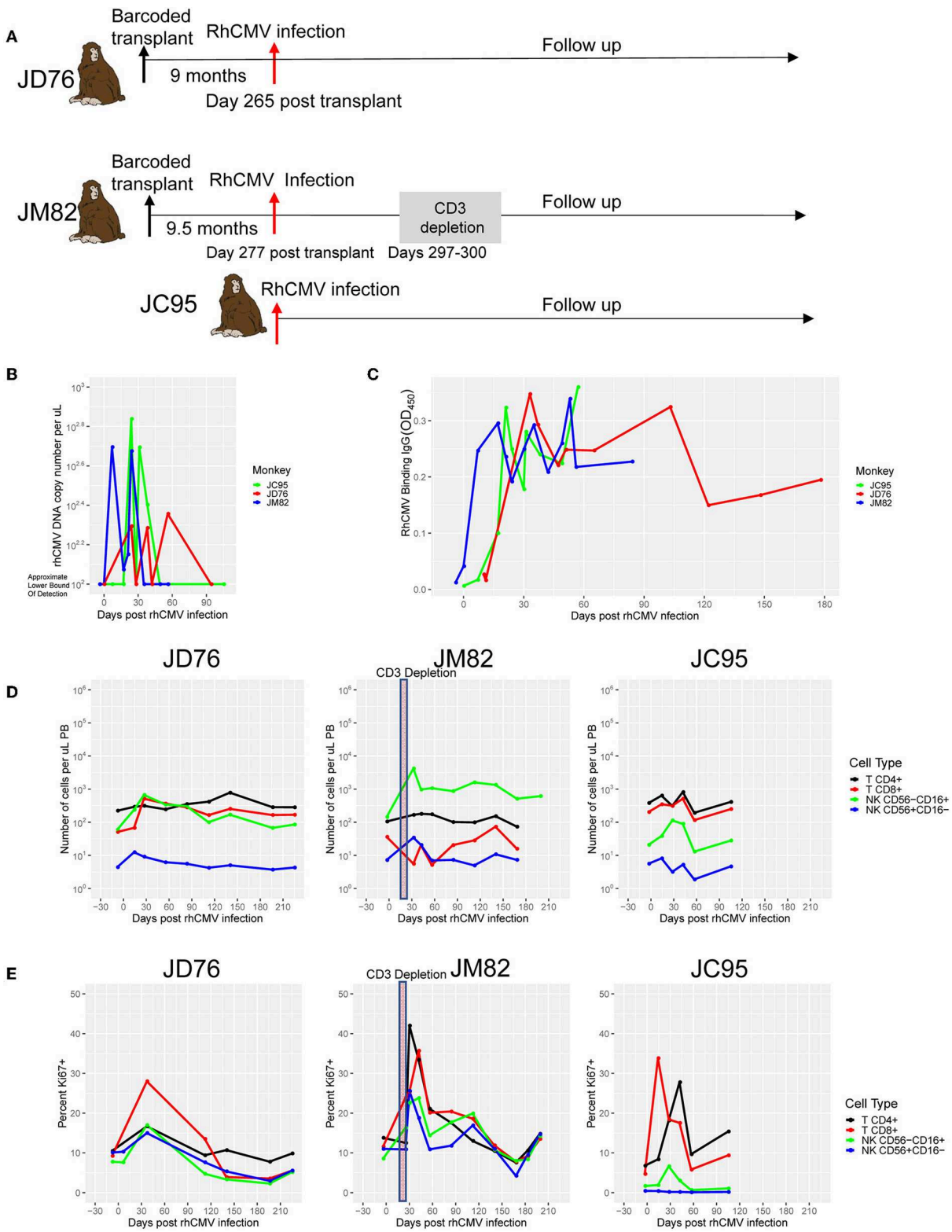

FIGURE 3 | Experimental RhCMV infection. **(A)** Experimental design for RhCMV infection. RMs were infused with RhCMV 9 and 9.5-months post-transplantation. JM82 was T-depleted on days 20-23 post-infection (days 297–200 post-transplant). RhCMV plasma viral load and IgG serologies were followed over time and blood samples were collected for barcode analysis. **(B)** RhCMV DNA copy number detection in plasma post-infection. The lower limit of detection is estimated by the

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FIGURE 3 | number of copies obtained that received a non-empty read from the qPCR machine on two of three replicates. **(C)** Anti-RhCMV IgG in plasma post-infection for the three investigated RMs. **(D)** Cell counts per ul post-infection for CD4+ and CD8+ T cells and $CD56^{-}CD16^{+}$ and $CD56^{+}CD16^{-}$ NK cells. **(E)** The $Ki67^{+}$ percentages in each cell population over time before and after RhCMV infection. The shaded boxes on JM82 plots in panel D and E indicate $CD3^{+}$ T depletion.

developed a very prominent T cell clonal response, with a number of T cell clones becoming dominant and strongly biased toward the T cell lineage, implying peripheral expansion, beginning at about 1-month post-infection (red stars in **Figure 4A**), coinciding with the proliferation and increase in total T cells observed in this compartment (**Figures 3D,E**). We confirmed that these expanding T cell clones were primarily $CD8^{+}$ T cells (**Figure S4C**). In contrast, following peri-infection T depletion in JM82, no marked changes in T cell clonality were observed at 1 month post-infection; however, groups of enlarging T cell clones did appear 3 months post RhCMV infection in JM82, upon later regeneration of the T cell compartment (**Figure 4A**, red stars on the left y axis).

In $CD56^{-}CD16^{+}$ NK cells following RhCMV infection, we did not observe any immediate (<1 month post infection) marked changes in the clonal contributions in JD76 examining the largest contributing clones on heatmap analyses (**Figure 4A**) despite an increase in NK $CD56^{-}CD16^{+}$ cell numbers in the PB (**Figure 3D**). No trackable clones increased in relative contribution more than 10-fold during the first month post-infection. Thus, the rapid proliferation and increase in numbers of circulating mature NK cells immediately following infection in this animal appeared to result from a polyclonal, clonally non-specific response. However, at 2 m post-infection (11 m post-transplantation), when mature NK numbers had stabilized, the clonal profile markedly changed, with multiple new NK-biased clones expanding in relative contribution and persisting for up to 8 m post-infection (green stars on the right of heatmaps in **Figure 4A**) and a relative decrease in contributions from some large clones present before infection (black stars in **Figure 4A**). These clonal shifts could have resulted from preferential proliferation or enhanced survival of specific expanding clones and/or exhaustion or differential contraction of the disappearing NK clones. The mature NK clonal pattern in JM82 (T cell depletion) also showed disappearance of a set of biased NK clones beginning 0.5 m following infection (black stars) and expansion of a new set of small clones beginning 2 m post-infection and persisting (green stars) (**Figure 4A**), but most of the marked proliferation and increase in NK numbers early after infection in this animal appears to have resulted from a non-specific, pan-clonal stimulation.

We analyzed autocorrelations (Spearman) of all clonal contributions to $CD56^{-}CD16^{+}$ NK over time (**Figure 4B**), comparing relatedness of clonal patterns to the immediately preceding sample. The $RhCMV^{pos}$ animals overall showed relative stability by 3–6 months post-transplant, indicating slow and steady clonal modulations, other than one marked change at 6 months in ZJ31. However, in both $RhCMV^{neg}$ animals, autocorrelations sharply dipped following RhCMV infection (marked by arrows **Figure 4B**), indicating shifts in clonal composition between adjacent time points. While these shifts could have occurred for other reasons, the timing is very suggestive for a link between the clonal shifts and RhCMV infection. These analyses suggest that RhCMV infection did significantly impact on mature NK clonal dynamics, resulting in exhaustion of some clones and relative expansion and persistence of new clones.

Clonal Pattern Following Re-infection With RhCMV

We studied potential adaptive/memory responses to RhCMV by re-inoculating JM82 with RhCMV at 10 months post the first RhCMV infection (at day 575, or 19.5 m post transplantation), immediately following T cell depletion (**Figure 5A**), resulting in clear but short-lived viremia (**Figure 5B**). Not surprisingly, RhCMV was cleared much more quickly than following the initial infection(9 days vs. 57 days post infection) (**Figures 3B**, **5B**). Both $CD56^{-}CD16^{+}$ and $CD56^{+}CD16^{-}$ NK cells again expanded and proliferated following re-infection (**Figures 5C,D**), with some increase in T cell numbers and marked residual T cell proliferation ($Ki67^{+}$) despite anti-CD3 immunotoxin administration. Notably, in the post-reinfection samples, the peak Ki67 percentage in $CD56^{-}CD16^{+}$ NK cells was much higher than post-initial infection (∼50 vs. 25%). We analyzed the clonal pattern following re-infection, and did not observe any major new clonal shifts, as analyzed by both clustering heat map analysis (**Figure 5E**) and autocorrelation analysis (**Figure 5F**), other than 3 defined expanded $CD56^{-}CD16^{+}$ NK clones appeared at 1 m post-reinfection from the existing clones (red stars on the right of heatmap, **Figure 5E**).

Increase in $NKG2C^{+}$ $CD16^{+}$ NK Cells Following RhCMV Infection

It has been hypothesized in the literature that NKG2C, an activating receptor, is a marker of NK cell adaptive responses in humans (22, 41), in contrast to NKG2A, the inhibitory isoform (42). Given the lack of antibodies that discriminate between the two isoforms in macaques, RNA probe single cell staining and analysis by FACS was recently shown to be a feasible alternative methodology for analysis of RM NK cells, and $RhCMV^{pos}$ macaques were shown to have a higher fraction of $NKG2C^{+}$ NK cells than $RhCMV^{neg}$ macaques (36). Using this approach, we analyzed NKG2C and NKG2A expression in $CD56^{-}CD16^{+}$ NK cells in two $RhCMV^{pos}$ macaques (**Figure 6A**), as well as in samples before and after RhCMV infection in the three $RhCMV^{neg}$ macaques (**Figure 6B**). The three $RhCMV^{neg}$ macaques greatly increased the fraction and absolute number of $NKG2C^{+}$ NK cells (**Figures 6B–D**), accounting for almost all of

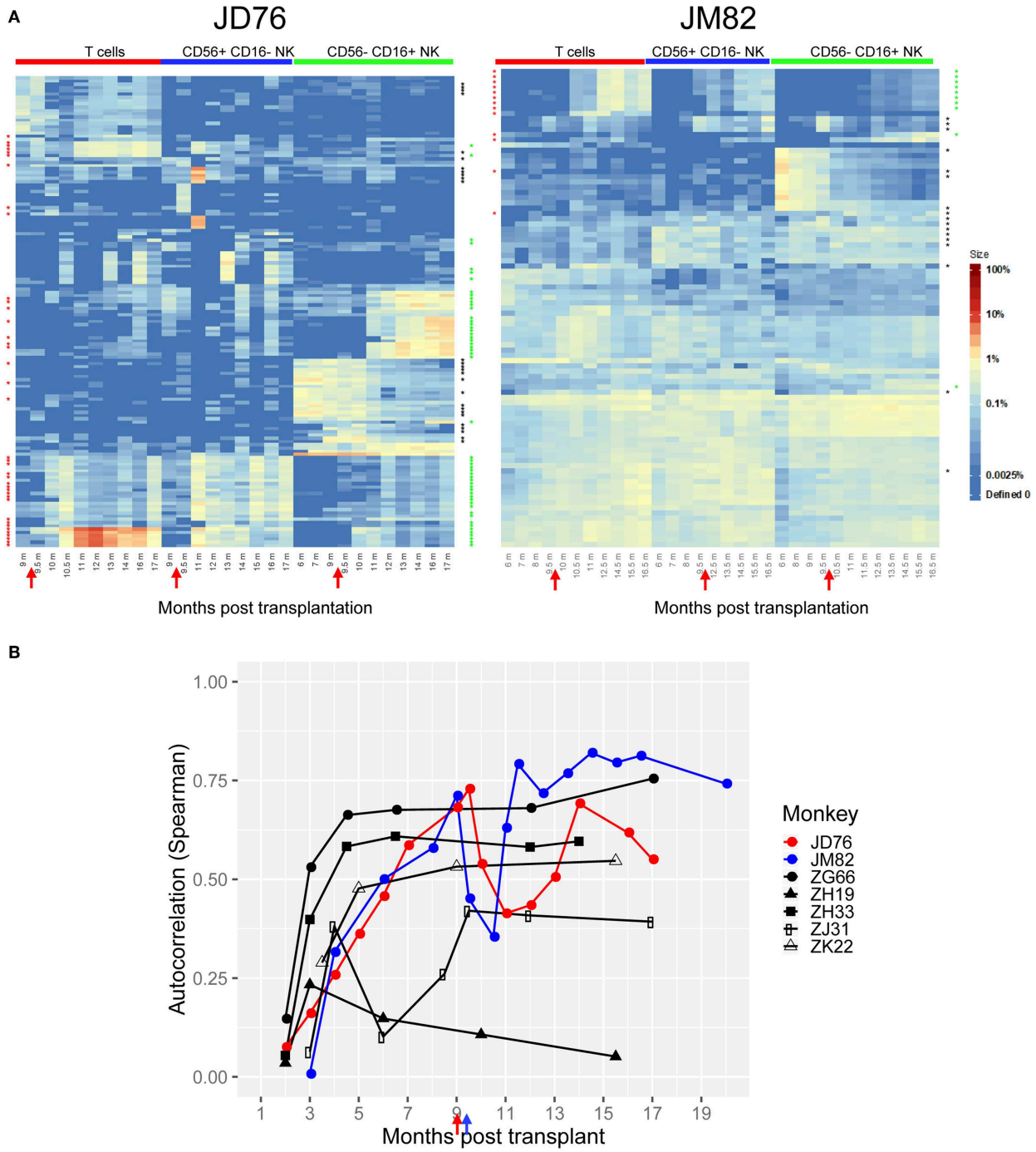

FIGURE 4 | Impact of RhCMV infection on immune cell clonality. **(A)** Heatmaps displaying the contributions from the top 10 clones from each sample plotted over time in animals JD76 and JM82 before and after RhCMV administration, constructed as explained in the legend to **Figure 2**. The red arrows on the x axis show when RhCMV was administered in relation to months post-transplantation. The color scale for fractional clonal contributions is shown on the right. Red stars to the left of each heat map designate barcodes (clones) that increased fractional contributions >10 fold to T cells between the pre-RhCMV time point and post-RhCMV time points. Green stars to the right of each heat map designate barcodes that increased fractional contributions >10 fold to $CD56^{-}CD16^{+}$ NK cells between the pre-RhCMV time point and post-RhCMV time points. Black stars to the right of each heat map designate barcodes that decreased fractional contributions >10 fold to $CD56^{-}CD16^{+}$ NK cells between the pre-RhCMV time point and post-RhCMV time points. **(B)** Autocorrelation plots display the Spearman correlation between clonal contributions to adjacent time points in $CD56^{-}CD16^{+}$ NK cells. Samples with close to identical clonal contributions will have an autocorrelation close to 1 and very dissimilar clonal contributions will have an autocorrelation near 0. $RhCMV^{pos}$ RM are shown in black and the two barcoded $RhCMV^{neg}$ RM are shown in red(JD76) and blue(JM82). The autocorrelation between two time points is plotted at the later of the two time points being compared. The arrows in red(JD76) and blue(JM82) on the bottom of X axis indicate the time of RhCMV infection.

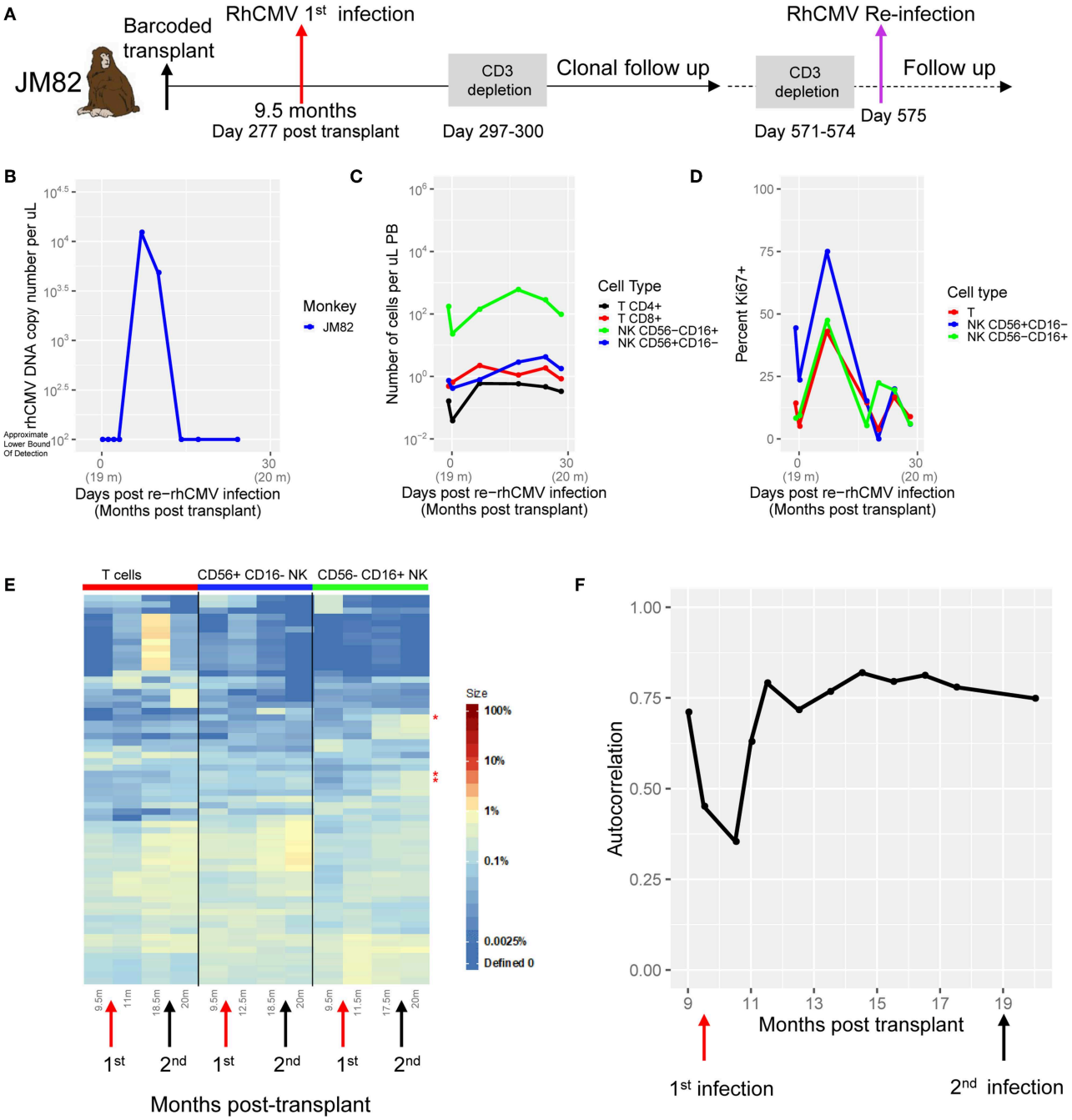

FIGURE 5 | RhCMV Reinfection. **(A)** Experimental design. Reinfection of JM82 at day 298 post-initial infection (day 575 post-transplant). T cells were depleted day 294–297 post-initial infection (571–574 post-transplant). **(B)** RhCMV DNA copies in plasma post-reinfection. **(C)** Cell counts of $CD4^+$ and $CD8^+$ T cells and $CD56^-CD16^+$ and $CD56^+CD16^-$ NK cells post-reinfection. **(D)** $Ki67^+$ percent in each lineage post-reinfection. **(E)** Heatmap for each lineage pre and post-infection. Colors represent percent contribution to a sample. Stars indicate NK cells that are 2 times expanded from baseline. **(F)** Autocorrelation plots display the correlation (spearman) between adjacent time points of the $CD56^-CD16^+$ NK lineage.

the increase in $CD56^-CD16^+$ NK cells in these animals following infection. This proportion stabilized or continued to increase over time post-infection up to 6 m post RhCMV infection. Of note, the fraction and absolute number of NKG2A/NKG2C double positive cells increased before the maximal level of $NKG2C^+$ cells in all three macaques, consistent with a model of transit through a double positive state before final maturation to $NKG2C^+$ adaptive NK cells (22, 36).

Human adaptive NK cells often express both inhibitory and activating killer immunoglobulin-like receptors (KIR) and NKG2C (10, 11). We stained the NK cells with both an anti-human KIR2D (clone NKVFS1) antibody which recognizes the rhesus KIR3DL01 (43, 44) and the NKG2C probes. As shown in **Figure 6E**, in the RhCMV^{pos} monkeys ZJ31 and RQ4753, more than 88% of the KIR3DL01^{+} NK cells express NKG2C as detected by KLRC2 probe. In both monkeys, within the KIR3DL01^{-} NK population, the fraction of cells expressing NKG2C is lower than in the KIR3DL01^{+} NK population. In the 3 RhCMV^{neg} monkeys, two (JD76 and JC95) express KIR3DL01^{+} on a fraction of their CD56^{-}CD16^{+} NK cells. Both KIR3DL01 positive and negtive NK cells increased NKG2C expression to a similar degree following CMV infection in JC95. In contrast in JD76, there appeared to be differential expansion of NKG2C^{+} cells expressing this particular KIR following RhCMV infection, suggesting this KIR potentially could interact specifically with RhCMV (**Figure 6F**).

DISCUSSION

We previously reported oligoclonal expansions of mature macaque NK cells appearing rapidly following transplantation, maintained independently of ongoing maturation from HSPC, and clonally-segregating with expression of specific KIR (18). In the current study, we hypothesized that RhCMV, reactivated following transplantation, might be driving the expansion and persistence of these expanded, long-lived NK clones. The overall pattern of NK cell recovery and phenotype following transplantation was similar in RhCMV^{pos} and RhCMV^{neg} animals. Expanded, NK-biased CD16+ mature NK clones still appeared in the two transplanted RhCMV^{neg} macaques following engraftment, however the size of individual clones and overall contributions appeared to be smaller than observed in the majority of RhCMV^{pos} macaques.

These observations provide some support for the hypothesis that RhCMV does play a role in the stimulation and maintenance of these expanded and persistent mature NK cell clones, but the appearance of CD56^{-}CD16^{+} expanded, NK-biased clones even in the CMV^{neg} animals suggest that other stimuli must contribute to their manifestation. We speculate that additional latent herpes viruses, such as lymphocryptoviral (LCV), the RM equivalent of Epstein Barr Virus (EBV), may be stimulating NK cell clonal expansions due to reactivation post transplantation. The two RhCMV^{neg} animals we transplanted in this study were from "specific pathogen free" colonies, defined as negative for tuberculosis, herpes B virus, type D simian retrovirus, STLV1 (simian equivalent of HTLV1), and SIV (the simian equivalent of HIV), but were both serologically LCV^{pos} at the time of transplantation. EBV reactivation occurs frequently following human HSPC autologous transplantation (45, 46). While recent work focuses primarily on the link between CMV and NK cells with adaptive properties, other studies have linked EBV to mature NK cell responses. For example, it was observed in the study that human CMV^{pos} students acquiring acute EBV infection expanded CD56^{dim} NK cells, albeit expressing the inhibitory receptor NKG2A, not the activating receptor NKG2C associated with CMV infection (47). A recent publication analyzed hematopoietic clonal diversity via insertion site retrieval in children with adenosine deaminase-deficient severe combined immunodeficiency treated with retroviral gene therapy, revealing massive expansion of a CD56^{dim} NK cell clone coincident with EBV reactivation post- transplantation (48).Other reports also suggest important roles for NK cells in response to EBV in human (49, 50). Whether EBV plays role in inducing mature clonal NK expansion would benefit from further investigation in our barcode model (16, 51).

When we experimentally induced primary RhCMV infection in the two barcoded RhCMV^{neg} macaques 9–10 months transplantation, we observed proliferation of T and NK cells in the PB at 0.5–1 months post-infection and an expansion in circulating cell numbers. During this acute phase, coinciding with viremia, clonal patterns in both T and NK cells in terms of relative contributions from individual clones did not markedly change, suggesting a non-specific homogeneous expansion/proliferation of these compartments in response to inflammatory stimuli such as cytokines. 1–2 months following infection, new expansions of both T cell clones and mature CD56^{-}CD16^{+} NK cell clones appeared. Although we observe clear changes in the clonal profile following infection and reinfection, it is difficult to discern if the changes observed are due to specific NK responses to RhCMV infection, given the observation of waxing and waning NK expanded clones in both RhCMV^{pos} and RhCMV^{neg} animals. However, the autocorrelation analyses presented in **Figure 4** suggest a marked specific change beyond underlying clonal fluctuations occurring following RhCMV primary infection in the RhCMV^{neg} animals. In addition, very rapid clearance of a second RhCMV challenge 9 months after the initial infection occurred coincident with CD56^{-}CD16^{+} NK cell proliferation, without major clonal NK shifts, suggesting long-term persistence of the NK RhCMV reactive clonal repertoire. It could be possible that the NK cells expansion is stimulated by other proliferating cell populations such as T cells post RhCMV infection, however, with T cells depletion in JM82 at the time of first RhCMV infection and prior to the second RhCMV infection, we still observed obvious CD56^{-}CD16^{+} NK expansions post each RhCMV infection.

The lack of antibodies able to distinguish NKG2A from NKG2C expression on RM NK cells (40) has hindered direct comparisons between putative human adaptive NK cell responses to RhCMV infection or reactivation, characterized by expansion of NKG2C^{+} mature NK cells in multiple studies (21, 22, 47). Using RNA probes able to distinguish expression of the two genes by flow cytometry (36), as previously used to uncover higher fractions of NKG2C-expressing cells in RhCMV^{pos} vs. RhCMV^{neg} animals, we documented increase expression in CD16^{+} NK cells in the three animals we monitored before and after infection, further supporting the relevance of our model to NK dynamics in humans following RhCMV infection. We also observed that about 90% of the KIR3DL01^{+}CD16^{+} NK cells were NKG2C^{+} in both RhCMV^{pos} and RhCMV^{neg} monkey post

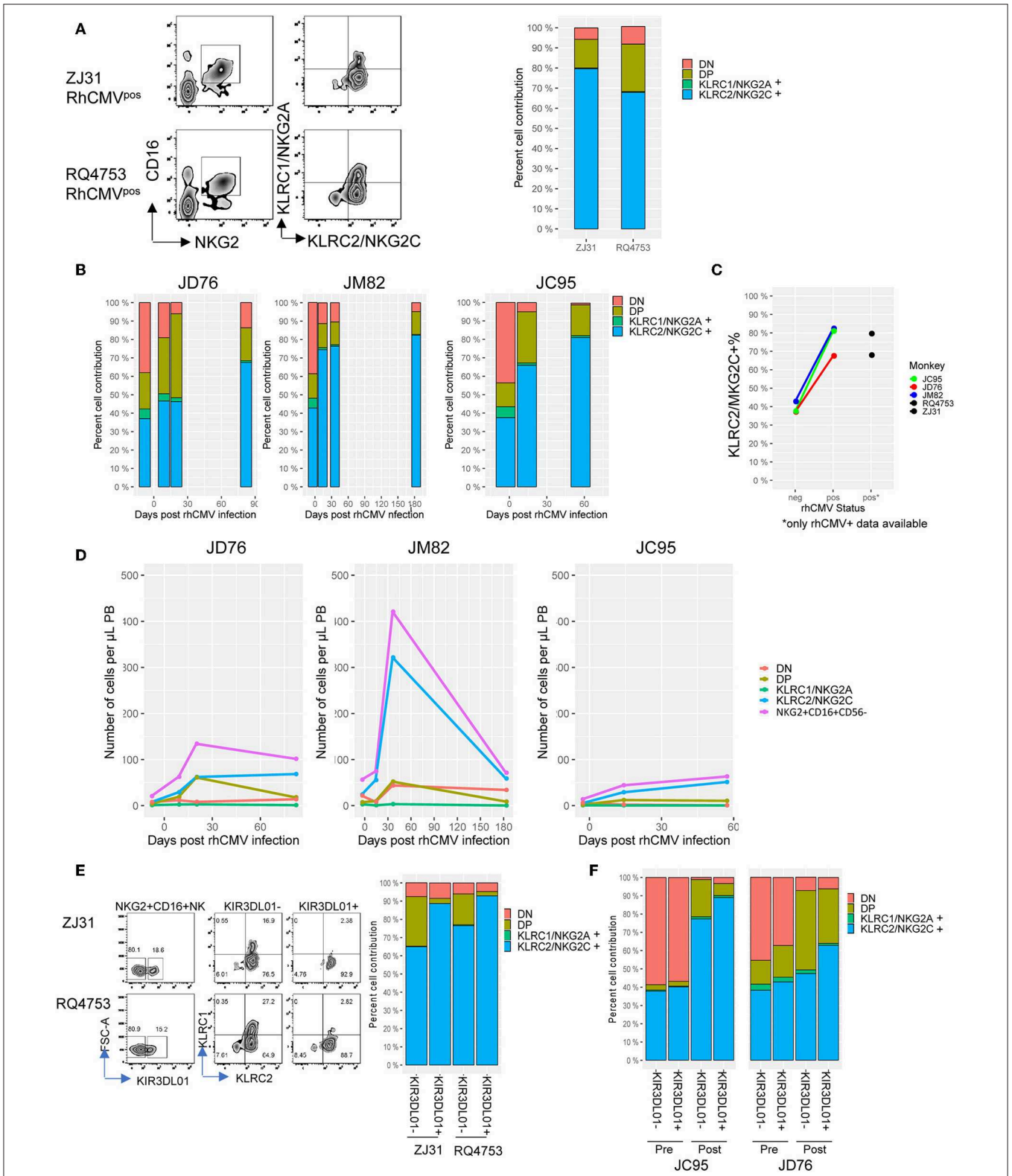

FIGURE 6 | Analysis of NKG2A vs. NKG2C transcript expression in CD56-CD16+ NK cells. **(A)** FACS plots for KLRC1 (NKG2A) and KLRC2 (NKG2C) RNA analysis for RhCMV^{pos} animals (barcode monkey ZJ31 and untransplanted RQ4753). The right panel shows a barplot for the two animals where the percent KLRC1/NKG2A^{+}, KLRC2/NKG2C^{+} double positive (DP) and double negative (DN) CD56^{-}CD16^{+} NK cells is plotted. **(B)**The NKG2A/C profile in CD56^{-}CD16^{+} NK cells over time

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FIGURE 6 | before and post RhCMV infection. **(C)** Comparison of the percent NKG2C positive $CD56^{-}CD16^{+}$ NK cells in $RhCMV^{neg}$ and $RhCMV^{pos}$ animals. Lines connect the pre-infection and latest post-infection sample for the initially $RhCMV^{neg}$ animals, and $RhCMV^{pos}$ animals are shown as black points. **(D)** Absolute cell numbers for each cell population in the PB post initial infection over time from JD76, JM82, and JC95. Total $CD56^{-}CD16^{+}$ NK cell numbers are shown in purple. **(E)** left panels show the FACS plots for the expression of KIR3DL01 and KLRC1/KLRC2 on $NKG2^{+}$ $CD56^{-}CD16^{+}$ NK cells from two $RhCMV^{pos}$ monkeys. Right panel shows the a barplot for the two animals where the percent contribution to $CD56^{-}CD16^{+}KIR3DL01^{+}$ and $CD56^{-}CD16^{+}KIR3DL01^{-}$ NK subpopulations is plotted for $KLRC1/NKG2A^{+}$, $KLRC2/NKG2C^{+}$, double positive (DP) and double negative (DN) cells. **(F)** Barplots for the two $RhCMV^{neg}$ animals (JC95 and JD76) where the percent contribution to $CD56^{-}CD16^{+}KIR3DL01^{+}$ and $CD56^{-}CD16^{+}KIR3DL01^{-}$ NK subpopulations is plotted for $KLRC1/NKG2A^{+}$, $KLRC2/NKG2C^{+}$, double positive (DP) and double negative (DN) cells, samples from pre- and post RhCMV infection are shown.

infection, this results strongly links these two adaptive memory markers together to provide further evidences for NK adaptive immune features.

In conclusion, by studying $RhCMV^{neg}$ animals and subsequently infecting them in a rhesus macaque model allowing tracking of individual NK cell clones, we have shown long-lasting clonal expansions arising in response to RhCMV, suggesting a clonal adaptive response with the potential to retain immunological memory. These analyses raise additional questions regarding NK dynamics in response to environmental cues with relevance to clinical adoptive NK cell transfer which we will examine in future barcoding experiments in the rhesus macaque model.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in github.com (https://github.com/dunbarlabNIH/CMV).

ETHICS STATEMENT

The animal study was reviewed and approved by the NIH Animal Care and Use Committee (ACUC).

AUTHOR CONTRIBUTIONS

Conceptualization: CW, CD, AK, LT, and DY. Analytics and statistical analyses: LT and DE. Investigation: LT, DY, DE, XF, DR, MM, DT, LS, and CW. Resources: AK, RR, and RD. Writing: LT, CD, and CW. Supervision: AK, CD, and CW.

FUNDING

This study was supported by NHLBI Divisions of Intramural Research; the Scientific Research Training Program for Young Talents sponsored by Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (DY); The TNPRC Base Grant NIH/OD OD011104 (AK); The NIH grant R01 DE026014 (to RR), and DR was also supported, in part, by NIH training grant T32 AI007387.

ACKNOWLEDGMENTS

We thank Naoya Uchida for providing the χHIV plasmid, Keyvan Keyvanfar for support of flow cytometric analyses, the NHLBI FACS and DNA Sequencing and Genomics Cores, and the NIH Biowulf High-Performance Computing Resource. We thank the animal care staff for careful maintenance of the $RhCMV^{neg}$ animals and support of all animal care and procedures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02381/full#supplementary-material

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

frontiers in Immunology

REVIEW
published: 11 October 2019
doi: 10.3389/fimmu.2019.02357

Natural Killer Cells in Myeloid Malignancies: Immune Surveillance, NK Cell Dysfunction, and Pharmacological Opportunities to Bolster the Endogenous NK Cells

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Natural killer (NK) cells are large granular lymphocytes involved in our defense against certain virus-infected and malignant cells. In contrast to T cells, NK cells elicit rapid anti-tumor responses based on signals from activating and inhibitory cell surface receptors. They also lyse target cells via antibody-dependent cellular cytotoxicity, a critical mode of action of several therapeutic antibodies used to treat cancer. A body of evidence shows that NK cells can exhibit potent anti-tumor activity against chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and myelodysplastic syndromes (MDS). However, disease-associated mechanisms often restrain the proper functions of endogenous NK cells, leading to inadequate tumor control and risk for disease progression. Although allogeneic NK cells can prevent leukemia relapse in certain settings of stem cell transplantation, not all patients are eligible for this type of therapy. Moreover, remissions induced by adoptively infused NK cells are only transient and require subsequent therapy to maintain durable responses. Hence, new strategies are needed to trigger full and durable anti-leukemia responses by NK cells in patients with myeloid malignancies. To achieve this, we need to better understand the interplay between the malignant cells, their microenvironment, and the NK cells. This review focuses on mechanisms that are involved in suppressing NK cells in patients with myeloid leukemia and MDS, and means to restore their full anti-tumor potential. It also discusses novel molecular targets and approaches, such as bi- and tri-specific antibodies and immune checkpoint inhibitors, to redirect and/or unleash the NK cells against the leukemic cells.

Keywords: NK cells, myeloid malignancy, cancer immunotherapy, drug development, NK cell dysfunction

OPEN ACCESS

Edited by:
Emily Mace,
Columbia University, United States

Reviewed by:
Evelyn Ullrich,
Goethe University Frankfurt, Germany
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: *02 August 2019*
Accepted: *19 September 2019*
Published: *11 October 2019*

Citation:
Carlsten M and Järås M (2019) Natural Killer Cells in Myeloid Malignancies: Immune Surveillance, NK Cell Dysfunction, and Pharmacological Opportunities to Bolster the Endogenous NK Cells. Front. Immunol. 10:2357. doi: 10.3389/fimmu.2019.02357

INTRODUCTION TO NATURAL KILLER CELLS, THEIR RECEPTORS, AND ROLE IN THE IMMUNE SYSTEM

The natural killer (NK) cell was discovered in the mid-1970s based on its ability to lyse certain tumor cells without prior sensitization of the host (1–4). Based on this, and the understanding that both T and B cells in contrast to NK cells need to undergo somatic gene rearrangement to become fully functional with specific immunity that quickly respond upon

recalling, NK cells have for long been considered innate immune cells. However, more recent data have challenged this perception by demonstrating that NK cells also can carry memory-like features (5). Today, NK cells are explored in a wide variety of contexts, including, but not limited to, infectious diseases, autoimmunity, pregnancy, and cancer. Thus, from an unknown cell type with undetermined biological meaning and significance in the mid-1970s, it has now more than 40 years later been recognized that NK cells are key components of our immune system.

NK cells have traditionally been classified as group 1 innate lymphoid cells and develop from hematopoietic stem cells (HSCs) while maturing outside the bone marrow compartment (6, 7). They have the capability to migrate to a number of tissues to launch immune responses to infections and cancer (8). The basis for target recognition by NK cells was revealed in the mid-1980s when the "missing-self" hypothesis was postulated (9). However, as predicted by the investigators at that time, activation signals are needed in addition to "missing-self" to trigger cytotoxicity (10). Today, we know that a delicate interplay between an array of germ-line encoded receptors expressed on the NK cell surface control NK cell degranulation (**Figure 1**) (11, 12), a cytotoxicity mechanism that lyses target cells via the release of substances such as perforin and granzymes. The key receptors controlling self-recognition by human NK cells are HLA class I-binding receptors, including the Killer Immunoglobulin-like Receptor (KIR) family as well as the Natural Killer Group 2A (NKG2A) and Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1, also referred to as LIR-1) (11). The inhibitory KIRs and the NKG2A receptor have also been shown to be involved in NK cell education, a functional maturation process that allows self-inhibited NK cells to become potent killers upon interaction with cells losing self-HLA class I expression (13). In contrast to the inhibitory receptors, an array of activation, co-activation, and adhesion receptors such as the natural cytotoxicity receptors (NCRs) NKp30 and NKp46 and the NKG2D, 2B4, and DNAM-1 receptors trigger NK cell activation following binding to ligands up-regulated on cells undergoing stress and/or infection (11). Under normal conditions when NK cells are not heavily activated by cytokines, at least two of these receptors need to be stimulated simultaneously to trigger degranulation (14). This is in contrast to the FcγRIIIA receptor (CD16a), that upon ligation to the Fc portion of an antibody bound to a target cell alone potently can trigger degranulation (15). This process is referred to as antibody-dependent cellular cytotoxicity (ADCC). Importantly, engagement of the LFA-1 receptor on the NK cell is required in most situations to direct the granulae release toward the target cell and thereby trigger efficient target lysis (15). The latter adds another layer to how NK cell cytotoxicity is regulated. In addition to target cell lysis via the release of granzymes and perforin, NK cells also kill cells via stimulation of death receptors on the target cell surface, which triggers caspase-dependent apoptosis (16). Both

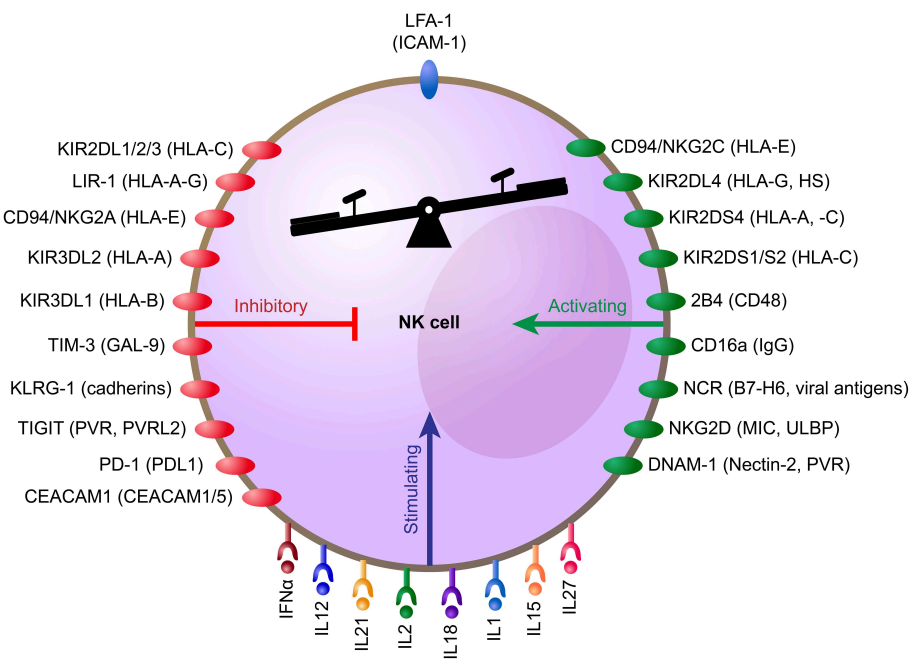

FIGURE 1 | NK cell receptors, their function, and ligands. Schematic illustration showing how NK cell activity and cytotoxicity are controlled by signals from cell surface receptors. Cytokines and corresponding cytokine receptors on the NK cell are shown at the lower part of the NK cell. Inhibitory signals triggered by receptors (red) upon engagement of their ligands (in brackets) are shown on the left side of the NK cell. Activating signals triggered by receptors (green) upon engagement of their ligands (in brackets) are shown on the right side. Binding of LFA-1 (blue) on NK cells to ICAM-1 on target cells direct the granulae release toward the target cell, which is needed for efficient target cell lysis.

TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) on the NK cell surface can trigger caspase-mediated apoptosis in target cells expressing TRAIL-R1 and/or -R2 and Fas, respectively (17). Importantly, NK cells do not only kill infected and tumor-transformed cells via these mechanisms, but also utilize these receptors to control immune responses by killing, i.e., T cells (18, 19).

NK cells have several functions in the immune system. Based on data from individuals with severe NK cell deficiencies and data from experimental animal models, it has been recognized that they are highly implicated in controlling Epstein–Barr virus (EBV) (20–22), but also involved in the defense against Herpes simplex virus (HSV) infections (23). Moreover, it is well-established that NK cells can react to cytomegalovirus (CMV) infection and prevent CMV reactivation following allogeneic stem cell transplantation (SCT) (24, 25). Beyond their role in viral infections, NK cells have an immunomodulatory role either by directly controlling other immune cells (18, 19) or by release of chemokines and cytokines that can attract and stimulate both innate and adaptive components of the immune system (26, 27). NK cells also have a documented role in pregnancy (28). Given the rapid advances in our understanding of NK cells, additional functions for these cells in the body will likely be unveiled in the near future.

The role for NK cells in cancer has been addressed since the discovery of this lymphocyte subset. Over the years, it has become clear that NK cells are involved in tumor immune surveillance (29). Indirect evidence comes from cohort studies showing that individuals with poor NK cell function early in life have a higher risk of presenting with cancer compared to matched controls (30). Clinical observations also indicate that a ligand repertoire on acute myeloid leukemia (AML) blasts favoring NK cell activation is positively linked to better outcome of patients undergoing chemotherapy (31). More direct evidence from animal models indicate that knock-out of key NK cell receptors such as NKG2D and DNAM-1 leads to higher incidence of tumor formation compared to in mice with wild-type expression of these receptors (32, 33). Another line of evidence comes from clinical studies on allogeneic SCT and adoptive NK cell infusion showing NK cells can be utilized to treat patients with cancers, including myeloid malignancies (34, 35). This has opened up a new field focusing on NK cell-based cancer immunotherapies that all aim to bolster the NK cell tumor targeting capacity to improve outcomes of patients with cancer (36). In parallel to this development, more and more studies also demonstrate that NK cells in patients with cancer are defective, and in some cases also few in numbers, indicating a potential breach of NK cell-mediated tumor immune surveillance that may facilitate disease progression. Dysfunctional NK cells have been reported in both solid tumors (37) and hematological malignancies, including myeloid malignancies (38, 39). For some of these cancers, it has also been proposed that restoration of the NK cell function after treatment with cytoreductive chemotherapy, or other targeted drugs, can re-establish NK cell-mediated cancer control. As will be discussed below, a prime example of this is chronic myeloid leukemia (CML), but there are also data reporting that this can occur in other myeloid malignancies such as AML and myelodysplastic syndromes (MDS) as well as in chronic myelomonocytic leukemia (CMML). Notably, in contrast to malignancies of the myeloid lineage, data on the role for NK cells in targeting B cell-derived leukemias such as acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) are less clear and will not be discussed in this review.

This review will focus on our current understanding of the role for NK cells in targeting malignant myeloid cells and thereby preventing the initiation and/or the progression of AML, MDS, and CML, and how malignant cells in these diseases can evade NK cell recognition. Methods to circumvent and/or restore this imbalance will be discussed. In the emerging era of immune checkpoint inhibitors and tumor targeting antibodies, including bi- and tri-specific killer engagers, the review will have a special focus on the mechanisms governing suppressed NK cell function in these diseases and means to restore the NK cell phenotype and function to define potential opportunities to use such drugs in clinical practice. As other reviews and articles have comprehensively covered the role of NK cells in settings of allogeneic SCT and adoptive cell transfer to treat AML, CML, or MDS, our review will only touch upon these topics. Instead, this review will have a particular focus on the endogenous NK cells and their therapeutic potential and limitations.

EVIDENCE FOR NK CELL-MEDIATED TARGETING OF MALIGNANT MYELOID CELLS AND DATA SUPPORTING A ROLE FOR NK CELLS IN THE TREATMENT AND CONTROL OF CML, AML, AND MDS

Introduction to CML, AML, and MDS—Biological and Clinical Similarities and Differences

Although originating from the myeloid lineage, CML with its 9;22 translocation that creates the BCR/ABL fusion gene is biologically and clinically very different from AML and MDS. From being a disease with high mortality following transformation to blast crisis, where allogeneic SCT was considered the only treatment option that could offer a potential cure, CML is now efficiently treated using tyrosine kinase inhibitors (TKIs) (40). Unfortunately, similar approaches have not been equally successful for MDS and AML, diseases that do not express the BCR/ABL tyrosine kinase fusion gene but are rather triggered and driven by multiple mutations. In contrast to CML, the more aggressive AML disease as well as high-risk MDS are generally associated with dismal outcome. Hence, there is an urgent need of identifying new and more efficient treatment options for these malignancies. To successfully design new therapies that induce durable responses, it is likely key to understand the underlying disease and how it potentially compromises the immune system. For deeper understanding of the CML, AML, and MDS diseases *per se*, please see references (41–43).

NK Cell-Mediated Targeting of Tumor-Transformed Myeloid Cells via Natural Cytotoxicity and Its Role in Treating Patients With Myeloid Malignancies

Preclinical studies have firmly demonstrated that NK cells can kill leukemic cells of the myeloid lineage. Data derive from studies using leukemia cell lines, but also freshly isolated leukemic blasts from patients with CML, AML, or MDS. In addition to these studies exploring the potential of primary human NK cells, studies have also demonstrated that NK cell lines can target primary as well as immortalized AML and CML cells (44).

The first series of experimental studies on this topic were conducted using AML and CML blasts and published just a few years after the NK cell was first described. In a small *ex vivo* study published already 1983, investigators were able to show that freshly explanted CML blasts could be lysed by interferon (IFN)-activated NK cells from healthy donors (45). As demonstrated in a paper from the group of Ronald Herberman a few years later (1989), the main basis for prevention of clonogenic growth of freshly explanted AML and CML blasts or cells from pre-leukemic patients (today called MDS) was cell-to-cell interaction, although soluble factors produced by the NK cells were also involved (46). Importantly, the anti-leukemia activity was only detectable in these experiments when enriched NK cell populations were used. The need for cell-to-cell contact to trigger NK cell-mediated inhibition of autologous CML blast growth has later been verified in other studies (47).

The more recent studies on this topic have mainly focused on targeting AML cells with NK cells *in vitro*. Most studies have addressed the potential of resting and overnight cytokine-activated [i.e., interleukin (IL)-2 or IFN] NK cells (39, 45). Other studies have explored the potential of *ex vivo* expanded NK cells (48, 49). The molecular specificity of NK cell-mediated cytotoxicity of leukemic cells is based on several receptor–ligand interactions. For instance, the NKG2D and DNAM-1 receptors as well as the NCRs have been reported important for the targeting of AML and CML blasts (50–52), whereas studies on freshly isolated MDS blasts have revealed that the DNAM-1 receptor is central with contributions from the NKG2D receptor and the NCRs NKp30 and NKp46 (39). It is also evident from the literature that blockade of inhibitory KIR, CD94/NKG2A, and LIR-1 augment NK cell-mediated killing of leukemic blasts (53), indicating that they express enough HLA class I to at least partially inhibit NK cells. The role for these activation and inhibition receptors in targeting of myeloid malignancies by NK cells will be discussed in more detail in section Means to Restore NK Cell Function and Trigger Their Cytotoxicity Against Myeloid Malignancies below.

Exploring Human NK Cells to Target CML, AML, and MDS Cells Implanted in Animal Models

Until today, the vast majority of xenografted mouse models used to explore the anti-leukemia potential of primary human NK cells have focused on human leukemia cell lines. One of the major reasons for this is that engraftment of primary AML, CML, and MDS cells has historically been difficult, with only recently reaching robust and reliable engraftment rates in optimized models (54–56). Furthermore, the use of human leukemia cell lines enables the researcher to introduce luciferase and/or fluorescent proteins (such as green fluorescent protein; GFP) to efficiently track the tumor burden in the mice. This is exemplified in several studies on human xenografted leukemia, which will be discussed below.

Ex vivo expanded peripheral blood NK cells can prevent leukemia development in severe combined immunodeficiency disease (SCID)-beige mice and NOD-*scid* IL2Rgamma^{null} (NSG) mice inoculated with K562 cells (49, 57). In line with this, investigators have also shown that NK cells generated from CD34^{+} hematopoietic stem cells *ex vivo* as well as from cord blood cells can clear K562 cells in mice (58, 59). Moreover, cytokine-induced killer cells, featuring a mixed NK and T-cell phenotype, were capable of mediating potent reduction of tumor burden in mice engrafted with the AML cell line THP-1 (60). In contrast to utilizing human leukemia cell lines as targets in the animal models, the ability of primary human NK cells to target xenografted primary myeloid leukemia in mice has only been highlighted in few studies. One example of the latter comes from a study that efficiently utilized *ex vivo* expanded human NK cells expressing a single KIR (61). There are also data addressing the role for primary human NK cells targeting primary xenografted autologous myeloid leukemia. As demonstrated by Siegler et al. (62), *ex vivo* expanded NK cells are able to target xenografted autologous AML blasts. In this study, the authors speculate that up-regulation of the NKG2D receptor and the NCRs following *ex vivo* expansion and activation of the NK cells prior to adoptive infusion into the mice was key to govern the anti-leukemic effects. Although several models have been used to establish that primary human NK cells can target leukemic cells implanted in mice, we predict that development of more advanced models will be valuable tools to explore how the leukemic cells can negatively affect the adoptively infused NK cells in detail.

Data on Utilizing NK Cells to Treat Patients With Myeloid Malignancies

Data supporting NK cell-mediated rejection and control of myeloid leukemia in patients have been generated from studies on allogeneic SCT. In 2002, Ruggeri et al. reported that KIR-ligand mismatching in the graft-vs.-host (GvH) direction of donor NK cells was key to prevent AML relapse following haploidentical SCT (34). In line with these data, Hsu et al. also demonstrated that the genomic lack of one or more ligands in the recipient for donor KIR was associated with improved outcome in AML and MDS in settings of T-cell-depleted HLA-identical sibling transplantations (63). Studies on large transplantation cohorts have also linked certain KIR genotypes and KIR–KIR-ligand genotype pairs that also include activating KIRs to post-transplant control of leukemia (64–66). More recent data also indicate the expansion of adaptive NK cell subsets post-transplantation is linked to improved outcome in AML, which

adds an additional layer to the role of NK cells in post-transplant relapse protection (67).

The potential of utilizing mature NK cells in setting of adoptive cell transfer to treat myeloid leukemia patients was demonstrated by Miller et al. (35). In this study, 19 patients with relapsed/refractory AML were treated with overnight IL-2-activated haplo-identical NK cells. In this patient population with very advanced high-risk disease, 5 out of 19 patients had a complete remission (CR). Remarkably, four out of the five responders had received donor NK cells with a KIR-ligand mismatch in the GvH direction. Following this publication, there has been an explosion of clinical trials demonstrating improved outcome of AML and MDS patients treated with NK cells in different settings (49, 68–73). Of note, most of these studies have not been able to demonstrate a beneficial effect of KIR-ligand mismatching. This may relate to the relative loss of cell surface HLA class I expression on the myeloid blasts compared to the lymphocytes. As demonstrated by Verheyden et al., the relative expression of HLA class I, and especially HLA-C, was markedly down-regulated on myeloid blasts compared to autologous T cells potentially leading to reduced inhibition by HLA-Bw4- and HLA-C-binding KIRs and thereby attenuation of the role for KIR-ligand mismatching (74). Instead, data indicate that outcomes following adoptive NK cell therapy are positively predicted by presence and expansion of donor NK cells and dampened host immune activation post NK cell infusion as well as removal of regulatory T cells prior to NK cell infusion (72, 75). As shown by Romee et al., adoptive infusion of memory-like NK cells can trigger anti-AML responses while leading to improved persistence of the NK cells (49). Another factor that has been highlighted in more recent studies is the dose of alloreactive NK cells. This has been demonstrated in the setting of adoptive NK cell infusion as post-consolidation therapy for elderly patients with AML (70), and also in the setting of pre-allogeneic SCT for patients with AML, MDS, or CML (71). Nevertheless, due to the relatively poor persistence of adoptively infused NK cells, objective clinical responses induced in these settings are only transient. Hence, these protocols can be used as a bridge to an allogeneic SCT or maybe to deepen responses in the post-consolidation setting, but not cure patients with myeloid malignancies.

Collectively, the capacity of NK cells to target AML, MDS, and CML blasts *in vitro* and in xenografted mouse models is well-documented with clear involvements of the NKG2D and DNAM-1 receptors, but also the NCRs. Based on data from CML, AML, and MDS patients undergoing allogeneic SCT, it is clear that NK cells do have a role in the clearance and control of myeloid malignancies in certain settings. Although adoptive NK cell transfer can be effective and adds to the notion that NK cells can be utilized to target myeloid malignancies, clinical remissions are only transient. An alternative approach that may induce durable remissions without the need of cellular therapy would be to bolster the anti-tumor potential of the endogenous NK cells. This approach has until now been relatively unexplored and likely been limited due to leukemia-induced dysfunction of the NK cells in these patients. With the increased knowledge, we predict that this approach will be a more viable option in the near future. NK cell dysfunction in myeloid malignancies and how to restore it will be described in the following sections of this review.

NK CELL FUNCTION AND MATURATION IN PATIENTS WITH MYELOID MALIGNANCIES AT DIAGNOSIS AND UPON TREATMENT

NK Cell Numbers and Function During Treatment and Disease Progression

The anti-leukemic activity of NK cells inversely correlates to disease progression in AML—the NK cells are suppressed at diagnosis, restored at remission, and again suppressed at relapse (76, 77). Similarly, in MDS, the cytolytic activity of NK cells is severely altered, even in the presence of IL-2 stimulation *in vitro*, as compared to NK cells from healthy donors (78). In CML, the NK cells decrease in number along disease progression, respond less to stimuli, and exhibit reduced cytolytic activity (79, 80). Similar to AML patients in CR, CML patients with a major molecular response (MMR) to TKIs have restored cytolytic functions of NK cells (81). In support of NK cells being involved in immune control of CML cells, patients with a high percentage of NK cells at the time of TKI discontinuation had a better long-term outcome (82). Also, the role for "missing-self" reactivity by endogenous uneducated NK cells has been highlighted in CML patients treated with TKI. Patients carrying non-interacting KIR3DL1 and HLA-B allele pairs, leading to less inhibition of NK cells upon interaction with CML blasts, have better outcome upon TKI treatment (83). In AML, higher cytolytic activity of NK cells predicts a better long-term outcome of patients at both diagnosis and in remission (84–87). In addition, high expression of the activating NK cell receptors NKp30 or NKp46 predicts a better outcome (38, 88–90). The role for "missing-self" genotypes has, like for CML, also been associated with an improved outcome in AML following post-consolidation treatment with dihydrochloride and low-dose IL-2 that activates NK cells (91). In a follow-up study, the investigators identified that the efficacy against AML was linked to a dimorphism in HLA-B at amino acid −21 that has an impact on NK cell education (92), again supporting a critical role for NK cells in this disease. In a separate study, the outcome following treatment of AML and high-risk MDS with the hypomethylating agent Azacytidine could be predicted by NK cell function after three to six cycles (93). Taken together, NK cell function is often suppressed upon diagnosis and at disease progression of myeloid malignancies, but restored in remission. Increased number of NK cells as well as more activated NK cells at diagnosis and following remission correlate with better outcome for patients treated with hypomethylating agents, TKI and IL-2. These findings suggest that NK cells have a central role in the control of myeloid malignancies by counteracting disease progression.

Altered Maturation of NK Cells in Myeloid Malignancies

Normal NK cell differentiation is defined by combinations of markers that include CD56, CD16a, CD57, KIRs, and

NKG2A (94). Immature NK cells ($CD56^{bright}CD16a^{-}CD57^{-}$) are cytokine-producing cells with low cytotoxic activity, whereas more mature NK cells ($CD56^{dim}CD16a^{+}CD57^{+}$) have higher cytotoxic activity (95). NK cell differentiation is characterized by down-regulation of NKG2A and up-regulation of KIR, which alter their reactivity given the HLA class I repertoire expressed on the target cell. In myeloid malignancies, NK cell maturation was suggested to be perturbed with a selective loss of an immature NK cell population in both AML patients and in leukemic mice (77, 96). This loss of primitive NK cells was accompanied by an increased percentage of phenotypically more mature ($CD56^{dim}KIR^{+}CD57^{+}$) NK cells in the peripheral blood of AML patients (97). However, opposing findings of a decreased proportion of mature NK cells ($CD56^{dim}CD16a/CD57^{bright}$) in AML and MDS have also been reported (98). Consistent with previous findings by Martner et al. (99), Chretien et al. divided the AML patients into three subtypes based on the NK cell maturation and found that patients with more immature NK cells had reduced relapse free and overall survival, suggesting that disease-induced alterations in NK cell maturation affect patient outcome (100). Therapies can also affect the differentiation stage of the NK cells. In first remission, an increased percentage of immature ($CD56^{bright}$) NK cells in AML patients has been observed, possibly because the NK cells are under reconstitution after intense chemotherapy (101). In CML, treatment with the TKI dasatinib is associated with differentiation of NK cells (102). Upon MMR or molecular response (MR), CML patients have more mature cytolytic NK cells ($CD57^{+}CD62L^{-}$), indicating restoration of NK cell function (81). Although several of the studies described above found that disease-induced mechanisms and certain treatments influence the maturation of NK cells in myeloid malignancies, interpretations of how the maturation stage of NK cells in this context affect their anti-leukemic activity is so far mainly based on correlative findings. Hence, more studies are needed to clarify how the maturation stage of NK cells in myeloid malignancies is perturbed and affected by treatment both in a short- and, more importantly, long-term perspective. Single-cell RNA-sequencing, which is an emerging methodology that recently has increased our understanding of NK cell regulation (103, 104), has the potential to further clarify how NK cell maturation is affected by treatment.

Collectively, disease-induced mechanisms in myeloid malignancies negatively affect core properties of NK cells such as their differentiation and cytotoxic potential correlating to disease progression. Moreover, the NK cell function during and after treatment is linked to treatment responses and outcome, suggesting that NK cells play a key role in controlling myeloid malignancies. By further characterizing the mechanistic basis for how NK cell dysfunctions arise and how NK cell differentiation and function is modulated by treatment may translate into new treatment opportunities for myeloid malignancies as discussed in more detail below.

THE IMPACT OF SHARED GENETIC ABERRATIONS BETWEEN NK CELLS AND MALIGNANT MYELOID CELLS

The cellular origin of myeloid malignancies is thought to be a normal HSC that first acquires genetic lesions that give rise to a pre-malignant clone (105–107). In support of this hypothesis, early genetic aberrations associated with clonal hematopoiesis and myeloid malignancies can be found in multiple hematopoietic lineages, including NK cells, affecting their function (**Figure 2A**). Although NK cells isolated from chronic phase CML patients were found to be *BCR/ABL1* negative (108, 109), Nakajima et al. observed $BCR/ABL1^{+}$ NK cells in advanced phases of the disease (110). The reason why $BCR/ABL1^{+}$ NK cells are found predominantly in advanced phases of the disease is currently unclear but might be due to an expansion of the malignant stem cell pool during disease progression that gradually outcompetes normal HSCs. By contrast, T cells were always *BCR/ABL1* negative, suggesting that the presence of *BCR/ABL1* is not compatible with T cell development (110). To evaluate the impact of *BCR/ABL1* on NK cell differentiation and function, *BCR/ABL1* was introduced into cord blood $CD34^{+}$ cells and the NK92 NK cell line (111). Enforced *BCR/ABL1* expression in cord blood $CD34^{+}$ cells resulted in altered NK cell differentiation (110), and in NK92 cells, a decreased cytotoxicity was observed (112). Consistent with these findings, $BCR/ABL1^{+}$ NK cells from CML patients grown in culture had reduced cytotoxic and proliferative capacity (113). In contrast, $BCR/ABL1^{+}$ dendritic cells selectively activate NK cells, demonstrating that NK cells can also be affected by other non-myeloid cell lineages that express *BCR/ABL1* (114).

Although early studies did not detect chromosomal aberrations in NK cells from MDS patients (115, 116), later studies reported aneuploid NK cells ranging from 20 to 60% in MDS (78, 117). In addition to acquired mutations shared with the malignant cells and NK cells in patients, certain congenital mutations that pre-dispose for MDS/AML are associated with defects in NK cells. One such example is SAMD9L gain-of-function mutations that pre-disposes for MDS and are associated with defects in myeloid cells, B and NK cells (118). Also, constitutive Gata2 mutations that pre-disposes for MDS/AML are associated with alterations in NK cells as evidenced by an accumulation of terminally differentiated NK cells (119). In AML, DNMT3A mutations, which are early and often initiating events associated with clonal hematopoiesis (120), are found in NK cells, but to a lesser extent in B and T cells (121).

Taken together, early genetic aberrations driving malignant transformation are detected in a substantial fraction of NK cells in patients with myeloid malignancies. Some of these aberrations as exemplified by enforced *BCR/ABL1* expression in NK cells negatively affect NK cell cytotoxicity and differentiation. Future studies combining genetic characterization by massive parallel sequencing of NK cells with functional NK cell assays are expected to further clarify the full functional impact of cancer-associated genetic aberrations co-existing in NK cells.

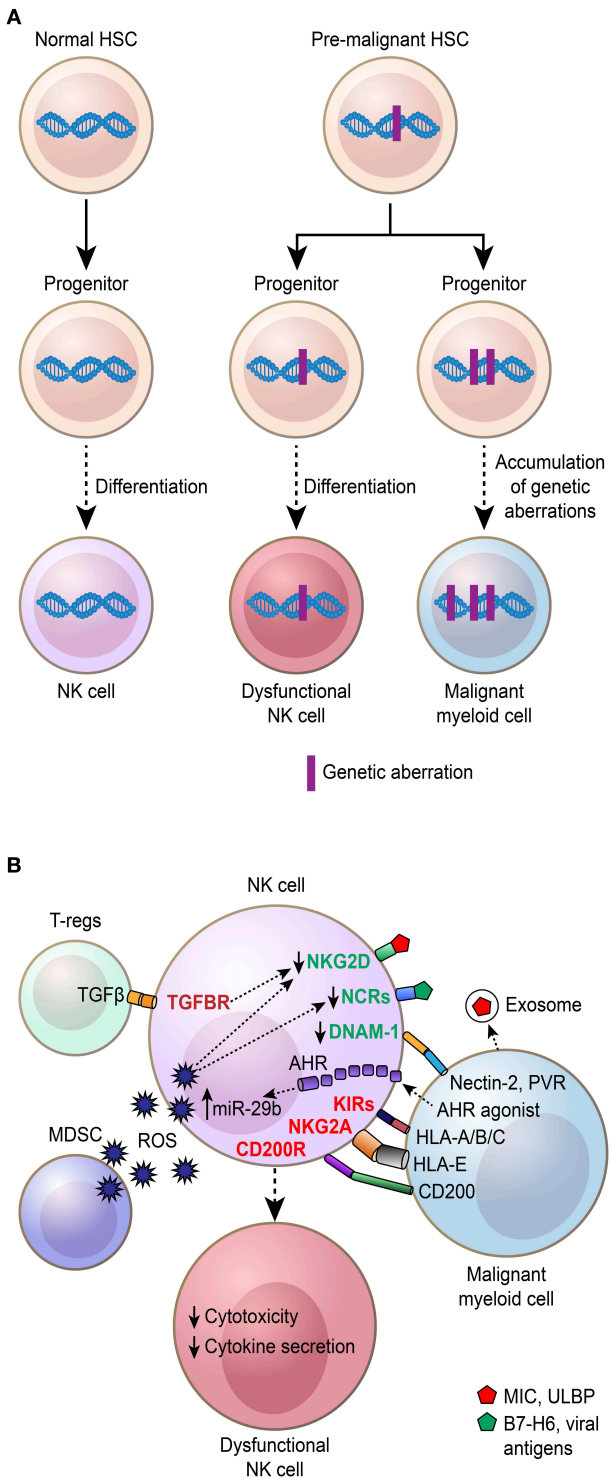

FIGURE 2 | Mechanisms behind NK cell dysfunction and phenotypic/maturation alterations in myeloid malignancies. Schematic illustration showing how various mechanisms contribute to NK cell dysfunction and phenotypic/maturation alterations. **(A)** NK cells arise from hematopoietic stem cells (HSCs) that through progenitor stages differentiate to mature NK cells (purple). Initiating genetic aberrations in myeloid malignancies are thought to arise in HSCs, referred to as pre-malignant HSCs, that among other cell

(Continued)

FIGURE 2 | types give rise to NK cells, which are dysfunctional in their cytotoxic capacity and have altered maturation. **(B)** NK cells are regulated by various secreted factors and cell–cell interactions affecting their cytotoxic and cytokine-secreting capacity. Regulatory T cells (Tregs), myelo-derived suppressor cells (MDSC), and malignant myeloid cells contribute to the suppression of NK cells that become dysfunctional with altered cytokine secretion and reduced cytotoxic capacity.

MECHANISMS SUPPRESSING NK CELLS IN MYELOID MALIGNANCIES AND MEDIATING ESCAPE FROM NK CELL RECOGNITION

As appreciated from the previous section, NK cells in patients with CML, AML, and MDS are often, if not always, dysfunctional compared to healthy control NK cells. An array of mechanisms has been identified, including but not limited to soluble factors, cell-to-cell interactions, and other regulatory elements in the tumor microenvironment (**Figure 2B**). As described above, mutations affecting the NK cell population can also contribute to poor function of these effector cells (118). Below, we will discuss the so far known mechanisms driving the development of dysfunctional NK cells in these diseases.

Several studies published today have linked poor NK cell function with altered NK cell subset composition, phenotype, and ability to form a fully functional immunological synapse (38, 39, 122–127). In some cases, these alterations have been linked to poor clinical outcome (38, 125). Most of these studies have highlighted down-regulation of key activation NK cell receptors such as NKG2D, DNAM-1, and the NCRs, down-regulations that do not seem to correlate with the subtype of AML or MDS (38, 39, 122–124, 128). Nevertheless, studies have shown that the loss of these receptors positively correlates to the leukemia burden in the patients (38, 39, 123) and that it can be fully, or at least partially, restored in patients achieving CR following chemotherapy (38). In fact, data show that NK cell-to-tumor cell interactions can trigger the loss of DNAM-1 and NCRs (37, 38, 126, 129). Receptor–ligand interactions, triggering internalization of the activation NK cell receptor, has been highlighted as one of the most critical mechanisms (37, 38, 129, 130). Loss of activating receptors, such as NKG2D, can also be triggered by the presence of soluble molecules in the tumor microenvironment. As shown by Boissel et al. and several other groups, soluble NKG2D ligands (NKG2D-Ls) including MICA, MICB, ULBP1, and ULBP2, shedded by the tumor cells *per se*, and tumor exosomes expressing NKG2D-Ls trigger the reduction of NK cell surface NKG2D (131–135). In this context, it should be highlighted that reports indicate that AML blasts, including AML stem cells, may also evade NK cell-mediated killing by expressing low or no NKG2D-Ls (52, 136, 137). The NKG2D receptor can also be down-modulated via cytokines such as TGF-β (138). In addition to these mechanisms governing suppressed NK cell function leading to poor NK cell-mediated targeting of leukemic cells, data from a pre-clinical animal model on *de novo* AML along with collected NK cell from patients with AML have

indicated that the microRNA (miRNA) miR-29b, a regulator of T-bet and Eomes, can be elevated in NK cells via AML cell-induced activation of the transcription factor aryl hydrocarbon receptor that directly up-regulates miR-29b expression resulting in incomplete maturation and poor cytotoxicity (96, 139). Other soluble mechanisms involve the release of Tim-3 that prevent the release of IL-2 while increasing the release of galectin-9 and thereby hamper NK cell cytotoxicity and targeting of primary AML cells (140). Despite that CD137 (4-1BB) is a therapeutic target for agonistic antibodies in clinical development that stimulate NK cells and T cells (141), stimulation of CD137 expressed on the surface of activated NK cells has been shown to suppress their function in AML (142). The CD137L was primarily identified in AML of the monocytic lineage, although it was found on other AML subtypes too (142). Further studies are needed to dissect the exact role for this interaction in regulating NK cell function in myeloid malignancies.

Several other mechanisms behind the escape of myeloid malignancies from NK cell recognition have also been described. Data show that down-regulation of ligands for DNAM-1 on the leukemic cell surface renders the cells resistant to NK cell targeting (143). Another study suggests that the leukemic blasts can avoid NK cell recognition by expressing low levels of NCR and NKG2D ligands, a resistance that can be reverted following exposure to differentiation-promoting myeloid growth factors and IFN-γ (136). In a separate study, expression of the oncogenic fusion proteins PML-RARA and AML1-ETO found in acute promyelocytic leukemia (APL) and some non-APL AMLs, respectively, was associated with the loss of the 2B4 ligand CD48 on the leukemia cell surface (144). Interestingly, CD48 expression was increased on APL cells following exposure to an HDAC inhibitor (HDACi). On the contrary, increased levels of IFN-γ in the tumor microenvironment may lead to up-regulation of HLA class I, and especially HLA-E, on the tumor cells leading to immune escape by inhibition of NK cells via the CD94/NKG2A receptor (145). Along these lines, up-regulation of the glycoprotein CD200 on AML cells resulted in escape from NK cell-mediated lysis via interaction with the CD200 receptor on the NK cell surface, a phenomenon that was restored using a CD200 inhibitory antibody (128).

Factors in the tumor microenvironment can also play a critical role. In addition to suppressed NK cell function, it has been demonstrated that NK cell proliferation can be inhibited by the tumor while not influencing the NK cell viability and cytotoxicity *per se* (146). As shown in CML, AML, and CMML, reactive oxygen species (ROS) can trigger both apoptosis of NK cells in the tumor microenvironment but also reduced NK cell function connected to reduced expression of activation NK cell receptors (147–149). Data also show that cell-to-cell interactions between AML cells and mesenchymal stromal cells render the AML cells less susceptible to NK cells (150). More details on the role for the tumor microenvironment learnt from other malignancies are not discussed in this review as they have been reviewed elsewhere (151).

In conclusions, an array of mechanisms has been proposed to trigger NK cell suppression, reduced NK cell numbers, and escape from NK cell-mediated recognition. Most of them have been addressed in studies on tissue samples from patients or in *ex vivo* experiments with NK cell co-cultures. Although shown in experimental animal mouse models (152), the loss of function of adoptively infused NK cells in human has not yet been systematically addressed. Nevertheless, understanding these mechanisms is key to developing new NK cell-based therapies against myeloid malignancies, especially those relying on endogenous NK cells and that may lead to long-term non-chemotherapy-based control of these diseases. The next section will discuss means to restore and/or trigger anti-leukemic responses and tumor control by NK cells.

MEANS TO RESTORE NK CELL FUNCTION AND TRIGGER THEIR CYTOTOXICITY AGAINST MYELOID MALIGNANCIES

Dysfunctions of NK cells associated with myeloid malignancies restrain tumor immune surveillance, but may also limit therapeutic options that depend on NK cells for their mode-of-action. In addition to drugs used in the clinic that restore NK cells such as TKI for CML and hypomethylating agents for MDS and AML, a number of pharmacological strategies to re-establish and/or bolster NK cell function, including cytokines, engineered antibodies, and small-molecule drugs, are currently being explored with the aim of utilizing the endogenous NK cells to clear and control myeloid malignancies (**Figure 3**).

Cytokines Including Histamine and IL-2

In 1998, high-dose IL-2 was the first immunotherapy approved for metastatic malignant melanoma and showed durable responses in a subset of patients (153). Although associated with significant toxicity, IL-2 therapy demonstrated that cytokine-induced activation of the immune system, including T cells and NK cells, can have long-term beneficial effects in certain cancers. IL-2 have in pre-clinical studies shown therapeutic efficacy by restoring NK cell receptor expression and bolster NK cell cytotoxicity against autologous AML blasts *in vitro* (48), but clinical studies evaluating IL-2 monotherapy in AML and MDS have been disappointing (154–156). However, in contrast to monotherapy, Brune et al. demonstrated that combining histamine with low-dose IL-2 treatment in AML results in improved leukemia-free survival (157). Histamine dihydrochloride acts by enhancing the immune-promoting properties of IL-2 by reducing production of immunosuppressive reactive oxygen species (ROS) (158), which leads to expansion of $CD56^{bright}$ NK cells (90, 159). For this therapy, a high expression of NKp30 and NKp46 on $CD16a^{+}$ NK cells before and during treatment predicted leukemia-free and overall survival (90). In addition to activating NK cells, it is a concern that IL-2 also stimulates Tregs, which are immunosuppressive and counteract NK cell activation (160). For histamine with low-dose IL-2 treatment, a promising observation was that the increase in Tregs was transient, whereas the increase in NK cells was more long-lasting (161). In contrast to IL-2, IL-15 that activates memory T cells and bulk NK cells is associated with less toxicity, suggesting that IL-15 has several advantages over IL-2 in a clinical setting

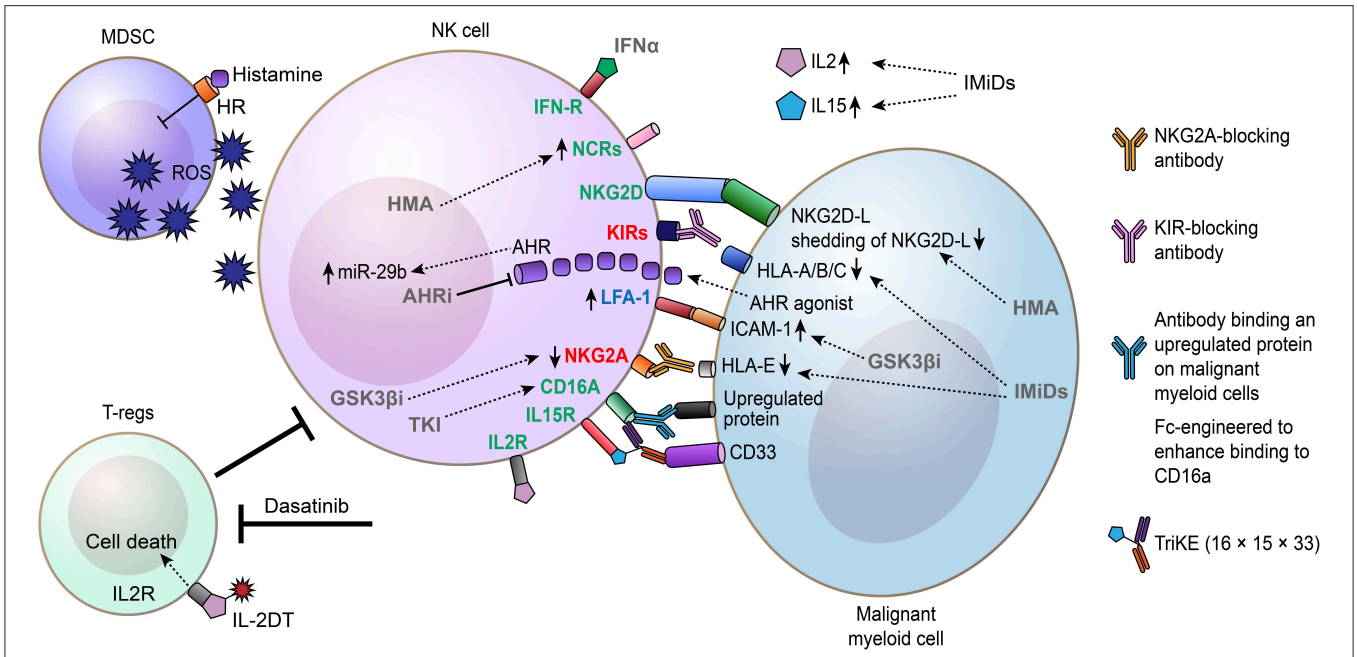

FIGURE 3 | Drugs and approaches explored to restore and augment the antileukemia-capacity of NK cells. Schematic illustration showing how drugs can restore, augment, and direct NK cell-mediated killing of malignant myeloid cells. Drugs promoting NK cell-mediated killing of myeloid malignant cells by directly affecting NK cells, inhibiting regulatory T cells (Tregs) or myelo-derived suppressor cells (MDSC), and/or affecting malignant myeloid cells are shown. IL-2DT, IL-2 diphtheria toxin fusion protein. IMiDs, immunomodulatory imide drugs (include thalidomide and analogs such as lenalidomide and pomalidomide). AHRi, aryl hydrocarbon receptor inhibitor; GSK3βi, GSK3β inhibitor; HMA, hypomethylating agents (includes azacytidine and decitabine).

(160). Partially, by inducing the expression of the activating NK cell receptor NKp30, IL-15 was found to enhance the cytotoxicity of NK cells from AML patients (162, 163). When expressed in a non-secreted form in NK cells, IL-15 stimulated autonomous NK cell growth and increased their cytotoxicity against leukemia and lymphoma cells in cultures and in mice (164). However, recent reports indicate that chronic or repetitive exposure of IL-15 to NK cells lead to NK cell exhaustion (165, 166), suggesting that the long-term effects of IL-15 should be carefully monitored in future studies.

In CML, interferon alpha (IFN-α) was used as a standard treatment prior to the TKI era. Although the full mechanistic basis for how IFN-α has antileukemic activity is unknown, IFN-α has been shown to boost the function of endogenous NK cells (167). Another cytokine that has been shown to bolster NK cells in CML is IL-2. In line with findings described above for IL-2 in AML, Cervantes et al. used IL-2 to stimulate autologous NK cells and demonstrated selective suppression of CML progenitor cells relative to corresponding normal progenitors (47).

Small-Molecule Drugs

As discussed in section Mechanisms Suppressing NK Cells in Myeloid Malignancies and Mediating Escape From NK Cell Recognition, one mechanism that has been put forward to explain impaired tumor immune surveillance by NK cells in myeloid malignancies is long-term exposure of soluble NKG2D ligands such as MICA, MICB, and ULBP2 secreted by the malignant blasts. Consistent with this notion, hypomethylating agents (azacytidine and decitabine) that are used to treat AML and MDS patients were found to decrease shedding of MICA, MICB, and ULBP2 and restore NK cell function (168). In line with these findings, Vasu et al. reported that decitabine enhances NK cell cytotoxicity induced by an anti-CD33 monoclonal antibody (mAb) against AML blasts associated with up-regulation of NKG2D (169). In an AML xenograft mouse model, decitabine treatment potentiated NK cell-mediated killing of the AML cells by NKp44 up-regulation, suggesting that hypomethylating agents are promising drugs for enhancing NK cell activity by multiple mechanisms (170). Complementary to decitabine, which up-regulates NKG2D, the HDACi valproic acid was found to induce the expression of NKG2D-Ls on AML cells, rendering them more sensitive to lysis by NK cells (171). Another approach to enhance NK cell function in AML is inhibition of glycogen synthase 3 kinase beta (GSK3β). Parameswaran et al. provided pharmacological and genetic evidence that inactivation of GSK3β restores NK cells from AML patients resulting in enhanced killing of autologous leukemic cells (172). Mechanistically, GSK3β inhibition promoted up-regulation of LFA-1 on NK cells and its partner ICAM-1 on AML cells, associated with increased AML–NK cell conjugates (172).

Another clinically approved drug that improves NK cell function is lenalidomide, used for treatment of multiple myeloma, 5q- MDS, and B-cell lymphomas (173). In patients with relapsed/refractory solid tumors or MDS, lenalidomide treatment was found to increase IL-2 and IL-15 levels accompanied by restoration of NK cell function (174). Similar to

AML, lenalidomide and its derivate pomalidomide potentiated NK cell function (175). The antileukemic activity of these drugs was associated with down-regulation of HLA class I molecules on the AML cells (175). Although lenalidomide has been shown to achieve anti-cancer activity by inducing degradation of essential proteins for 5q- MDS and multiple myeloma cells (176, 177), the mechanistic basis for how lenalidomide activates NK cells is currently unclear.

In CML, a somewhat unexpected finding is that several TKIs (imatinib, dasatinib, and nilotinib), dasatinib in particular, induces expansion of NK cells from diagnostic values, indicating that these therapies promote tumor immune surveillance mediated by NK cells (178, 179). Moreover, TKI therapy results in improved NK cell function and killing of leukemic cells (180). Recent findings revealed that the restored NK cell function by dasatinib treatment is coupled to down-regulation of the NK cell inhibitory receptor NKG2A (181). A positive effect of dasatinib on the immune system was suggested to persist even long-term after stopping treatment, as a CML patient remained in MR several years post-treatment, associated with cellular immunity by memory and effector cytotoxic T lymphocytes and NK cells (182).

Antibody-Based Therapies That Depend on NK Cells for Eradicating Myeloid Malignancies

Therapeutic antibodies can achieve anti-tumor responses not only by modulating the activity of their protein targets but also by redirecting effector cells of the immune system to the cancer cells. By targeting cell surface proteins up-regulated on the malignant cells, a selective immune response can be activated against the cancer cells. In particular, NK cells are critical effector cells for eliciting ADCC. Therapeutic antibodies designed to induce ADCC are predominantly of IgG1 isotype and bind to an antigen on cancer cells and to the low-affinity CD16a on NK cells with their Fc domain. In addition to physically linking the malignant cells and NK cells together, binding of the antibody to CD16a is sufficient to activate the NK cells and induce ADCC, even without additional activation signals (12, 15). One such example is Rituximab, which targets CD20 on B cells, and is used today for treatment of several forms of B cell malignancies (183). Consistent with NK cells playing a key role in mediating ADCC upon Rituximab treatment, patients homozygous for the single-nucleotide polymorphism CD16a-158V, which bind IgG1 with higher affinity than CD16a-158F, showed improved clinical response to Rituximab (184, 185).

For myeloid malignancies, there is a strong rationale to target a chemotherapy-resistant reservoir of self-renewing leukemia cells, referred to as leukemia stem cells, as these are associated with disease relapse after initial responses to therapy (107, 186, 187). Consistent with this hypothesis, antibodies directed to IL3Rα (CD123), which is up-regulated on AML stem-cell-enriched cells, showed anti-leukemic activity in pre-clinical models of AML (188, 189). To enhance the binding to CD16a, an Fc-engineered anti-CD123 antibody was developed that showed superior NK-cell mediated killing of leukemia stem cells in AML and CML (190–192). Similarly, an antibody that binds to CD133 on myeloid cells and with amino acid substitutions (S293D/I1332E) in the Fc domain for enhanced binding to CD16a induced strong degranulation and lysis of CD133-expressing AML cells in the presence of either autologous or allogeneic NK cells (193). Interleukin 1 receptor accessory protein (IL1RAP) is another candidate therapeutic target up-regulated on leukemia stem cells in myeloid malignancies (194–196). Consistent with IL1RAP being up-regulated on leukemia stem cells vs. normal hematopoietic stem and progenitor cells, IL1RAP-targeting antibodies with enhanced CD16a-binding capacity induced selective NK cell-mediated ADCC when exposed to candidate leukemia stem cells (196). Moreover, Ågerstam et al. demonstrated that IL1RAP-targeting antibodies exhibited potent antileukemic efficacy in CML and AML xenograft models (197, 198).

Another promising approach to direct the immune system to kill cancer cells is the use of bispecific antibody-based modalities that can be designed to bind one antigen on the cancer cell and a separate antigen on a cytotoxic immune cell. By using a Bispecific Killer Engager (BiKE) consisting of a single-chain variable fragment (scFv) targeting CD16a on NK cells and a scFv targeting CD33 on AML cells, NK cell-mediated cytotoxicity and cytokine release could be effectively triggered (199). With the aim to boost NK cell activity and persistence, as a further development of the 16 × 33 BiKE targeting CD16a and CD33, IL-15 Trispecific Killer Engagers (TriKE) referred to as 16 × 15 × 33 TriKEs have been developed (200). When compared to the 16 × 33 BiKE, Vallera et al. demonstrated that the 16 × 15 × 33 TriKE induced superior NK cell cytotoxicity and cytokine release when exposed to AML cells (200).

Prevent Suppression From the Microenvironment

Certain types of immune cells are immune suppressive and can restrain immune-mediated attacks against malignant cells. Both Tregs and myeloid-derived suppressor cells (MDSCs) have been shown to restrain NK cells, hence, therapeutic interventions aimed at depleting either of these cells have the potential to enhance NK cell activity (201). One approach to deplete MDSCs is the use of the 16 × 15 × 33 TriKEs, which, in addition to killing $CD33^+$ malignant cells, are also effective in killing $CD33^+$ MDSCs, leading to restoration of NK cell function in MDS (202, 203). In CML patients, dasatinib treatment is associated with inhibition of Tregs. Consistent with this hypothesis, the response rate after 18 months' treatment with dasatinib was significantly better in CML patients with low numbers of Tregs that inversely correlated with NK cell counts, indicating that inhibition of Tregs by dasatinib enhances NK cell-mediated killing of leukemic cells (102). The TNF family member receptor activator for NF-KB ligand (RANKL) is mainly known as a regulator of bone remodeling but also regulates immune functions. Activation of RANKL signaling in AML cells result in secretion of immune-modulatory factors that impaired NK cell function (204). Consistent with this finding, treatment of AML cells with Denosumab, an

inhibitory RANKL antibody, resulted in enhanced NK cell function (204).

Checkpoint Inhibition

Immune checkpoint inhibitors targeting the PD1/PDL1 interaction have been clinically validated and show remarkable response rates in several forms of cancer. Mechanistically, the selective anti-tumor effect of the T cells is based on the recognition of tumor neo-antigens presented on HLA class I molecules. With a high mutational burden in certain cancers, more tumor neo-antigens are formed and recognized by the T cell receptors. As myeloid malignancies have a relatively low mutational burden, immune checkpoint inhibitors for T cells are expected to be less effective in disorders such as AML. However, recent data by Hsu et al. proposed that NK cells express PD1 and that blockade of the PD1/PDL1 interaction also activates NK cells that are indispensable for the therapeutic effect of these therapies (205). Hence, blocking PD1/PDL1 may show unexpected therapeutic efficacy in myeloid malignancies by activating NK cells, possibly in combinations with other therapies, a route that warrants further investigations.

As postulated by the "missing-self" hypothesis (9), NK cells are regulated by inhibitory HLA class I molecules that bind to their cognate KIRs on NK cells. To enhance NK cell activity, the mAb 1-7F9 that cross-reacts with KIR2D molecules and block the interaction with virtually all HLA-C molecules was developed (206). In the presence of NK cells, 1-7F9 induces selective killing of HLA-C expressing AML cells vs. normal peripheral blood mononuclear cells (206). When evaluated in a phase I study in AML, increased expression of the activation marker CD69 on NK cells was observed and relapse-free survival compared favorable to historical data from comparable patient cohorts (207). Blocking KIRs also augments ADCC induced by antibodies binding to CD20 and CD33, suggesting that KIR blockade can enhance the efficacy of therapeutic antibodies that rely on ADCC for killing of cancer cells (206, 208). However, based on data claiming that the anti-KIR antibody can rapidly detune NK cell function *in vitro* and in cancer patients (209), thereby limiting its therapeutic efficacy, and given the pre-clinical data indicating that KIR blockade augments ADCC (206, 208), better responses are likely to be achieved when combining KIR blockade with other drugs that boost NK cell cytotoxicity. In addition to tumor-targeting antibodies, drugs such as lenalidomide that is reported to boost NK cell function *per se*, and maybe also decitabine or HDACi as discussed above, may be relevant. Further studies are needed to fully delineate the efficacy of such approaches and if it induces durable remissions. In addition to KIR, a subset of NK cells expresses the inhibitory receptor NKG2A that bind to HLA-E on healthy and cancer cells. In line with a key role for NKG2A in immune checkpoint regulation, Ruggeri et al. demonstrated that targeting of NKG2A with a blocking antibody resulted in strong NK-cell mediated anti-leukemic activity in mice engrafted with primary leukemia cells (210). Similar data for KIR and NKG2A have also been generated in *ex vivo* experiments by others (53). Again, it should be highlighted that targeting these receptors alone may have limited efficacy due to the risk of detuning of baseline NK cell cytotoxicity and that combination therapies may generate better results.

In summary, several clinically approved drugs and drugs in pre-clinical development can be utilized to improve NK cell function by distinct mechanisms. Hence, identifying beneficial combinations of these therapies in a disease- and genotype-specific manner has the potential to not only restore tumor immune surveillance in patients with myeloid malignancies, but also further enhance NK cell activity over normal baseline levels. If further combined with other immunotherapies or targeted therapies that neutralize oncogenic drivers, multiple therapies can be used simultaneously to attack the malignant cells, a strategy that will minimize the risk for resistance mechanisms to arise and may ultimately lead to cure of patients.

CONCLUDING REMARKS AND FUTURE OUTLOOK

In recent years, significant advances have been made in our understanding of the role for NK cells in myeloid malignancies. We have become aware of the idea that NK cells in patients with MDS, AML, and CML most often are dysfunctional, but also that their phenotype and function can be partially restored following administration of tumor-targeting drugs such as TKI, chemotherapy, and hypomethylating agents, and also by immunostimulatory agents such as cytokine-based therapies. Data also demonstrate that such restoration of the endogenous NK cell function can be key in achieving durable responses in subgroups of patients. Although therapeutic strategies involving adoptive NK cell infusions hold promise, with objective clinical response rates of 30–50% in patients with advanced disease such as relapsed and/or refractory AML and high-risk MDS, these results are only transient and non-curative today. Therefore, a tempting and, in many ways, more natural approach to achieve long-term remissions would be to redirect the endogenous NK cells to target and control the disease. This notion is based on the ample support for NK cell-mediated immunosurveillance of myeloid malignancies along with the abovementioned data demonstrating that endogenous NK cells can be key to attain durable remissions, a phenomenon that is in line with that observed for donor NK cells in preventing leukemia relapse in certain settings of allogeneic SCT. Identifying therapies that redirect endogenous NK cells is especially of interest given the aging population, in which more and more patients are ineligible for an allogeneic SCT or even to high-intensity chemotherapy. In addition to its simplicity and potential to serve the broader population, the current high costs for SCT and cellular immunotherapies favor this alternative approach.

However, to be able to develop effective therapies that rely on endogenous NK cells, we need to better understand what factors that cause tumor evasion from NK cells and identify drugs that prevent or neutralize them. We also need to recognize what drugs can be utilized to selectively augment the tumor killing capacity of the endogenous NK cells *per se* and the temporal aspects of using these drugs. Moreover, to achieve durable disease control, we need to identify therapies that not only

activate the NK cells short-term and potentially exhaust them, but more importantly to develop drugs or approaches/protocols that stimulate the NK cells for enhanced tumor immune surveillance long-term. To this end, combinational therapies and/or sequential therapies may be required for achieving significant clinical responses. Nevertheless, it will be critical to, in more detail, understand the processes that govern NK cell development and how it is perturbed in disease, findings that may translate into new therapeutic opportunities. Lessons may also be learnt from studies on ALL, as this leukemia seems to be less vulnerable to targeting by NK cells compared to AML. Such studies could potentially improve our understanding of the molecular specificity of NK cell killing of leukemic cells in general but also evasion mechanisms employed by the ALL cells *per se* as well as factors in the bone marrow environment of that disease in particular. As mentioned in this review, both cytokines, antitumor antibodies, including BiKEs, and TriKEs, and checkpoint inhibitors hold promise for the treatment of myeloid malignancies but need to be studied in greater detail until their full potential can be expected. We also need to identify new molecules to target in order to explore new therapeutic opportunities as well as biomarkers to monitor NK cell function during treatment. While this is explored, we will likely start receiving the first insights into the potential role for CAR-NK cells in treating cancer, which hopefully will contribute to our understanding while adding another layer of immunological pressure to retain the myeloid malignancy in remission. Compared to CAR-T cells that can induce toxic and even lethal cytokine release syndromes and neurotoxicity, the CAR-NK cells are expected to be better tolerated, but their potential short persistence in patients might limit their clinical use. Several molecular targets expressed on myeloid leukemia cells, such as CD123 and CD33 but also NKG2DLs and CD7, are currently being explored in the CAR field and more efficient protocols for CAR-NK cell development are being established. However, the discovery of additional and potentially more suitable molecular targets is needed to more selectively target the malignant myeloid cells while sparing normal cells. Another important aspect is also that the suppressed autologous NK cells in myeloid malignancies used for reprogramming to CAR-NK cells need to have restored or ideally enhanced function prior to reprogramming and that mechanisms potentially dysregulating the CAR-NK cells following re-infusion need to be controlled. This also applies if using IPS- or cord blood-derived CAR-NK cells. Hence, drugs and approaches discussed in this review are utterly important and need further attention also in relation to CAR-NK cells against myeloid malignancies to induce and maintain durable remissions.

Based on the data presented in this review, we strongly believe that new unique opportunities to better utilize NK cells to induce long-term remissions in patients with myeloid malignancies will be a reality in the near future.

AUTHOR CONTRIBUTIONS

MC and MJ have contributed equally in the outline and writing of the manuscript as well as for the design of the figures.

FUNDING

This work was supported by funding from the Swedish Childhood Cancer Foundation, the Swedish Cancer Society, the Swedish Research Council, the Crafoord Foundation, the Royal Physiographic Society of Lund, the Swedish Society for Medical Research, and Wallenberg Clinical Fellow. The authors declare that this study received funding from BioCARE. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. Fredrik B. Thorén, who has critically reviewed the content of this review.

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

frontiers in Immunology

MINI REVIEW
published: 17 October 2019
doi: 10.3389/fimmu.2019.02456

Transcriptional, Epigenetic and Pharmacological Control of JAK/STAT Pathway in NK Cells

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Differentiation of Natural Killer (NK) cells is a stepwise process having its origin in the bone marrow and proceeding in the periphery, where these cells follow organ specific trajectories. Several soluble factors and cytokines regulate the distinct stages of NK cell differentiation, and ultimately, their functional properties. Cytokines activating the Janus kinases (JAKs) and members of the signal transducer and activator of transcription (STAT) pathway control distinct aspects of NK cell biology, ranging from development, terminal differentiation, activation, and generation of cells with adaptive properties. Here, we discuss how the recent advances of next generation sequencing (NGS) technology have led to unravel novel molecular aspects of gene regulation, with the aim to provide genomic views of how STATs regulate transcriptional and epigenetic features of NK cells during the different functional stages.

Keywords: NK cells, innate lymphoid cells, JAK, STAT, cytokine, transcriptome, transcription factor

OPEN ACCESS

Edited by:
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Francisco Borrego,
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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: *23 July 2019*
Accepted: *01 October 2019*
Published: *17 October 2019*

Citation:
Scarno G, Pietropaolo G, Di Censo C, Gadina M, Santoni A and Sciumè G (2019) Transcriptional, Epigenetic and Pharmacological Control of JAK/STAT Pathway in NK Cells. Front. Immunol. 10:2456. doi: 10.3389/fimmu.2019.02456

INTRODUCTION

Natural Killer (NK) cells are the founding members of the ILC family and represent the innate counterpart of cytotoxic T lymphocytes (1, 2). Like $CD8^+$ T cells, NK cells are able to kill infected or transformed cells in a perforin and granzyme dependent manner, as well, these cells are able to mount a rapid type-1 response by releasing the eponymous cytokine, interferon (IFN)-γ (3, 4). NK cells share the ability to produce type-1 cytokines with a distinct "helper" prototypical innate subset, termed ILC1 (5, 6). NK cells differ from ILC1 for their cytotoxic abilities, for a higher propension to circulate in the bloodstream and for the expression of lineage defining transcription factors (LDTFs) (7–9). In this regard, both NK cells and ILC1 are regulated by transcription factors (TFs) of the T-box family; however, while Eomes is expressed and required only by NK cells, T-bet (encoded by *Tbx21*) is expressed by both prototypical subsets (10–14). Expression of T-bet is fundamental for the generation of ILC1, and it also has non-redundant roles in regulating NK cell turnover, effector functions and egression from bone marrow (10, 11, 15).

Cytokines and other soluble factors regulate several aspects of NK cell biology, acting through signal-dependent TFs (SDTFs). In particular, cytokines activating the Janus kinases (JAKs) and members of the signal transducer and activator of transcription (STAT) pathway control NK cell development, terminal differentiation, acquisition of effector phenotype up to generation of cells with adaptive features able to provide secondary responses (16, 17). Mammalian genomes contain four genes encoding for JAKs, namely JAK1, JAK2, JAK3, and TYK2; and seven genes for STATs,

STAT1-4, STAT5A, STAT5B, and STAT6 (18, 19). Activation of the JAK tyrosine kinases occurs upon receptor engagement, and the juxtaposition of JAKs and STATs allows, after phosphorylation, STAT dimers to dissociate from the membrane complex and to migrate into the nucleus, where they bind specific DNA-motifs modulating gene expression (20).

The role of the JAK/STAT dependent signals on NK cells and other ILCs has been discussed in recent reviews (16, 17, 21); herein, we focus on the molecular mechanisms underlying NK cell differentiation in physiological and pathological contexts. We discuss how the advances of next generation sequencing (NGS) technology and the establishment of novel mouse models have led to a better definition of the genes regulated by STATs, and their transcriptional and epigenetic control of NK cells during differentiation and host defense. Finally, we provide an overview of the JAK inhibitors currently approved for the treatment of immune-mediated disorders and their possible implication on NK cells.

STAT5 AS A CENTRAL NODE FOR DEVELOPMENT, IDENTITY AND HOMEOSTASIS OF NK CELLS

The bone marrow is the main site for NK cell and ILC development in the adult, containing distinct progenitors and precursors able to give rise to cells having different fates (22, 23). Differentiation proceeds with a pool of circulating progenitors which move to the periphery, where NK cells and other ILCs follow organ specific trajectories and acquire distinct effector functions (24). In the current model, NK cells have a dedicated pathway of differentiation comprising a pool of committed NK cell precursors (NKps) (25–27). Their differentiation follows a stepwise process encompassing distinct developmental and/or functional stages, discriminated through the expression of CD27 and CD11b levels in mice and CD56 and CD16 in humans [redefined recently by single cell RNA-seq approach (28, 29)].

The cytokines IL-7 and IL-15 are critical for lymphoid development by transmitting their signals through the common IL-2 γ-chain receptor (CD132) and by activating JAK3, JAK1, and STAT5 (30). Deletion of *Jak3* in mice is associated with reduced numbers of lymphoid and ILC precursors, in contrast to an accumulation of NKp (31). This evidence is in line with previous findings demonstrating that IL-15 was required for the NKp to proceed toward the next maturation stages (25). Similarly, mice carrying conditional deletion of *Jak1* in *Ncr1*-expressing cells (*Jak1*$^{fl/fl}$ *Ncr1Cre*) show profound defects in NK cell differentiation and homeostasis; *Jak2* deletion, instead, does not affect NK cell development and survival (32).

JAK3 and JAK1 mainly activate STAT5, which represents a key multi-lineage TF (MLTF) controlling development of both adaptive and innate lymphocytes (33, 34). Ablation of the entire *Stat5* locus, comprising both *Stat5a* and *Stat5b*, results in a high perinatal lethality, due to the pleiotropic role of this TF; however, the few viable *Stat5*$^{-/-}$ mice show absence of NK cells (35). Conditional deletion of *Stat5* in Ncr1-expressing cells allows to eliminate the confounding effects related to lymphopenia and inflammation observed in mice carrying germline ablation; in these settings, both development and survival of NK cells remain highly impaired (36).

Due to the massive effect of STAT5 deletion on NK cells, our understanding of how this SDTF works at the molecular level has remained elusive; the use of mice bearing only one allele of STAT5 has helped to clarify this aspect. Between the two paralogs, *Stat5b* is more expressed than *Stat5a* in innate and adaptive lymphocytes, and its deletion has broad effects on NK cell differentiation (37–39). Transcriptomic analyses performed on NK cells retaining only one *Stat5* allele (*Stat5a*$^{-/-}$ *Stat5b*$^{+/-}$) have shed light on the homeostatic impact of this TF on NK cells, which consists on regulation of over 400 genes (39). The residual NK cells present in these mice show a developmental block associated with an accumulation of CD11b^{low} cells, and a drastic decrease of the expression of the anti-apoptotic gene, *Bcl2*. Along with defects in development and survival, STAT5 sustains the expression of most of the genes (52 out of 76) defining NK cell identity, including NKG2D, perforin and granzymes, and the LDTF T-bet (39). These findings have helped to discriminate between the instructive role of STAT5 during NK cell differentiation and its permissive function in regulating survival.

Upon activation, STAT5 can form dimers but also tetramers having distinct ability to interact with DNA-regulatory elements (40, 41). While STAT5 dimers bind to canonical GAS (IFN-γ activation site, TTCN3GAA) motif, STAT5 tetramers bind to divergent motifs having an optimal spacing of 2–27 base pairs between GAS and GAS-like sequences. The relative importance of STAT5 dimers vs. tetramers in NK cells has been evaluated by the generation of a mouse model carrying genes encoding for tetramer defective mutant STAT5 proteins (40, 42). In these mice, the impaired STAT5 binding to the *Bcl2* locus, and the consequent lower mRNA and protein expression, leads to a more rapid cell death of NK cells compared to wild type cells (40). Interestingly, transgenic expression of Bcl2 is able to rescue the effect of *Stat5* deficiency on the homeostatic pool of NK cells (43). These "*Bcl2*-rescued" NK cells undergo a functional switch from tumor-suppressive to tumor-promoting cells, since loss of STAT5 determines upregulation of the pro-angiogenic factor VEGFA, which sustains tumor growth (43). Thus, while STAT5 represents a central node in NK cell development, acquisition of cell identity, and homeostasis (**Figure 1**), the involvement of other STATs in regulating these processes appears limited. Of note, type I IFNs and STAT1 can have distinct indirect effects on NK cell homeostasis: including the regulation of MHC class I expression (44), as well as the regulation of the production and trans-presentation of IL-15 on accessory cells (45–47).

MULTIPLE STATs UNDERLIE EFFECTOR FUNCTIONS OF NK CELLS

Effector functions of NK cells depend both on cytokines and on a complex equilibrium between activating and inhibitory receptors, which bind molecules present on healthy and stressed

Abbreviations: ILC, innate lymphoid cell; IFN, interferon; LDTF, lineage defining transcription factor; SDTF, signal dependent transcription factor; NK, natural killer; STAT, Signal Transducer and Activator of Transcription.

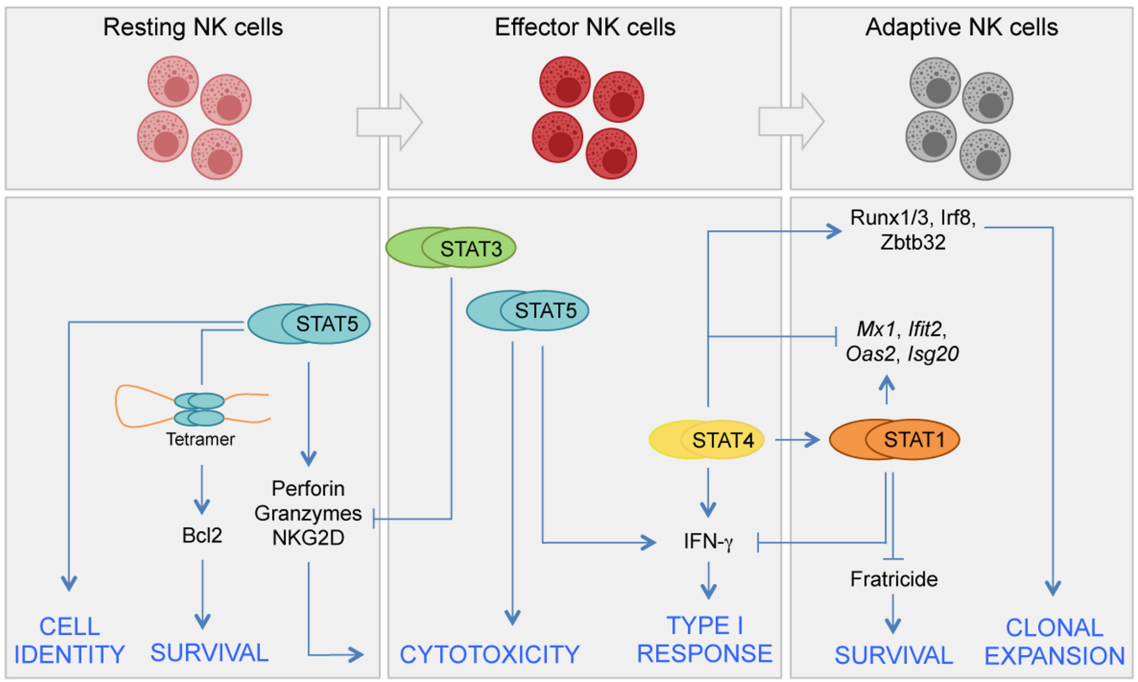

FIGURE 1 | Distinct requirements for STATs in NK cell differentiation. JAK/STAT signals control several aspects of NK cell biology, including development, terminal differentiation, acquisition of effector functions, and generation of adaptive NK cells. NK cell development begins in the bone marrow from committed precursors and it is driven by signals inducing STAT5 activation. In homeostatic conditions STAT5 sustains NK cell survival by direct regulation of Bcl2 expression. STAT5 is also required for terminal differentiation and acquisition of NK cell identity. STAT4 and STAT1 have both specific and shared roles during viral infection. STAT4 controls a network of TFs required for clonal expansion of NK cells during proliferation. STAT4 and STAT1 compete at genomic level for the expression of IFN-γ and other genes. STAT3 has a role in restraining NK cell effector functions by inhibiting perforin, granzyme B, and NKG2D expression.

cells including MHC class I and adhesion molecules (48–52). The ability of NK cells to sense environmental changes and rapidly release their effector potentials is favored by a primed epigenetic and transcriptional state, leading to high basal expression of cytokine receptors, LDTFs, and SDTFs, including STATs (53). Although distinct STATs can be easily linked to particular effector and helper functions, it is now clear that activation of NK cells, like the other ILCs, can be influenced by complementary actions of multiple STATs (54–56).

Acting downstream of IL-12, STAT4 is necessary to mount a proper innate response against pathogens by actively regulating NK effector functions, including both IFN-γ production and cytotoxic response (57). The global impact of STAT4 in NK cell activation has been recently tackled using transcriptomic and epigenetic approaches (58, 59). Upon cytokine stimulation, over 300 differentially expressed genes are bound by STAT4 within or in proximity of the locus (59). Along with direct regulation of key effector genes, STAT4 controls the expression of several TFs required for a proper antiviral response, including *Zbtb32*, *Runx1*, *Runx3*, and *Irf8*. At molecular level, STAT4 binds to the promoter and intergenic regions of the gene locus of these TFs, leading to an increase of the permissiveness of the transcription through modification of the chromatin state, via trimethylation of histone H3 lysine 4 (59). Mouse models carrying selective deletion of these TFs have helped to unravel their impact on the cell cycle program of NK cells during viral infection. The effects of Zbtb32 on the proliferative burst and protective ability of NK cells are mediated by antagonizing the anti-proliferative effects of the TF Blimp-1 (encoded by *Prdm1*) (60); Irf8, instead, regulates proliferation acting upstream of Zbtb32 (61). During the course of viral infection, the expression of STAT4 and STAT1 follows an opposite fate. Indeed, while STAT4 expression is down-regulated, STAT1 results progressively up-regulated (58, 62). This differential expression pattern affects the signaling downstream of type I IFNs, which mainly activates STAT4 in the early phases of infection and STAT1 in later phases. The increased levels of STAT1 cause a displacement of STAT4 from type I IFN receptors, this switch induces a STAT1 dependent down-regulation of IFN-γ production in NK cells (62).

The role of STAT3 on NK cells has been dissected by employing distinct mouse models, showing differential effects whether deletion of *Stat3* gene occurs before or after NK cell development (63, 64). When $Stat3^{fl/fl}$ mice are crossed with *Tie2-Cre* mice, the effects of *Stat3* deletion extend to the whole hematopoietic compartment. In these settings, NK cells show a decreased expression of NKG2D and impaired effector functions (63). In line with these findings, NK cells from subjects with dominant-negative STAT3 mutations show an impaired expression of NKG2D both at steady state and after cytokine stimulation (63). On the other hand, specific

deletion of *Stat3* in differentiated NK cells, using *Ncr1iCre Stat3*$^{fl/fl}$ mice, leads to an increased expression of DNAM-1, Perforin, and Granzyme B, and enhanced anti-tumor activity, as the result of the possible repressive functions of STAT3 on these cells (64). Considering these conflicting findings, genome-wide studies aimed at dissecting the transcriptomic impact of *Stat3* deletion on NK cells would be particularly relevant to discriminate between the direct and indirect roles of this TF in regulating differentiation and effector functions.

Beyond the homeostatic requirement in sustaining the expression of NK effector molecules, cytokines activating STAT5 have been used to stimulate NK cell functions *in vitro*, for decades (65, 66). Genomic maps of STAT5 distribution obtained by ChIP-seq analysis have revealed a widespread DNA binding in untreated and IL-15-treated NK cells. However, the acute stimulation with IL-15 induces a redistribution of this TF to a new set of DNA regulatory elements. In these settings, STAT5 binding occurs on almost half of the differentially expressed genes. Gene set enrichment analysis (GSEA) have confirmed a positive enrichment for IL-2/STAT5 signaling in STAT5 bound genes (39). In contrast, unbound genes show a positive enrichment for downstream targets of the mTOR pathway, which has been shown to mediate IL-15-dependent functions in NK cells, including proliferation and terminal differentiation, by regulating CD122 (IL-2Rβ) and CD132 (IL-2Rγ) expression; as well as metabolism, and acquisition of cytolytic features (67, 68).

SPECIFIC ROLES FOR STATs DURING FORMATION OF ADAPTIVE NK CELLS

In the context of viral infection, NK cells are able to provide secondary immune responses by following a differentiation path which leads to generation of long-lived cells, named "memory" or "adaptive" NK cells (69, 70). Changes of chromatin accessibility of NK cells have been tracked *in vivo* up to 35 days after MCMV infection, by ATAC-seq (58). This analysis has revealed that the epigenetic landscape of NK cells is highly dynamic, with the majority of chromatin remodeling occurring in the first 2 weeks. These modifications pave the way for a further acquisition of the transcriptional adaptive state, observed at later time points (58). Genomic maps of STAT4 and STAT1 distribution in cytokine-stimulated NK cells have shown a differential DNA occupancy, being STAT4 mainly localized at putative enhancer sites and STAT1 at promoter regions (58). In line with these results, during MCMV infection the chromatin accessibility of putative enhancer sites and promoters remains less accessible in NK cells deficient for STAT4 and STAT1, respectively. Moreover, due to the existing competitive effects between STAT4 and STAT1, deletion of *Stat1* in NK cells leads to an increased DNA accessibility of non-promoter regions; as well as, to an increased expression of selected STAT4 regulated genes, such as *Ifng*. Conversely, the expression of several STAT1 targets, including *Mx1*, *Ifit2*, *Oas2*, and *Isg20*, is upregulated in absence of *Stat4* (58).

The interplay between STATs and LDTFs is a further mechanism underlying acquisition of specific functions in innate lymphocytes, including the generation of the adaptive phenotype in NK cells. This is the case for the cross-regulation occurring between STATs and T-bet (39, 71, 72); while STAT5 induces T-bet expression in homeostatic conditions (39), STAT4 binds to *Tbx21* locus at a distal enhancer site and promotes T-bet expression during MCMV infection (72). T-bet and Eomes are both necessary for NK cell proliferation; however, the IL-12/STAT4/T-bet axis plays a non-redundant role for the maintenance of adaptive NK cells (72). We have discussed in the previous section the network of TFs induced by STAT4, namely Zbtb32, Runx1, Runx3, and Irf8, which are all necessary to enhance proliferation and clonal expansion of NK cells (59–61). As well, expression of STAT1 has a non-redundant role for survival, regulating a Bcl2-independent mechanism enabling NK cells to evade cell death after viral infection. In particular, type 1 IFNs and STAT1 are required to prevent a mechanism of NK cell mediated fratricide, occurring via NKG2D and perforin (73). Overall, these findings shed light on the complex network of TFs and molecules regulated by STATs, required for the acquisition of the adaptive traits by NK cells.

CONCLUSION: TRANSLATIONAL RELEVANCE OF TARGETING THE JAK/STAT PATHWAY IN INFLAMMATION AND CANCER

Manipulation of cytokine signaling in NK cells and other ILCs is drawing a growing interest for the treatment of inflammatory diseases and cancer (74, 75). In particular, harnessing NK cell effector functions against cancer by interfering with cytokine signaling has led to promising results in several mouse models (76–79). In this context, the suppressor of cytokine signaling (SOCS) proteins are a class of natural regulators of the activity of STATs. The SOCS protein CIS (encoded by *Cish*) is at the top among the genes induced by STAT5 activation, and acts as a negative regulator of IL-15 signaling, preventing excessive activation (77). Targeting *Cish* has a huge impact in enhancing NK cell dependent tumor immunity in several mouse models (77, 79); thus, given its primary role in restraining NK cell functions, CIS represents a novel immune checkpoint for these cells.

On the other hand, several small molecules capable to inhibit JAKs enzymatic activity have been recently developed. At least five JAK inhibitors (also known as JAKinibs) are now approved by various regulatory agencies to treat immune-mediated disorders. These first-generation JAKinibs comprise ruxolitinib, a JAK1 and JAK2 inhibitor, approved for myeloproliferative malignancies; tofacitinib, a JAK1, JAK2, JAK3 inhibitor, approved for rheumatoid arthritis, psoriatic arthritis, and ulcerative colitis; baricitinib, a JAK1 and JAK2 inhibitor, approved for rheumatoid arthritis; peficitinib, a pan-JAK inhibitor approved (only in Japan) for the treatment of rheumatoid arthritis; and oclacitinib, a JAK1 and JAK2 inhibitor, approved for allergic dermatitis in dogs (80).

The impact of ruxolitinib in NK cell homeostasis and functions has been evaluated in humans in distinct contexts. Myelofibrosis patients undergoing ruxolitinib treatment show a defect in NK cell number and differentiation, as well as,

impaired functions upon cytokine stimulation; these effects have been related to the increased rates of infection observed in these patients (81). Ruxolitinib also inhibits the generation and functions of cytokine-induced memory-like NK cells by interfering with both IL-15 and IL-12 signaling (82). Finally, Ruxolitinib administration can limit STAT1 activation in patients carrying STAT1 gain of function mutations. In these patients, the prolonged STAT1 activation leads to an impaired NK cell maturation and function, associated with lower STAT5 phosphorylation downstream of IL-15 stimulation, and with lower levels of perforin. These defects are partially reverted by ruxolitinib administration (83).

More selective agents have been developed and are currently being tested. These next-generation inhibitors may possess the advantage of a reduced toxicity. For example, selective targeting of JAK1 would spare interfering with many of JAK2-dependent cytokines involved in hematopoiesis, including Epo, Tpo, G-CSF, GM-CSF, IL-3, and IL-11. Conversely, their efficacy could also be limited. Recently, immunogenomic analysis of mice administered with several JAKinibs, including both first- and second-generation inhibitors, have highlighted the impact of blocking either one or both JAK1 and JAK3 on NK cell homeostasis. Moreover, the JAK1-specific inhibitor (PF-02384554) was more efficient than the JAK3-specific (PF-06651600) in blocking the secondary autocrine response to IFN-γ induced in IL-2 activated NK cells (84).

The optimal degree of JAK inhibition required for an individual cell type in any given tissue remains unknown. To this end, selective JAKinibs may be the key to provide new mechanistic insights in the modulation of the JAK/STAT pathway in NK cells. This approach could be more effective than the use of JAK-deficient mice, in which developmental defects can mask the functional relevance of each JAK. Finally, we are now aware that JAKinibs can affect the structure of the epigenome and preferentially impact genes with super-enhancer structure (85). Notably, several genes encoding for cytokines or their cognate receptors are located within loci with super-enhancer architecture. Therapeutically, it will be important to understand how these drugs, alone or in combination with other chemotherapeutic agents, can be used to effectively, and safely, regulate these critical loci and, in turn, immune as well as non-immune cells.

AUTHOR CONTRIBUTIONS

GSca, GP, CD, MG, AS and GSci wrote the manuscript. CD designed the figure and made the necessary edits. The final manuscript was a result of the joint efforts of all the authors.

FUNDING

GSci and AS were supported by the Italian Association for Cancer Research (AIRC), MFAG-21311; 5x1000-21147, respectively; by Institut Pasteur (France), Transversal Research Program, PTR-113-17 program. GSci was supported by Istituto Pasteur Italia—Fondazione Cenci Bolognetti (Under 45-213).

ACKNOWLEDGMENTS

We would like to thank Dr. Lorenzo Cuollo and Dr. Silvia Piconese for the helpful criticism and discussion.

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

frontiers in Immunology

REVIEW
published: 12 November 2019
doi: 10.3389/fimmu.2019.02590

JAK/STAT Cytokine Signaling at the Crossroad of NK Cell Development and Maturation

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Natural Killer (NK) cells are cytotoxic lymphocytes of the innate immune system and play a critical role in anti-viral and anti-tumor responses. NK cells develop in the bone marrow from hematopoietic stem cells (HSCs) that differentiate through common lymphoid progenitors (CLPs) to NK lineage-restricted progenitors (NKPs). The orchestrated action of multiple cytokines is crucial for NK cell development and maturation. Many of these cytokines such as IL-2, IL-7, IL-12, IL-15, IL-21, IL-27, and interferons (IFNs) signal via the Janus Kinase / Signal Transducer and Activator of Transcription (JAK/STAT) pathway. We here review the current knowledge about these cytokines and the downstream signaling involved in the development and maturation of conventional NK cells and their close relatives, innate lymphoid cells type 1 (ILC1). We further discuss the role of suppressor of cytokine signaling (SOCS) proteins in NK cells and highlight their potential for therapeutic application.

Keywords: NK cell, ILC1, development, maturation, cytokine, JAK, STAT, SOCS

OPEN ACCESS

Edited by:
Ewa Sitnicka,
Lund University, Sweden

Reviewed by:
Roland Jacobs,
Hannover Medical School, Germany
Laurel L. Lenz,
University of Colorado Denver School of Medicine, 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: *14 August 2019*
Accepted: *18 October 2019*
Published: *12 November 2019*

Citation:
Gotthardt D, Trifinopoulos J, Sexl V and Putz EM (2019) JAK/STAT Cytokine Signaling at the Crossroad of NK Cell Development and Maturation. Front. Immunol. 10:2590. doi: 10.3389/fimmu.2019.02590

INTRODUCTION

Innate lymphoid cells (ILCs) comprise a variety of cell types with the morphological characteristics of lymphoid cells, but unlike adaptive immune cells, ILCs completely lack rearranged antigen receptors. In analogy to the classification of T cell subsets, ILCs can be sub-divided into three groups according to their dependence on distinct transcription factors and to their cytokine expression repertoire (1). Group 1 ILCs include two major members, conventional NK cells and ILC1s, both of which are characterized by the ability to produce T helper-1 (Th1) cell signature cytokines (e.g., interferon-gamma, IFN-γ) and by their functional and developmental dependence on the transcription factor T-BET. Group 2 cells (ILC2s) produce Th2 cell-type cytokines (e.g., IL-4, IL-5, IL-9, and IL-13) and depend on GATA3, whereas group 3 cells (ILC3s) are potent producers of IL-22 and/or IL-17A and are characterized by RORγt expression (1, 2).

NK cells account for 8–15% of circulating cells in the human blood or 2–6% in mouse blood, and are found throughout the body, in particular in lymphoid organs, lung, liver, uterus and gut (3). Similar to $CD8^+$ cytotoxic T cells, NK cells are important in the defense against tumors and the spread of viral infections by producing pro-inflammatory cytokines such as IFN-γ and TNF-α. However, unlike T cells NK cells do not require prior sensitization and lack antigen-specificity allowing them to patrol and eliminate a broad range of altered and transformed cells. To do so, the activity of NK cells is controlled by a delicate balance of inhibitory and activating receptors, which interact with surface ligands and either prevent or trigger the lysis of a target cell (4, 5). Whereas, NK cells recirculate via blood and lymph vessels and have a license to kill, ILC1s are mostly

tissue-resident and show low cytotoxic potential. Besides their common feature of being highly efficient IFN-γ producers, NK cells and ILC1s share many surface markers as well as transcription factors that complicates their discrimination especially under conditions of inflammation or in cancer (6, 7).

In humans, the identification of specific markers for ILC1s remains challenging (6, 8). In mice, surface expression of CD49b and expression of the transcription factor Eomesodermin (EOMES) merge cells under the umbrella of conventional NK cells. In contrast, CD49a expression and the absence of EOMES expression assigns cells to the ILC1 lineage (7, 9). However, to add a layer of complexity it was shown that CD49a expression can be induced on conventional mouse NK cells *in vivo* upon viral (10) and parasite infection (11) and in the tumor microenvironment (12, 13). Treatment of mouse splenic NK cells with IL-2 and TGF-β induces the expression of ILC1-associated markers, such as CD49a and TRAIL (12). On the other hand, expression of EOMES under the control of the *Tbx21* (T-BET) locus induces ILC1s to acquire an NK cell-like phenotype (14).

The high plasticity within group 1 ILCs and the reversible trans-differentiation of group 2 and 3 ILCs into ILC1s (15) complicate the task to dissect the impact of aberrant cytokine signaling or expression of signaling molecules on those cells. It might thus be necessary to re-evaluate some previously published literature on NK cells to determine whether conventional NK cells and/or ILC1s have been analyzed.

NK CELL DEVELOPMENT AND MATURATION

NK cells originate from common lymphoid progenitors (CLPs) in the bone marrow and may traffic to secondary lymphoid tissues, where they undergo terminal maturation and exit to the circulation (16, 17). The α-lymphoid progenitor (α-LP) and the early ILC progenitor (EILP) are the first progenitors with restricted lineage potential for all ILC subsets (18, 19). Downstream of EILPs are NK precursors (NKPs) giving rise to conventional NK cells and common helper-like innate lymphoid precursors (CHILPs), the ancestors of all other ILC subsets including ILC1s (15). The most distinct characteristic of NKPs is the acquisition of CD122 (IL2Rβ) expression, which is pivotal in the transduction of IL-15 signals via JAK1/3 and STAT5. Loss of one of these components unequivocally precludes NK cell development (20–23). This already highlights the central role of the JAK/STAT signaling cascade in NK cell development and maturation.

Human NK cells, classified as $CD3^-CD56^+NKp46^+$ cells, can be further subdivided based on the expression of the low affinity Fc-receptor CD16 in $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$ cells. $CD56^{bright}CD16^-$ NK cells are more responsive to stimulation by inflammatory cytokines and are thought to be immature precursors of $CD56^{dim}CD16^+$ mature NK cells, which show a higher cytotoxic capacity. The development of human NK cells can be stratified to five stages (16). The final maturation of human NK cells is accompanied by the loss of CD94/NKG2A and CD226 (DNAM1) expression, the acquisition of killer immunoglobulin-like receptors (KIRs) and CD57, and the change in the expression pattern of homing molecules such as CD62L (24, 25). Recently though, several studies have challenged this traditional model and suggested that $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^-$ NK cells may arise from separate lineages (26).

Mouse NK cells are defined as $CD3^-CD49b^+NKp46^+$ cells and in C57BL/6 mice additionally $NK1.1^+$. Their maturation in the periphery is associated with the upregulation of CD11b, CD43, KLRG1, and Ly49 receptors, and the downregulation of CD27 (17). Although the acquisition or loss of these surface markers is happening on a continuous scale, it has become customary to distinguish three subsets of immature ($CD27^+CD11b^-$), semi-mature ($CD27^+CD11b^+$) and mature ($CD27^-CD11b^+$) NK cells (27, 28).

In general, compared to their more immature counterparts, mature NK cells produce less cytokines, show a reduced proliferative capacity, but become more cytotoxic against target cells. However, in the process of terminal differentiation NK cells gradually lose their effector functions as well as the expression of the activating receptor DNAM1 (24, 28).

JAK/STAT SIGNALING

Most cytokines that influence group 1 ILC development or functions signal via the Janus kinase / signal transducer and activator of transcription (JAK/STAT) pathway (see **Figure 1**). Depending on the cell type, developmental status and microenvironment, JAK/STAT signaling contributes to the regulation of differentiation, proliferation, migration, survival or cytotoxicity in response to more than 50 cytokines, growth factors and hormones (29–31). Many of these cytokines are crucial for NK cells; their signal transduction and downstream effects are summarized in **Figure 2**. To allow this enormous complexity, the JAK/STAT signaling cascade

Abbreviations: BRAF, rapidly accelerated fibrosarcoma isoform B; CHILP, common helper-like innate lymphoid precursor; CIS, cytokine inducible SH2-containing protein; *Cish*, gene coding for CIS protein; CLP, common lymphoid progenitor; CLPD, chronic lymphoproliferative disorder; DC, dendritic cell; DNAM1, DNAX accessory molecule 1 (CD226); EILP, early innate lymphoid cell progenitor; EOMES, Eomesodermin; γc, common gamma chain; GATA3, GATA-binding protein 3; GH, growth hormone; GOF, gain-of-function; HSC, hematopoietic stem cell; HSV-1, herpes simplex virus 1; ID2, inhibitor of DNA binding 2; IFN, interferon; IFN-I, type 1 interferon [e.g., IFN-α (alpha), IFN-β (beta)]; IFN-II, type 2 interferon [IFN-γ (gamma)]; IFN-III, type 3 interferon [IFN-λ (lambda)]; IFNAR, interferon-α/β receptor; IL, interleukin; ILC, innate lymphoid cell; ILCP, innate lymphoid cell precursor; IRF9, interferon regulatory factor 9; ISGF3, interferon-stimulated gene factor 3; JAK, Janus kinase; *Klrk1*, gene coding for NKG2D protein; LCMV, lymphocytic choriomeningitis virus; LIF, leukemia inhibitory factor; LOF, loss-of-function; mAB, monoclonal antibody; MCMV, mouse cytomegalovirus; MEK, mitogen-activated protein kinase kinase; MHC, major histocompatibility complex; NK, natural killer; NKG2D, natural killer receptor group 2, member D; NKP, NK lineage-restricted progenitor; NKTCL, NK/T-cell lymphoma; OSM, oncostatin M; PMA, phorbol 12-myristate 13-acetate; PYK2, protein tyrosine kinase 2; SCID, severe combined immunodeficiency; SH2, Src homology 2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; T-BET, T-box expressed in T cells; *Tbx21*, gene coding for T-BET; TGF-β, transforming growth factor beta; Th1, T-helper cell type 1; Th2, T-helper cell type 2; T-LGL, T-cell large granular lymphocytic; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TYK2, tyrosine kinase 2; RORγt, retinoic acid-related orphan receptor gamma t; U-STAT, unphosphorylated STAT; VEGF-A, vascular endothelial growth factor A.

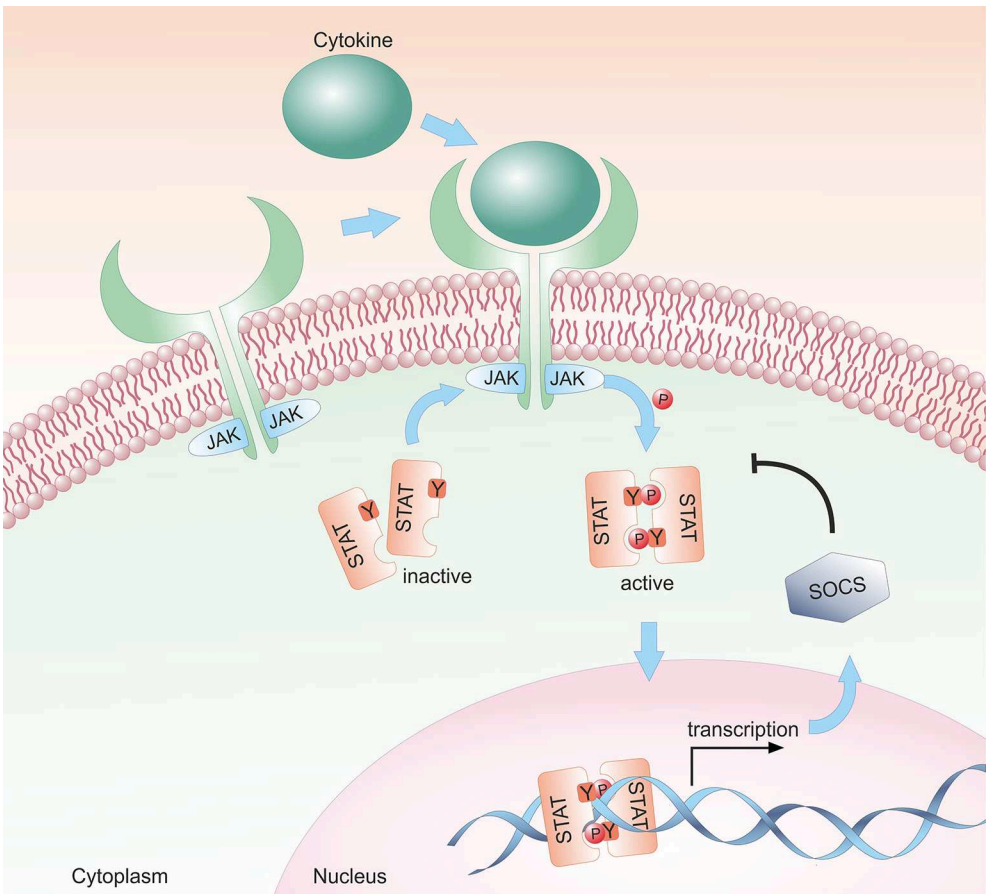

FIGURE 1 | Schematic representation of the canonical JAK/STAT signaling pathway. The JAK/STAT pathway transmits extracellular cytokine signals to the nucleus. Upon binding of a cytokine to its transmembrane receptor, receptor-associated JAKs are activated and phosphorylate STAT proteins. Activated STAT proteins translocate as either homo- or hetero-dimers to the nucleus and modulate target gene transcription. In a negative feedback loop, SOCS proteins are expressed and inhibit the JAK/STAT signaling cascade by suppressing JAK kinase activity, by competing with STAT proteins for binding to the receptor and/or by proteasomal degradation of the proteins.

transports extracellular signals from the cell membrane to the nucleus via various steps. In the canonical signaling cascade, extracellular binding of a cytokine to its corresponding multimeric receptor leads to conformational changes of the receptor chains. Receptor-associated JAK kinases come into close proximity, and sequentially phosphorylate each other and the intracellular portion of the receptor. This creates docking sites for STAT proteins that are recruited to the receptors and phosphorylated on their tyrosine residues by JAK kinases. STAT phosphorylation provokes detachment from the receptor, the formation of homo- or hetero-dimers with other STAT proteins and nuclear translocation. In the nucleus STATs regulate target gene transcription by binding to promotor or enhancer motifs or other non-coding intra- and intergenic regions (29–31) (see **Figure 1**). In addition, several non-canonical pathways have been described; these include kinase-independent functions of JAKs, the formation of higher order STAT tetramers or multifactorial complexes with other transcription factors, and pathways building on unphosphorylated STAT proteins (U-STATs) (31, 32). In NK cells, non-canonical functions have so far been described for TYK2, STAT1 and STAT5 (see below).

The JAK/STAT pathway is highly conserved among species. Mammals express four members of the JAK family (JAK1-3 and TYK2) and seven STAT proteins (STAT1-4, STAT5A, STAT5B, and STAT6). STAT5A and STAT5B are highly homologous but encoded by distinct genes located on the same chromosome directly adjacent to the *Stat3* gene locus indicating that these three genes derived from the duplication of a common primordial gene (33, 34). Although distinct members of the JAK/STAT cascade share high homology, their specific functions vary considerably. Gene-targeted mice have deepened our understanding of distinct roles of individual JAK and STAT proteins (see **Figure 3**). Deficiency of *Jak2* (35) and *Stat3* (36) precludes embryonic development, whereas *Jak1*- (37) and *Stat5a/b*-deficiencies (38) lead to perinatal lethality. Loss of the other members of the JAK/STAT pathway does not interfere with viability of the animals, but reveals distinct phenotypes including the absence of lymph nodes and/or high sensitivity to infections (39).

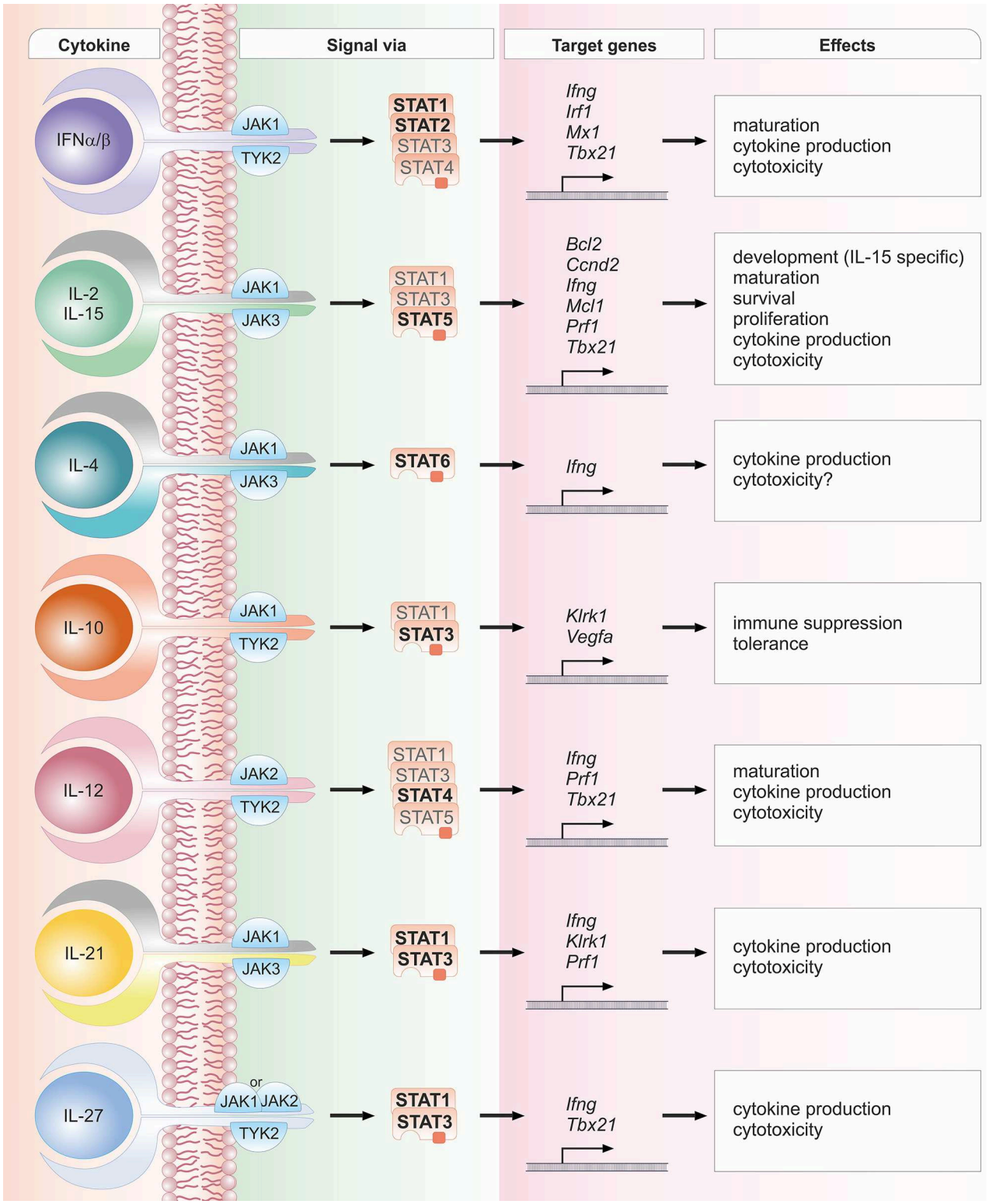

FIGURE 2 | Schematic overview of crucial cytokines in NK cell biology, their associated JAK and STAT proteins, exemplary target genes and biological effects. One cytokine can lead to the activation of several STAT proteins. STAT proteins predominantly activated by the respective cytokine are depicted in bold font; STAT proteins that have been reported to be activated to a lesser extent are depicted in light font. The details and references for distinct cytokine signaling cascades and the functional responses can be found in the corresponding sections of the main text.

JAK1

JAK1 is involved in the signal transduction of several cytokines crucial for NK cell biology, for instance the IL-2 family cytokines including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Most importantly, IL-15 represents the major cytokine regulating NK cell development, maturation and function (17, 40). Additionally, as the major component downstream of IFNs and IL-10, JAK1 plays a pivotal role in NK cell biology (41). It should also be mentioned that JAK1 associates with the IL-4 receptor family (transmitting IL-4 and IL-13 signals) and the gp130 receptor family (transmitting, e.g., IL-6, IL-11, IL-27, LIF, and OSM signals) (41).

Given the fundamental role of IL-15 and other IL-2 family cytokines transmitted via the common γ (γc) receptor, it is not surprising that complete loss of *Jak1* leads to perinatal lethality in mice, accompanied by a strong reduction in the number of thymocytes and B cells (37). These observations were recently confirmed in adult mice: inducible deletion of *Jak1* leads to impaired hematopoietic stem cell homeostasis and a pronounced decrease in immature $B220^+$ NK cells (42).

Using mice with $NKp46^+$ cell-specific deletion of *Jak1* uncovered the crucial role of JAK1 in NK cell development and survival (21). *Jak1* deficiency reduces the numbers of NK cells and ILC1s in a dose-dependent manner. This indicates that other JAK family members fail to compensate for the loss of JAK1. The consequences of *Jak1* deletion within the NK cell compartment exceed the effects seen upon loss of the JAK1 downstream effector STAT5 (21, 22). Different half-lives of JAK1 and STAT5 proteins may contribute to the difference in NK cell frequency. One may also reason that the more pronounced depletion of NK cells results from the combined loss of STAT3 and STAT5-mediated signals in *Jak1*-deficient animals.

To the best of our knowledge, no reports on *JAK1*-deficient individuals exist so far, suggesting that like in mice, it might lead to embryonic lethality in humans. A patient harboring a biallelic *JAK1* germline mutation leading to a partial loss of kinase activity has been reported. The resulting functional impairment was associated with a mild immunodeficiency, recurrent atypical mycobacterial infections and early onset metastatic bladder carcinoma (43).

JAK2

JAK2 is a critical mediator of growth hormone (GH), erythropoietin and IFN-II signaling and thus plays a pivotal role in hematopoiesis (35, 44). In both NK and T cells, IL-2 signals via JAK1/3 and STAT1/3/5 inducing NK and T cell proliferation and enhancing NK cell cytotoxicity. However, unlike in T cells, in NK cells IL-2 additionally activates JAK2 and STAT4 (45). JAK2 in combination with TYK2 mediates the signal transduction of the IL-12 family members: IL-12 activates STAT4 and to a lesser degree STAT1, STAT3 and STAT5 (46); IL-23 activates mainly STAT3 and STAT4, and IL-27 signals mainly via STAT1 and STAT3 (47). Although it was assumed that IL-15 signals exclusively via JAK1/3, a recent study described an IL-15-mediated JAK2 activation in murine NK cells (48).

Germline deletion of *Jak2* results in embryonic lethality at day 12.5 due to impaired hematopoiesis (35). Studies using *Jak2*-conditional knockout mice uncovered a mild defect in NK cell maturation in the absence of JAK2 in the hematopoietic system (49). In line, treatment of mice with the JAK2 inhibitor BSK805 reduces NK cell numbers due to decreased proliferation and an immature maturation profile resulting in an increased metastatic burden (49). In contrast, deletion of *Jak2* in mature NK cells does not impact on NK cell numbers or maturation

Cytokines	Protein	MHC I	NK cell phenotype: in complete (c) or hematopoietic cell-restricted (h) KO mice				NK cell phenotype: in $NKp46^+$ cell-restricted KO mice				References
			DEV	MAT	IFN-γ	CYTO	DEV	MAT	IFN-γ	CYTO	
IFN, IL-2, -4, -7, -10, -15, -21, -27	JAK1		h	h							21, 37, 42
IL-2, -12, -27	JAK2		h	h							21, 49
IL-2, -4, -7, -15, -21	JAK3		c								23
IFN-I, IL-10, -12, -27	TYK2		c	c	c	c					59, 62, 65
IFN, IL-10, -12, -15, -21, -27	STAT1		c	c	c	c					69, 76, 77, 103
IFN	STAT2		c	c	c						99, 103, 104
IFN, IL-10, -12, -15, -21, -27	STAT3		h	h		h					111, 114
IFN, IL-2, -12	STAT4		c	c	c	c					132-134
IL-2, -7, -12, -15	STAT5A/B		h	h	h	h					22, 122, 152, 153, 157
IL-4, -13	STAT6		c	c	c	c					177, 182
IL-2, -15	CIS		c	c	c	c					57
IFN, IL-12	SOCS1		c			c					57, 197
IL-15	SOCS2		c		c	c					48
IL-12, -15, -27	SOCS3		h	h							57, 204

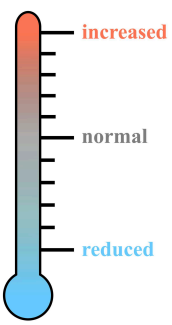

FIGURE 3 | The roles of distinct JAK/STAT and SOCS family members in NK cells. NK cell development, maturation and function are tightly regulated by a plethora of cytokines, which most prominently use the JAK/STAT pathway for their signal transduction. This figure summarizes the available literature about each member of the JAK/STAT signaling pathway and some of their negative regulators (SOCS1-3 and CIS), relevant upstream cytokines and the NK cell phenotypes observed in complete or conditional knockout mice. The individual cells are coded by color: compared to wild-type reduced (blue), unchanged (gray) or increased (red); blank cells indicate not determined yet. c, complete knockout mice; CIS, cytokine induced SH2-containing protein; CYTO, cytotoxicity; DEV, development; h, hematopoietic cell-restricted knockout mice; IFN, interferon; IL, interleukin; IFN-γ, IFN-γ production; JAK, Janus kinase; KO, knockout; MAT, maturation; MHC, major histocompatibility complex; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TYK2, tyrosine kinase 2.

(21). It is conceivable that JAK2 is crucial for development and maturation of early NK cell progenitor stages in the bone marrow. Alternatively, JAK2-inhibition or deletion may interfere with other cell types to alter NK cells extrinsically by changing the cytokine milieu. JAK2 has been reported to be required for the development of dendritic cells (DCs) (50), which are potent producers of IL-15 and thus indispensable for proper NK cell priming (51). DC-mediated NK cell priming is potentially impaired upon JAK2 inhibition or deletion. Support for this concept stems from the observation that IL-15 treatment overcomes the JAK2 inhibitor-mediated increase of tumor metastasis (49).

JAK3

Together with JAK1, JAK3 transmits signals downstream of the γc cytokines IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 resulting in phosphorylation of STAT1, STAT3, STAT4, STAT5 and STAT6 (41, 52). *Jak3*$^{-/-}$ mice are immunocompromised, display severe developmental defects in the lymphoid lineage and lack NK, T and B cells (23, 53). The *in vivo* administration of Tofacitinib, which predominantly inhibits JAK3 and to a lesser degree JAK1 and JAK2, depletes all NK cell subsets in the periphery of rhesus macaques (54). Likewise, human patients harboring *JAK3* mutations suffer from severe combined immunodeficiency (SCID) lacking NK and T cells (55). A previous study in mice having a spontaneous *Jak3* mutation disclosed the association of an impaired JAK3 signaling with a differentiation block of NK and ILC1s at the pre-NKP and ILCP stage (56). In summary, these findings define a non-redundant role of JAK1 and JAK3 for NK cell development and the differentiation of EILPs.

Interestingly, quantitative mass spectrometry analysis in mature NK cells demonstrated a predominance of JAK1 protein compared to JAK3 (57). It was further proposed that JAK1 dominates JAK3 in the signal transduction of γc cytokines. While loss of the JAK1 kinase function completely abrogates downstream signals, loss of the kinase activity of JAK3 in human cell lines only diminishes STAT5 phosphorylation. It was thus suggested that JAK3 functions by activating JAK1, but does not directly induce STAT5 phosphorylation (58). The details of the molecular interactions in NK cells remain to be determined. It is currently unclear what effect the conditional deletion of *Jak3* in mature NK cells will have and how it will affect their proliferation and effector functions.

TYK2

TYK2 associates with the IFN-I (IFNAR1), IL-10Rβ, IL-12Rβ1, and IL13Rα1 receptors and is thus involved in the signal transduction of a large number of cytokines including IFN-I, IL-10, IL-12, IL-23, and IL-27 (46). Despite its broad activity, *Tyk2*-deficiency does not preclude survival of mice (59, 60).

NK cells derived from *Tyk2*$^{-/-}$ mice display impaired IL-12-mediated signaling resulting in reduced STAT1, STAT3, and STAT4 activation (61, 62). Although the development of NK cells in the bone marrow is unaltered, the final maturation in the periphery is severely impaired as evidenced by fewer CD27^{-}CD11b^{+} and KLRG1^{+} cells in *Tyk2*-deficient mice. This translates into impaired IFN-γ production and cytolytic responses (62) in line with the involvement of STAT4 in the regulation of IFN-γ and perforin expression, respectively (63, 64). Mice expressing a kinase-inactive version of TYK2 (*Tyk2*^{K923E}) show a milder defect in NK cell maturation and cytotoxicity compared to *Tyk2*$^{-/-}$ mice, indicating that TYK2 has kinase-independent functions (62). Using mice with NKp46^{+} cell-specific deletion of *Tyk2* revealed that the impact of TYK2 on NK cell maturation and tumor surveillance is cell-extrinsic and depends on the presence of TYK2 in dendritic cells (65). Accordingly, the defects in NK cell maturation and cytotoxicity related to *Tyk2*-deficiency are reversed upon treatment with recombinant IL-15/IL-15Rα. However, NK cell-intrinsic TYK2 is required for IL-12-induced IFN-γ production and the defense against *Listeria monocytogenes* (65).

Tyk2-deficient mice and patients with autosomal recessive *TYK2* mutations are susceptible to infections. NK cells from *TYK2*-deficient patients have an impaired, albeit not completely abrogated, IL-12-mediated IFN-γ production (60). This may explain why their susceptibility to viral infections is less severe when compared to patients harboring a *STAT1*-deficiency (66). A thorough analysis of peripheral NK cell maturation in the reported *TYK2*-deficient patients is pending.

STAT1

STAT1 is the predominant transcription factor activated by IFNs, irrespective of the subtype. Whereas, type II IFN (IFN-γ) induces the homo-dimerization of STAT1, type I IFN (IFN-α/β/ε/κ/ω) and type III IFN (IFN-λ 1-3) signaling triggers the formation of the ISGF3 complex consisting of STAT1, STAT2, and IRF9. In addition, STAT1 transmits signals from IL-6, IL-10, IL-12, IL-15, IL-21, IL-27, and IL-35 (31, 67–71).

IFNs play a pivotal role in NK cell maturation, as they provide the necessary signals for IL-15 trans-presentation by DCs (51) and MHC class I expression (37, 72, 73). *Stat1*-deficient NK cells show profound defects in NK cell maturation, cytokine-induced IFN-γ production, cytolytic capacity and memory formation (69, 74–77). In line, NK cells from *Ifnar1*-deficient animals display an immature phenotype (78) as well as defects in basal (79) and virus-induced cytotoxicity (80). The maturation defect seen in complete *Ifnar1*-knockout mice was not recapitulated upon NKp46^{+} cell-specific *Ifnar1* gene deletion (78), suggesting that NK cell-extrinsic factors, such as the presentation of MHC class I and/or IFN-mediated trans-presentation of IL-15, play a crucial role in proper NK cell licensing and maturation. Accordingly, the transfer of *Stat1*-deficient bone marrow into wild-type mice provided sufficient signals for proper NK cell maturation *in vivo* (76).

In addition to IFNs, the STAT1/3 activating (70) cytokine IL-21 is known to drive the maturation of mouse and human NK cells (81, 82). Recombinant IL-21 treatment not only increases CD8^{+} T cell functions (83) but also the cytotoxicity and cytokine production of NK cells by inducing the expression of perforin and IFN-γ, respectively (82, 84). However, mice lacking the IL-21R do not display any defect in NK cell numbers or maturation (85) arguing against a profound effect of IL-21 on NK cell maturation under homeostatic conditions *in vivo*.

Analogous to IL-21, IL-27 signals mainly via STAT1 and STAT3 (47, 86). Whereas, IL-27 treatment alone does not have a major impact on NK cells, co-stimulation with IL-27 and IL-2, IL-12 or IL-18 leads to enhanced NK cell activation, cytokine production and cytotoxicity (47, 86–88). Accordingly, loss of IL-27R in mice leads to reduced NK cell-mediated IFN-γ production and T-BET expression after influenza virus infection (89). *Il27ra*-deficient mice are characterized by an unusual maturation profile, represented by fewer mature NK cells in the bone marrow, while more mature NK cells are found in the spleen (89). This phenotype does not reflect the situation in *Stat1*-deficient mice and may suggest an altered dissemination of NK cells rather than a maturation defect.

However, *Stat1-Y701F* knock-in mice lacking the tyrosine residue essential for STAT1 translocation and transcriptional activity do mirror the impaired maturation phenotype of NK cells seen in *Stat1*$^{-/-}$ mice (90). Although the function of STAT1 was considered to depend on the tyrosine phosphorylation, *Stat1-Y701F* expressing NK cells are more cytotoxic against tumor cells than *Stat1*$^{-/-}$ NK cells. A novel non-canonical function of STAT1 at the immunological synapse of NK cells regulating tumor surveillance and cytotoxicity may account for that effect (90). Whereas STAT1-Y701 phosphorylation is triggered by cytokine stimulation, non-stimulated primary mouse NK cells display a constitutive CDK8-mediated phosphorylation of STAT1-S727 (69). The introduction of a point-mutation (*Stat1-S727A*) that prevents the serine phosphorylation event results in NK cells that produce less IFN-γ upon stimulation, and have a mild defect in KLRG1 and NKG2A/C/E expression. Nevertheless, these *Stat1-S727A* NK cells show enhanced cytotoxicity against tumors *in vitro* and *in vivo*, which correlates with increased perforin and granzyme B levels (69), once more highlighting the existence of non-canonical STAT signaling (32).

Like *Tyk2*-deficient mice, *Stat1*-deficient mice are highly susceptible to bacterial and viral infections (80, 91–93). Biallelic *loss-of-expression* or *loss-of-function* (*LOF*) *STAT1*-deficiency in humans is detrimental, with most patients succumbing to lethal infections with mycobacteria or herpes simplex virus 1 (HSV-1) encephalitis before the age of two years (66), which is accompanied by a profound effect on NK cell cytotoxicity (94). Unexpectedly, *STAT1 gain-of-function (GOF)* mutations are likewise associated with increased susceptibility to infectious diseases, such as chronic mucocutaneous candidiasis, bacterial and viral infections, autoimmune diseases and even cancer (95). *STAT1 GOF* patients have fewer and highly immature CD56^{dim} NK cells in the periphery showing reduced cytotoxicity, IFN-γ production and cytokine-induced proliferation (96, 97). This defect was partially rescued by treatment with the JAK1/2 inhibitor ruxolitinib (96) and improved the patients' clinical picture (98). The mechanism of how *STAT1 GOF* mutations result in hyporesponsive NK cells is not fully understood, but it was paralleled by decreased activation of STAT5 (96), which is a master regulator for NK cell functions. These observations indicate that STAT1 signaling needs to be tightly controlled and neither reduced nor excessive pathway activation is beneficial for NK cell maturation and function.

STAT2

STAT2 together with STAT1 and IRF9 are activated in response to IFN-I and IFN-III. This turns STAT2 into a crucial mediator of antiviral defense. Depending on the viral challenge, *Stat2*$^{-/-}$ mice are more (99, 100) or less (101, 102) susceptible to infection compared to *Stat1*$^{-/-}$ mice. In the course of lymphocytic choriomeningitis virus (LCMV) infections, STAT1 and STAT2 are both required for optimal viral control, but STAT2 plays a subordinate role compared to STAT1: although both *Stat1*- and *Stat2*-deficient NK cells produce increased amounts of IFN-γ early after LCMV infection, this exclusively drives bodyweight loss in the absence of STAT1, but not STAT2 (103). *Stat2*$^{-/-}$ mice are highly susceptible to MCMV infection and succumb within the first week after infection (100). In line with a crucial role of IFN-I signaling in NK cell expansion and memory formation in the context of MCMV, NK cells from *Stat1*-, *Stat2*-, and *Irf9*-deficient mice are defective in their ability to expand (74, 104). As shown in *Stat1*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice, also *Stat2*$^{-/-}$ NK cells have a defect in NK cell maturation, which could be rescued in bone marrow chimeras (104), again suggesting an NK cell-extrinsic role of IFN-I in NK cell maturation.

STAT2-deficient human patients present a higher incidence of distinct viral infections with astounding variation ranging from asymptomatic adult carriers of the mutation to infants succumbing to viral illness. In particular, fatal prolonged febrile encephalitic illness following measles/mumps/rubella vaccination has been reported in six vaccinated children with a *STAT2* deficiency (105–107). Unlike STAT1, STAT2-mediated signaling seems to be dispensable for host defenses against most viral childhood diseases such as respiratory syncytial virus bronchiolitis or HSV-1 as well as infections with intracellular bacteria. This can be partially explained by an unaltered response to IFN-II in *STAT2*-deficient patients, and the observation that depending on the bacterial infection IFN-I can play adverse roles (108, 109).

Besides its role in antiviral responses, IFN-I has been implicated in anti-tumor immunity. While the contribution of STAT2 for T cell-mediated tumor surveillance has been unequivocally documented (110), the role of STAT2 in NK cell-mediated tumor surveillance is still enigmatic.

STAT3

Cytokines such as IL-2, IL-10, IL-12, IL-15, IL-21, IL-27, and IFN-I induce STAT3-Y705 phosphorylation in NK cells (111, 112). While most of these cytokines positively regulate NK cell maturation and/or activation, IL-10 is classified as immunosuppressive cytokine (113).

Several studies reported constitutive STAT3 phosphorylation of tumor-infiltrating immune cells including NK cells (114, 115). STAT3 phosphorylation is considered to be driven by inflammatory and immunosuppressive cytokines and growth factors produced by both tumor and tumor-infiltrating cells including IL-6, IL-10, or VEGF-A. STAT3 activation in the tumor stroma has been associated with an impaired tumor immune surveillance of both NK and CD8^{+} T cells (116, 117). High IL-10 levels in the liver also dampen hepatic NK cell responses and restrain the expression of Ly49 receptors (118). In light of

recent advances in the discrimination of NK cells and ILC1s, these observations could potentially indicate a specific role of IL-10 in ILC1s, which lack most of the Ly49 receptors (119). This suppressive role is of particular importance in the liver, where IL-10 ensures that liver NK cells/ILC1s remain immune-tolerant, but is undesirable in the context of tumor surveillance (113, 115). Under certain conditions NK cells themselves (120) and the recently described regulatory ILCs have been reported to produce IL-10, which inhibits cytokine-induced IFN-γ production of ILC1s (121).

Studies in mice with constitutive or NKp46$^+$ cell-specific *Stat3*-deficiency indeed show that STAT3 suppresses NK cell-mediated tumor surveillance in melanoma and leukemia models (111, 114). Loss of *Stat3* does not alter classical NK cell maturation but is paralleled by increased expression of the activating receptor and maturation marker DNAM1 as well as increased expression of STAT5 and its downstream targets perforin and granzyme B (111). It is thus attractive to speculate that STAT3 represses STAT5-mediated signaling in wildtype NK cells. As described below, STAT5 represents a master regulator of NK cell function. The fact that IL-15 stimulation induces both STAT3 and STAT5 activation in NK cells (111, 113) endorses the hypothesis that STAT3 is crucial to control IL-15/STAT5-mediated NK-cell cytotoxicity to prevent detrimental hyperactivity. This concept warrants testing of a combined treatment with IL-15 and anti-STAT3 inhibitors in the context of anti-cancer immunotherapy.

Alternatively, STAT3 acts downstream of the cytokine IL-10 (111), which has been shown to transcriptionally induce the tumor promoting factor VEGF-A in NK cells (122). It is thus attractive to speculate that STAT3 activation in NK cells promotes tumor progression by dampening their cytolytic activity and driving tumor angiogenesis.

Besides suppressing cytotoxicity, STAT3 regulates the expression of the activating receptor NKG2D. IL-10 and IL-21 treatment induces NKG2D expression in a STAT3-dependent manner in human and mouse NK cells (71, 123). In line, human NK cells derived from hyper-IgE syndrome patients carrying *STAT3 LOF* mutations show a pronounced decrease of NKG2D expression (71).

STAT3 GOF mutations in NK cells can be found in patients with chronic lymphoproliferative disorders of NK cells (CLPD-NKs) (124) as well as aggressive NK cell leukemia (125) and extranodal NK/T-cell lymphoma (NKTCL) (126, 127). The identified *STAT3* mutations enhance the levels of phosphorylated STAT3 protein and provide a growth advantage to the affected cells. These findings support the concept that STAT3 has an oncogenic potential in NK cells and highlight the importance of tight controls and negative feedback regulators.

STAT4

In contrast to other immune cells such as CD8$^+$ T cells, IL-2 stimulation induces JAK2 and STAT4 activation in NK cells and enhances IL-12 signaling by upregulating the expression of the IL-12R (128, 129). IL-12 is the main driver of STAT4 activation and crucial for IFN-γ production in NK cells and ILC1s (129–131). Under steady-state conditions, *Stat4*- as well as *Il12r*-deficient mice harbor an unaltered NK cell repertoire in the periphery. Due to its rather restricted action downstream of IL-12, *Stat4*-deficiency in mice manifests in reduced IL-12-induced NK cell proliferation, IFN-γ production and cytotoxicity (132, 133). This can be explained by the fact that STAT4 regulates the induction of T-BET, a transcription factor important for NK and ILC1s that induces the transcription of important key players of the cytotoxic machinery, such as IFN-γ, granzyme B and perforin (131, 134). STAT4 and T-BET are also necessary for the generation and maintenance of MCMV-specific memory NK cells (135, 136). In line with the lessons learnt from mice, a heterozygous missense mutation in *STAT4* leading to a defect in IL-12-dependent IFN-γ immunity was identified in two patients suffering from acute chronic fungal infections (137).

IL-12 also has a unique and detrimental role in adipose tissue, as diet-induced obesity is associated with IL-12 production and the proliferation and subsequent accumulation of adipose-resident ILC1 and NK cells. IL-12/STAT4 signaling is required for the increased proliferation and IFN-γ production of all group 1 ILC subsets in the adipose tissue driving M1 macrophage polarization and obesity-associated insulin resistance (138).

Apart from IL-12, IFN-I has been reported to induce phosphorylation and dimerization of STAT4 amongst all other STAT proteins (139). NK cells have particularly high basal STAT4 levels pre-bound to IFNAR1 (103). During the early phase of viral infections, STAT4 becomes activated initiating a fast IFN-γ response followed by STAT1 activation, which replaces STAT4 at the IFNAR receptor decreasing the ability to produce IFN-γ (103). These data exemplify how one cytokine activates several STAT molecules enabling a tight regulation of cellular responses.

STAT5A and STAT5B

Of all STAT proteins, STAT5 is the major regulator of NK cell development, maturation, survival and function and is activated by cytokines such as IL-2, IL-7 and IL-15. Compared to IL-15, IL-2, and IL-7 play a minor role in the development and survival of NK cells and ILC1s (140, 141). STAT5 is also implicated in the development, survival and memory formation of CD8$^+$ T cells, which is regulated by IL-2, IL-7, and IL-15 signaling (142, 143). IL-2 plays a crucial role in activating CD8$^+$ T and NK cells against target cells *in vivo* (144, 145) and it is therefore commonly added to *in vitro* culture systems. Although mouse NKPs express high levels of CD127 (IL-7Rα) (146), NK cell development and function are unaltered in the absence of IL-7 signaling in mice (147). The only exception are thymic NK cells, whose development depends on IL-7 and the transcription factor GATA3 (148). By contrast, in humans IL-7 controls the survival of immature CD56^{bright} NK cells (149).

Knock-out mice lacking IL-15 or its receptor subunits are devoid of NK cells proving the indispensable role of IL-15 for NK cell development (141, 150, 151). IL-15 trans-presentation by DCs is crucial to prime NK cell maturation and function (51). As STAT5 is a critical transcription factor downstream of IL-15, impaired STAT5 signaling impacts strongly on NK cell viability and function (152, 153).

In general, STAT5 is an umbrella term for two distinct transcription factors: STAT5A and STAT5B sharing 96%

sequence homology. Despite largely redundant functions, several non-redundant and tissue-specific roles have been described (154–156). *Stat5a/b*-deficiency in mice is perinatally lethal due to anemia and hematopoietic failure (38). Early on, STAT5 has been described to be essential for NK cell development, as the first STAT5 knockout mice that express an N-terminally truncated version of *Stat5a/b* are viable but devoid of peripheral NK cells (153). Single knockout mice for *Stat5a* or *Stat5b* verified the impact of STAT5 for NK cell development, maturation and cytoloytic capacity also indicating non-redundant functions of STAT5A and STAT5B. STAT5B is the dominant isoform for NK cells as its deletion has a significantly larger impact than deletion of STAT5A (152, 157). This is explained by a higher abundance of STAT5B over STAT5A transcripts in NK cells (157). Mice expressing only one allele of either *Stat5a* or *Stat5b* ($Stat5a^{+/-}Stat5b^{-/-}$ and $Stat5a^{-/-}Stat5b^{+/-}$) have drastically diminished numbers of NK and ILC1 progenitors, splenic NK cells as well as intestinal and liver NK cells (157). Liver-resident ILC1s and bone marrow NK cells are less sensitive to reduced STAT5 expression levels. STAT5 was also verified as an upstream regulator of the transcription factor T-BET (122, 158) and a recent study showed that both transcription factors co-localize throughout the genome (157).

The cell-intrinsic role of STAT5 in NK cells was studied using mice where *Stat5a/b* deletion is restricted to $NKp46^{+}$ cells. This results in a severe reduction of peripheral NK cells (22) as NK cell survival relies on the expression of anti-apoptotic STAT5 target genes such as *Mcl1* or *Bcl2* (122, 159). The residual NK cells found in the bone marrow of $Stat5^{fl/fl}Ncr1iCre^{Tg}$ mice harbor an immature phenotype and a major developmental block at the NKP stage (22). Enforced expression of the anti-apoptotic factor Bcl-2 rescues survival of *Stat5a/b*-deficient NK cells, but does not allow proliferation, maturation and reconstitution of effector functions (122). Apart from the central role of STAT5 driving the expression of transcription factors pivotal for NK cell development (ID2, EOMES and T-BET) and regulating the expression of crucial effector molecules (perforin, granzymes and IFN-γ), STAT5 has been reported to suppress the expression of the pro-angiogenic factor VEGF-A in NK cells (122). Further research is necessary to verify if STAT5 directly acts as a transcriptional suppressor or competes with the binding of other activating transcription factors.

Decidual NK cell-derived VEGF-A has a positive impact on neo-angiogenesis and placenta development during pregnancy (160–162). In contrast, the expression of VEGF-A in tumor-infiltrating NK cells promotes tumor formation (122). VEGF-A-secreting tumor-associated NK cells have also been reported in patients and are associated with poor disease outcome (163–165).

In line with observations in mice, patients with a *STAT5B LOF* mutation harbor significantly reduced NK cell numbers (166–168). *STAT5 GOF* mutations are found in malignancies of innate and innate-like lymphoid cells (125, 127, 169) and drive tumorigenesis in mouse NKT cells (170).

STATs in general, but STAT5 proteins in particular, are known to form higher order tetramers. A recent study highlighted the importance of STAT5 dimers for NK cell development, while the formation of STAT5 tetramers is a prerequisite for proper NK cell maturation and survival. The authors speculate that interfering with STAT5 tetramer formation could be used therapeutically to restrict the growth of NK cell leukemia and lymphomas (171).

To summarize, STAT5 is a master regulator of NK cells ensuring their development and survival and regulating maturation, proliferation, cytotoxicity and their precarious production of VEGF-A.

STAT6

STAT6 is activated by IL-4 and IL-13 and is involved in Th2 polarization and the development of allergic inflammation (172). Allergies and IL-4 signaling have been suggested to protect from cancer development. Indeed, IL-4 overexpression in combination with phthalic anhydride-induced allergy induction in mice enhances NK cell activity and reduces tumor burden (173). The effect of IL-4 on NK cells is highly controversial, as it was shown that IL-4 treatment of purified NK cells diminishes their cytotoxic capacity (174), while it enhances NK cell cytotoxicity and IFN-γ production when applied *in vivo* (175). IL-4 synergizes with IL-12 and/or IL-2 to induce IFN-γ production, which was shown to be partially dependent on STAT6 (176). *In vitro* stimulation of mouse NK cells with a mixture of phorbol 12-myristate 13-acetate (PMA), ionomycin, IL-2, IL-4, and anti-IFN-γ mAb induces IL-5 and IL-13 production in a STAT6-dependent manner (177). Also human NK cells possess the ability to produce IL-5 and IL-13 upon IL-4 stimulation (178, 179). In various allergic diseases, such as asthma and allergic rhinitis, NK cell-derived Th2 cytokine production contributes to eosinophil infiltration and thereby promotes allergic inflammation (180, 181). Although the involvement of STAT6 in this signaling is highly probable, it still awaits formal proof.

It was previously shown that loss of STAT6 in mice does not impact on NK cell development or maturation (177). However, *Stat6*-deficiency is associated with higher cytotoxic activity of NK cells and increased resistance to ectromelia virus infection (182). The seeming opposing results showing enhanced cytotoxicity of IL-4/STAT6-activated as well as STAT6-deficient NK cells certainly call for a more detailed analysis of the role of STAT6 in NK cells in the context of anti-tumor and anti-viral immunity.

SOCS PROTEINS

The family of suppressor of cytokine signaling (SOCS) proteins has eight members including SOCS1-7 and CIS which represent important negative regulators of the JAK/STAT signaling pathway (183, 184). SOCS proteins are characterized by a central SH2 domain and an extended SH2 sub-domain, a highly conserved C-terminal SOCS box and a variable N-terminal region. SOCS proteins exert their eponymous function by three means: (i) Via their SH2 domain, SOCS proteins bind to phosphotyrosine residues on cytokine receptors thereby competing with STAT binding and activation. (ii) Via the SOCS box they recruit an E3 ubiquitin ligase complex that leads to proteasomal degradation of signaling molecules including cytokine receptors and JAK kinases. (iii) The N-terminal domain of SOCS1 and SOCS3 contains a kinase inhibitory region serving as pseudo-substrate for JAKs consequently blocking their activity (183, 184).

Immunomodulatory effects of SOCS proteins on NK cells have been reported and suggest them as attractive candidates for immunotherapies (185). SOCS1, 2, 3 and CIS are rapidly induced upon cytokine (57, 186) or GH (187) stimulation. In contrast, little is known about the residual family members SOCS 4-7 that are constitutively expressed in unstimulated cells (185). SOCS4 and SOCS5 are crucial regulators of anti-viral immunity in the context of influenza infection (188, 189). SOCS6 negatively regulates JAK/STAT3 signaling and is epigenetically silenced in NK cell lymphomas (190). *Socs7*$^{-/-}$ mice suffer from a severe cutaneous disease due to hyperactive mast cells and the increased production of pro-inflammatory cytokines (191).

CIS

The cytokine induced SH2-containing protein (CIS, *Cish*) is induced by IL-2 and IL-15 and provides a negative feedback loop to inhibit JAK/STAT5-mediated signaling in NK cells (57). CIS interacts with JAK1 to target it for proteasomal degradation and thereby abrogates IL-15-induced signaling. In line with the crucial function of the IL-15/STAT5 axis for NK cell biology, hyperactive IL-15 signaling in *Cish*$^{-/-}$ mice translates to enhanced NK cell proliferation and cytotoxic function. This ultimately leads to resistance toward experimental metastasis (57) and chemically-induced sarcoma (192). *Cish*$^{-/-}$ mice react to IL-2 treatment with a further decrease of tumor burden in models that are usually unaffected by IL-2 treatment. Additive effects were also observed when CIS-deficiency was combined with targeted immunotherapies such as BRAF and MEK inhibitors, immune checkpoint blockade antibodies, or IFN-I treatment (192). These data suggest that CIS represents a promising target in immunotherapy especially in combination with other immunomodulatory agents (185).

SOCS1

SOCS1 negatively regulates signaling of IFNs and IL-12 (193, 194) and plays an important role in DC and T cells suppressing antigen-presentation and antitumor immunity (195). *Socs1*$^{-/-}$ mice die shortly after birth due to severe inflammation and uncontrolled IFN-γ signaling (196). *Socs1*$^{-/-}$*Ifng*$^{-/-}$ double-knockout mice survive until adulthood (196) and IL-12-treated NK cells isolated from these mice display an enhanced capacity to lyse YAC-1 target cells (197). However, *Socs1*$^{-/-}$*Ifng*$^{-/-}$ mice seem to have slightly reduced NK cell numbers in the periphery and hampered NK cell proliferation in response to IL-15 (57). A detailed analysis of the role of SOCS1 in NK cell development, maturation and function is pending.

SOCS2

SOCS2 is closely related to CIS and induced by STAT5-activating cytokines, such as GH and IL-15 in mouse and human NK cells (57, 186, 187). SOCS2 represses NK cell development, as *Socs2*$^{-/-}$ mice have increased NK cell numbers in bone marrow and spleen while T-, B- and myeloid cell numbers are unaltered (48). The increased NK cell numbers translate into enhanced tumor surveillance. In contrast to the situation in *Cish*-deficient mice and against the expectations, *Socs2*-deficiency does not enhance the cytotoxicity or IFN-γ production of NK cells. Intriguingly, the absence of SOCS2 boosts IL-15-induced JAK2/STAT5 activation in NK cells (48), which has commonly been believed to signal via JAK1 and JAK3.

In contrast to murine NK cells, knockdown of *SOCS2* has no impact on the IL-15-induced *in vitro* differentiation of primary human NK cell precursors, but severely diminishes the cytotoxic function of primary NK cells and the human NK cell line NK-92. The reduced cytotoxicity was assigned to impaired degradation and accumulation of the focal adhesion kinase PYK2 (198), which is involved in the formation of the NK/target cell synapse upon killing (199). Although it is clear that SOCS2 interferes with NK cell functions, the distinct roles of SOCS2 in human and mouse NK cells remain enigmatic.

SOCS3

Socs3$^{-/-}$ mice are embryonically lethal due to placental defects (200) and impaired fetal liver hematopoiesis (201). SOCS3 counteracts inflammation by inhibiting a variety of pro-inflammatory signaling pathways (202). Most prominently, SOCS3 inhibits gp130 receptor/STAT3 signaling by direct inhibition and/or ubiquitin-mediated degradation of the receptors or their associated JAK kinases (downstream of IL-6, IL-11, IL-27, OSM and LIF). SOCS3 was also shown to negatively regulate IL-12-induced STAT4 activation by blocking the IL-12Rβ2 subunit via its SH2 domain (203).

In mouse NK cells, SOCS3 is induced upon IL-15 signaling (57) and is a direct target gene of the helix-loop-helix protein ID2 (204). *Id2* deletion in NKp46^{+} cells leads to a complete absence of peripheral NK cells due to impaired IL-15 signaling. NK cell numbers are rescued by additionally deleting *Socs3* (204). However, loss of *Socs3* alone in the presence of ID2 does not alter the development, maturation or IL-15-induced proliferation of mouse NK cells (57, 204). CRISPR/Cas9-mediated disruption of *SOCS3* in human NK cells promotes proliferation and cytotoxicity (205), thereby suggesting that SOCS3 may be a useful target for NK cell-based immunotherapy. However, taking into account the detrimental effect of *Socs3*-deficiency in mice, any envisaged inhibitor treatment will have to be applied specifically on NK cells to avoid generic adverse side-effects.

CONCLUDING REMARKS

The JAK/STAT pathway is evolutionary highly conserved and transmits extracellular signals to the nucleus modulating target gene transcription. Members of this pathway are frequently altered in cancer including malignancies of innate lymphocytes, making them an attractive target for drug development. Several JAK inhibitors are already used for the treatment of rheumatoid arthritis, psoriasis and myelofibrosis, and entered phase 2 and 3 clinical trials for the treatment of other inflammatory diseases and cancer. While the first clinically used inhibitors such as Ruxolitinib or Tofacitinib, proved to target multiple JAK kinases, more specific compounds found their way into clinical trials (206, 207).

We and others have previously shown that treatment with the JAK1/2 inhibitor Ruxolitinib substantially impairs NK cell functions leading to increased susceptibility to viral infections

and tumor metastasis (49, 208). In line, in a mouse B cell lymphoma model Ruxolitinib treatment promotes tumor progression by enhancing NK cell-derived VEGF-A expression (122). On the other hand, Ruxolitinib treatment significantly reduces disease burden in the context of $CD56^+$ T-cell large granular lymphocytic (T-LGL) leukemia (170) and restores impaired NK cell functions in patients harboring *STAT1 GOF* mutations (96).

JAK inhibitor treatment shall be carefully evaluated to identify the complex interplay and potential opposing effects on target and immune cells. While in the context of inflammatory and immune-related diseases JAK inhibitor-induced dampening of NK cell functions may be advantageous, NK cell malfunction in metastatic cancers should be precluded. Besides blocking JAK kinases, considerable effort is undertaken to develop specific STAT inhibitors. This could be of particular interest for the field of immunotherapy, as treatment with STAT3 or STAT6 inhibitors may enhance NK cell cytotoxicity. Finding ways to efficiently improve NK cell functions will promote the use of adoptively transferred NK cells in everyday clinics.

Targeting the negative regulators of the JAK/STAT pathway also holds great promise as novel immunotherapeutic strategy. In particular, CIS was shown to be a checkpoint in NK cell-mediated tumor control making it an attractive candidate for anti-tumor therapy. The expanding knowledge of immune checkpoints and potential drug candidates opens a new avenue for immunotherapy, yet the next challenge is to develop specific and stable compounds suitable for clinical use.

AUTHOR CONTRIBUTIONS

JT generated the figures. DG, VS, and EP drafted the figures and wrote the manuscript.

FUNDING

The authors are supported by grants from the Austrian Science Fund (FWF): doc.fund DOC 32-B28 and F6107 awarded to VS, the stand-alone project P32001-B awarded to EP.

ACKNOWLEDGMENTS

We would like to thank our laboratory members and the entire JAK/STAT community for numerous discussions and K. Klein for proofreading of the manuscript and to apologize to all investigators whose research was not cited.

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 November 2019
doi: 10.3389/fimmu.2019.02692

29-Color Flow Cytometry: Unraveling Human Liver NK Cell Repertoire Diversity

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Recent studies have demonstrated extraordinary diversity in peripheral blood human natural killer (NK) cells and have suggested environmental control of receptor expression patterns on distinct subsets of NK cells. However, tissue localization may influence NK cell differentiation to an even higher extent and less is known about the receptor repertoire of human tissue-resident NK cells. Advances in single-cell technologies have allowed higher resolution studies of these cells. Here, the power of high-dimensional flow cytometry was harnessed to unravel the complexity of NK cell repertoire diversity in liver since recent studies had indicated high heterogeneity within liver NK cells. A 29-color flow cytometry panel allowing simultaneous measurement of surface tissue-residency markers, activating and inhibitory receptors, differentiation markers, chemokine receptors, and transcription factors was established. This panel was applied to lymphocytes across three tissues (liver, peripheral blood, and tonsil) with different distribution of distinct NK cell subsets. Dimensionality reduction of this data ordered events according to their lineage, rather than tissue of origin. Notably, narrowing the scope of the analysis to the NK cell lineage in liver and peripheral blood separated subsets according to tissue, enabling phenotypic characterization of NK cell subpopulations in individual tissues. Such dimensionality reduction, coupled with a clustering algorithm, identified CD49e as the preferred marker for future studies of liver-resident NK cell subsets. We present a robust approach for diversity profiling of tissue-resident NK cells that can be applied in various homeostatic and pathological conditions such as reproduction, infection, and cancer.

Keywords: natural killer cells, liver immunology, tissue-resident cells, high-dimensional, flow cytometry

OPEN ACCESS

Edited 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: *30 August 2019*
Accepted: *01 November 2019*
Published: *19 November 2019*

Citation:
Filipovic I, Sönnerborg I, Strunz B, Friberg D, Cornillet M, Hertwig L, Ivarsson MA and Björkström NK (2019) 29-Color Flow Cytometry: Unraveling Human Liver NK Cell Repertoire Diversity. Front. Immunol. 10:2692. doi: 10.3389/fimmu.2019.02692

INTRODUCTION

The last five decades have seen extraordinary developments in the understanding of natural killer (NK) cell biology. NK cells are innate lymphocytes originally discovered as cells capable of killing tumor cells and later virally-infected cells (1, 2). One of the major pathways of cell death mediated by NK cells involves secretion of cytolytic molecules like perforin and granzymes, which makes NK cells functionally related to their adaptive counterpart, cytotoxic T lymphocytes (CTLs) (3, 4). However, the mechanisms which trigger the killing of the target cells are fundamentally different

between these two lineages. NK cells use an array of germline-encoded receptors to carry out their main tasks associated with the recognition of non-self: tumor surveillance and clearance of viral infections (5, 6). Engagement of distinct activating and inhibitory receptors expressed on the surface of NK cells by their respective ligands determines the functional response. Importantly, genetic and environmental determinants shape the overall diversity of these receptors (7).

Since their discovery, it has become clear that NK cells are found not only in circulation, but also in lymphoid organs as well as non-lymphoid organs like uterus and liver (8). The liver is instrumental in regulating systemic homeostasis, and represents an organ with a dynamically changing microenvironment (9). Notably, it is also highly enriched in immune cells and has a distinct immune composition: NK cells are among the most abundant, representing 30–40% of human intrahepatic lymphocytes compared to the 10–15% typically observed in peripheral blood (10). The microenvironment of the liver has a complex anatomical organization (11) and is essential in maintaining tolerance toward antigens derived from the gut, including the diverse gut microbiome, via the gut-liver axis (12). Unsurprisingly, a subset of liver NK cells with antigen-specific memory was described in the mouse (13). These cells express CXCR6 which, although not required for antigen recognition, reliably labels this subset of liver NK cells in mouse, but also a subset residing in human liver (14). Similarly, mouse parabiosis studies demonstrating existence of liver-resident CD49a^{+} NK cells (15) led to the first characterization of a human counterpart (16). Other studies have shown that liver Eomes^{hi}T-bet^{lo} NK cells are absent from blood but also that they do not overlap entirely with previously identified CD49a^{+} subset (14, 16–18). Yet another report, using cytometry by time-of-flight (CyTOF) followed by flow cytometry validation, identified for the first time CD49e^{-} NK cells as the human liver-resident NK cell population (19). Altogether, this suggests an underlying heterogeneity within liver NK cell subsets.

Given the limited extent to which tissue residency in human liver samples can be investigated compared to mouse models, and given the clinical implications for immune responses such as tolerance and disease, detailed phenotypic characterization of human liver NK cells is essential. One of the main challenges in reaching a consensus when comparing literature on liver NK cells comes from a limited number of markers one could analyze by conventional flow cytometry. To overcome this, we here designed a 29-color tissue NK cell-focused panel, demonstrated its potential on liver, peripheral blood and secondary lymphoid tissue, and performed deep profiling of liver NK cell diversity in comparison to peripheral blood NK cells.

MATERIALS AND METHODS

Human Samples

Blood samples used in this study were peripheral blood mononuclear cells (PBMCs) derived from buffy coats from blood donations of healthy human volunteers from the local hospital blood bank. Liver samples were obtained from human adult liver tissue during resection surgery for primary or secondary liver malignancies. Human pediatric and adult uninfected tonsils were obtained from patients undergoing tonsillectomy due to sleep-disordered breathing or obstructive sleep apnea syndrome. All samples were from Karolinska University Hospital, Huddinge, Sweden. None of the samples were matched. All blood and tissue donors gave oral and written informed consent conforming to the provisions of the Declaration of Helsinki. The regional Ethics Committee in Stockholm, Sweden, approved all the protocols involving collection of blood, liver, and tonsil samples.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using density gradient centrifugation. The blood was diluted with Phosphate Buffered Saline (PBS, Sigma) and layered onto the Ficoll-Hypaque media solution (GE Healthcare). Samples were centrifuged at room temperature, with brakes turned off, for 20 min at 2,000 revolutions per minute (rpm). The mononuclear cell layer was carefully removed from the interface and washed twice with PBS. Cells were frozen in CoolCell containers (Corning) in heat-inactivated Fetal Bovine Serum (FBS; Sigma) supplemented with 10% dimethyl sulfoxide (DMSO; Sigma) and stored in liquid nitrogen until use.

Tissue Dissociation and Cell Isolation

Mononuclear liver cells were isolated from the tumor non-affected area of the liver tissue as previously described (20). In brief, the tissue underwent a series of flushing steps to remove excess sinusoidal blood, followed by a three-step perfusion protocol in which the final step involved enzymatic processing (with collagenase XI, Sigma). Supernatant obtained through these steps was washed and layered onto the Ficoll-Hypaque media solution for the density gradient centrifugation to isolate leukocytes in the same way as PBMCs. Whole tonsils were mechanically processed by cutting and passing through a 100 μm strainer, followed by a 40 μm straining step, and finally a density gradient centrifugation in the same way as liver and blood samples. Post-isolation, cells from liver and tonsil were frozen in FBS supplemented with 10% DMSO and stored in liquid nitrogen, similar to PBMC.

Flow Cytometry

Vials with cryopreserved mononuclear cell suspensions isolated from peripheral blood, liver, and tonsil were thawed rapidly in a water bath at 37°C, and transferred carefully to complete cell medium (RPMI with 10% FBS, L-glutamine, Penicillin/Streptomycin). After two washes, cells were resuspended in FACS buffer (PBS with 2 mM EDTA and 2% FBS), filtered through a 40 μm strainer (BD Falcon), counted and stained immediately in 96-well V-bottom plates. All staining steps were performed at room temperature in the dark and all washing steps were performed by centrifuging plates for 2 min at 1,800 rpm at room temperature, unless otherwise stated. Cells were incubated with antibodies against surface antigens diluted accordingly in 50 μl FACS buffer for 20 min (see **Table 1** for dilution details) followed by two washes with 150–200 μl FACS buffer. In the second staining step cells were stained with the

LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Fisher) and fluorescently conjugated streptavidin for 20 min. This was again followed by two washes. Next, samples were fixed for 45 min in freshly prepared fixation/permeabilization working solution from eBioscience Foxp3/Transcription Factor Staining Buffer set (Thermo Fisher). Fixing solution was removed by centrifugation and washing once in 1× permeabilization buffer from the same fix/perm kit. Finally, cells were stained with antibodies against intracellular antigens diluted in 1× permeabilization buffer from the same set for 30 min. Samples were then washed twice in 1× permeabilization buffer and resuspended in 200 μl FACS buffer. To remove potential clumps in the cell suspension, the cells were transferred into 5 ml polystyrene round-bottom tubes (BD Falcon) through the 35 μm strainer cap. The cells were acquired on a FACSymphony A5 instrument (BD Biosciences). Importantly, in all three steps where fluorescently conjugated antibodies were added, BD Horizon Brilliant Stain Buffer Plus (BD Biosciences) was supplemented at 1:5 to minimize staining artifacts commonly observed when several BD Horizon Brilliant dyes are used. Single-stained UltraComp eBeads Compensation Beads (Thermo Fisher) were used according to manufacturer's instructions to prepare compensation controls by incubating with fluorescently conjugated antibodies used in experiments. The FACSymphony A5 flow cytometer used in this study was equipped with the following lasers: UV (355 nm), violet (405 nm), blue (488 nm), yellow/green (561 nm), and red laser (637 nm). The yellow/green, blue, and violet lasers were tuned at 200 mW, the red laser was tuned at 140 mW, and the UV laser was tuned at 60 mW. An instrument cleaning program and FACSDiva Cytometer Setup and Tracking (CST) software were run daily with the CST beads, to ensure optimal cytometer performance. PMT voltages were automatically updated by applying previously created "application setting" for this study. This allowed for a rigorous and reproducible approach to panel optimization. Further information on individual filters and cytometer configuration, can be found in **Table 1**, in addition to details of antibodies used in this study.

Flow Cytometry Analysis

After acquisition on FACSymphony A5 flow cytometer, FCS3.0 files were exported from the BD FACSDiva software and imported into FlowJo v.10.6.0 (BD Biosciences). Automated compensation was calculated by FACSDiva software using single-stained compensation beads. This 29-color compensation matrix was analyzed in detail in FlowJo through investigating N-by-N view feature as well as the pairwise expression of all proteins stained for in this study. Fluorescence minus one (FMO) experiments were run prior to this study, which also aided the optimization of the compensation matrix. Based on this, the compensation matrix was adjusted where necessary due to over- or under-compensation by the automated algorithm. After the compensation matrix was adjusted, samples were concatenated and analyzed using FlowJo plugins (https://flowjo.com/exchange/#/), namely: Downsample (v.3.0.0), UMAP (v2.2), and PhenoGraph (v.0.2.1). UMAP was run using the default settings (Euclidean distance function, nearest neighbors: 15 and minimum distance: 0.5). PhenoGraph was run using the default number of nearest neighbors ($K = 30$). Parameters for running UMAP and PhenoGraph were selected depending on the experimental question and are specified in the accompanying text and figure legends. Graphs were made in Prism 8, v8.2.0 (GraphPad Software Inc.). **Figure 1A** was prepared in BioRender and all figures were put together in Illustrator CC 2019 (Adobe).

RESULTS

Design of a 29-Color Human NK Cell-Focused Flow Cytometry Panel

NK cells in all tissues are classified as $CD56^{high}CD16^{-}$ and $CD56^{low}CD16^{+}$ NK cells, commonly referred to as $CD56^{bright}$ and $CD56^{dim}$ NK cells, respectively (8). These subsets of NK cells are identified both in circulation and in the liver but in different frequencies within total NK cells. Peripheral blood is rich in the $CD56^{dim}$ population and there is generally a lower percentage of circulating $CD56^{bright}$ NK cells. Contrasting this the liver is rich in the $CD56^{bright}$ NK cell subset, similarly to other non-lymphoid (e.g., uterus) and secondary lymphoid organs (e.g., tonsils). When found outside of circulation, the $CD56^{bright}CD16^{-}$ NK cell population is typically considered to be the tissue-resident population (8). Yet, with respect to human liver, and as alluded to in the introduction, the tissue-resident NK cell population within this organ has been defined in multiple distinct ways suggesting a high degree of heterogeneity among these cells. This was a strong rationale for the current study, where we aimed to compare the identification of liver NK cells from different published reports.

We harnessed the power of technical advances within high-end flow cytometry and designed a comprehensive 29-color NK cell-focused flow cytometry panel to compare the diversity of tissue-resident and circulating NK cells. As a starting point, this was applied to NK cells from three tissue types to demonstrate its potential: liver, peripheral blood, and tonsil. Details of the antibodies used in panel design can be found in **Table 1**. We carefully considered all aspects of panel design when selecting fluorochromes for distinct antibodies (21). These considerations included: (1) titration of every antibody used in the panel, (2) application of appropriate fluorescence minus one (FMO) and isotype controls to aid in detecting fluorochrome aggregates and setting accurate positive gates, (3) alignment of the fluorochrome brightness with the antigen expression density within a cell, and (4) avoiding, when possible, high spectral overlap between fluorochromes on co-expressed markers. In total, we used 32 antibodies, in addition to the dead cell marker (DCM), to detect 29 fluorescent parameters. The focus of the panel were surface and intracellular proteins associated with tissue residency as well as those describing the functional potential of an NK cell (activating and inhibitory receptors, effector proteins, activation and differentiation markers, chemotaxis, and proliferation). The panel was designed to exclude main myeloid lineages and B cells (Lin channel: DCM, CD14, CD19, CD123) from future analysis. Since tissue residence is not only a property of NK cells and resident T cells display similar phenotypes (22), we assigned separate fluorophores to main T cell subsets

TABLE 1 | Antibodies used in this study.

Antigen	Clone	Fluorophore	Laser line	BD FACSymphony filter	Dilution used	Custom conjugate	Company	Function
CCR5	2D7/CCR5	BUV395		379/28	25	No	BD biosciences	Cell trafficking
CD16	3G8	BUV496		515/30	200	No	BD biosciences	NK cell subsets
CD56	NCAM16.2	BUV563	UV (355 nm)	580/20	200	No	BD biosciences	NK cell subsets
CD49a	SR84	BUV615		605/20	25	Yes	BD biosciences	Tissue residency/cell retention
CD38	HIT2	BUV661		670/25	25	No	BD biosciences	Maturation/activation
CD69	FN50	BUV737		735/30	50	No	BD biosciences	Tissue residency/cell retention/activation
CD45	HI30	BUV805		810/40	100	No	BD biosciences	Common lymphoid identity
CD49e	REA686	VioBright FITC		530/30	100	No	Miltenyi biotec	Tissue residency/cell retention
NKG2C	REA205	Biotin		N/A	100	No	Miltenyi biotec	Activating receptor
Streptavidin	N/A	BB630		610/20	400	Yes	BD biosciences	N/A
CD103	Ber-Act8	BB660	Blue (488 nm)	670/30	50	Yes	BD biosciences	Tissue residency/cell retention
NKG2A	131411	BB700		710/50	25	No	BD biosciences	Inhibitory receptor
Perforin	δG9	BB755		750/30	200	Yes	BD biosciences	Effector function/cytotoxicity potential
Granzyme B	GB11	BB790		810/40	100	Yes	BD biosciences	Effector function/cytotoxicity potential
Eomes	WD1928	eFluor 660		670/30	25	No	Thermo Fisher	Transcription factor
Ki-67	B56	AF700	Red (637 nm)	730/45	100	No	BD biosciences	Proliferation marker
CD57	TB03	APC-Vio770		780/60	50	No	Miltenyi Biotec	Maturation
Tim-3	7D3	BV421		450/50	50	No	BD biosciences	Co-inhibitory receptor/immune checkpoint
CD14	M5E2	V500		525/50	100	No	BD biosciences	Non-NK cell lineage exclusion
CD19	SJ25C1	BV510		525/50	100	No	BD biosciences	Non-NK cell lineage exclusion
CD123	6H6	BV510		525/50	50	No	Biolegend	Non-NK cell lineage exclusion
LIVE/DEAD Dead Cell Stain	N/A	Fixable Aqua		525/50	100	No	Thermo fisher	Exclusion of dead cells
CD8	RPA-T8	BV570	Violet (405 nm)	586/15	50	No	Biolegend	T cell subsets
CD161	DX12	BV605		605/40	25	No	BD biosciences	Maturation/NK cell subsets
CX3CR1	2A9-1	BV650		677/20	50	No	Biolegend	Cell trafficking
CXCR6	13B 1E5	BV711		710/50	50	No	BD biosciences	Cell trafficking
CD3	SK7	BV750		750/30	100	No	Biolegend	Non-NK cell lineage exclusion/T cell subsets
NKp46	9E2/NKp46	BV786		810/40	25	No	BD biosciences	Activating receptor
PLZF	R17-809	PE		586/15	50	No	BD biosciences	Transcription factor
T-bet	4B10	PE-Dazzle 594		610/20	25	No	Biolegend	Transcription factor
CD4	OKT4	PE-Cy5		670/30	200	No	Biolegend	T cell subsets
KIR2DL2, KIR2DL3, KIR2DS2	GL183	PE-Cy5.5	Yellow-green (561 nm)	710/50	50	No	Beckman coulter	Activating and inhibitory receptors
KIR2DL1, KIR2DS1	EB6	PE-Cy5.5		710/50	50	No	Beckman coulter	Activating and inhibitory receptors
CD127	A019D5	PE-Cy7		780/60	50	No	Biolegend	T cell subsets/innate lymphoid cells
Brilliant Stain Buffer Plus	N/A	N/A	N/A	N/A	5	N/A	BD biosciences	N/A

Color-coding indicates different lasers and their colors.

to allow for relevant comparisons. Cryopreserved cells from non-matched liver, peripheral blood, and tonsil donors were stained with this 29-color panel and acquired flow cytometry data were then processed and analyzed (**Figure 1A**). After optimizing compensation (see section Materials and Methods), two "clean-up gates" were included in the gating strategy to remove super-fluorescent fluorochrome aggregates (**Figure 1B**). It is important to note that the addition of a specific buffer

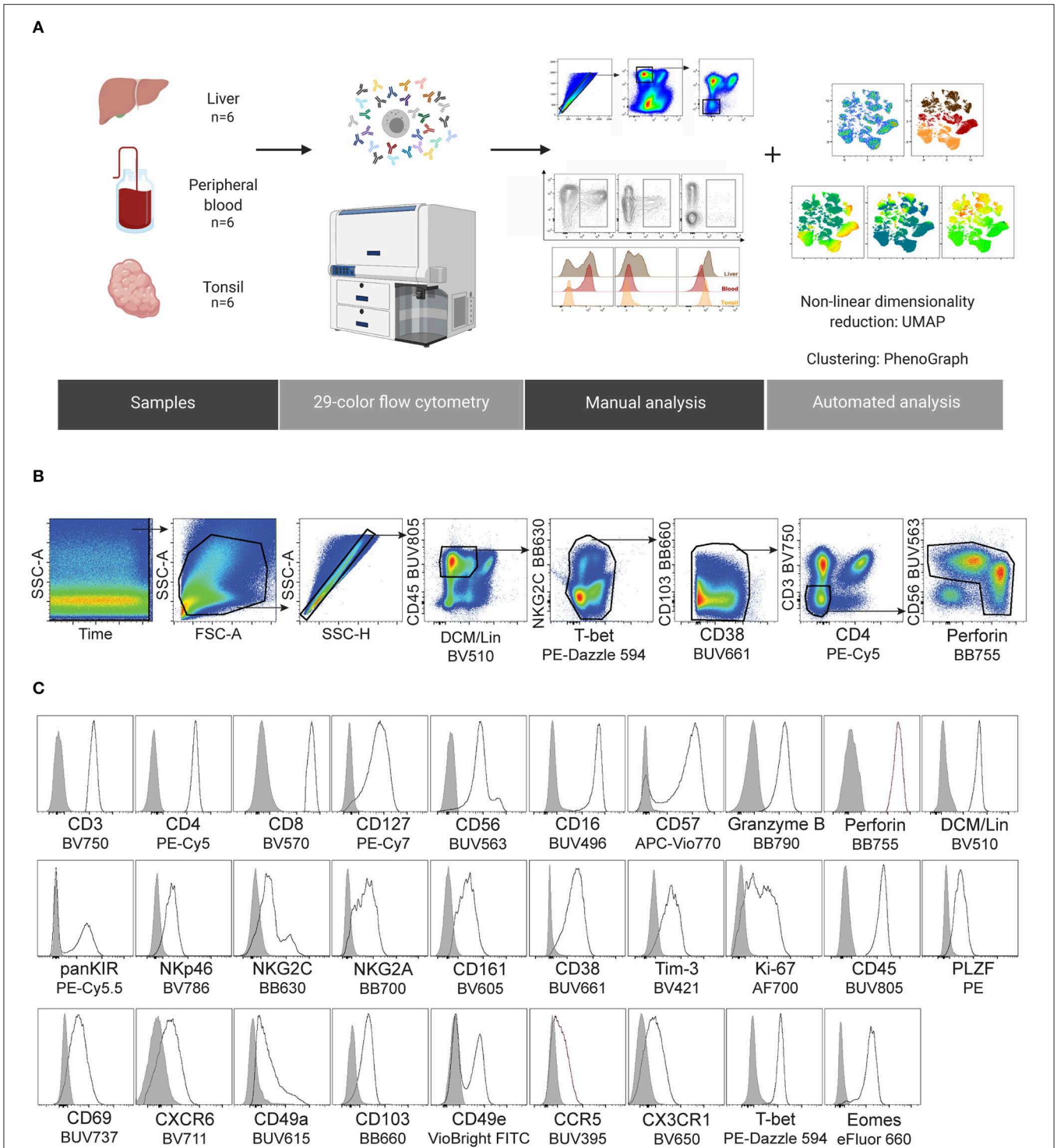

FIGURE 1 | Design of a 29-color human NK cell-focused flow cytometry panel. **(A)** Summary of the experimental workflow. **(B)** Gating strategy used for identification of NK cells and downstream analysis. Two clean-up steps were performed (NKG2C BB630 vs. T-bet PE-Dazzle 594 and CD103 BB660 vs. CD38 BUV661) to remove fluorochrome aggregates. **(C)** Representative histograms for the indicated proteins (black line), including an internal negative control for each (gray shaded histogram). DCM, Dead Cell Marker; Lineage (Lin), CD14/CD19/CD123.

drastically decreased the amount of these aggregates (see section Materials and Methods). Moreover, their presence was sample-dependent and likely due to differences in quality when samples were isolated and frozen. Finally, we observed that populations with low protein expression levels for a particular antigen could be successfully distinguished from negative

populations, which validated the usefulness and efficacy of our panel (**Figure 1C**).

Distinct T Cell, ILC, and NK Cell Clusters Are Robustly Separated by Non-linear Dimensionality Reduction

To capture the non-linear structure of our single cell data, we performed dimensionality reduction using a FlowJo implementation of the recently developed uniform manifold approximation and projection (UMAP) algorithm (23). UMAP was performed on live $CD45^+$ cells (gated as single, live, Lin^-CD45^+ cells, **Figure 1B**). $CD45^+$ cells from individual samples were down-sampled to 25,000 events per sample, individual samples were electronically barcoded, and finally concatenated for downstream analyses. A total of 18 samples were included in the analysis, six for each source material. UMAP was run using all compensated parameters except the previously gated CD45 and DCM/Lin. Several clusters were identified in the resulting UMAP maps. These were pulled together predominantly according to the defining lineage markers rather than the tissue of origin (**Figures 2A,B**). All clusters contained populations found in liver, peripheral blood, and tonsil or the combination of the two, apart from one cluster which appeared to be liver-specific (**Figures 2A,B**). To determine what defined these clusters, we analyzed the expression of lineage markers displayed on the UMAP coordinates. There were two clearly separated clusters of $CD3^+$ cells, one uniformly co-expressing CD4, and the other one with more variable levels of CD8. The IL-7 receptor (CD127) was variably expressed in both of these two clusters. It was also highly expressed in a small $CD3^-$ cluster close to T cells (**Figure 2B**, top row), suggesting that these were innate lymphoid cells (ILCs). Furthermore, the cluster located in close proximity to the ILCs was characterized by a high expression of CD56 and absence of CD3. Within this cluster, a sub-cluster was $CD16^{high}$, indicating that it may contain $CD56^{dim}$ (and possibly $CD56^{bright}CD16^+$) NK cells. We color-mapped UMAP plots by the remaining (NK-focused) parameters in our panel, which validated our notion that the $CD3^-CD56^+$ UMAP cluster contained NK cells (**Figure 2B**, bottom row and **Supplementary Figure 1A**). The above-mentioned liver-specific cluster localized within the $CD3^-CD56^+$ UMAP cluster and was shown to contain cells expressing high levels of CD49a, CD69, CXCR6, and Eomes compared to other CD56-positive cells, as well as low expression of T-bet and CD49e (**Figure 2B**, bottom row).

Through manual gating analysis during panel optimization for this study, we observed a degree of donor-to-donor variability, particularly in the expression of tissue residency markers (data not shown). We tested the overall robustness of UMAP, as well as how successful it was in detecting such variability through three different approaches. Firstly, we deconvoluted individual donor samples in the concatenated file and displayed them on the UMAP embeddings of $CD45^+$ cells (**Figure 2C**). Liver samples 3 and 5, for example, had sections of the liver-specific cluster missing, while the other $CD56^+CD16^+CD3^-$ UMAP cluster demonstrated an even higher level of variability between non-matched blood and liver donors (**Figure 2C**). Secondly, we performed manual flow cytometry gating for NK cells, $CD4^+$ and $CD8^+$ T cells in the concatenated file in all tissues as well as in each individual tissue and overlaid cells from this analysis on the UMAP map (**Figure 2D**, **Supplementary Figure 1B**). Manually gated subsets shared the UMAP coordinates with automatically detected clusters across all tissues. Thirdly, given the focus on NK cells in our panel, we ran a UMAP analysis similar to the one in **Figure 2A** excluding CD56 as a clustering parameter. Reassuringly, the combined expression of all other parameters from the panel was specific enough to identify T cells and NK cells, resulting in nearly identical clustering (**Supplementary Figure 1C**).

PhenoGraph Distinguishes Populations of Tissue-Enriched Lymphocytes and Their Diversity Across Individuals

To identify cell subsets within our high-dimensional data visualized with UMAP, we ran PhenoGraph on the $CD45^+$ population (24). PhenoGraph clustering identified 36 populations of lymphocytes (**Figure 3A**). We labeled the previously generated two-dimensional UMAP projection of $CD45^+$ cells by these results and observed that most PhenoGraph populations were found within $CD4^+$ and $CD8^+$ UMAP clusters. Two populations (#7 and #33) were identified in the cluster between cells marked by high expression of CD4 and CD56 and another two (#10 and #28) were spanning two UMAP clusters (**Supplementary Figure 2A**). Four out of the 36 populations had a majority of cells (>91%) falling within the $CD3^-CD56^+$ UMAP cluster (**Figure 3B** left plot and **Figure 3D**, indicated by the arrows). Displaying all 36 PhenoGraph populations' frequencies as a proportion of the total $CD45^+$ population within each individual sample showed a high level of diversity in the lymphocyte repertoire between samples and across tissues analyzed (**Figure 3C**). The liver was the most heterogeneous, with major differences in the $CD4^+$ cluster, but also in the $CD3^-CD56^+$ cluster (**Figure 3C**). Analysis of PhenoGraph populations within the $CD3^-CD56^+$ UMAP cluster revealed that 3 of them were present only in liver and blood, while population #27 was the only one that was also present in tonsil (**Figure 3D**).

This analysis shows that PhenoGraph, combined with a dimensionality reduction technique such as UMAP, represents a powerful approach to visualize the general diversity of immune cells within an individual, and to assess tissue distribution of immune cell subsets.

Detection of Human Liver-Enriched NK Cell Populations in High-Dimensional Space

The scope of this study was to describe the heterogeneity between tissue-resident and circulating NK cells. We showed that NK cells from tonsils contributed to the total number of cells in the $CD3^-CD56^+$ UMAP cluster (**Supplementary Figure 2B**) and the inclusion of tonsil tissue aided in panel design and validation. However, the NK cell frequency is low in tonsil compared to liver and blood (**Supplementary Figure 2C**) and our panel and study

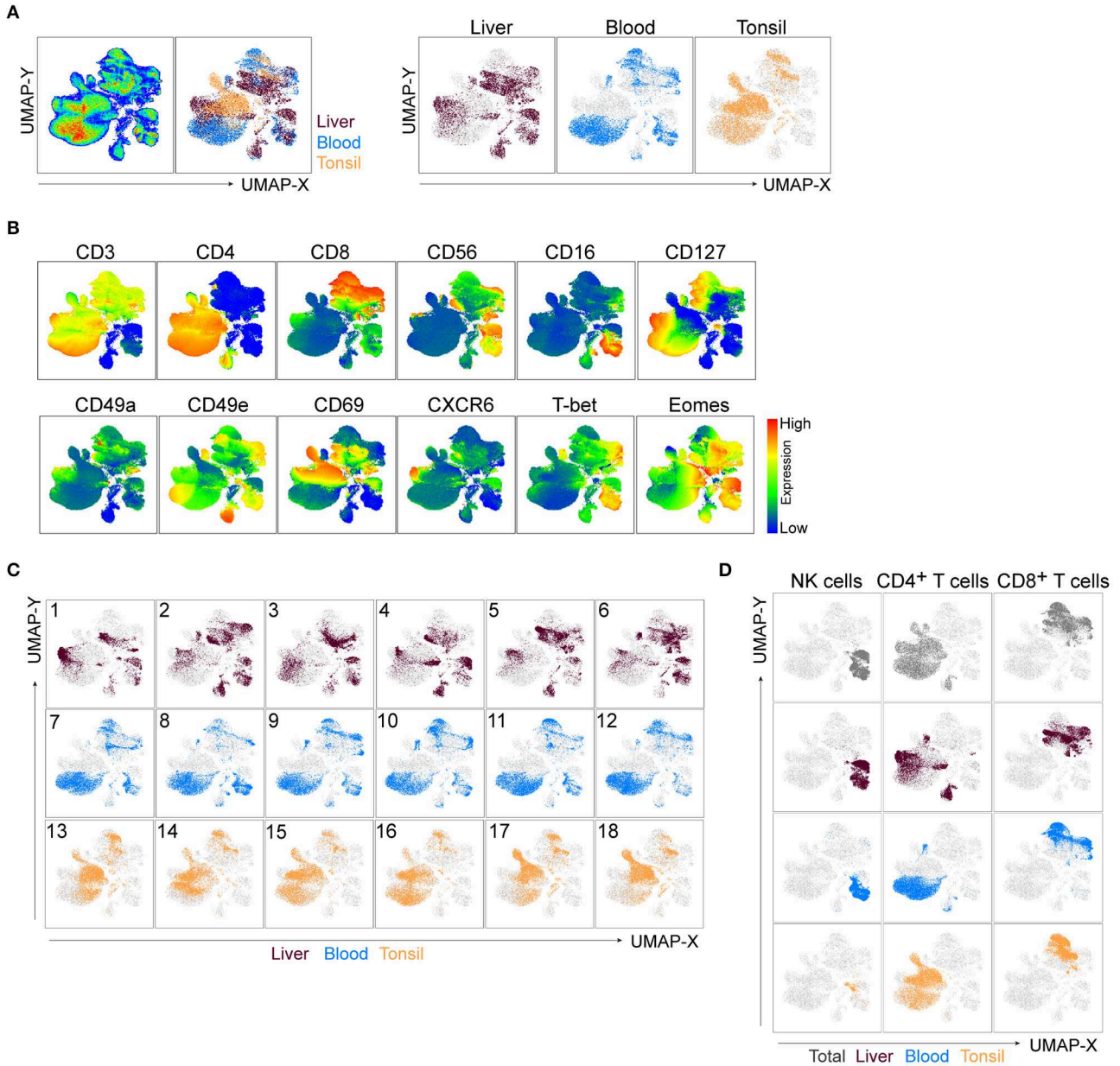

FIGURE 2 | UMAP robustly embeds CD45$^+$ cells from distinct tissues and identifies main clusters of lymphocytes. **(A)** UMAP plot of CD45$^+$ cells. Live, Lin$^-$CD45$^+$ cells from liver, blood, and tonsil tissue were gated (**Figure 1B**), down-sampled to 25,000 cells per sample which were barcoded and concatenated. Eighteen samples were included in the analysis (six per source material). CD45 and DCM/Lin were excluded from the list of UMAP running parameters. The resulting UMAP projection is colored according to the tissue of origin (combined in left panel or individually in right panel). **(B)** UMAP plots showing expression intensities of lineage markers (top row), as well as some of the markers distinguishing liver-enriched NK cells from peripheral blood population (bottom row). See **Supplementary Figure 1A** for compiled plots of all other parameters. **(C)** UMAP embeddings from **(A)**, colored by the tissue of origin and displayed for each individual donor, labeled 1-18. **(D)** Events in the UMAP embeddings were overlaid with manually gated NK cells, CD4$^+$, and CD8$^+$ T cells and displayed for all tissues in the concatenated file (top row), or for each tissue separately (following three rows). Representative gates can be found in **Supplementary Figure 1B**.

aim was not to distinguish and analyse tonsil NK cells in relation to other ILC1 populations found in this tissue as this has been reported elsewhere (25). Thus, for the subsequent downstream analysis of NK cells, we focused on liver and peripheral blood and performed further UMAP analysis of these cells (gated as in **Figure 3B**, left plot). UMAP again separated liver-specific NK cells from the other big cluster of cells shared between blood and liver (**Figure 4A**). Such clustering appeared to be driven by proteins with specific expression patterns associated with tissue residency (expression of CXCR6 and CD69 but absence of CD49e, T-bet, and CD16) since their expression levels displayed the highest difference between the two big clusters

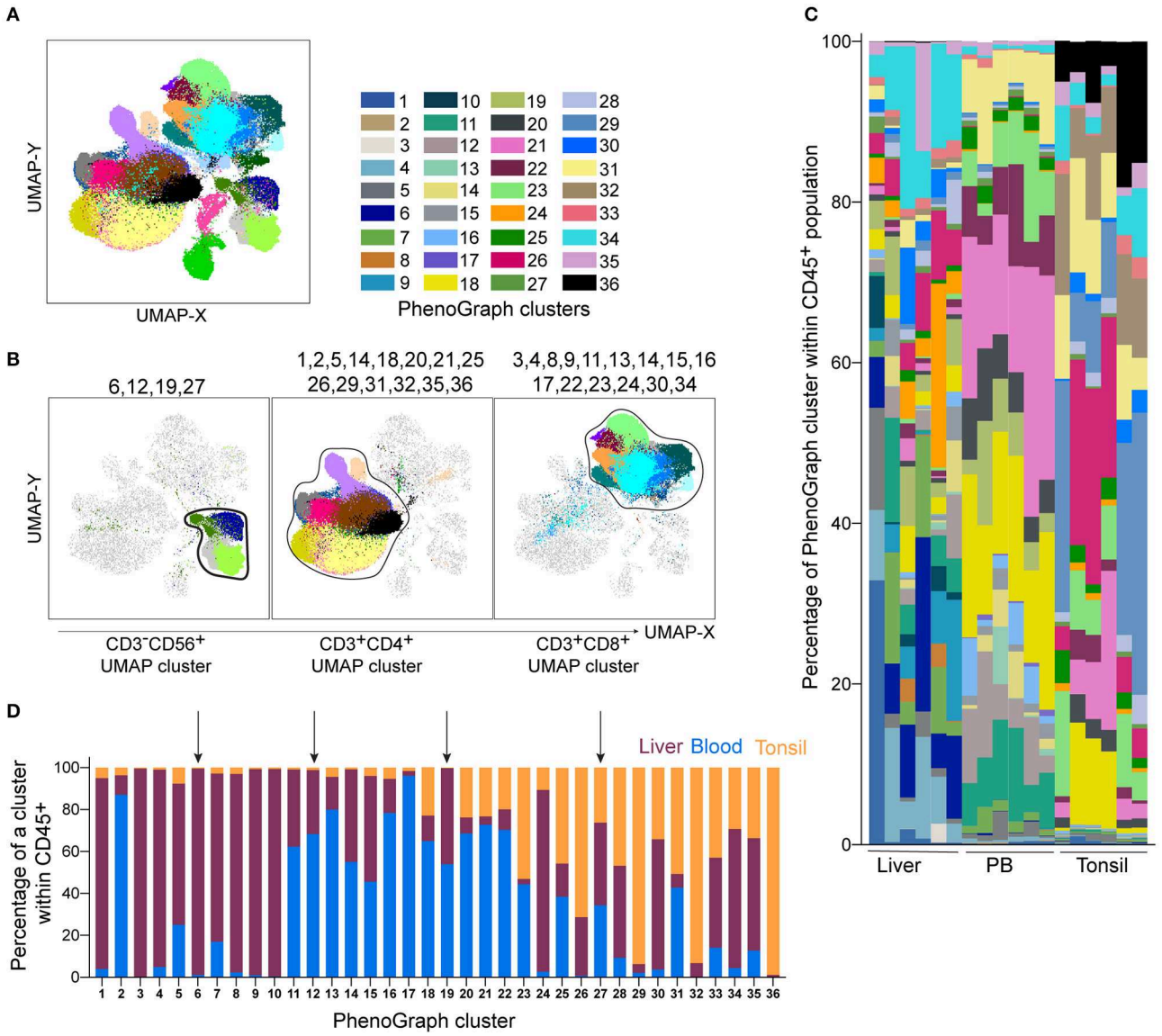

FIGURE 3 | PhenoGraph analysis of the Lin$^-$CD45$^+$ population identifies tissue-enriched clusters and demonstrates their diversity across individuals. PhenoGraph clustering was performed on Lin$^-$CD45$^+$ barcoded and concatenated cells from all samples, CD45 and DCM/Lin were excluded from the list of running parameters. **(A)** Plot of all 36 identified PhenoGraph clusters overlaid on the UMAP projection. **(B)** Selected PhenoGraph clusters displayed over UMAP embeddings. Previously identified main lineage UMAP clusters are indicated by black lines (CD3$^-$CD56$^+$ cluster, CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cluster). **(C)** Stacked bars showing relative abundance of every PhenoGraph cluster within CD45$^+$ cells in each liver, peripheral blood (PB), and tonsil sample. Color coding same as in **(A)**. **(D)** Relative abundance of liver, blood, and tonsil CD45$^+$ cells within each detected PhenoGraph cluster. Arrows indicate PhenoGraph clusters within the CD3$^-$CD56$^+$ UMAP cluster shown in **(B)**.

(low-to-high expression) (**Figure 4B**). Most of the other proteins (i.e., NKG2A, CD38, CD161, Tim-3, PLZF) were expressed at various intermediate-to-high levels in the clusters. (**Figure 4B**). As before, we next applied PhenoGraph on total liver and peripheral blood NK cells. Eighteen populations were identified, each one with a different contribution to the total population of NK cells in liver and blood (**Figures 4C,D**). Populations #1, #2, #3, #4, #5, #6, and #15 were most highly enriched in liver over blood (>95% found in liver; **Figure 4D**). Populations #14 and #18 were present at almost equal frequencies between liver and blood ("shared" clusters). Populations #9, #10, #11, #12, and #13 were found at higher frequencies in blood compared to liver and populations #16 and #17 were almost exclusively detected in blood (**Figure 4E**). CD127 was highly expressed in a separate cluster connecting CD49e$^-$ (liver-enriched) and CD49e$^+$ (liver and blood) UMAP clusters.

PhenoGraph revealed the underlying heterogeneity between blood and liver CD3$^-$CD56$^+$ cells and demonstrated a higher

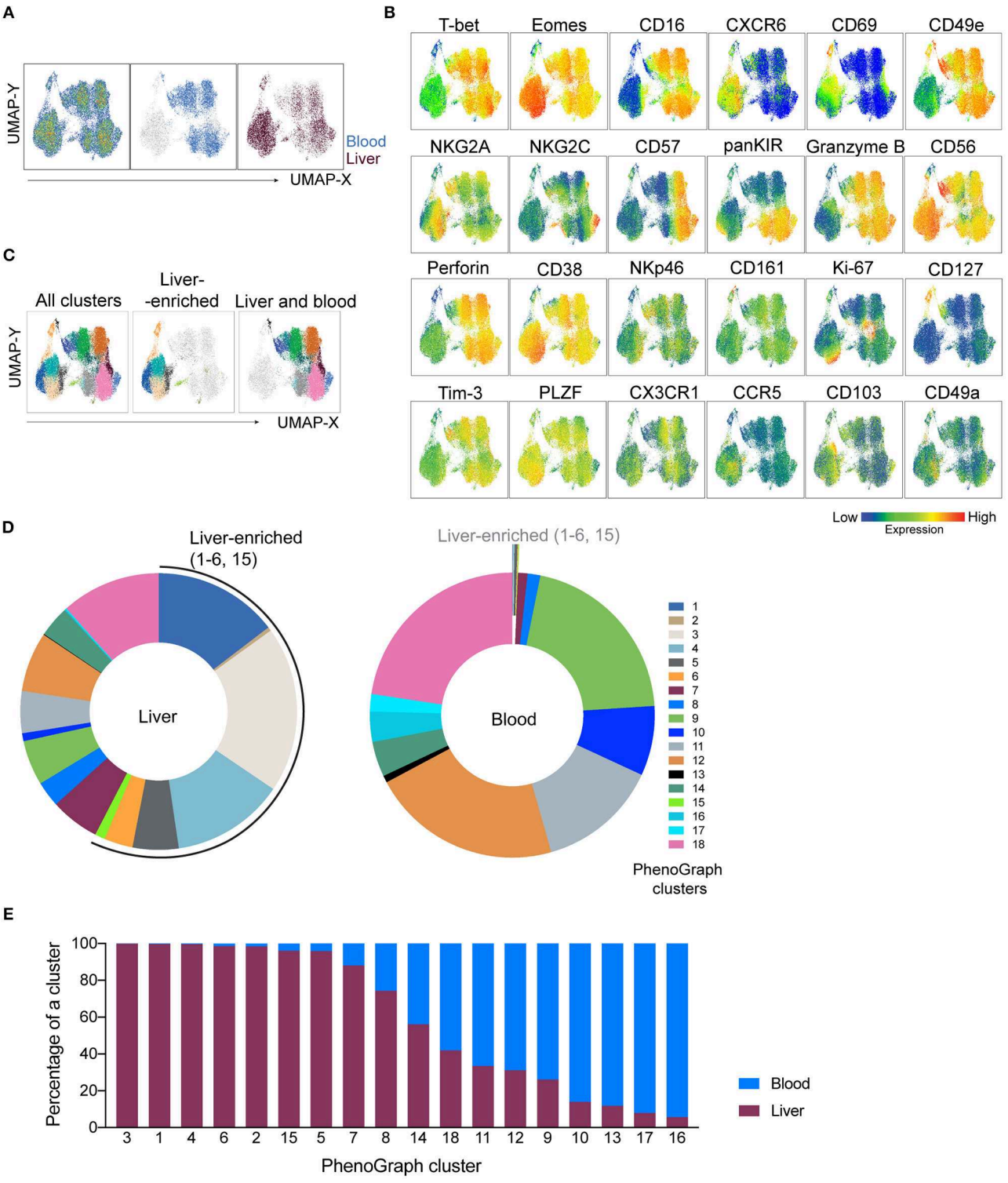

FIGURE 4 | NK-cell focused panel coupled with dimensionality reduction and clustering techniques detects liver-enriched clusters. UMAP and PhenoGraph were run on $CD3^{-}CD56^{+}$ UMAP cluster as gated in **Figure 3B**, containing cells from liver and blood. CD45, DCM/Lin, CD3, and CD4 were excluded from the list of running parameters for UMAP and PhenoGraph. **(A)** UMAP projection of concatenated $CD3^{-}CD56^{+}$ cells from non-matched liver ($n = 6$) and blood ($n = 6$) samples, either as a pseudocolor plot combining all samples (left plot) or colored according to the tissue of origin (plots on the right). **(B)** Resulting UMAP embeddings, colored according to the expression of markers from the NK-cell focused panel. **(C)** UMAP embedding from **(A)**, overlaid with 18 identified PhenoGraph clusters. Color-coding is the same as indicated by the legend in **(D)**. **(D)** Donut plots showing frequency of 18 identified PhenoGraph clusters within liver and blood $CD3^{-}CD56^{+}$ cells. Liver-enriched clusters are indicated in both sample sources. **(E)** Relative abundance of liver and blood $CD3^{-}CD56^{+}$ cells within every detected PhenoGraph cluster.

diversity of NK cell subsets in liver when compared to blood, with seven main phenotypes enriched in the liver.

Assessment of Phenotypic Diversity Within Liver-Enriched NK Cells

Having established that numerous NK cell phenotypes exist in liver and blood and that they differ we next systematically analyzed the phenotype of NK cells identified in the 18 PhenoGraph-derived populations. To this end, we summarized expression levels of all proteins in our 29-color panel for each population (**Figure 5A**). One of the most differentially expressed proteins between these two subsets was CD49e. In fact, CD49e was the only protein with an expression pattern that reliably recapitulated clustering according to the tissue of origin. This was underlined as sorting of the PhenoGraph clusters according to the increasing levels of CD49e organized the subpopulations similarly to what was obtained by analyzing tissue-enrichment (**Figures 4E**, **5A**). Finally, and in contrast to the other more variable tissue residency markers, CD49e displayed a more uniform expression. Thus, we could recapitulate previously described phenotypes: $CD49e^+$ NK cells, predominantly found in blood or in blood and liver, expressed high levels of T-bet, CD16, perforin, and granzyme B, for example (#9, #11, #18). Our panel allowed us for the first time to observe the simultaneous expression of these proteins on the same cell, together with additional markers that also appeared to be differentially expressed between blood and liver. For instance, $CD49e^-$ NK cells displayed generally lower levels of Tim-3, CX3CR1, and NKp46. However, even within the $CD49e^-$ cells, we observed clusters with a high expression of certain markers typically associated with the $CD49e^+$ cells, and vice versa. For example, population #8 was $CD103^+$, while population #15 expressed granzyme B. Similarly, CD49a expression was found in two $CD49e^+$ populations. One of them was #13, which expressed CD127, several tissue residency markers (CXCR6, CD49a, CD103, CD69) and low-to-none of the conventional NK markers. Together with #6, they appeared to contain the majority of blood and liver ILCs, respectively.

Next, we compared NK cell populations with different tissue origins: one liver-enriched population (#3), one which was present in similar frequencies in blood and liver ("shared," #18), and two blood-enriched populations, #10 and #12, which appeared phenotypically as $CD56^{bright}$ and $CD56^{dim}$ NK cells, respectively (**Figure 5B**). The liver-enriched population was different from the other three populations phenotypically, and although #18 and #12 were phenotypically very similar and resembling $CD56^{dim}$ NK cells with respect to CD57, granzyme B, and perforin expression, population #18 was KIR-positive whereas #12 was KIR-negative (**Figure 5B**).

Finally, we observed a high level of heterogeneity within liver-enriched subsets. Interestingly, variable levels of expression were characteristic of proteins most commonly associated with tissue residency. For example, although populations #4, #5, and #15 were all more common in the liver, #4 and #5 were $CXCR6^+CD103^+CD69^+$, while #15 was $CXCR6^-CD103^-CD69^{low}$. Additionally, only #5 had a low level of CD49a expression, but population #15 was distinguished from the other two by being $NKG2C^+CD38^{low}KIR^+PLZF^-$ (**Figure 5C**). The only two populations showing clear signs of proliferation, indicated by a high Ki-67 expression, were #3 (liver-enriched, $CD49e^-$) and #14 (blood-enriched, $CD49e^+$) (**Figure 5D**).

Together, these data showed that although commonly recognized tissue residency markers in liver NK cells were highly expressed in liver-enriched NK cell subsets compared to blood, they were not uniformly expressed at high levels when a detailed analysis was performed on populations of these cells. By contrast, our analysis found CD49e to robustly separate liver-enriched from blood- and liver-shared NK cell populations.

DISCUSSION

Flow cytometry is a widely-adopted technology used to investigate the dynamics of immune responses. In the present study, we implemented a 29-color state-of-the-art flow cytometry panel to investigate the diversity of human liver tissue-resident NK cells. The panel encompasses proteins involved in tissue residency, transcription factors, maturation, and effector functions (activating and inhibitory receptors, cytotoxicity potential, activation). We employed a non-linear dimensionality reduction technique to visualize the high-dimensional dataset generated, and used it in conjunction with a clustering approach to detect cellular phenotypes associated with tissue residency. We demonstrate that this approach is robust and can be used to explore NK cell diversity in tissues such as liver and tonsil, but it can also be applied to other organs (e.g., uterus, lung, skin, spleen, salivary gland), with only minor alterations. The analysis framework described here can also be readily adapted to study tissue-resident NK cells in settings of disease where clinical parameters can be included as additional parameters in the analysis.

The technological advances that have led to a significantly increased resolution in the study of single cells brought with them the curse of dimensionality (26). This has been the case with RNA-sequencing methods over the past decade, resulting in the development of many tools for the analysis of high-dimensional data (27). An explosion of bulk and single-cell RNA-sequencing methods has multiple implications for flow cytometry. Firstly, thousands of protein-coding genes and their expression levels can be quantified, and researchers now have more gene candidates than ever to investigate further, including the downstream biological functions of putative proteins. Flow cytometry is the first port of call for such experiments, due to well-established sensitivity and robustness. Secondly, next generation of flow cytometry analyzers has brought the curse of dimensionality into the flow cytometry field. On the other hand, the same tools (or their adaptations) which were developed for RNA-sequencing analysis can also be used in high-dimensional cytometry analysis, such as UMAP used in this study. Thirdly, recently developed methodologies such as CITE-seq and REAP-seq (28, 29) enable concurrent investigation of transcript and protein levels, which can mitigate the shortcomings of RNA-sequencing methods alone, such as weak correlation between

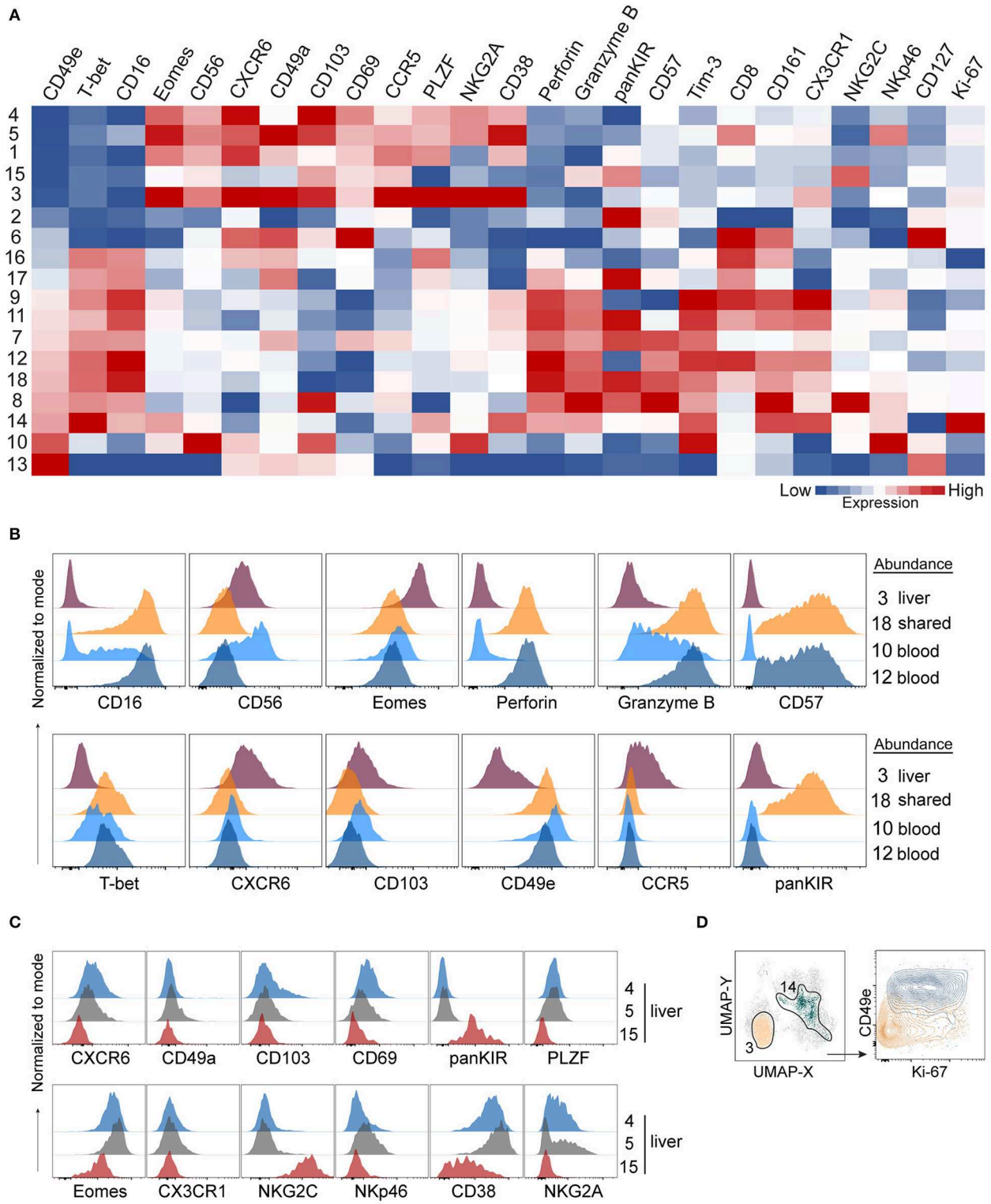

FIGURE 5 | A 29-color NK-cell focused panel captures phenotypic diversity within liver-enriched NK cells. **(A)** Heatmap displays Z-score transformed median expression values for each of the parameters within 18 identified PhenoGraph clusters as described in **Figure 4**. Color scale was determined for each column separately, based on the lowest and highest Z-score value of that parameter. **(B)** Histograms displaying expression levels of selected proteins in PhenoGraph clusters. Legend indicates whether a cluster is predominantly enriched in liver (#3), present in similar frequencies in liver and blood (shared, #18) or enriched in blood (#10, #12). **(C)** Histograms displaying expression levels of selected proteins from **(A)** in selected liver-enriched clusters (#4, #5, #15). **(D)** Identification of the two most proliferating PhenoGraph clusters (#3 and #14) according to their Ki-67 expression level based on analysis in **(A)**, displayed against CD49e.

the levels of a detected transcript and its translated protein (30–32). Information obtained from these novel experimental pipelines can in powerful ways describe the immune landscape and emphasizes the shift towards the need for high-dimensional flow cytometry.

Along these lines, CyTOF has become a useful tool for immunologists in recent years, since it had emerged as technology enabling investigation of more parameters than possible with conventional flow cytometers at the time (33). While it is a powerful method, CyTOF implementation in the experimental workflow may not always be feasible, depending on the experimental question. Metal isotopes used in CyTOF essentially mitigate compensation-caused issues during data analysis, but cells are destroyed during ionization and cannot be sorted for downstream experiments. Therefore, a flow cytometry panel informed by CyTOF findings still might have to be optimized, should one decide to investigate live cells in downstream applications. Most importantly, flow cytometry is the highest throughput approach in single-cell analysis, as tens of thousands of cells can be run per second, at a low cost of operating (34).

The human NK cell repertoire is highly diverse within and between individuals (7). The conventional classification of NK cells into $CD56^{bright}$ and $CD56^{dim}$ subsets captures only the major differences in the subset-associated phenotypes. However, this is insufficient to explain the different functions that phenotypically similar subsets can exhibit in different tissues. CD56 has an unclear function itself and its "brightness" is not a good discriminator when it comes to implications of surface expression on NK cell functions. $CD56^{bright}$ NK cells are considered to be non-cytotoxic and with immunoregulatory functions, but they can also exhibit enhanced cytotoxicity and degranulation against viral and tumor antigens (35). Along these lines, even absence of CD56 on NK cells marks a specific subset of $CD56^{neg}$ NK cells resembling $CD56^{dim}$ with moderate responsiveness and differential expression of several granule proteins (36, 37). This demonstrates the necessity to assess NK cell phenotypes (and consequently their biological functions) as a set of markers, rather than relying on individual bimodal-expression-based classifications. The panel we designed represents a collection of markers most commonly described to be differentially expressed on liver NK cells. Our findings here substantiate the major findings of previous studies phenotypically describing liver NK cells (38), but also combine them and additionally identify novel differences within liver NK cells.

In more detail, performing dimensionality reduction of $CD45^{+}$ cells data in all three tissues ordered events according to their lineage, instead of the tissue of origin. This was sufficient to assess the general landscape of T cells, non-NK ILCs, and NK cells. However, narrowing the scope of the analysis to the NK cell lineage in liver and blood robustly separated subsets based on the relative enrichment in the tissue and suggested that distinct cellular phenotypes drove this separation. Out of all markers in our panel, CD49e expression most reliably ordered NK cell populations according to their tissue origin, as liver-enriched populations were all CD49-negative, corroborating previous CyTOF findings (19). This suggested that CD49e should be included in future studies of liver-enriched NK cells, and validated the importance of this marker in studies of tissue-resident subsets through another experimental approach. Future work should also address the exact role of CD49e and what the lack of expression means for the function of intrahepatic NK cells. All other liver-enriched populations expressed higher levels of CXCR6, CD49a, CD103, and CD69 as well as CCR5 compared to NK cells enriched in blood or shared between blood and liver.

We took a conservative approach when interpreting our unbiased clustering results in the context of studies that used manual gating to quantify and describe NK cells. Nonetheless, we still detected phenotypic similarities to populations described in those studies. For example, cluster #10 (**Figures 4D,E**, **5A**) appeared to resemble previously described cytokine-induced $CXCR6^{+}$ blood NK cells since this cluster was $CD56^{bright}CD69^{+}$ and also expressed higher levels of NKG2C than non-$CXCR6^{+}$ blood-enriched clusters (e.g., cluster #9) (39). An elegant study demonstrated that liver microenvironment TGF-β is required to induce and maintain a liver-resident phenotype (40). However, liver-conditioned media used in that study could not induce CXCR6 on blood NK cells, in contrast to earlier findings with cytokines (39). The panel we propose, addressing tissue resident surface markers as well as transcriptional program associated with acquisition/loss of tissue residency, will be a valuable tool in future studies of how tissue residency is maintained as it aids identification of exact subpopulations in response to dynamic changes in the microenvironment, given the heterogeneity of tissue resident subsets. Our results also corroborate recent findings that liver $CXCR6^{+}$ NK cells contain a high percentage of educated NK cells, considered to be $NKG2A^{+}$ when compared to blood and liver $CXCR6^{-}$ counterpart (41). In our dataset, liver-enriched $CXCR6^{+}$ clusters #3, #4, and #5 are also highly $NKG2A^{+}$, while $CXCR6^{-}$ liver-enriched cluster #2 has a high KIR expression and low levels of NKG2A (**Figure 5A**). We also identify cluster #15 in the liver with the highest expression of NKG2C and lowest expression of CXCR6, similar to previous studies (41). However, cluster #1 that we identified in liver was $CXCR6^{+}$ but $NKG2A^{-}$ as well as KIR^{low}. Our panel could therefore be adapted to investigate the relationships between these clusters in context of education in future studies.

In general, a variable pattern of expression of a majority of tissue residency markers examined calls for caution when interpreting the results, but also suggests the existence of differential gene regulation pathways in distinct liver-enriched clusters. Multiple levels of gene regulation could be analyzed. It would be valuable to obtain transcriptome data for these clusters, for example by performing CITE-seq on single cells with antibodies used in this study, conjugated to oligonucleotides. This information could help determine the relationship between populations we identified here, reveal potential differentiation trajectories, and answer questions such as why some CD49e-negative subsets had low levels of CXCR6. However, since these populations have relatively similar phenotypes, it might be that posttranscriptional gene regulation is more important in regulating the functional potential of various subsets of liver NK cells, in a manner recently suggested to explain increased granzyme B levels in human educated peripheral blood NK

cells (42). A limitation of our study is that we used non-matched samples as well as liver samples from non-affected areas of patients undergoing liver resection surgery for primary or secondary tumors. This type of samples has been routinely used in earlier work describing liver-resident NK cell subsets (14, 16, 17). However, it may be that the heterogeneity of NK cell subsets we observed in our study between similarly obtained individual liver samples, for example, originates from a different stage of malignancy and consequently an altered microenvironment between individual donors. Since we demonstrated that our workflow can detect this variability, future studies applying this approach on larger cohorts of patients with well-defined underlying pathologies will be useful in addressing the cause of such variability.

In summary, and in addition to the 29-color panel developed here, we carried out our analysis via a user-friendly interface, although thorough understanding of the nature of high-dimensional data, data transformation methods, and clustering approaches are still required. We used algorithms that minimized bias and maximized unsupervised analysis of the data with caution. Biological knowledge is still essential to avoid data misinterpretation that might originate from algorithms attributing fluorochrome aggregates to rare subsets, for example. Thus, manual analysis is far from obsolete and will remain essential for the foreseeable future (21). In the present study, we examined only six samples per sample source. Regardless, our workflow was robust enough to demonstrate intra- and inter-sample diversity of cellular phenotypes even among these samples. In the future, such a workflow can be applied to large cohorts to give enough statistical power to confidently identify phenotype metaclusters associated with disease states or correlating with other molecular biomarkers. Patient samples usually come with the caveat of limited sample material, and time and simplicity of experimental manipulation are often of essence. Therefore, high-dimensional flow cytometry holds great promise to be a major tool in investigation of complex immune responses, due to the excellent sensitivity and high-throughput nature of the approach. We anticipate similar workflows to the one we describe here to become a routine in investigating NK cells residing in other lymphoid and non-lymphoid organs and the immune responses they are involved in, both during normal homeostasis and in disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethics Committee of Stockholm, Stockholm, Sweden. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IF designed the study, performed experiments, acquired and analyzed data, and drafted the manuscript. IS, MC, and LH contributed to the data analysis and the discussion. BS contributed to the data analysis, discussion, and edited the manuscript. DF provided clinical samples. NB and MI designed the study, performed data analysis, edited the manuscript, and supervised the work.

FUNDING

This work was supported by the Swedish Research Council, the Swedish Cancer Society, the Swedish Foundation for Strategic Research, the Cancer Research Foundations of Radiumhemmet, Knut and Alice Wallenberg Foundation, the Novo Nordisk Foundation, the Center for Innovative Medicine at Karolinska Institutet, Region Stockholm, SRP Diabetes Karolinska Institutet, StratRegen Karolinska Institutet, and Karolinska Institutet. IF and MI are funded by the Wenner-Gren Foundation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02692/full#supplementary-material

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 November 2019
doi: 10.3389/fimmu.2019.02728

Dynamic Changes in Natural Killer Cell Subset Frequencies in the Absence of Cytomegalovirus Infection

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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: 22 August 2019
Accepted: 07 November 2019
Published: 22 November 2019

Citation:
Gyurova IE, Schlums H, Sucharew H, Ambroggio L, Ochayon DE, Win HT, Bryceson YT, Bernstein DI and Waggoner SN (2019) Dynamic Changes in Natural Killer Cell Subset Frequencies in the Absence of Cytomegalovirus Infection. Front. Immunol. 10:2728. doi: 10.3389/fimmu.2019.02728

Individuals lacking functional natural killer (NK) cells suffer severe, recurrent infections with cytomegalovirus (CMV), highlighting the critical role of NK cells in antiviral defense. Therefore, ongoing attempts to develop an efficacious vaccine to prevent CMV infection should potentially aim to elicit NK-cell antiviral responses as an accessory to conventional T- and B-cell based approaches. In this regard, CMV infection provokes marked phenotypic and functional differentiation of the NK-cell compartment, including development of adaptive NK cells that exhibit enhanced antiviral activity. We examined longitudinal blood samples collected from 40 CMV-seronegative adolescents to ascertain whether a CMV glycoprotein B (gB) vaccine in the absence of CMV infection can stimulate differentiation or expansion of CMV-associated subsets of NK cells. Study participants uniformly lacked the CMV-dependent NKG2C^{+} subset of NK cells, suggesting that an adjuvanted CMV gB vaccine alone is an inadequate stimulus for sustained expansion of these cells. In contrast, we observed unexpected dynamic fluctuations in the frequency of NK cells lacking FcRγ, EAT-2, and SYK, which were independent of vaccination or CMV infection. Whereas, FcRγ^{neg} NK cells in CMV infection are reported to express increased levels of the maturation marker CD57, the FcRγ^{neg} NK cells observed in our CMV-negative vaccine cohort express less CD57 than their FcRγ^{+} counterparts. The FcRγ^{neg} NK cells in CMV-negative individuals were also functionally distinct from this subset in CMV infection, exhibiting comparable IFN-γ production and degranulation as FcRγ^{+} NK cells in response to cytokine or antibody-dependent stimuli. These results suggest that frequencies of some NK cell subsets may increase in response to

unknown environmental or inflammatory cues distinct from that which occurs after CMV infection. Greater understanding of the nature of the signals driving CMV-independent accumulation of these subsets should permit development of mechanisms to facilitate vaccine-driven expansion of CMV-reactive NK cells.

Keywords: immunization, CMV, innate lymphoid cells, NK cells, memory, FcRγ, CD56, CD57

INTRODUCTION

Cytomegalovirus (CMV) is a significant global cause of morbidity with manifestations of infection ranging from subclinical disease to death. Congenital infection and the infection of immunocompromised patients, including transplant recipients, result in the most severe consequences of CMV in the human population. Congenital CMV accounts for roughly 400 deaths and more than 5,000 developmentally impaired children each year in the United States (1). Therefore, effective strategies to prevent or control infection are desperately needed.

Unfortunately, CMV has proven to be a challenging target for vaccine development. To date, most CMV vaccine efforts focus on elicitation of antibodies against viral glycoproteins or generation of antiviral T-cell responses (2). Administration of a MF59-adjuvanted CMV glycoprotein B (gB) vaccine to CMV-seronegative adolescent girls induced strong gB-specific antibody responses and afforded 43% protective efficacy (3). The same vaccine conferred short-lived, 50% protection, against CMV infection in seronegative post-partum women (4) and reduced post-transplant viral load when given to patients awaiting a kidney or liver transplant (5). While promising, these results indicate that humoral responses against gB may be insufficient to effectively prevent CMV infection in many individuals. DNA vaccines aimed at eliciting CMV-reactive T cells have also afforded minimal protection in a transplant patient-based clinical trial (6). These advances prompted development of new vaccines aimed at eliciting both humoral and cellular immunity (7), but it remains unclear whether other arms of the immune response must be engaged to effectively prevent CMV infection.

Natural killer (NK) cells are critical antiviral effectors that produce IFN-γ (8), lyse virus-infected cells (9), and regulate adaptive immune responses (10–15). NK cells play an important role in control of CMV infection in both mice and humans (16, 17). Since NK cells lack the somatically rearranged antigen-specific receptors characteristic of T and B cells, and because they were previously thought to be short-lived cells (18), vaccine triggering of NK cells has historically been considered of little value. However, recent data suggests that the innate immune system makes important contributions to vaccine-elicited protection against infection (19, 20). Specifically, long-lived populations of adaptive NK cells with antigen-specific features similar to those of memory T cells have emerged as potential new targets of vaccines aimed at preventing CMV infection (21–24).

Immunological memory in virus-specific NK cells is widely described in the context of murine CMV. In C57BL/6 mice, a mouse CMV gene product engages an activating NK cell receptor, Ly49H (*Klra8*), promoting clonal expansion and contraction of the Ly49H-expressing subset of NK cells (25–29). Thereafter, a subset of memory Ly49H$^+$ NK cells with enhanced antiviral effector functions persists indefinitely (30). Similar types of adaptive NK cells develop in response to hapten sensitization (31), vaccinia virus infection (32), and virus-like particle immunization of mice (33). Likewise, simian immunodeficiency virus-reactive memory NK cells develop in rhesus macaques after virus infection or immunization (34). Collectively, animal studies point to existence of long-lived, virus-dependent subpopulations of memory NK cells that are likely better antiviral effectors than their naïve counterparts.

Several types of memory NK cells have been characterized in humans. These include memory NK cells induced by cytokines (35), varicella zoster virus exposure (36), antibody-mediated stimulation (37), or CMV-derived peptides (38). High frequencies of NK cells expressing the activating receptor NKG2C are frequently observed in CMV seropositive individuals (39). These NKG2C$^+$ cells undergo proliferative expansion during primary CMV infection in transplant patients (40) and in response to CMV-infected fibroblasts (41), IL-12-producing infected monocytes (42), and CMV UL40-derived peptides (38). CMV-associated adaptive NK cells expressing NKG2C display altered DNA methylation patterns and reduced expression of signaling molecules, including FcRγ, spleen tyrosine kinase (SYK), and EWS/FLI1-associated transcript 2 (EAT-2) (43, 44). These FcRγ^{neg}, SYK^{neg}, and/or EAT-2^{neg} NK cells also generally lack expression of the transcription factor promyelocytic leukemia zinc finger protein (PLZF) (44). These phenotypic changes are linked to more potent antibody-dependent activation, expansion, and function of these adaptive NK cells relative to other NK-cell subsets. NK cells with reduced expression of FcRγ, SYK, or EAT-2 are also detected in CMV seronegative individuals, with a minor fraction (10%) of individuals displaying significant expansions of this population (44).

The crucial function of NK cells in immune defense against CMV coupled with the discovery that distinct subsets of NK cells emerge after infection, collectively suggest that targeted induction of these subsets of NK cells during immunization may provide enhanced protection against CMV infection. The capacity of existing vaccines to elicit transient or sustained expansion of CMV-associated human NK cells has not been reported. In this study, we interrogate longitudinal peripheral blood mononuclear cell (PBMC) samples collected from MF59-adjuvanted CMV glycoprotein B (gB) vaccine or placebo recipients who locally participated in clinical trial NCT00133497 (3). Our study reveals vaccine-independent oscillation of FcRγ^{neg}

NK cell frequencies, but not those of NKG2C^{+} NK cells, in the blood of CMV seronegative individuals. Phenotypic and functional characterization of FcRγ^{neg} NK cells in this CMV seronegative cohort reveals distinct features from those reported for FcRγ^{neg} NK cell in individuals infected with CMV. These finding provoke re-evaluation of the paradigm concerning NK-cell subset dynamics in humans.

MATERIALS AND METHODS

CMV Vaccine Trial

This study was approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board and conducted by the Cincinnati Vaccine and Treatment Evaluation Unit (VTEU) as part of CMV vaccine trial NCT00133497. Study participants were 12- to 17-year-old healthy adolescent females confirmed CMV seronegative at the start of the study. Only samples from the Cincinnati site of this clinical trial were available for the purposes of the present study. Furthermore, only those subjects with available samples spanning trial duration were used for experimental analyses. As a result, a total of 40 participants were randomized into two groups ($n = 20$/group) receiving either three doses of CMV gB subunit vaccine in MF59 adjuvant (20 μg gB and 10.75 mg MF59, Sanofi Pasteur) or sterile saline (Sodium chloride 0.9%) placebo by intramuscular injection in the deltoid on days 0, 30, and 180 of protocol (3). Urine, saliva and blood were collected throughout time course to assess CMV infection by PCR and seroconversion to non-vaccine CMV antigens, respectively. The 40 subjects evaluated longitudinally in the present study remained CMV negative throughout sampling period. Three additional vaccine trial participants who were part of the placebo group and became positive for CMV infection during longitudinal sampling period were used to examine NK-cell subset frequencies at time points subsequent to natural acquisition of CMV infection. Peripheral blood mononuclear cells (PBMC) were collected and cryopreserved at screening and various time points (days 0, 1, 30, 60, 180, and 210) of trial (3).

NK-Cell Phenotypic Analyses

PBMC were concomitantly stained and assessed by flow cytometry during a single experimental run (or block). A volunteer blood donor with a high percentage of NKG2C^{+} NK cells extraneous to vaccine trial was selected as a positive control for NKG2C staining and included in each block of vaccine trial participant samples to benchmark stain validity and reproducibility. Expression of FcRγ, SYK, and EAT-2 are benchmarked against CD4 T cells in the same sample, where the latter cells do not express these proteins (44). Phenotypic analyses of PBMCs were performed using fluorochrome-conjugated antibodies. Cells were stained for surface markers using CD3 (OKT3, Biolegend), CD19 (HIB19, BD Biosciences), CD4 (RPA-T4, BD Biosciences), CD14 (M5E2, BD Biosciences), CD56 (N901, Beckman Coulter), NKG2C (REA205, Miltenyi Biotech), NKG2A (Z199, Beckman Coulter), CD57 (HCD57, Biolegend), CD16 (3G8, BD Biosciences), Ki-67 (Ki-67, Biolegend), and a fixable live-dead stain (Pacific Green, Invitrogen) in FACS buffer (HBSS supplemented with 5% fetal bovine serum and 2 μm EDTA). Following surface staining, cells were fixed in 2% paraformaldehyde (Fisher Scientific) and permeabilized with 0.04% Triton X-100 (Sigma Aldrich). Intracellular staining in FACS buffer with 2% bovine serum albumin was then performed to identify FcRγ (polyclonal rabbit, Millipore), EAT-2 (polyclonal rabbit, ProteinTech Group), SYK (4D10.1, eBioscience) markers. Intracellular EAT-2 staining was followed by secondary staining with polyclonal anti-rabbit IgG (Invitrogen).

NK-Cell Functional Analyses

PBMC samples were thawed rapidly in a 37°C water bath and cell number and viability were determined using 0.4% Trypan Blue (Thermo Fisher Scientific). Cells were cultured at 5×10^5 per well in a 96 well U-shaped plate (Corning Life Sciences) at 37°C in 5% CO_2. Control wells received only media [RPMI 1640 media (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum], while cytokine-stimulated wells received a combination of IL-12 (10 ng/ml), IL-15 (100 ng/ml), and IL-18 (100 ng/ml) (44). After 18 h of culture, Golgi Plug (BD Biosciences) and Golgi Stop (BD Biosciences) were added for an additional 6 h at final concentrations of 1 μg/ml and 2 μM, respectively. To assess antibody dependent cell cytotoxicity (ADCC), a third well of 5×10^5 PBMC for each sample were mixed with 1.25×10^5 P815 cells [2:1 effector to target (E:T) ratio] pre-incubated with 2.5 μg/ml anti-CD32 (Clone 2.4G2, Bio-X-Cell). Cells were incubated in the presence of Golgi Stop and Golgi Plug for a total of 6 h (45). Anti-CD107a (H4A3, Biolegend) at 1:200 dilution was added to all cells in the final 6 h of stimulation. Intracellular staining in FACS buffer was performed to assess IFN-γ (4S.B3, Biolegend) production. Flow cytometric data for all phenotypic and functional analyses were obtained using an LSR Fortessa instrument (BD Biosciences) and analyzed via FlowJo_v10 software (Treestar).

T-Distributed Stochastic Neighbor Embedding (t-SNE) Analyses

The tSNE algorithm of FlowJo_v10 was used to visualize dimensionality of NK cell subsets over time. For each donor, the data at individual time point was down sampled (gated on CD56^{dim} NK cells) and then concatenated to create three dimensionally reduced t-SNE plots. Populations expressing or lacking various proteins were overlaid on t-SNE plots to identify subset clusters.

Statistical Analyses

Differences between placebo and vaccine recipients were compared using mixed effects two way ANOVA with restricted maximum likelihood. Changes over time (0, 6, 7, 10, and 13 months) and treatment group (placebo and vaccine) in the proportion of CD56^{bright} and CD56^{dim} cells were evaluated using generalized linear mixed models with a Poisson distribution, log link function, and an offset of the logarithm of the total NK cell count specified. A random intercept and a random slope and an interaction term between time and treatment group was included in the model. Correlations between NK cell markers were determined by linear regression analysis. Phenotypic differences between groups were determined by

Student's *t*-test and functional differences were assessed by two-way ANOVA. Graphs were generated using GraphPad Prism and statistical tests were performed in Prism and SAS 9.4 (SAS Institute Inc., Cary NC).

RESULTS

CMV gB Vaccine Trial Cohort

To determine whether CMV vaccination strategies can trigger emergence of CMV-associated NK cell subsets, we examined a longitudinal series of PBMC from a subset ($n = 40$, **Table 1**) of CMV vaccine trial participants (NCT00133497) for whom a full set of samples were available. Half of the study participants received three intramuscular injections of CMV gB in MF59 adjuvant while the placebo group was administered sterile saline in place of the vaccine (**Figure 1A**). Vaccine recipients exhibited a robust gB-specific antibody response (**Figure 1B**). None of the selected 40 study participants acquired CMV infection during the study period, as measured by PCR for CMV in urine and for seroconversion against non-vaccine CMV antigens (3).

Minimal Variation in Total NK-Cell Frequencies Over Time

We first assessed the proportion of total NK cells ($CD56^+$ $CD3^-$ $CD19^-$ $CD14^-$ $CD4^-$) in PBMC. **Figure 1C** depicts the gating scheme used to identify NK cells in our samples. There was a broad range (2.0–21.5%) of NK-cell proportions across study participants (**Figure 1D**). The mean proportion of NK cells across all time points is similar in groups receiving placebo or vaccine (Placebo = 7.4%, Vaccine = 8.6%, $p = 0.16$), while the changes in NK cell proportions over time between the placebo or vaccine group were not statistically significantly different ($p = 0.71$).

NK cells can be stratified based on CD56 expression into $CD56^{dim}$ and $CD56^{bright}$ subsets (**Figure 2A**) that exhibit distinct phenotypic and functional characteristics (46). The $CD56^{dim}$ subset comprises a mean 88.8 ± 1.14% (average of all time points) of circulating NK cells in study participants (**Figure 2B**), where the ratio between $CD56^{bright}$ and $CD56^{dim}$ cells in vaccine and placebo groups is relatively consistent over study time points (**Figure 2C**). Specifically, time did not modify the effect between the placebo and vaccine groups regarding $CD56^{dim}$ cell counts ($p = 0.38$). In addition, neither time ($p = 0.97$) nor treatment group [mean vaccine: 6.50% (95% CI: 5.34, 7.91%); mean placebo: 5.51% (95% CI: 4.51, 6.74%), $p = 0.24$] were independently associated with $CD56^{dim}$ cell counts. For $CD56^{bright}$ cell counts, time modified the effect of placebo and vaccine groups ($p = 0.01$); wherein $CD56^{bright}$ count increased by a factor of 1.25 (95% CI: 1.07, 1.46%, $p = 0.01$) in the vaccine group but the change in the placebo group was not statistically significant (**Table 2**), which is consistent with other vaccine studies (47–49).

TABLE 1 | Study participant demographics.

	gB/MF59	Placebo	Total
Number of subjects	20	20	40
Age category at vaccination, *n* (%)			
12–15 years old	14 (70)	11 (55)	25
15–17 years old	6 (30)	9 (45)	15
Race, *n* (%)			
Black	7 (35)	7 (35)	14
Caucasian	12 (60)	11 (55)	23
Other	1 (5)	2 (10)	3
Ethnic origin, *n* (%)			
Hispanic/latino	1 (5)	0 (0)	1
Not Hispanic/latino	19 (95)	20 (100)	39

Age, race, and ethnicity of female subjects who received either three doses of placebo or gB/MF59 vaccine (n = 20 per group).

Absence of CMV-Dependent $NKG2C^+$ NK-Cell Subset

High frequencies of NKG2C-expressing NK cells have been almost exclusively observed in CMV seropositive individuals (39). This subset expands after CMV reactivation in organ or tissue transplant recipients (40), and reflects activation of this subset by CMV UL40-derived peptides coupled with pro-inflammatory cytokines (38). Due to the confirmed CMV negative status of vaccine trial participants throughout the duration of the vaccine study and the absence of UL40 antigens in the vaccine formulation, we hypothesized that $NKG2C^+$ NK cell frequencies would be low at all time points. Using a positive control PBMC sample known to contain $NKG2C^+$ NK cells (44), we confirmed that our staining protocol can effectively detect this subset (**Figure 3A**). As expected, $NKG2C^+$ NK cells were largely undetectable in all vaccine trial participants at baseline and the average absolute change in frequency from baseline proportions of this subset hardly varied across time in both placebo (0.046–0.41% range of mean absolute change from baseline visit) and vaccine (−0.83–0.96% range of mean absolute change from baseline visit) recipients (**Figure 3B**). Analysis of additional samples from vaccine trial participants ($n = 3$) at time points after natural acquisition of CMV infection (confirmed by PCR/seroconversion to non-vaccine CMV antigens) remained negative for $NKG2C^+$ NK cells over the study timeframe (data not shown).

CMV- and Vaccine-Independent Dynamic Changes in NK-Cell Subset Frequencies

Expanded subsets of NK cells that lose expression of FcRγ, EAT-2, and/or SYK are expanded in approximately half of CMV seropositive individuals but can also be observed in seronegative donors, albeit less commonly (≤10% of individuals) and at much lower frequencies (43, 44). As these subsets can expand upon Fc receptor engagement by antibody (37, 43), we hypothesized that robust antibody responses against vaccine antigens may trigger accumulation of these subsets after vaccine prime and boost administration. We could detect NK cells within the $CD56^{dim}$ subset that exhibited loss of FcRγ expression (**Figure 4A**).

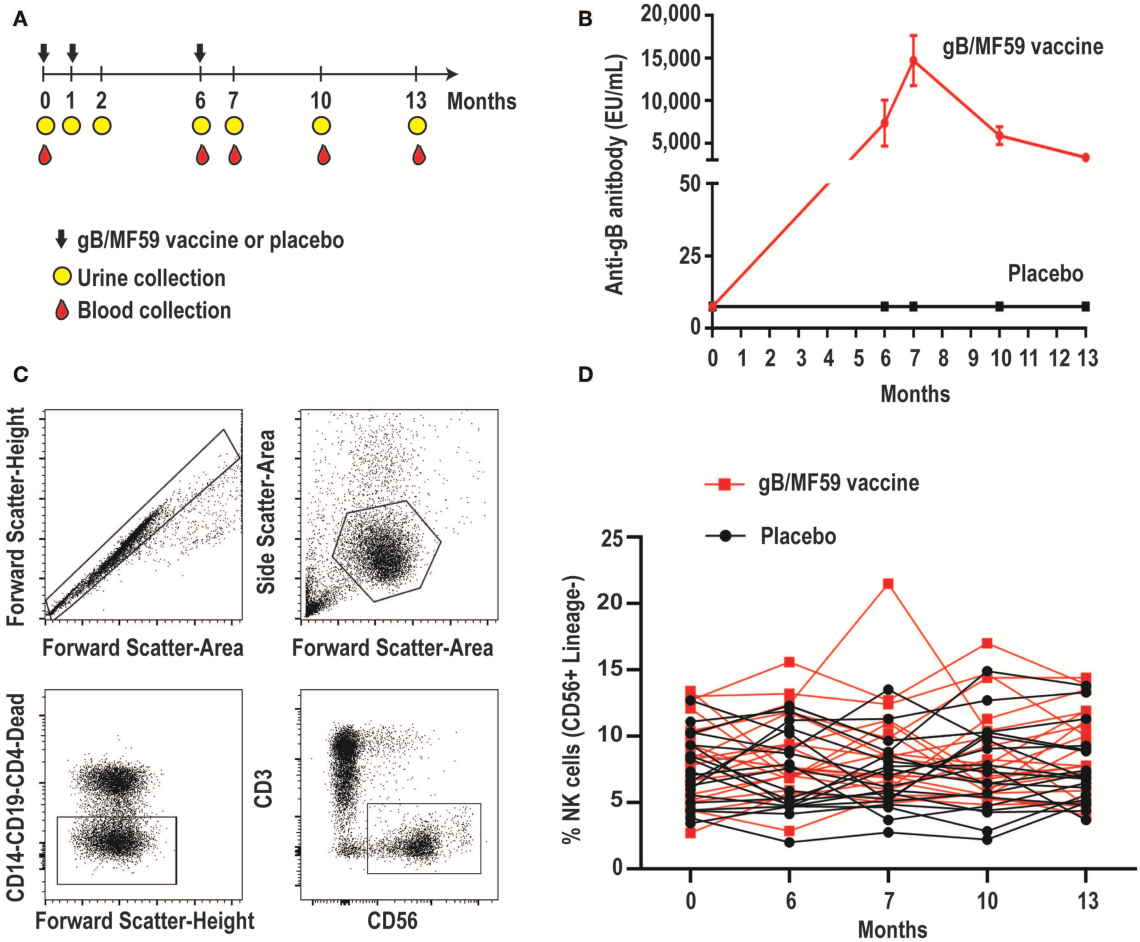

FIGURE 1 | Longitudinal antibody and NK-cell responses of vaccine study participants. **(A)** Schematic representation of the timeline of vaccine trial depicting three administrations of gB/MF59 vaccine or placebo (sterile saline) and timing of urine and blood samples collection. **(B)** Sera anti-gB antibody titers for placebo ($n = 20$) and vaccine ($n = 20$) recipients analyzed in present study. **(C)** Representative flow cytometry gating of singlets, live lymphocytes, lineage-negative (CD19, CD14, CD4, and CD3) CD56$^+$ NK cells based on forward scatter (height and area), side scatter (area), viability dye uptake, and surface marker expression. **(D)** Resulting frequencies of gated NK cells in individual vaccine and placebo recipients over sampling period.

These FcRγ^{neg} NK cells concomitantly lacked EAT-2 and SYK expression in most study participants relative to their FcRγ$^+$ NK cell counterparts (**Figure 4A**).

Interestingly, we detected a progressive increase in the frequency of FcRγ^{neg} NK cells following prime and boost immunization in a subset of vaccine recipients (**Figure 4B**). However, a similar pattern was observed in some placebo recipients. Moreover, the majority of individuals given vaccine ($n = 11$) or placebo ($n = 10$) exhibited transient elevations and depressions in the frequency of FcRγ^{neg} NK cells over time (**Figure 4C**). A smaller number of individuals in both groups demonstrated FcRγ^{neg} NK cells at baseline that disappeared over time, or lacked this subset of cells entirely (**Figure 4C**). High-dimensional analysis with t-SNE confirmed FcRγ^{neg}, EAT-2^{neg}, and SYK^{neg} NK cells largely cluster as one subset, the frequency of which changes over time within the selected study participant (**Figure 4D**).

Variations in Frequencies of FcRγ^{neg} NK Cells Are Not Associated With Proliferation

In addition to increases and decreases in the proportion of FcRγ^{neg} populations among NK cells, the frequency of these cells among total blood leukocytes (PBL) shows similar patterns of expansion and contraction (**Figure 5A**). The marked increases in the number of FcRγ^{neg} NK cells in some individuals over time are potentially attributable to periods of proliferative expansion. In fact, adaptive subsets of NK cells that accumulate during acute CMV infection of solid organ transplant recipients are characterized by heightened expression of Ki-67, an indication that these cells are highly proliferative (50). Analysis of Ki-67 expression over time in a subset of six vaccine trial participants exhibiting ebb-and-flow representative of FcRγ^{neg} NK-cell within the NK-cell repertoire revealed relatively stable, low-level expression of Ki-67 on FcRγ^{neg} NK cells (**Figure 5B**). There was no clear visual relationship between Ki-67 expression

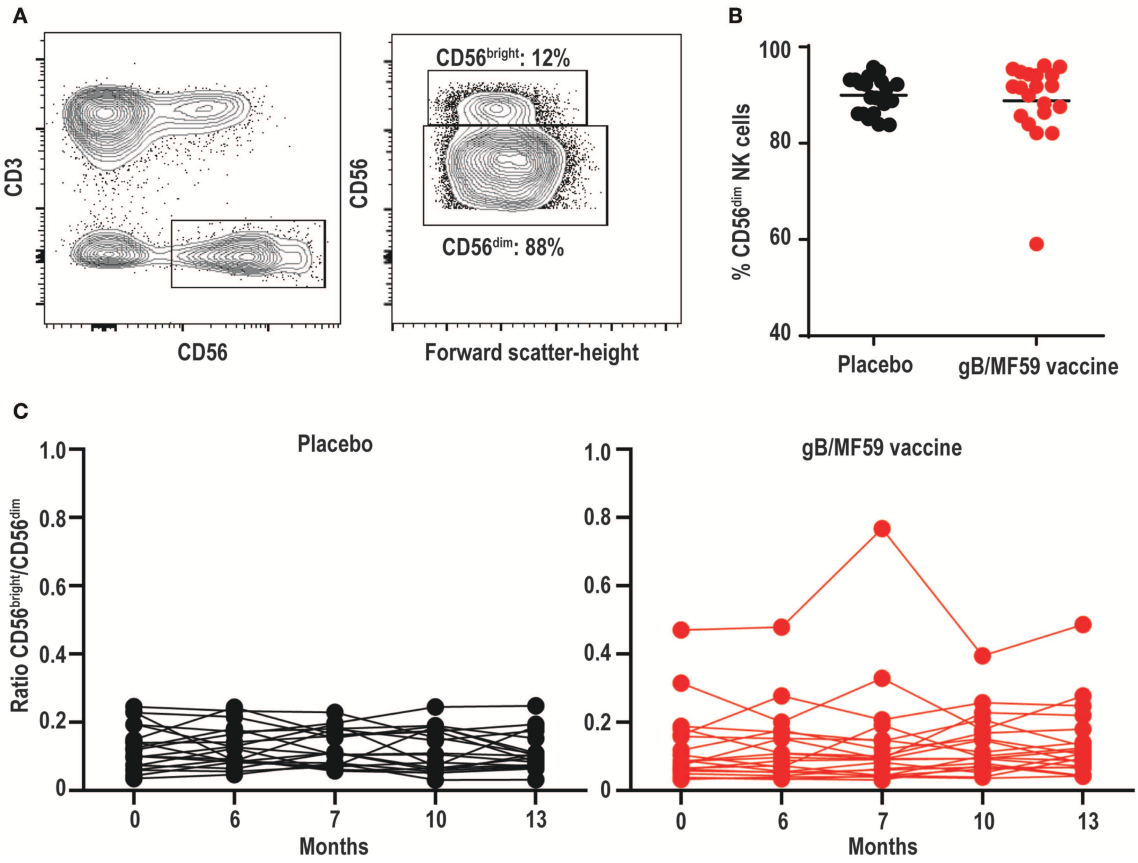

FIGURE 2 | Stable proportions of $CD56^{dim}$ and $CD56^{bright}$ NK cells over time. **(A)** Representative gating of $CD56^{bright}$ and $CD56^{dim}$ events within the $Lineage^{-}CD3^{-}CD56^{+}$ NK cell gate, and **(B)** percentage of $CD56^{dim}$ NK cells in each study participant averaged across all time points. Bar represents mean among treatment group ($n = 20$/group). **(C)** Ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells in each study participant across vaccine trial time points. Statistically significant changes in subset proportion over time evaluated using generalized linear mixed model as described in Methods, with results of analysis presented in **Table 2**.

and changes in frequency of $FcR\gamma^{neg}$ NK cells among blood leukocytes (**Figure 5B**), and linear regression analysis of all time points analyzed revealed absence of significant relationship between the proportion of Ki-67-expressing $FcR\gamma^{neg}$ NK cells and the fraction of NK cells that are $FcR\gamma^{neg}$ (**Figure 5C**). Thus, within the limitations of these measurements and our sampling intervals, our data provide little evidence in support for the hypothesis that variations in $FcR\gamma^{neg}$ NK cell frequencies are attributable to proliferative expansions of these cells.

Distinct CD57 Expression on $FcR\gamma^{neg}$ NK Cells in Absence of CMV

While the proportions of $FcR\gamma^{neg}$ $EAT\text{-}2^{neg}$ SYK^{neg} NK cells vary among individuals and at different time points, the percentage of NK cells expressing other receptors associated with CMV infection, including CD57 (range 10–54%) or NKG2A (range 20–84%), exhibited little variation across time (**Figure 6A**). In fact, no statistically significant differences in CD57 ($p = 0.96$) or NKG2A ($p = 0.75$) expression were observed over time between placebo and vaccine groups. The temporally stable but heterogeneous expression of CD57 and NKG2A among individuals in the present study is consistent with prior observations (51). As CMV infection is associated with increased expression of CD57 and down-regulation of NKG2A (52), most notably among $FcR\gamma^{neg}$ (37) and $NKG2C^{high}$ (50) NK cells, the expression of these receptors was examined on the NK-cell subsets in vaccine trial participants (**Figure 6B**). $FcR\gamma^{neg}$ NK cells detected in CMV-negative individuals in this study segregated as $NKG2A^{low}$ relative to $FcR\gamma^{+}$ cells (**Figure 6B**), consistent with previous studies (37, 44). However, $FcR\gamma^{neg}$ NK cells were not enriched for expression of the maturation marker CD57 (**Figure 6B**). In fact, the totality of $FcR\gamma^{neg}$ NK cells observed across time points and individuals in this study expressed less CD57 than their $FcR\gamma^{+}$ counterparts (**Figure 6C**). Thus, $FcR\gamma^{neg}$ NK cells are more prevalent in the NK-cell repertoire in this longitudinally examined study cohort and frequently exhibit dynamic changes in frequency over time as well as distinct CD57 expression patterns relative to $FcR\gamma^{neg}$ NK cells in CMV infected individuals.

No Functional Impact of $FcR\gamma$ Loss in CMV Seronegative Subjects

Signaling alterations of adaptive NK cells in CMV positive individuals lead to distinct functional capacities as compared to

conventional NK cells (53). In particular, FcRγ^{neg} NK cells in CMV infected individuals exhibit reduced IFN-γ production in response to cytokine stimulation (44), but elevated antibody-dependent effector function (37). Functional responses of FcRγ^{neg} NK cells in the absence of CMV were examined at a total of 18 samples from a subset of six vaccine trial participants scoring positive for FcRγ^{neg} NK cells. In contrast to observations in CMV seropositive individuals, IL-12 and IL-18 cytokine stimulation did not lead to statistically significant differences in IFN-γ production ($p = 0.41$) or degranulation as measured by CD107a exposure ($p = 0.67$) between FcRγ^{neg} and FcRγ^{+} NK cells in the CMV seronegative vaccine cohort (**Figure 7A**). FcRγ^{neg} and FcRγ^{+} NK cells also exhibited comparable degranulation ($p = 0.58$) and IFN-γ production ($p = 0.38$) when stimulated with P815 cells pre-incubated with α-CD16 antibody (**Figure 7A**). Of note, FcRγ^{neg} NK cells in vaccine recipients produced slightly more IFN-γ but displayed similar degranulation in response to α-CD16-bound P815 relative to the same cells in individuals receiving placebo (data not shown).

CD57^{+} NK cells are also differentially sensitive to cytokine and antibody-dependent stimulation compared to CD57^{neg} NK cells (54). FcRγ^{neg} NK cells in the present vaccine cohort exhibit a distinct CD57 expression pattern compared to FcRγ^{neg} NK cells in CMV seropositive individuals (37, 52). Therefore, functional responses of CD57^{neg} FcRγ^{neg} and CD57^{+} FcRγ^{neg} NK cells were compared within CMV seronegative vaccine trial participants. CD57^{neg} and CD57^{+} FcRγ^{neg} NK cells exhibited similar IFN-γ production after stimulation with IL-12 and IL-18 ($p = 0.51$)

TABLE 2 | Change in NK cell CD56^{bright} and CD56^{dim} subsets over time.

	Placebo mean (95% CI)	Vaccine mean (95% CI)
CD56^{DIM} (Interaction $p = 0.38$)		
0 month	5.95% (4.73, 7.50%)	6.20% (4.95, 7.76%)
6 months	5.41% (4.29, 6.81%)	6.34% (5.07, 7.94%)
7 months	5.15% (4.09, 6.49%)	6.99% (5.58, 8.75%)
10 months	5.41% (4.30, 6.82%)	6.45% (5.15, 8.07%)
13 months	5.67% (4.49, 7.16%)	6.53% (5.21, 8.20%)
CD56^{BRIGHT} (Interaction $p = 0.01$)		
0 month	0.62% (0.50, 0.77%)	0.58% (0.47, 0.72%)
6 months	0.61% (0.49, 0.76%)	0.60% (0.48, 0.74%)
7 months	0.60% (0.49, 0.75%)	0.69% (0.56, 0.86%)
10 months	0.55% (0.44, 0.68%)	0.70% (0.57, 0.87%)
13 months	0.59% (0.47, 0.73%)	0.72% (0.58, 0.90%)

Mean (95% confidence interval) percentage of CD56^{dim} and CD56^{bright} cells by time and treatment group.

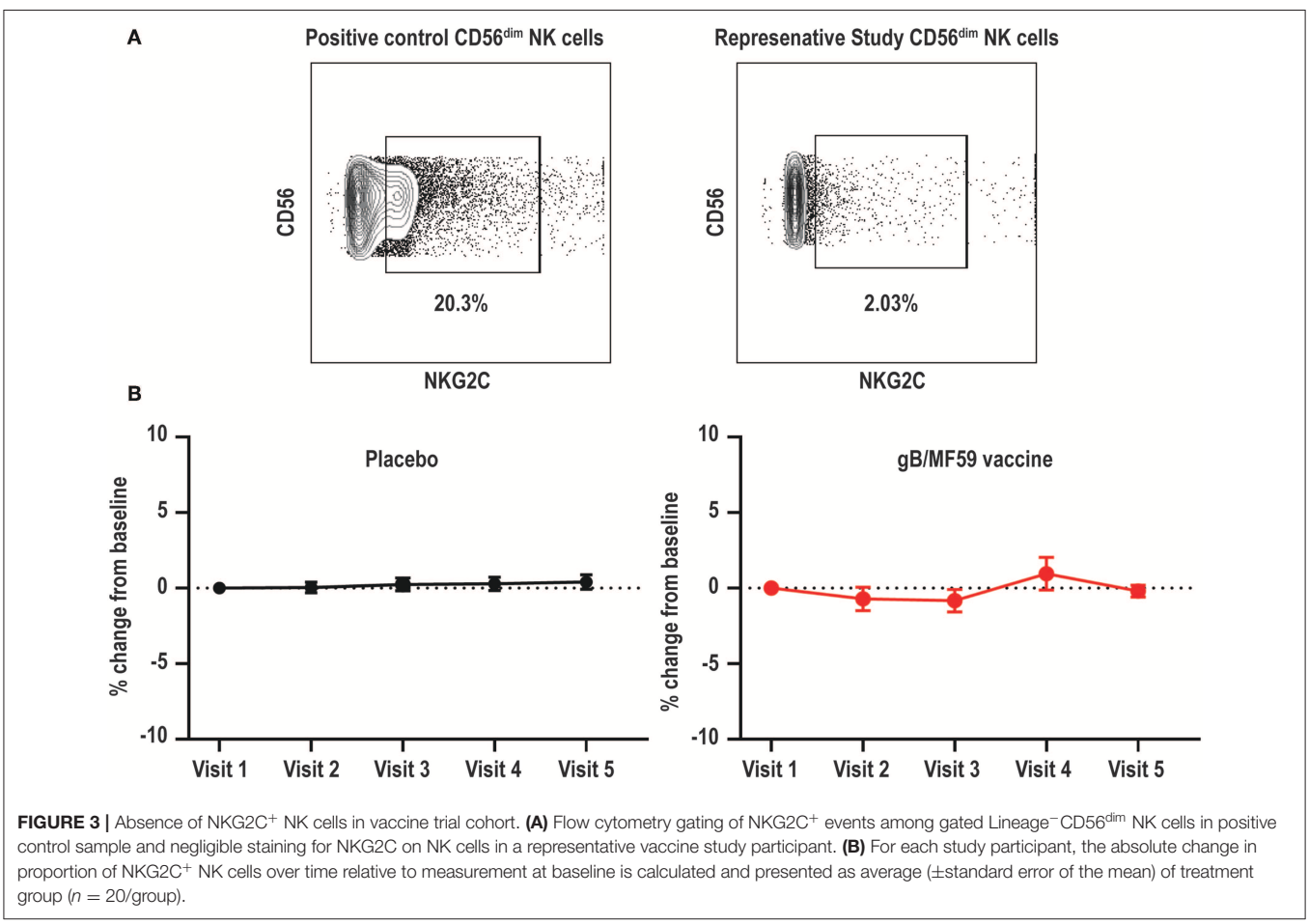

FIGURE 3 | Absence of NKG2C^{+} NK cells in vaccine trial cohort. **(A)** Flow cytometry gating of NKG2C^{+} events among gated Lineage^{-}CD56^{dim} NK cells in positive control sample and negligible staining for NKG2C on NK cells in a representative vaccine study participant. **(B)** For each study participant, the absolute change in proportion of NKG2C^{+} NK cells over time relative to measurement at baseline is calculated and presented as average (±standard error of the mean) of treatment group ($n = 20$/group).

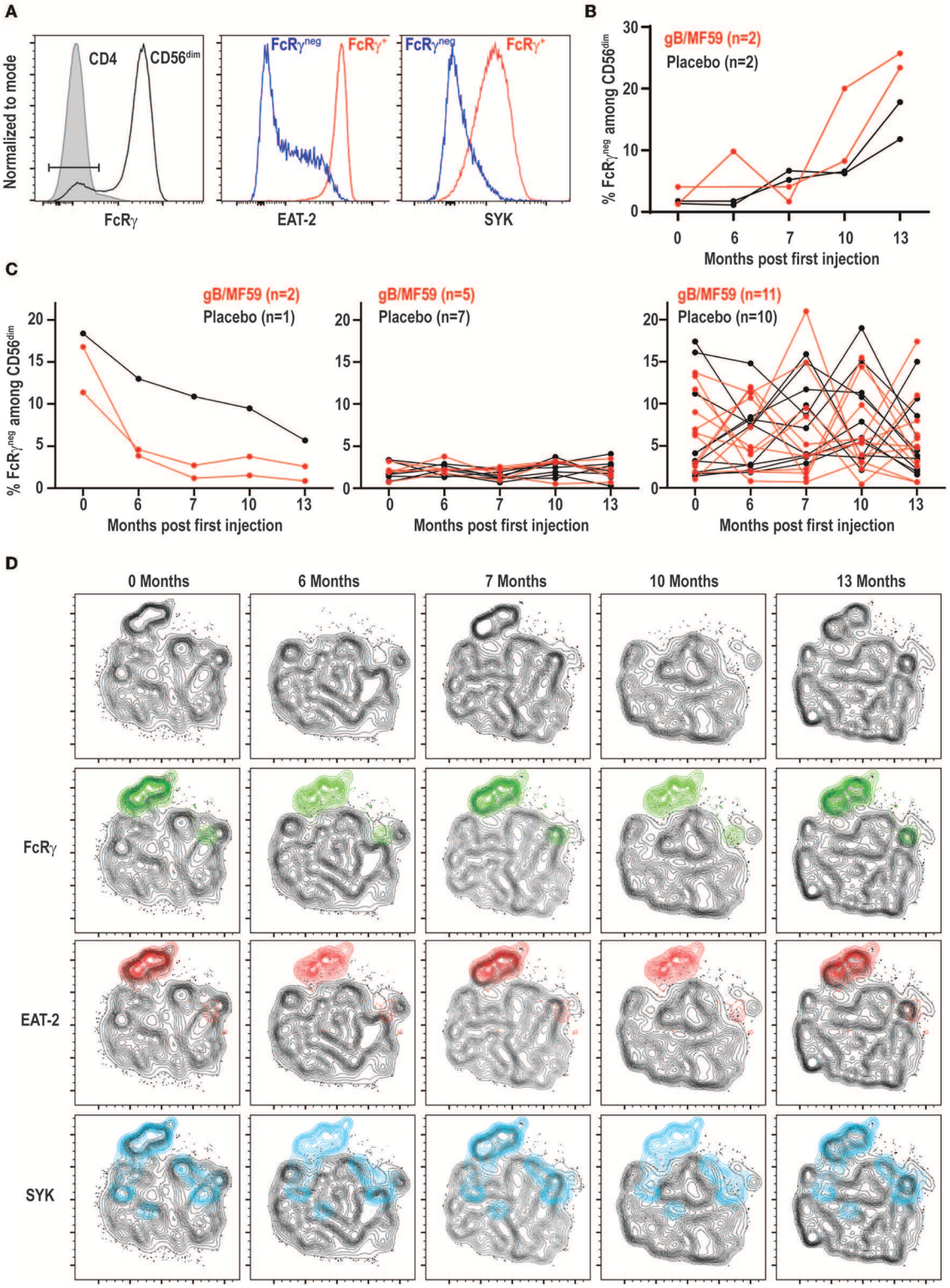

FIGURE 4 | Dynamic vaccine-independent changes in NK-cell subset representation within repertoire over time. **(A)** Representative gating of FcRγ^{neg} CD56^{dim} NK cells (open histogram) relative to CD3^{+}CD4^{+} T cells (shaded histogram) in the same sample. EAT-2 and SYK expression on gated FcRγ^{neg} (blue histogram) and FcRγ^{+} (red histogram) subsets of CD56^{dim} NK cells. **(B)** Proportions of FcRγ^{neg} CD56^{dim} NK cells over time in a subset of gB-MF59 (red) or placebo (black) recipients revealing expansion of these cells within the repertoire. **(C)** Proportions of FcRγ^{neg} CD56^{dim} NK cells over time in remaining study participants grouped based on pattern of subset contraction (left), absence of subset (middle), or ebb-and-flow changes in repertoire. **(D)** Location of each adaptive NK-cell subset in t-SNE distribution of a single study participant CD56^{dim} NK cell repertoire over time course is highlighted in green (FcRγ^{neg}), red (EAT-2^{neg}), and blue (SYK^{neg}).

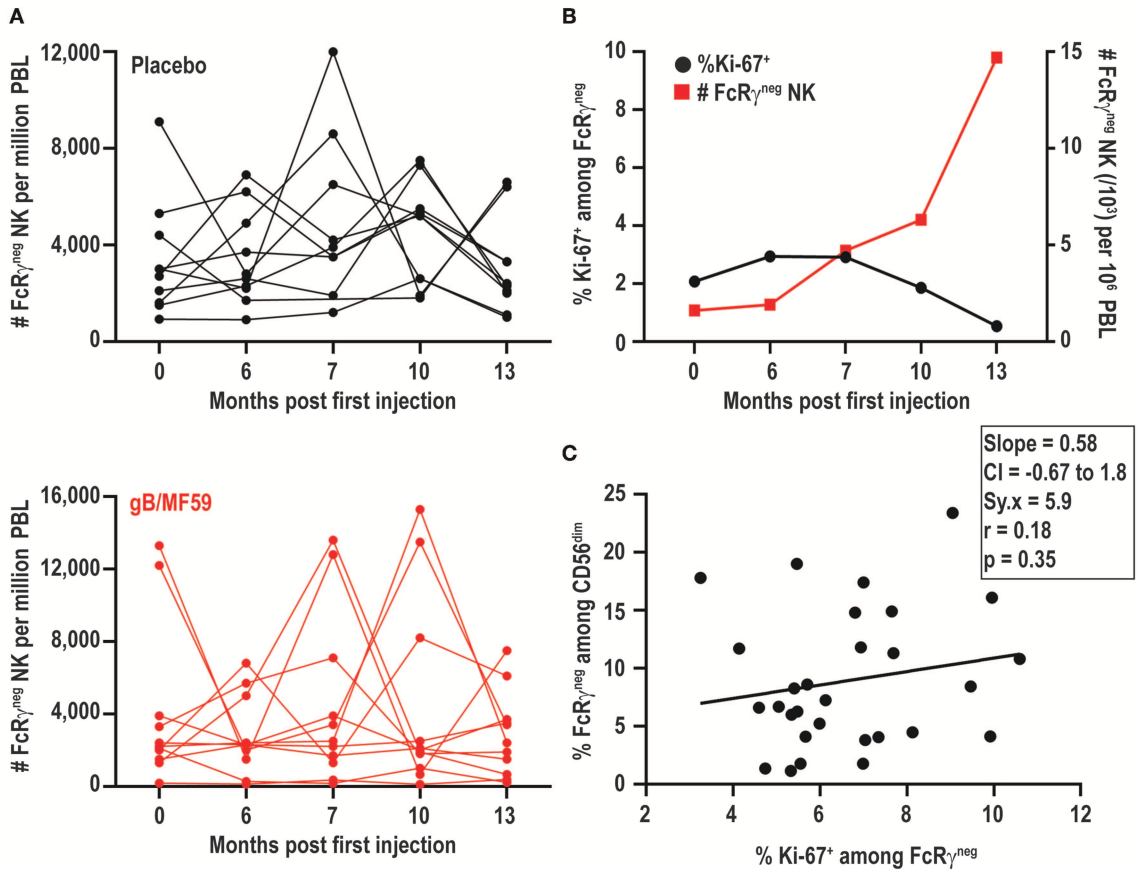

FIGURE 5 | FcRγ^{neg} NK cell subset expansions in absence of increased Ki-67. **(A)** Frequencies of FcRγ^{neg} CD56^{dim} NK cells among PBL over time in a subset of gB-MF59 (red) or placebo (black) recipients exhibiting ebb-and-flow changes in repertoire. **(B)** Relationship between percent of FcRγ^{neg} NK cells staining Ki-67^{+} and expansion of FcRγ^{neg} NK cell subset over time. One representative individual is shown from among six vaccine trial participants with marked fluctuations in FcRγ^{neg} NK cell frequencies that were analyzed in this experiment. **(C)** Regression analysis of the linear relationship between the proportions of Ki-67^{+} cell within the FcRγ^{neg} NK cell subset and total FcRγ^{neg} NK cells. Slope, confidence interval (CI), residual standard error (Sy.x), correlation (r), and significant deviation of slope from zero (p) are shown.

or α-CD16 antibody bound P815 cells ($p = 0.14$) (**Figure 7B**). However, CD57^{+} FcRγ^{neg} NK cells degranulated more robustly than their CD57^{neg} FcRγ^{neg} NK cell counterparts in response to either cytokine or P815+α-CD16 stimulation (**Figure 7B**).

DISCUSSION

Past cross-sectional analyses suggest that adaptive subsets of NK cells are rarely present in the absence of CMV infection, whereas the frequencies of these adaptive NK cells are markedly increased in the majority of CMV seropositive individuals (43, 44, 50, 55, 56). The present longitudinal analysis of NK cells in healthy CMV-negative individuals affirms the lack of NKG2C-expressing NK cells in CMV-naïve persons (44), yet challenges the paradigm that the FcRγ^{neg} NK cell subset is infrequent or absent in CMV seronegative individuals. In contrast to the current prototype, the majority (28 of 40, 70%) of the healthy, demonstrably CMV-negative adolescent women profiled in this clinical study exhibit measurable frequencies of FcRγ^{neg} NK cells during at least one study time point, with dynamic changes in the frequency of these cells among circulating NK cells over time. Changes in frequencies of these subsets did not correlate with vaccine administration or vaccine-antigen-specific antibody titers (data not shown), suggesting that undefined environmental factors promote oscillations in the representation of these subsets among total circulating NK cells.

The low frequencies of NKG2C-expressing NK cells across all time points in the 40 vaccine trial participants is consistent with absence of CMV infection of these individuals and the purported link between CMV gene products and expansion of this subset of NK cells (57–59). Likewise, we observed very low (0.22 ± 0.15% of live lymphocytes) but highly stable frequencies of infection-associated CD56^{neg} CD16^{+} NK cells in our cohort (data not shown), consistent with absence of CMV and other viruses linked to this unusual NK-cell population. Within the limitations of our sampling scheme, our results support the hypothesis that CMV gB and MF59 are insufficient to stimulate differentiation or accumulation of NKG2C^{+} NK cells. Since CMV UL40-derived peptides presented by HLA-E are critically required

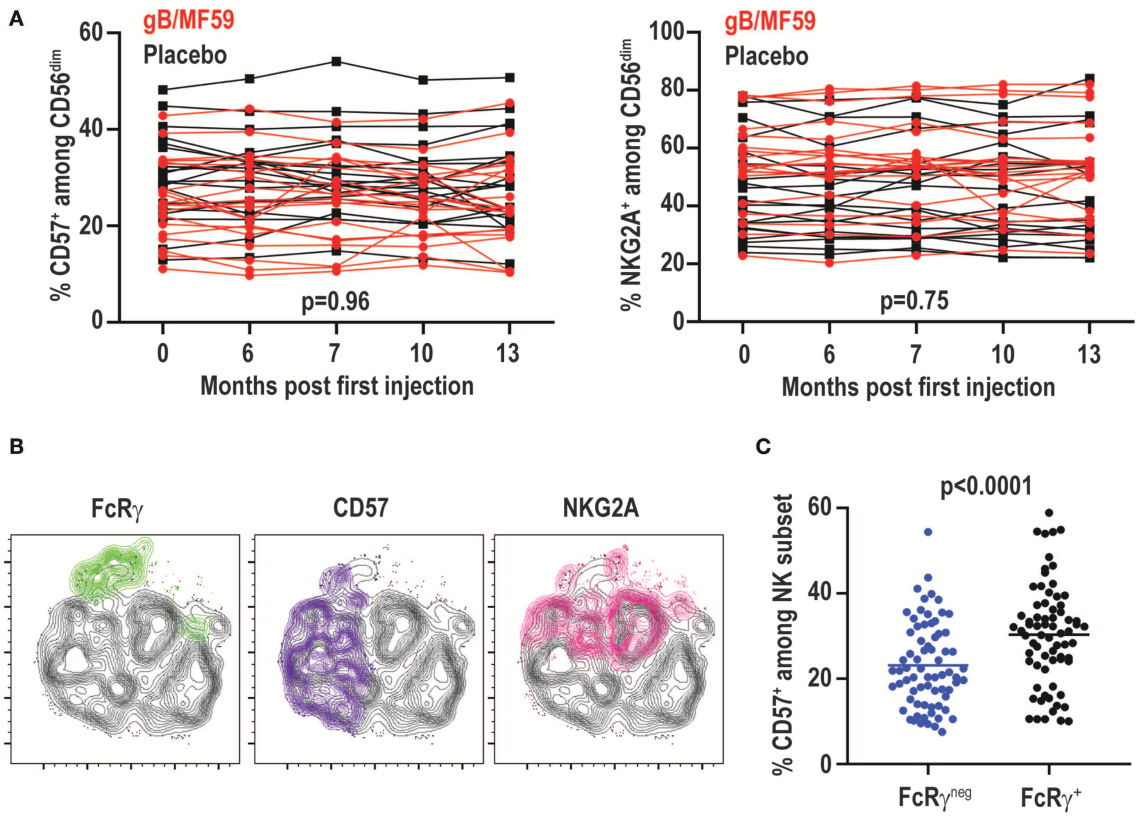

FIGURE 6 | No change in CD57 and NKG2A over time. **(A)** Proportions of $CD57^+$ and $NKG2A^+$ $CD3^-CD56^{dim}$ NK cells in individual gB/MF59 vaccine (red) and placebo (black) recipients over time. Mixed effects two way ANOVA with restricted maximum likelihood was used to compare mean differences over time between both placebo and vaccine groups for each marker. **(B)** Representative location of $FcR\gamma^{neg}$ (green), $CD57^+$ (purple), and $NKG2A^+$ (pink) cells at single time point in t-SNE distribution of a single study participant $CD56^{dim}$ NK cell repertoire. **(C)** Expression of CD57 on $FcR\gamma^{neg}$ and $FcR\gamma^+$ $CD56^{dim}$ NK cells across all time points and study participants where the $FcR\gamma^{neg}$ subset was detectable (≥5% of $CD56^{dim}$ NK cells). Statistical significant differences between repeated measures determined by Student's *t*-test.

for HCMV-driven NKG2C expansion (38), incorporation of UL40 into next generation vaccines may more effectively elicit $NKG2C^+$ memory NK cell expansion.

In contrast to both the tight link between CMV and $NKG2C^+$ NK cells and the reported rarity of $FcR\gamma^{neg}$ NK cells in CMV-seronegative individuals, the present longitudinal data suggest that the latter NK cell subset may be commonly present in some NK-cell repertoires and can exhibit dynamic changes in frequency. There was no correlation between numeric increases in $FcR\gamma^{neg}$ NK cells and expression of the proliferation marker Ki-67, suggesting that release of this subset from tissues may be a greater factor in these dynamic changes than proliferative expansion. However, the timing of experimental sampling in the present study likely precludes precise determination of a link between proliferation and $FcR\gamma^{neg}$ NK cell accumulation. The majority (28 of 40, 70%) CMV seronegative individual in our study exhibited populations of $FcR\gamma^{neg}$ NK cells >10% during at least one of the five time points analyzed over a year-long study period. These data contrast a previous cross-sectional studies which found expansions of NK cells lacking FcRγ, EAT-2, and/or SYK in 6 out of 69 CMV seronegative adults (44). The fraction of study participants scoring positive for $FcR\gamma^{neg}$ NK cell subsets at any given time point in our study ranged from 30 to 45%, suggesting that additional factors may distinguish the two study populations. Moreover, the $FcR\gamma^{neg}$ NK cells measured in this study appear to differ from those observed in CMV-infected individuals with regards to expression of the maturation marker CD57 (37, 44, 50, 54).

In addition to their distinct phenotype, the $FcR\gamma^{neg}$ NK cells measured here differ in their functional activity as compared to their counterparts in CMV positive subjects (44). Namely, the $FcR\gamma^{neg}$ NK cells in the present study exhibit similar capacity to make IFN-γ and degranulate as $FcR\gamma^+$ NK cells in responses to cytokines or antibody-dependent stimuli. We speculate that CMV-independent $FcR\gamma^{neg}$ NK cells are unlikely to bear hypomethylation at the *IFNG* locus as a consequence of CMV infection (44). As CD57 expression on NK cells is putatively linked to increased cytolytic potential, decreased sensitivity to inflammatory cytokines, and reduced proliferative potential (60), this phenotypic disparity of $FcR\gamma^{neg}$ populations of NK cells in the absence of CMV may reflect important functional distinctions as well. The observed increase in degranulation

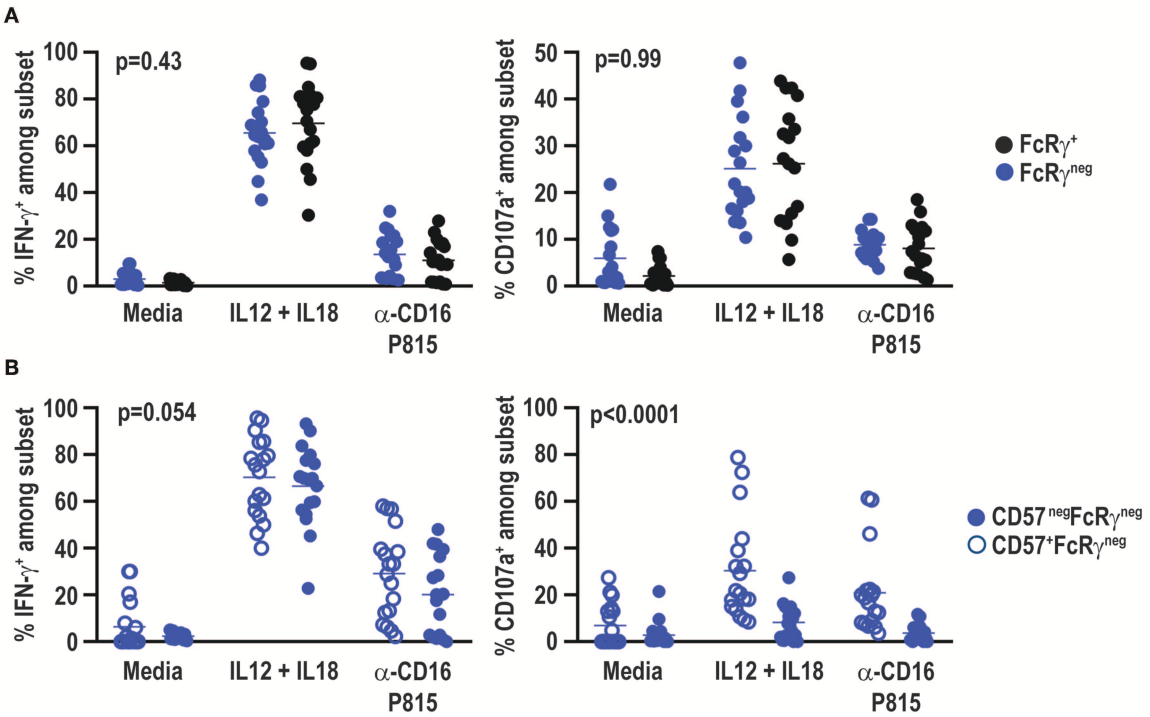

FIGURE 7 | No functional impact of FcRγ deficiency in HCMV-negative individuals. Eighteen samples from six vaccine trial participants (3 gM/BF59 and 3 placebo) scoring positive for FcRγ^{neg} NK cells were stimulated with either IL-12+IL-18 for 24 h or P815 cells labeled with α-CD16 antibody for 6 h. Degranulation and IFN-γ production assessed by addition of fluorochrome-labeled α-CD107a antibody as well as GolgiPlug and GolgiStop during final 6 h of incubation. Proportions of CD107a^{+} and IFN-γ^{+} events among **(A)** FcRγ^{neg} (blue) and FcRγ^{+} (black) CD3^{neg} CD56^{dim} NK cells or **(B)** CD57^{+} (open circles) and CD57^{neg} (closed circles) FcRγ^{neg} NK cells. Statistical significant differences between groups was determined by two-way ANOVA.

of CD57^{+} FcRγ^{neg} relative to CD57^{neg} FcRγ^{neg} NK cells we observe consistent with the notion that CD57^{+} cells are more differentiated and have a distinct transcriptional signature in comparison to CD57^{-} NK cells (54).

A major distinction of the present study population is the restriction to analysis of adolescent females. The influence of puberty-associated hormones and other pediatric variables on adaptive NK cell subsets is unknown. Therefore, it is possible that the present longitudinal study reveals dynamics of NK cell subsets that are unique to adolescents, or even adolescent females, that are not shared by adult CMV seronegative populations. Of note, NK cells express the alpha and beta estrogen receptors (ERα and ERβ) and exhibit function alterations in response to estrogen (61, 62). Moreover, while KIR, CD57, and NKG2A expression on NK cells remains stable across menstruation cycles (51, 63), the stability of the FcRγ^{neg} NK cell subset in this setting is less clear. Therefore, increased prevalence of CMV-associated NK cells or dynamic variation in the frequencies of the cells may reflect hormonal changes or environmental influences that are unique to or more common in adolescent females.

Besides these differences in age and gender of our study population, the participants in the CMV vaccine trial also exhibited a greater degree of racial diversity than was represented in previous cross-sectional studies (44). Specifically, 35% of our vaccine trial participants were Black (i.e., African American). Although race assuredly impacts the NK-cell repertoire in the context of highly polymorphic receptors, including killer-cell immunoglobulin-like receptors (KIR), the effects of race on CMV-reactive NK cells and receptors associated with these subsets are less well-defined. Intriguingly, 100% (15 of 15) of Black study participants demonstrated detectable FcRγ^{neg} NK cells at one or more time points of study, whereas only 58% (14 of 24) of Caucasian study participants exhibited FcRγ^{neg} NK cells in their repertoire. Thus, gender, race, genetics, and local environmental factors may all contribute to the distinct observations of adaptive NK cell frequencies in our study.

A key unanswered question concerns the nature of the stimuli provoking longitudinal changes in frequency of NK cell subsets. A recent study of barcoded hematopoietic cells in rhesus macaques noted significant fluctuations in the clonal composition of NK cells over time (64). Our study stringently controlled for CMV exposure via urine and blood analyses (3). Moreover, the results do not support a relationship between CMV gB vaccination or gB-specific antibody titers and altered frequencies of NK cell subsets. The present results contrast with marked change in NK-cell phenotype and function observed following protein subunit or inactivated virus vaccine administration in CMV seropositive individuals (48, 65–68). Nonetheless, other subclinical acute infections, vaccinations (e.g.,

seasonal influenza vaccine), inflammatory events, environmental exposures (i.e., allergens), or shifts in microbiota composition could alter the composition of the NK-cell repertoire. We speculate that these environmental stimuli or associated immune responses (i.e., antibody elaboration) provoke the expansion, differentiation, or release of FcRγ^{neg} NK cells into the circulation. The elevated frequency of these NK cell subsets in CMV-positive individuals may reflect an altered tempo or magnitude of these natural oscillations, or a greater regularity of the instigating stimulus. Alternatively, as the frequencies of these subsets appear to be more stable in CMV-seropositive individuals (40), aspects of the inflammatory environment during chronic CMV infection may more efficiently maintain these populations. Given that the MF59 adjuvant used in this CMV vaccine is designed for optimal stimulation of T and B-cell responses, future studies aimed at ascertaining the nature of inflammatory cues promoting adaptive NK cells will yield key insights into the types of adjuvants that may be applied to intentional promote sustained expansion of these NK cell subsets in next generation vaccines.

Our results, to our knowledge, represent the first longitudinal study of CMV-associated NK-cell subsets in healthy CMV seronegative individuals. Here, we had the unique ability to gain insight into the intra-individual variation in the frequency of NK-cell subsets following gB/MF59 vaccination. We show that the lack of change in NKG2C expression was consistent with absence of CMV infection, confirming the stringent association of this virus with NKG2C^{+} NK cells. However, we also present evidence suggesting that presence of FcRγ^{neg}, EAT-2^{neg}, and SYK^{neg} NK cells in the repertoire may be more temporally dynamic and CMV-independent than previously thought. These data also reveal potentially important functional differences between CMV independent FcRγ^{neg} NK cells and those accumulating in the context of CMV infection. Future work examining age and gender related differences as well as longitudinal analyses of post-transplant patients may give further insight into the variegated expression of CMV-associated NK-cell subsets.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cincinnati Children's Hospital Medical Center Institutional Review Board. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

IG: conception and design of study, execution of experiments, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. HSc: analysis and interpretation of data and critical revision of the manuscript. HSu and LA: statistical analyses and critical revision of the manuscript. DO and HW: execution of experiments. YB: analysis and interpretation of results and critical revision of the manuscript. DB: conception and design of study, sample collection and regulatory approvals, and critical revision of manuscript. SW: conception and design of study, analysis and interpretation of data, drafting and critical revision of the manuscript, obtained funding, and study supervision.

FUNDING

This research was supported by the Cincinnati Children's Research Foundation and the National Center for Advancing Translational Sciences of the National Institutes of Health (NIH) under Award Number UL1 TR001425. Investigators on this project are supported by NIH grants AI125413 (LA), DA038017, AI148080, and AR073228 (SW). The original clinical trial was supported by NIH contract HHSN272200800006C (DB). The Cincinnati Children's Flow Cytometry Core was supported by NIH grants AR047363, AR070549, DK078392, DK090971, S10OD025045, and S10OD023410. DO was supported by a fellowship from the American Heart Association. YB was supported by European Research Council under the European Union's Seventh Framework Program (FP/2007-2013)/ERC Grant Agreement no. 311335, the Swedish Research Council, Norwegian Research Council, Swedish Foundation for Strategic Research, Wallenberg Foundation, Swedish Cancer Foundation, Swedish Childhood Cancer Foundation, and the Stockholm County Council and Karolinska Institutet Center for Innovative Medicine.

ACKNOWLEDGMENTS

We would like to thank G. Hart (U. Minnesota) for insights into the staining protocol for FcRγ^{neg} EAT-2^{neg} SYK^{neg} NK cells.

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

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 December 2019
doi: 10.3389/fimmu.2019.02972

JAK Inhibition Differentially Affects NK Cell and ILC1 Homeostasis

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Janus kinase (JAK) inhibitors are widely used in the treatment of multiple autoimmune and inflammatory diseases. Immunologic and transcriptomic profiling have revealed major alterations on natural killer (NK) cell homeostasis associated with JAK inhibitions, while information on other innate lymphoid cells (ILCs) is still lacking. Herein, we observed that, in mice, the homeostatic pool of liver ILC1 was less affected by JAK inhibitors compared to the pool of NK cells present in the liver, spleen and bone marrow. JAK inhibition had overlapping effects on the transcriptome of both subsets, mainly affecting genes regulating cell cycle and apoptosis. However, the differential impact of JAK inhibition was linked to the high levels of the antiapoptotic gene Bcl2 expressed by ILC1. Our findings provide mechanistic explanations for the effects of JAK inhibitors on NK cells and ILC1 which could be of major clinically relevance.

Keywords: JAK/STAT, cytokines, NK cells, ILC, differentiation, kinase inhibitors

OPEN ACCESS

Edited by:
Yenan Bryceson, Karolinska Institutet (KI), Sweden

Reviewed by:
Timotheus You Fu Halim, University of Cambridge, United Kingdom
Stephen Noel Waggoner, Cincinnati Children's Hospital Medical Center, 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: *05 August 2019*
Accepted: *03 December 2019*
Published: *19 December 2019*

Citation:
Vian L, Le MT, Gazaniga N, Kieltyka J, Liu C, Pietropaolo G, Dell'Orso S, Brooks SR, Furumoto Y, Thomas CJ, O'Shea JJ, Sciumè G and Gadina M (2019) JAK Inhibition Differentially Affects NK Cell and ILC1 Homeostasis. Front. Immunol. 10:2972. doi: 10.3389/fimmu.2019.02972

HIGHLIGHTS

- JAK inhibition has distinct impacts on the homeostatic numbers of NK cells and ILC1.
- Tofacitinib has redundant effects on the transcriptomic programs of NK cells and ILC1.
- Basal expression level of Bcl2 underlies the differential impact of tofacitinib in NK cells and ILC1.

INTRODUCTION

Cytokines are pivotal in the maintenance of an appropriate immune system homeostasis, but dysregulation of their activity underlies multiple immune-related disorders (1). The elucidation of the role of the Janus kinase (JAK) family of intracellular tyrosine kinases in the signaling cascade downstream of cytokine receptors has highlighted this class of molecules as potential therapeutic targets. Indeed, inhibition of JAK enzymatic activity has proved successful for several immune-mediated pathologies and these drugs are now approved and prescribed to thousands of patients around the world (2). Given the clinical relevance of the drugs that target these enzymes,

more complete knowledge is clearly needed. As such, the pharmacological manipulation of JAKs represents an interesting strategy to study the homeostatic requirements of cytokine signals in different immune cell types ranging from T and B cells to innate lymphoid cells (ILCs).

ILCs provide rapid immune protection through an array of effector functions mirroring those associated with T cells (3). Based on this similarity, ILCs have been divided into five prototypical subsets: natural killer (NK) cells, ILC1, ILC2, ILC3, and lymphoid tissue inducer cells (3). NK cells and ILC1 are able to quickly release the signature cytokine interferon (IFN)-γ and, for this reason, were initially included within the group of type-1 ILCs (4, 5). The ontogeny of ILC1 and NK cells is thought to have both overlapping and independent routes (6, 7); among the latter, evidence in mice shows a distinct usage of T-box transcription factors with a selective expression and function for Eomes in NK cells in contrast to a specific requirement for T-bet in ILC1 (8).

In the context of ILC biology, JAK inhibitors (JAKinibs) have been employed to track different functional outputs, including cytokine production and cell proliferation upon cytokine stimulation *in vitro* (9). Notably, when used *in vivo*, JAKinibs have led to a reduction of the number of mouse NK cells (10). Likewise, patients treated with JAKinibs display a dose-dependent loss of peripheral blood NK cells (11–13). However, no information is currently available about the effects of *in vivo* treatment of JAKinibs on the phenotype of NK cells or other ILCs in distinct tissues.

Development and homeostasis of both NK cells and ILC1 depend on the functions of cytokines, primarily IL-15 and IL-7, which signal through the JAK/STAT pathway (14–16). Observations in humans, corroborated by studies using animal models, have shed light on the importance of the downstream signaling events induced upon activation of JAK3, JAK1, and STAT5 in the development and effector functions of ILCs (17). In this regard, patients carrying *JAK3* mutations develop severe combined immunodeficiency associated with loss of T and NK cells as well as the entire ILC system (18, 19). In mice, *Jak3* deficiency blocks NK/ILC differentiation in the bone marrow (BM) at the ILC precursor and the pre-NK cell progenitor stage; thus, no ILCs are preserved in these mice (20). Similarly, ablation of both *Stat5a* and *Stat5b* leads to almost total loss of NK cells (21). This phenotype is also observed when the entire *Stat5* locus or *Jak1* are deleted in *Ncr1*-expressing cells (22, 23). Selective preservation of *Stat5* alleles (*Stat5b* or *Stat5a*) has revealed a critical role of *Stat5b* more so than *Stat5a* in regulating ILC functions (24, 25), as well as a differential susceptibility among ILCs to tolerate deprivation of STAT5 signals, with NK cells and ILC1 being the most sensitive (25). The profound effects on lymphoid development leading to loss of ILC populations reveal a major limitation in using *Jak3*, *Jak1*, and *Stat5* deficient mice. Because many of the downstream effects of the JAK/STAT pathway affect the functions of the immune system, distinct compounds capable of blocking JAK enzymatic activity have been developed as selective immunosuppressant to be used in immune-mediated diseases (26).

Herein, we studied the impact of JAKinibs on the homeostasis of two prototypical ILC subsets: NK cells and ILC1. We assessed the effects of *in vivo* administration of a JAK1/3 inhibitor, tofacitinib, vs. a more selective JAK3 inhibitor, PF-06651600, focusing on NK cells from spleen, liver and BM and ILC1 from liver. Our data revealed differential effects of these JAKinibs on the NK cell and ILC1 numbers, the latter subset being less sensitive to JAK inhibition. By using a transcriptomic approach, we identified a major cell cycle block in both subsets after *in vivo* treatment with tofacitinib, associated with a decreased expression of antiapoptotic genes, including *Bcl2*. By using a pharmacological approach, we demonstrated that the high expression levels of *Bcl2* in ILC1 were associated with the differential impact of JAK inhibition observed between the two subsets, arguing for divergent dependence of the homeostasis of these populations on cytokine signals.

MATERIALS AND METHODS

Mice and Inhibitors

BALB/c and *Rag2*$^{-/-}$ mice were purchased from Jackson Laboratory. All animal studies were performed according to NIH guidelines for the use and care of live animals and were approved by the NIAMS Institutional Animal Care and Use Committee. JAKinibs were resuspended in 0.5% methyl cellulose and animals were dosed orally twice daily with vehicle or 30 mg/kg of tofacitinib (kindly provided by Pfizer) or 20 mg/kg of PF-06651600 (provided by the National Center for Advancing Translational Sciences (NCATS), NIH) for 1 week (or 3 days, where indicated) (27). ABT-199 (Venetoclax, Selleckchem) was resuspended in 60% Phosal 50PG, 30% PEG 400, and 10% EtOH. Animals were dosed orally once a day with vehicle or 90 mg/kg for a week.

Cell Isolation, Flow Cytometry, and Cell Activation Assays

Cells from spleen, liver and BM were isolated as previously described (28). Antibodies are listed in **Supplemental Figure 1**. Samples were acquired using LSR Fortessa cytometer (BD Biosciences) and BD FACSDiva software (v.8.0.1, BD Biosciences) and analyzed with FlowJo software (Tree Star). Cell sorting was performed using FACSAria III (BD Biosciences). For the evaluation of IFN-γ expression, cells were left untreated or stimulated with PMA/Ionomycin (Sigma-Aldrich) for 2 h or IL-2 (1,000 U/ml, Hoffmann-La Roche Inc.) and IL-12 (10 ng/ml), or IL-12 (10 ng/ml) and IL-18 (100 ng/ml) (R&D Systems) for 6 h (with the addition of GolgiPlug, from BD Biosciences).

RNA Sequencing and Transcriptomic Analysis

Cells isolated from spleen and liver were sorted (95–99% post-sort purity) as described in **Supplemental Figures 1A,B**. RNA-seq was performed according to manufacturer's protocol (NEBNext Ultra II RNA Library Prep, E7770L). Barcoded sequencing libraries were sequenced on Illumina HiSeq3000. 50-bp single end reads were demultiplexed to FastQ using bcl2fastq 2.17.1 and mapped onto mouse genome build mm10 using TopHat 2.1.1. Gene expression values (RPKM, reads per kilobase exon per million mapped reads) were calculated with Partek

Genomic Suites 7.18.0723. RPKM values were log2 transformed (with a 0.1 offset) and ANOVA was performed to find differentially expressed genes. Expressed genes having an average absolute RPKM > 2 were listed in **Supplemental Tables 1, 2** for NK and ILC1, respectively (*miRs* and *Snors* were excluded) and used for further analyses. Volcano plots were generated using R 3.6.0; heatmaps were generated using Morpheus software (Broad Institute). DAVID bioinformatics resource was used for GO analysis.

Statistics

Unpaired *t*-test and ANOVA were used to quantify statistical deviation between experimental groups, as indicated in figure legends. Asterisks denote significant differences $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Distinct Impact of JAK Inhibition on ILC1 and NK Cell Homeostatic Numbers

Immunologic and transcriptomic analysis performed on a wide range of adaptive and innate immune cells in mice have revealed a major impact of JAKinibs on the homeostatic pool of splenic NK cells (10). Building on these findings, we sought to dissect how prototypical liver ILC1 were affected by JAKinibs in relations to NK cells present in the liver, spleen and BM.

We used, as a model, mice treated with oral administration of a JAK1/3 or JAK3/TEC family (29) kinase-selective inhibitors, tofacitinib and PF-06651600, respectively, for a week, twice daily at doses comparable to the range approved for clinical use and which do not provide a total block of JAK3/1 activity (10). We analyzed lymphocytes isolated from liver, spleen and BM by flow cytometry and assessed the relative number of NKp46^{+} cells (gating strategies in **Supplemental Figure 1A**). Treatment with both JAKinibs led to a marked and significant reduction of the number (represented as ratio relative to control) of NKp46^{+} cells in all tissues analyzed (**Figure 1A**). Whereas, splenic and BM NKp46^{+} cells mainly comprise NK cells, the liver contains similar proportions of tissue resident ILC1 and NK cells. When we dissected liver NKp46^{+} cells by CD49b (DX5) and Eomes expression, we observed profound and significant changes of NK/ILC1 ratios (**Figure 1B**). This phenotype was associated with a differential effect in maintaining the homeostatic pools of ILC1 and NK cells. Indeed, while both NK cell and ILC1 numbers were reduced, NK cells were affected to a greater degree than ILC1 (**Figure 1C** and **Supplemental Figure 1B**). The differential impact of JAK inhibition on NK cells and ILC1 was independent by the presence of T and B lymphocytes, since similar results were obtained in *Rag2*$^{-/-}$ mice (**Supplemental Figure 1C**). Moreover, while evidence proving a similar efficacy of inhibiting JAK3 alone or both JAK1 and JAK3 has remained controversial (10, 30), our results showed that selectively targeting JAK3 (and TEC kinases), with PF-06651600, was as efficient as targeting multiple JAKs using tofacitinib in terms of their impact on the homeostatic pools of NKp46^{+} cells.

Together with the effects in liver and spleen, our data provided evidence for the impact of JAKinibs on the pool of BM NK cells

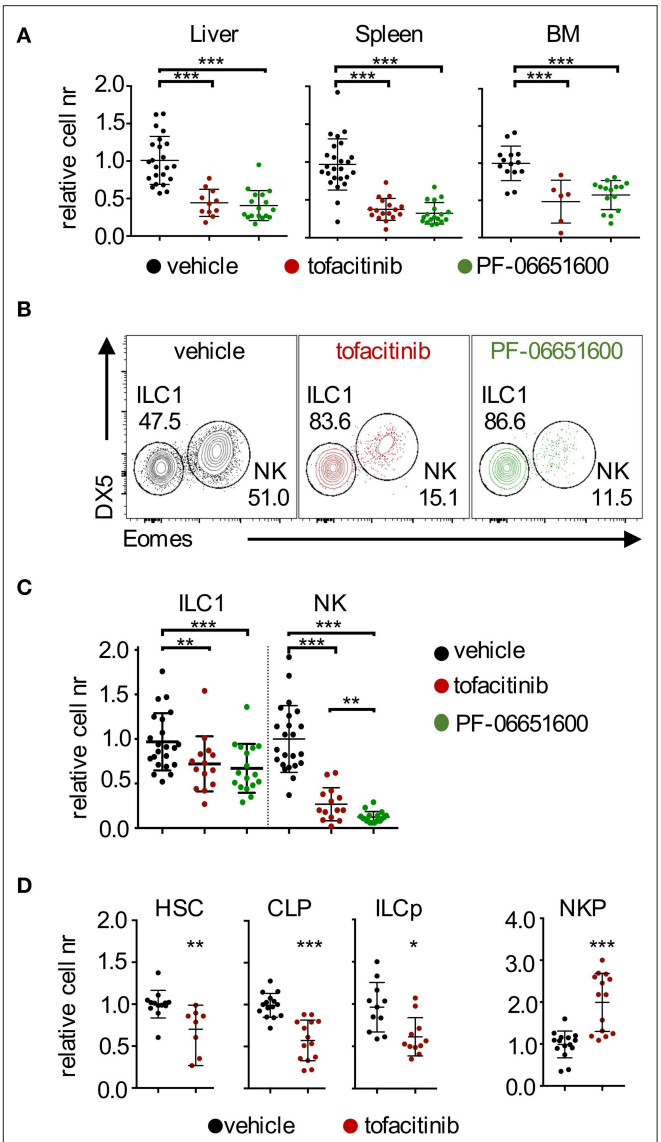

FIGURE 1 | Effects of tofacitinib and PF-06651600 on ILC1, NK cells, and BM progenitors. BALB/c mice were dosed orally with tofacitinib, PF-06651600 or vehicle twice a day for 7 days. **(A)** Relative cell numbers for CD3ε^{-} NKp46^{+} cells from liver (left panel), spleen (middle panel) and BM (right panel) are shown. ANOVA one-way test was applied. **(B)** Subsets of liver NKp46^{+} cells were distinguished by the expression of Eomes and DX5/CD49b. ILC1 were defined as DX5^{-}Eomes^{-} cells and NK cells as DX5^{+}Eomes^{+} cells. Percentages depicted in dot plots are representative. **(C)** Relative cell numbers for liver ILC1 and NK cells are shown. ANOVA one-way test was applied. **(A–C)** Five independent experiments were combined, and values were normalized to the mean of vehicle-treated mice in the corresponding experiment. **(D)** Relative cell numbers of HSC, CLP, ILCp, and NKP progenitors are shown. Student's *t*-test statistics are comparing samples to vehicle. Three independent experiments were combined, and values were normalized to the mean of vehicle-treated mice for each corresponding experiment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(**Figure 1A**), which led us to evaluate whether JAK inhibition affected NK cell/ILC progenitors present in this tissue. As shown in **Figure 1D** (gating strategies in **Supplemental Figure 1D**), treatment with tofacitinib resulted in decreased numbers of HSC,

CLP, and ILCp, as well as an accumulation of NKP, pointing to a developmental block at this stage. These findings were in agreement with a previous report showing that selective ablation of the *Stat5* locus in NK cells led to increased numbers of NKP (22).

Altogether, our data showed that administration of JAKinibs at doses within the range approved for clinical use, affected the homeostatic pool of both ILC1 and NK cells, although the impact of JAK inhibition was greater on NK cells. This effect was independent from the presence of adaptive immune cells. In addition, we showed that the effects of tofacitinib extended to BM precursors and ILC/NK progenitors.

Tofacitinib Administration Inhibits the Expression of Genes Regulating Cell Cycle and Survival in NK Cells

We have previously shown that NK cells with reduction in *Stat5b* or *Stat5a* levels exhibit a loss of their signature traits associated with a maturation block (25). Therefore, we investigated whether treatment with JAKinibs could also affect NK cell identity and/or differentiation by coupling transcriptomic analysis and flow cytometry.

At the transcriptional level, we observed that the impact of the 7-days treatment with tofacitinib mainly consisted of a

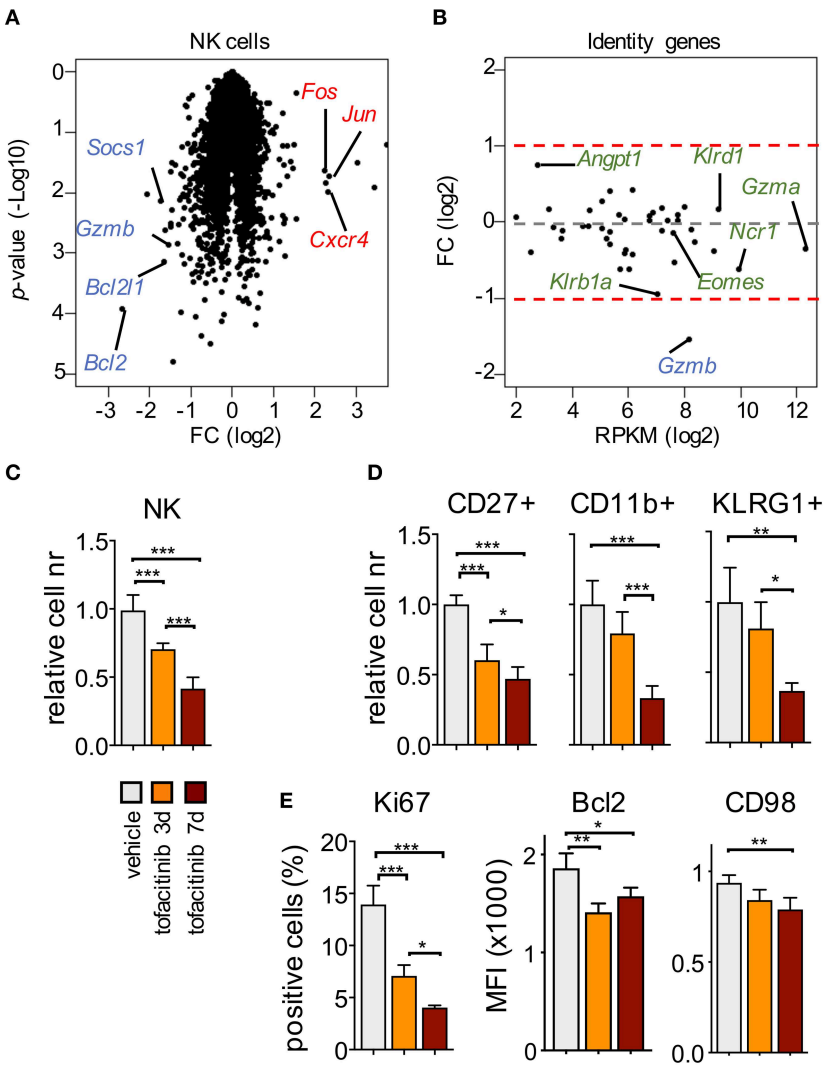

FIGURE 2 | Impact of tofacitinib on the transcriptome and phenotype of splenic NK cells. **(A)** Volcano plot for genes expressed by splenic NK cells isolated from mice treated or not for 1 week with tofacitinib. Representative down-regulated (blue) and up-regulated (red) genes are highlighted. Two mice for treated and three for vehicle were pooled together for each replicate. **(B)** MA plot shows transcript abundance (x axis, mean RPKM in NK cells receiving vehicle) and FC (y axis, log2 of tofacitinib ÷ vehicle) for genes associated with NK cell identity. **(C)** Relative cell number for splenic NKp46$^+$ and **(D)** NK cell subsets, defined by CD27, CD11b, or KLRG1 expression, at 3 and 7 days of tofacitinib treatment are shown. **(E)** Expression levels evaluated by flow cytometry for Ki67 (percentage of positive cells), Bcl2 [Mean Fluorescence Intensity (MFI)], and CD98 (MFI) in NK cells at 3 and 7 days of tofacitinib treatment are shown. **(C–E)** Statistics were performed using one-way ANOVA. Two independent experiments were combined (vehicle $n = 8$; 3-days treatment $n = 5$; 7-days treatment $n = 6$), and values were normalized to the mean of vehicle-treated mice for each corresponding experiment. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

reduction in gene expression in splenic NK cells (**Figure 2A** and **Supplemental Table 1**), which included down-regulation of genes involved in NK cell survival (*Bcl2* and *Bcl2l1*), proliferation (*Mki67*), and function (*Gzmb*), in agreement with a previous report (10). On the other hand, genes defining NK cell identity [gene list described by the Immgen project (31)] were not affected by the treatment, except for *Gzmb*, which was the only down-regulated gene with a fold-change (FC) higher than 2 (**Figure 2B**). Similarly, no significant differences in *Eomes* and *Tbx21* (encoding T-bet) were observed after *in vivo* treatment with tofacitinib.

To evaluate the effect of tofacitinib treatment on terminal differentiation, we measured the number of NK cells expressing markers associated with distinct maturation stages, namely CD27, CD11b, and KLRG1 (32, 33). To rule out the effects of possible mechanisms of cell adaptation or selection which could occur after the 7-days treatment, mice also received the drug for only 3 days. As shown in **Figure 2C**, NK cell numbers already started to decrease at the early time point, and to a greater extent at day 7. This reduction was associated with a global alteration of all the NK cell subsets analyzed (**Figure 2D**), and led, after 7 days of treatment, to a selective decrease of the frequency of terminally differentiated NK cells expressing KLRG1 (**Supplemental Figure 2**), suggesting that JAK inhibition could have cumulative effects during time either on differentiation or turn-over of this subset.

Finally, we evaluated by flow cytometry the expression levels of selected downregulated genes present in our dataset (**Supplemental Table 1**), including Ki67 (encoded by *Mki67*) to track cell cycle, Bcl2 for survival and CD98 (as a surrogate for Slc7a5 expression). As shown in **Figure 2E**, expression of these proteins was significantly affected both at day 3 and 7 after tofacitinib treatment. Among all the parameters analyzed, only Bcl2 was downregulated at higher degree at day 3 than day 7, suggesting that the effect on survival could occur earlier than the effects on proliferation and differentiation.

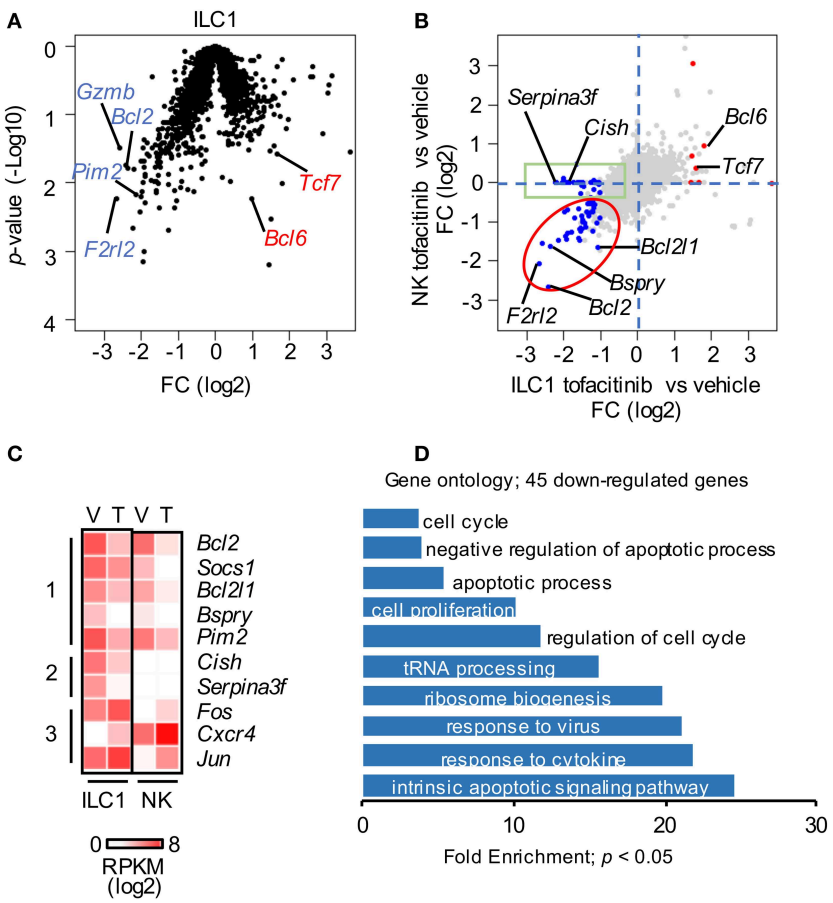

FIGURE 3 | Redundant effect of tofacitinib on ILC1 and NK cell transcriptomes. **(A)** Volcano plot for genes expressed by liver ILC1 isolated from mice treated or not for 1 week with tofacitinib. Representative down-regulated (blue) and up-regulated (red) genes are highlighted. **(B)** Scatter plot comparing the effects of tofacitinib on NK and ILC1. Genes significantly ($p < 0.05$) up-regulated (FC > 2) and down-regulated (FC < 0.5) in ILC1 are highlighted in red and blue, respectively. **(C)** Expression of selected genes in liver and splenic NK cells is depicted by heatmap, comparing mice administered with vehicle or tofacitinib. **(D)** Forty-five down-regulated genes in ILC1 having RPKM > 5; FC < 0.5; *p*-value < 0.05 were selected for GO. Only GO terms with a *p*-value < 0.05 are represented. More than 10 mice for each group (vehicle and tofacitinib) were pooled.

Our results showed that, in contrast to the previously employed genetic models, acute pharmacological inhibition did not alter NK cell identity suggesting differential requirements for acquisition of signature genes vs. homeostasis. Moreover, except for Bcl2, the impact of JAK inhibition on NK cells appeared to be cumulative during the time frame analyzed and encompassed effects on cell cycle as well as survival.

Redundant Effects of Tofacitinib on the Transcriptional States and Functions of ILC1 and NK Cells

To discriminate possible mechanisms underlying the differential sensitivity to JAKinibs on the homeostatic pool of liver ILC1 and NK cells, we explored the impact of tofacitinib on the transcriptome of ILC1 (**Supplemental Table 2**). Similar to what observed in NK cells, transcriptomic changes in ILC1 mainly consisted of a reduction in gene expression (**Figure 3A**, and **Supplemental Table 2**). Among the few significantly up-regulated genes, only *Tcf7*, a transcription factor expressed by ILC progenitors and required for the development of the whole ILC compartment (34), reached high expression levels of 54 RPKM and a FC higher than 2. Similarly to what we observed in NK cells, genes involved in survival (*Bcl2*), function (*Gzmb*), and proliferation (*Mki67*) were mainly down-regulated in ILC1.

To define distinct and shared genes affected by JAK inhibition on ILC1 and NK cells, we compared the two datasets highlighting the genes up- and down-regulated in ILC1 (FC $>$ 2; p-value $<$ 0.05). As shown in **Figure 3B**, most of the genes down-regulated in ILC1 followed a similar trend in NK cells (red ellipse), including *Bcl2*, *Bcl2l1*, or *Bspry* (**Figure 3C**, group 1). Moreover, we observed a discrete fraction of genes that was selectively down-regulated in ILC1 (green box). This group was enriched with genes specifically expressed on ILC1. Among these genes, *Cish*, which encodes for cytokine-inducible SH2 containing protein (CIS), was constitutively expressed on ILC1 and decreased upon *in vivo* treatment with tofacitinib (**Figure 3C**, group 2). Among the up-regulated genes, *Bcl6* was also induced in NK cells; and vice-versa, genes up-regulated in NK cells, such as, *Fos* and *Jun* followed a similar trend in ILC1 (**Figure 3C**, group 3).

Gene ontology (GO) analysis showed that transcripts regulating the response to cytokines/virus, cell cycle and the apoptotic pathway were enriched among the tofacitinib targets in ILC1 (**Figure 3D**). Thus, we sought to measure whether administration of tofacitinib for 7 days differentially affected the ability to produce IFN-γ, as well as, the expression of Ki67 and Bcl2 in liver ILC1 and NK cells. To evaluate the production of IFN-γ, liver cells were isolated both from untreated and tofacitinib-treated mice and stimulated with PMA/Ionomycin, IL-2/IL-12, or IL-12/IL-18. As shown in **Figure 4A**, a 7-days treatment with tofacitinib reduced the ability of NK cells to produce IFN-γ upon PMA/Ionomycin stimulation, while the potential of NK cells and ILC1 to respond to cytokines was not altered. These data suggest that the pharmacological block has limited effects on the cell intrinsic abilities to produce IFN-γ (**Figure 4A**).

Next, we tracked the levels of Ki67 and Bcl2 expression on liver ILC1 and NK cells after 7-days treatment with tofacitinib. Contrary to the impact observed on the homeostatic pools of both subsets, tofacitinib inhibited Ki67 expression at greater degree in ILC1 than in NK cells (**Figure 4B**). As shown above for splenic NK cells, the effects of tofacitinib on liver ILC1 were already detectable at day 3 and greater at day 7 after treatment (**Supplemental Figure 3A**). These data provide evidence for the role of JAKinbs in regulating the overall proliferative states of both ILC1 and NK cells, *in vivo*. Relative to Bcl2 expression, we observed that tofacitinib inhibited this protein in both subsets (**Figure 4C**). However, the expression levels of Bcl2 in liver ILC1 isolated from both untreated and tofacitinib-treated mice exceeded those observed in untreated NK cells, suggesting that the relatively high levels of Bcl2 on ILC1 might be responsible for the limited effect of JAKinibs on the homeostatic pool of these cells.

Altogether, we showed that the effects of JAK inhibition on ILC1 and NK cells appeared redundant at the transcriptional and functional level, with genes involved in survival and proliferation being mainly affected.

Basal Expression Levels of Bcl2 Are Linked to the Outcome of JAKinibs on the Homeostatic Numbers of ILC1 and NK Cells

The impact of Bcl2 family members in regulating NK cell survival has been previously addressed using genetic models, which have demonstrated that *Bcl2* and *Mcl1* have non-redundant roles in regulating NK cell survival (35–37), while *Bcl2l1* is dispensable (35). In NK cells, Bcl2 is down-regulated when STAT5 signaling is altered (25, 38), and its overexpression can rescue the effects on the NK cell pool associated with *Stat5* deficiency (39). In our dataset, the antiapoptotic genes *Bcl2* and *Bcl2l1* were both downregulated in ILC1 and NK cells, while *Mcl1* expression was not altered by tofacitinib treatment, implying differential mechanisms of homeostatic regulation for these three members of the Bcl2 family.

Given that Bcl2 protein was expressed at higher levels by liver ILC1, from both untreated and tofacitinib-treated mice, compared to untreated NK cells, we hypothesized that the differential effect of tofacitinib on the size of the two subsets was dependent on their distinct ability to survive after perturbation of Bcl2 function. To test this hypothesis, we treated mice with oral administration of a Bcl2 specific blocker, namely ABT-199 (Venetoclax) for 7 days (40, 41). We first analyzed whether the pharmacological block of Bcl2 had parallel effects compared with those observed in genetic models by evaluation the impact of ABT-199 on splenic NK cells. As shown in **Figure 5A**, ABT-199 administration induced a global decrease of the number of splenic NK cells. In addition, treatment mainly affected the number of more differentiated subsets defined according CD27 and CD11b expression (**Figure 5B**). These observations were in line with the

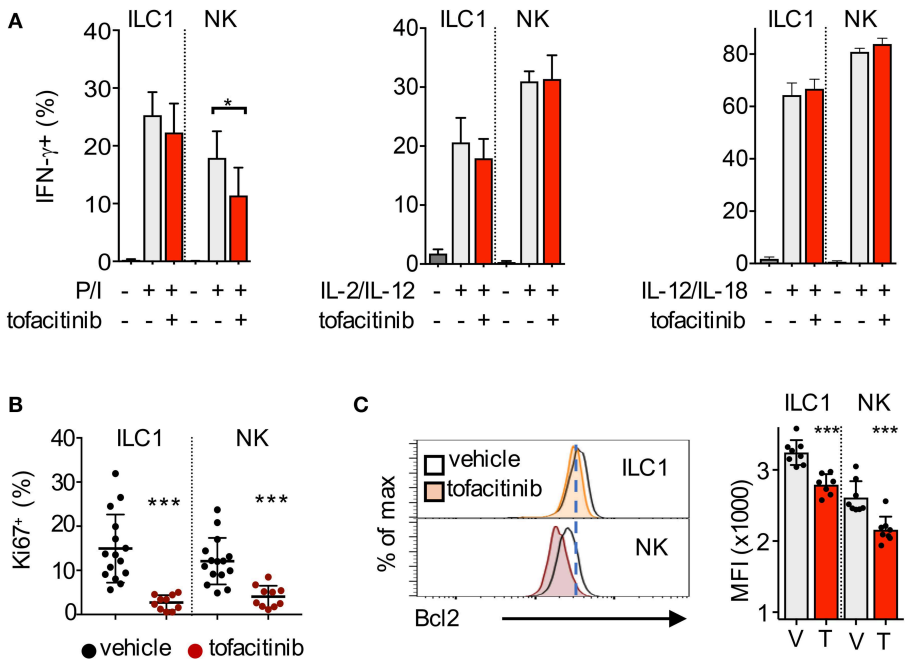

FIGURE 4 | Impact of tofacitinib treatment on the functions of NK cells and ILC1. **(A)** Bar graphs show the percentage of IFN-γ-positive ILC1 and NK cells, quantified by flow cytometry, after 2 h stimulation with PMA/Ionomycin (P/I), or 6 h stimulation with IL-2/IL-12 or IL-12/IL-18, in mice treated or not for 1 week with tofacitinib. Two experiments were combined. **(B)** Percentage of Ki67-positive ILC1 and NK cells isolated from liver after 7 days administration of tofacitinib or vehicle. **(C)** Representative histogram plots (left panel) and bar graphs (MFI, right panel) show Bcl2 protein expression in ILC1 and NK cells from mice treated with tofacitinib or vehicle. $^{*}P < 0.05$; $^{***}P < 0.001$.

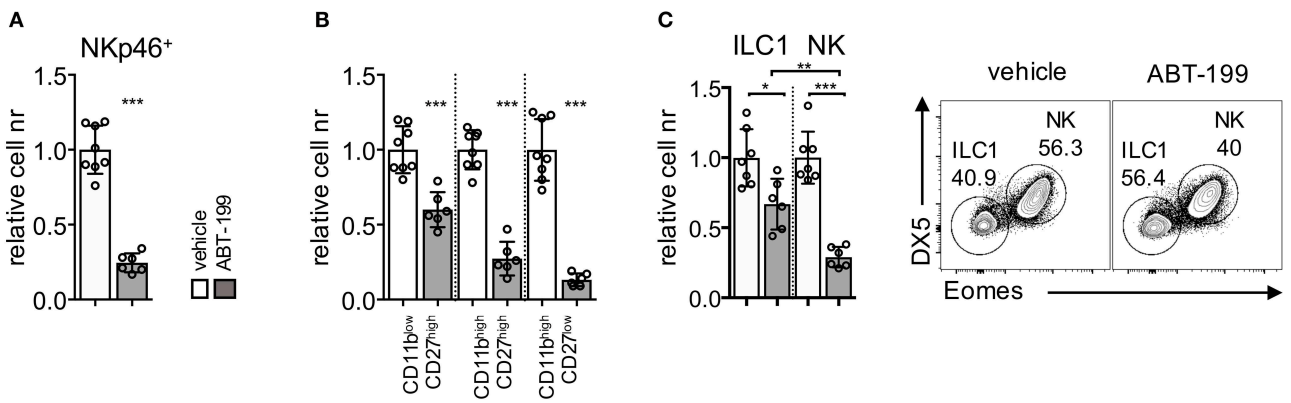

FIGURE 5 | ABT-199 treatment differentially affects the homeostatic pool of liver ILC1 and NK cells. Mice were dosed orally with ABT-199 or vehicle daily for 7 days. **(A)** Relative cell numbers of splenic NK cells and **(B)** NK cell subsets (dissected based on CD27 and CD11b expression) are shown. **(C)** Liver ILC1 and NK cells in mice untreated or treated for 7 days with ABT-199 (Venetoclax) or vehicle are depicted. Two experiments were combined (vehicle $n = 8$; ABT-199 $n = 6$), and values were normalized to the mean of vehicle-treated mice for each corresponding experiment. One-way ANOVA was applied. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

low Bcl2 expression levels observed in mature CD11b^{+} NK cell subsets (**Supplemental Figure 3B**) and with previous evidence in mice showing major defects in mature NK cells associated with the ablation of *Bcl2* gene.

Having established the impact of ABT-199 administration on splenic NK cells, we next analyzed its effect on the liver subsets. As shown in **Figure 5C**, treatment with ABT-199 led to a significant decrease of both liver ILC1 and NK cells. However, the number of the NK cell pool was affected at a higher degree in comparison to the number of ILC1, indicating that the higher levels of Bcl2 present on ILC1 provided an advantage in term of survival as compared to NK cells.

Altogether we showed that pharmacological inhibition of Bcl2 in mice recapitulated the effects observed using genetic models targeting Bcl2, consisting in a major loss of more differentiated NK cells. Moreover, we provided evidence for a differential effect of Bcl2 inhibition in ILC1 and NK cells, the latter being more sensitive to ABT-199 treatment.

DISCUSSION

To better understand the impact of JAK inhibition in regulation of NK cells and ILC homeostasis, we have administered JAKinibs to mice at doses comparable to the range approved for clinical use. The first unexpected finding we observed was the differential impact of JAKinibs on the homeostasis of NK cells and ILC1. This was unanticipated because both subsets were highly affected when *Jak3* and *Stat5* were targeted by genetic approaches in mice (20, 25). Mechanistically, we speculated that the limited effect of JAKinibs on the pool of ILC1 was linked to their higher expression levels of Bcl2 compared to NK cells. This hypothesis is supported by the higher sensitivity of NK cells compared to ILC1, in respect to the pharmacological block of Bcl2.

Along with survival, the cell cycle of both NK cells and ILC1 was highly affected by tofacitinib treatment; the drastic reduction of cells in the G1-S-M phase associated with the low frequency of $Ki67^+$ cells outline the pivotal role of JAK signals in regulating the proliferation of these prototypical subsets *in vivo*, and can also contribute to the decrease of their homeostatic number. Interestingly, this aspect, although inferred by results obtained using *in vitro* systems, had remained unexplained by employing genetic approaches, *in vivo*.

The effects observed on the pool of bone marrow NK cells and their precursors represent another possible factor contributing to the to the decreased number of NK cells in other organs. In this regard, tofacitinib treatment induces an increase of the chemokine receptor CXCR4, which could alter mechanisms of bone marrow retention of NK cells (42, 43). Despite the differences on the homeostatic numbers, the effect of JAK inhibition at the transcriptional level was similar for both NK cells and ILC1, with a main reduction of the expression of JAK-targets. No major changes in genes defining the NK cell identity were observed, indicating a differential role for JAK-dependent signals in regulating acquisition of identity and homeostasis. Among the few upregulated genes, we found the transcription factors *Bcl6*, *Tcf1*, *Fos*, and *Jun*. These TFs could be usually repressed by JAK-signals or, alternatively, up-regulated after tofacitinib treatment allowing cells to adapt to deprivation of JAK-dependent signals. The expression of Bcl6 has been related to mechanisms of ILC plasticity, involving suppression of ILC3 genes and promotion of NK/ILC1 specific programs (44). Although transitions of NK cells toward an ILC1-like phenotype occur both under physiological and pathological conditions, we did not observe alterations of ILC1 markers, such as Trail and CD49a, in NK cells after treatment (data not shown). Thus, the higher expression of Bcl6 in both NK cells and ILC1 after tofacitinib treatment might be part of a circuit reinforcing NK/ILC1 phenotypes in absence of proper levels of JAK-dependent signals.

While most of the genes followed a common transcriptional trend in NK cells and ILC1 upon tofacitinib treatment, *Cish* represented one of the few exceptions, being differentially targeted by tofacitinib in the two type 1 subsets. As recently reported, the homeostatic expression of *Cish* is very low in NK cells but increases rapidly following IL-15 stimulation (45). Since *Cish* levels were downregulated after *in vivo* treatment with tofacitinib, the constitutive expression of *Cish* in ILC1, instead, could be dependent on the high levels of JAK-dependent signals acting on these tissue-resident cells.

Moreover, we noticed that most of the effects observed in NK cells at day 7 after treatment were also present at an earlier time point. However, the impact of JAK inhibition on NK cells appeared to be cumulative, within the time frame analyzed, in terms of cell numbers, maturation stages and proliferation, with Bcl2 representing the only exception. Thus, our data suggest that the limited effects of tofacitinib on NK cell transcriptome could be independent from mechanisms of adaptation occurring during the treatment, which may select or generate cells resistant to JAK inhibition.

Finally, our study showed that this approach represents both an opportunity to better understand ILC biology as well as a strategy to modulate ILC functions during diseases. Since pharmacological inhibition of JAKs is now successfully utilized for the treatment of several immune-mediated pathologies, our study sheds light on the potential effects on immune cells when this pathway is targeted.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO: GSE135116.

ETHICS STATEMENT

The animal study was reviewed and approved by National Institute of Arthritis and Musculoskeletal and Skin Diseases, Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

LV performed the *in vitro*, *in vivo*, and RNA-seq experiments. ML, NG, CL, JK, GP, SD, and YF helped to perform the experiments. SB processed the transcriptomic data and performed the computational analyses. GS performed the transcriptomic analysis and data visualization. JO'S contributed to the project design, data interpretation, and manuscript writing. CT provided the critical reagents and helped to write the paper. LV, GS, and MG designed the project and wrote the manuscript with input from all authors.

FUNDING

This work was supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (ZIG AR041168-12; ZIC AR041169-12; ZIH AR041173-12, ZIC AR041181-11, and 1 ZIC AR041207-04). CT gratefully acknowledges support from the NCATS and NCI intramural programs. GS was supported by the Italian Association for Cancer Research (AIRC), MFAG 2018 (Project Code: 21311); and Institut Pasteur (France), Transversal Research Program; PTR-113-17.

ACKNOWLEDGMENTS

We would like to thank Dr. Yohei Mikami for critical reading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02972/full#supplementary-material

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Conflict of Interest: The National Institutes of Health hold patents on targeting JAKs. National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) (JO'S and MG) and Pfizer have a Collaborative Research and Development Award (CRADA).

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.

frontiers in Immunology

REVIEW
published: 25 February 2020
doi: 10.3389/fimmu.2020.00309

Transcriptional Regulation of Mouse Tissue-Resident Natural Killer Cell Development

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Natural killer (NK) cells are cytotoxic innate lymphocytes that are well-known for their ability to kill infected or malignant cells. Beyond their roles in tumor surveillance and anti-pathogen defense, more recent studies have highlighted key roles for NK cells in a broad range of biological processes, including metabolic homeostasis, immunomodulation of T cells, contact hypersensitivity, and pregnancy. Consistent with the breadth and diversity of these functions, it is now appreciated that NK cells are a heterogeneous population, comprised of specialized and sometimes tissue-specific subsets with distinct phenotypes and effector functions. Indeed, in addition to the conventional NK cells (cNKs) that are abundant and have been well-studied in the blood and spleen, distinct subsets of tissue-resident NK cells (trNKs) and "helper" Group 1 innate lymphoid cells (ILC1s) have now been described in multiple organs and tissues, including the liver, uterus, thymus, adipose tissue, and skin, among others. The cNK, trNK, and/or helper ILC1 populations that co-exist in these various tissues exhibit both common and distinct developmental requirements, suggesting that a combination of lineage–, subset–, and tissue–specific differentiation processes may contribute to the unique functional properties of these various populations. Here, we provide an overview of the transcriptional regulatory pathways known to instruct the development and differentiation of cNK, trNK, and helper ILC1 populations in specific tissues in mice.

Keywords: natural killer cells, tissue-resident NK cells, transcriptional regulation, transcription factors, group 1 innate lymphoid cells

OPEN ACCESS

Edited by:
Yenan Bryceson, Karolinska Institutet (KI), Sweden

Reviewed by:
Barbara L. Kee, University of Chicago, United States
Georg Gasteiger, Julius-Maximilians-Universität, 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: *07 November 2019*
Accepted: *07 February 2020*
Published: *25 February 2020*

Citation:
Valero-Pacheco N and Beaulieu AM (2020) Transcriptional Regulation of Mouse Tissue-Resident Natural Killer Cell Development. Front. Immunol. 11:309. doi: 10.3389/fimmu.2020.00309

INTRODUCTION

Natural killer (NK) cells are cytotoxic innate lymphocytes that were first identified in 1975 based on their capacity to spontaneously kill tumor cell lines without prior immunization (1, 2). Over the past 45 years, our understanding of NK cell biology has grown and evolved, and it is now clear that NK cells play important roles in diverse biological processes, ranging from tumor surveillance and anti-pathogen defense to metabolic disorders, inflammatory diseases, stem cell transplantation, neuronal pruning, and pregnancy (3–9).

The balance of evidence suggests that mouse NK cells and helper ILC1s are distinct lineages in mice, arising from separate lineage-committed progenitors under homeostatic conditions (10). They do, however, share extensive phenotypic and functional similarities, including expression of many markers historically associated with NK cells such as NKp46, robust production of interferon gamma (IFN-γ) upon activation, and expression of the T-box transcription factor, T-box expressed

in T cells (T-bet) (10). In certain tissues and inflammatory settings, NK cells and helper ILC1s can possess such similar phenotypes that they have been difficult to distinguish, particularly in the absence of lineage-tracing experiments. These challenges, along with the historical lag in recognizing NK cells and helper ILC1s as distinct lineages and the fact that few truly lineage-discriminating markers have been described, have resulted in a confusing body of literature in which NK cells and helper ILC1s have not always been separately identified or consistently defined. For consistency within this review, we will discriminate mouse NK cells from mouse helper ILC1s on the basis of Eomesodermin (Eomes) expression, a common convention notwithstanding the field's limited understanding of conditions under which NK cells might lose, or helper ILC1s might gain, Eomes.

While most studies on NK cells have focused on population(s) abundant in the blood and spleen, now commonly referred to as conventional NK cells (cNKs), unique tissue-specific and/or tissue-resident NK cell (trNK) populations have recently been described in diverse tissues, including the uterus, thymus, intestine, adipose, skin, peritoneal cavity, and salivary, lacrimal, and mammary glands. As described below, many of these unique trNK populations exhibit distinct tissue-specific phenotypes, functions, and developmental requirements. In particular, recent studies have highlighted notable differences in the transcriptional regulation of trNK, cNK, and helper ILC1 development, suggesting that distinct differentiation processes support the unique functional properties of tissue-specific trNKs. Here, we review current literature on the transcriptional pathways known to control the development of various trNK populations in mice, with a particular focus on regulatory mechanisms that are unique to trNKs as compared to cNKs and helper ILC1s in each tissue.

OVERVIEW OF cNK DEVELOPMENT IN THE BONE MARROW

The bone marrow is the primary, but not exclusive, site of cNK development in adults (11–13). Like T cells and B cells, cNKs develop from precursor populations with pan-lymphocyte potential—e.g., common lymphoid progenitors (CLPs) and lymphoid-primed multipotent progenitors (LMPPs)—via a stepwise differentiation process in which multi-lineage potential progressively diminishes as the NK cell fate becomes established (14, 15). Early innate lymphoid progenitors (EILPs) and alpha-lymphoid progenitors (αLPs) are among the earliest developmental intermediates capable of generating NK cell-committed NK progenitors (NKPs) and helper ILC-committed ILC precursors (ILCPs), but not T cell- or B cell-committed precursors (16–18). Mouse NKPs were originally reported to exist within a pool of $Flt3^{-}2B4^{+}CD27^{+}Id2^{hi}IL\text{-}7Ra^{+/-}$ cells in the bone marrow that lacked all mature immune cell lineage markers (Lin^{-}), including classical NK cell markers such as NKp46 (19–21). These included very early NKPs (e.g., pre-NKPs and pre-pro-NKPs) that lacked the IL-15 receptor β-chain, CD122, as well as more differentiated "refined" NKPs (rNKPs) that expressed CD122 and were thus responsive to IL-15, a cytokine known to critically regulate diverse aspects of cNK development and function (20, 21). NKPs were shown to give rise to immature NK cells (iNKs), which had acquired expression of the NK activing receptors NKp46 and, in some mouse strains, NK1.1 (19). [Of note, later lineage-tracing studies demonstrated that the markers originally used to identify NKPs, rNKPs, and iNKs in the bone marrow did not fully exclude all helper ILC lineage cells, especially helper ILC1s (22)]. Upregulation of CD49b, additional NK receptors (e.g., Ly49 receptors), and effector molecules such as perforin and granzymes mark the later stages of differentiation into mature NK cells (mNKs) (19–21). mNKs continue to mature in the bone marrow and peripheral tissues, a process marked by downregulation of CD27 and upregulation of CD11b, with $CD27^{+}CD11b^{-}$ cells being less mature (but more proliferative) and $CD27^{-}CD11b^{+}$ cells being most mature (23–25).

TRANSCRIPTIONAL REGULATION OF cNK DEVELOPMENT IN THE BONE MARROW

cNK development is controlled by the sequential and coordinated activities of multiple transcriptional regulators. Among these are the transcription factors T cell factor 1 (TCF-1) and Nuclear factor interleukin-3 regulated (Nfil3), both of which are expressed at or prior to the NKP-ILCP developmental branch point and are important for proper cNK and helper ILC lineage differentiation (17, 18, 26–34). Mice lacking TCF-1 have fewer pre-NKPs, rNKPs, and mNKs in the bone marrow. And, although peripheral cNK numbers are only modestly impacted in non-chimeric TCF-1-deficient mice, they are severely reduced in a competitive mixed bone marrow chimera environment (18, 34). Notably, TCF-1-deficient NK cells have an unusual hypermature but pro-apopotic phenotype linked to granzyme B overexpression, suggesting that TCF-1 controls cNK development by modulating the timing of maturation and effector gene expression (34).

Nfil3-deficient mice also have severe and early defects in NK cell development, reflected in a near-complete loss of cNKs in the periphery and significantly reduced numbers of NKPs, iNKs, and mNKs in the bone marrow (17, 26, 27, 31, 33). The requirement for Nfil3 appears to be restricted to the earliest stages of cNK development, as loss of Nfil3 at or after the iNK stage has little impact on cNK numbers or function (35). Nfil3 itself regulates expression of several other transcription factors important for NK differentiation and maturation, including Inhibitor of DNA binding 2 (Id2) and Eomes (discussed below) (26, 31, 32). Id2, which acts to inhibit E-box family proteins that support B and T cell differentiation, is indispensable for cNK development. Id2-deficiency leads to a severe reduction in the peripheral cNK compartment, owing to its critical roles in promoting cNK maturation, effector functionality, and sensitivity to IL-15 signaling (36–39).

Like Nfil3, the transcription factors, ETS proto-oncogene 1 (Ets1) and Signal transducer and activator of transcription 5 (Stat5), the histone H2A deubiquitinase, Myb-like, SWIRM and MPN domains 1 (Mysm1), and the long non-coding RNA (lncRNA), RNA-demarcated regulatory region of Id2 (*Rroid*) also critically regulate cNK development and are important for

maintaining proper Id2 expression in differentiating cNKs (40–43). Genetic deficiencies in Ets1, Mysm1, or *Rroid* all impair maturation of bone marrow cNKs, resulting in fewer, less mature, and less functional cNKs in the periphery (42, 43). Similarly, mature peripheral cNKs are severely reduced in mice lacking Stat5b, and to a lesser extent Stat5a (43–45), and Stat5 tetramerization was recently shown to support cNK maturation in the bone marrow and spleen (46).

Other important regulators of cNK development include the T-box family transcription factors, T-bet and Eomes. Deficiencies in either factor result in impaired cNK maturation in the bone marrow, leading to fewer and less mature cNKs in the periphery (47–52). T-bet in particular is important for modulating proliferation and supporting survival in maturing cNKs (47). Eomes and T-bet have both unique and overlapping functions in developing cNKs. For example, T-bet-deficiency only moderately impacts peripheral cNK numbers, and has little impact on bone marrow cNK abundance, whereas Eomes-deficiency substantially reduces both bone marrow and peripheral cNK numbers (50, 52). Moreover, compound deficiencies in both factors are far more deleterious than deficiencies in either factor alone, resulting in a near-complete loss of cNKs in the bone marrow and peripheral organs (48, 50).

Additional transcription factors known to regulate later stages of cNK cell differentiation and maturation include Kruppel-like factor 2 (KLF2), GATA binding protein 3 (Gata-3), Runt-related transcription factor 3 (Runx3), and Zinc-finger E homeobox-binding 2 (Zeb2). Similar to T-bet, KLF2 restricts abnormal proliferation and supports survival in maturing cNKs, and KLF2-deficiency reduces the number of mature cNKs in the periphery (53). Gata-3 helps sustain Id2, T-bet, and Nfil3 expression in maturing cNKs, and cNKs lacking Gata-3 exhibit defects in maturation and bone marrow egress (54). Similarly, Runx3 promotes later stages of cNK maturation, possibly through cooperative regulation with T-box and Ets family transcription factors, and cell-specific deletion of Runx3 or its co-regulator Cbf-β leads to a reduction in the peripheral cNK compartment (55, 56). And finally, Zeb2 has been shown to act downstream of T-bet to critically regulate the maturation, survival, and egress of mature cNKs from the bone marrow. Mice lacking Zeb2 have more immature cNKs in the bone marrow, and fewer mature cNKs in the periphery (57).

DEVELOPMENT OF TISSUE-SPECIFIC OR TISSUE-RESIDENT NK CELLS AND HELPER ILC1s

Liver

In addition to circulating $CD49a^{-}CD49b^{+}Eomes^{+}$ cNKs, the liver harbors a unique population of $CD49a^{+}CD49b^{-}Eomes^{-}$ ILC1s that are tissue-resident in parabiotic mice (58, 59). [Different groups refer to these tissue-resident cells as either liver trNKs or liver ILC1s; here, we will use the ILC1 designation since these cells are $Eomes^{-}$]. Liver ILC1s reside in the liver sinusoids and have been shown to mediate memory-like immune responses in models of contact hypersensitivity (CHS) and viral infection (59–62).

Phenotypically, liver ILC1s resemble immature cNKs in having low or no expression of killer cell lectin-like receptor G1 (KLRG1), CD11b, CD122, and Ly49 receptors such as Ly49A, Ly49D, Ly49G2, and Ly49H (50, 51, 63, 64). However, liver ILC1s are transcriptomically distinct from both immature and mature cNKs and exhibit an activated phenotype at steady state, characterized by high expression of CD69, CD44, and CD160, and low expression of CD62L (also known as L-selectin) (51, 59, 64, 65). They also express high levels of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and CD127, as well as chemokine receptors such as CXCR3 and CXCR6 that support residence in the liver sinusoids (50, 51, 59, 64, 66, 67). Although activated liver ILC1s retain cytotoxic functionality against target cells, they differ from cNKs in their higher production of TNF-α, IL-2, and granulocyte-macrophage colony-stimulating factor (GM-CSF), their preferential expression of granzyme C instead of granzyme B, and their reduced expression of perforin (51, 59, 64, 65). Liver ILC1s also express molecules involved in immune regulation, including PD-L1, LAG3, CD39, and CD73, and were recently shown to inhibit T cell function via the PD-1–PD-L1 axis (68).

The unique phenotype, function, and transcriptome of liver ILC1s, as well as the finding that they do not give rise to $Eomes^{+}$ NK cells following adoptive transfer into intact (un-irradiated) hosts (51), support their identity as a distinct lineage. Consistent with this, liver ILC1s and cNKs exhibit both common and distinct developmental requirements. Commonalities include their shared dependence on IL-15 but not IL-7 signaling for development, notwithstanding constitutive expression of CD127 by liver ILC1s (51). Additionally, both liver ILC1s and liver cNKs require TCF-1, Gata-3, Runx3 and its co-factor Cbf-β, and T-bet for development, although liver ILC1s are more severely impacted by T-bet-deficiency than liver cNKs (18, 50–52, 54, 56, 64).

Distinct developmental requirements include findings that liver ILC1s require Promyelocytic leukemia zinc finger (PLZF) and the Aryl hydrocarbon receptor (AhR) for development, two transcription factors that are dispensable for cNK development (22, 69). Loss of PLZF significantly impairs liver ILC1 development, similar to its impact on many other helper ILC sublineages (22). Likewise, mice lacking AhR have reduced numbers of liver ILC1s, but normal numbers of liver cNKs, owing to a role for AhR in limiting turnover and susceptibility to cytokine-induced death in liver ILC1s (69).

Nfil3 has been reported as dispensable for liver ILC1, but essential for liver cNK, development in some studies (32, 64, 70), although Nfil3-deficient liver ILC1s were recently reported to be competitively disadvantaged in mixed bone marrow chimeric mice (52). Liver ILC1s also do not require KLF2 or Eomes for development, unlike liver cNKs (50, 52, 53).

Development of liver ILC1s also uniquely depends on Homolog of Blimp-1 (Hobit), a transcription factor that supports T cell tissue-residency, in part by suppressing genes involved in tissue egress (71). Notably, although liver ILC1s are severely reduced in Hobit-deficient mice, NK and helper ILC1

populations in other organs remain largely unaffected, suggesting that Hobit is a liver-specific regulatory factor for ILC1s (52, 71).

Uterus

NK cells are one of the most abundant immune cell types in the uterus at steady state and during early- and mid-pregnancy. Uterine NK cells have been shown to regulate diverse processes in female reproductive biology, and are particularly critical for remodeling of the uterine vasculature during early pregnancy [Reviewed in Croy et al. (72)]. At least three distinct NK and/or ILC1 populations exist in the uterus, each with unique transcriptional signatures: CD49a$^-$CD49b$^+$Eomes$^+$ cNKs, tissue-resident CD49a$^+$CD49b$^-$Eomes$^+$ trNKs, and CD49a$^+$CD49b$^-$Eomes$^-$ helper ILC1s (73–76). The frequency and distribution of these populations vary with respect to sexual maturity and reproductive state (73, 75–77). Uterine helper ILC1s are most abundant in pre-pubertal mice, and exhibit preferential expansion during repeat pregnancies, whereas sexual maturity is associated with a decrease in the frequency of helper ILC1s and an increase in trNKs and cNKs (75). During pregnancy, trNKs further proliferate *in situ* and remain abundant throughout early decidualization (76). Placentation and mid-pregnancy are associated with a decrease in trNK cells and an increase in cNKs, with the latter comprising the majority through birth and weaning (75).

The origin and developmental requirements of uterine NK cells are only partially understood, although some appear to arise from *in situ* progenitors and others from recruited cells (77–79). Uterine trNKs develop normally in athymic mice and do not express CD127 at steady state, indicating that their development is distinct from thymic NK cells (discussed below) (64). Uterine NK cell development has been reported to be IL-15-dependent, although whether uterine trNKs, cNKs, and helper ILC1s all require IL-15 signaling to the same extent remains to be determined (80, 81).

With respect to transcriptional regulation of uterine NK and helper ILC1 development, Nfil3 has been implicated in some but not all studies, likely owing to differences in strategies used to identify cNKs, trNKs, and helper ILC1 populations in the uterus. Nfil3-deficiency is consistently associated with significantly reduced numbers of cNKs in the uteri of both virgin and pregnant mice (64, 74, 82). However, Nfil3-deficient mice were reported to have normal numbers of uterine CD49a$^+$CD49b$^-$ cells – a population that includes trNKs and helper ILC1s—in one study (64), but reduced trNK and normal helper ILC1 numbers in another study (74). The latter study showed that trNKs in Nfil3-deficient mice were able to expand in response to pregnancy, although defects in decidual vascularization and placentation persisted, possibly due to the persisting deficit in uterine cNKs and Group 2 ILCs (ILC2s) (74, 82).

In addition to Nfil3, T-bet and Runx3 have also been evaluated for their impact on uterine NK cell development. T-bet-deficiency does not alter the overall abundance of uterine CD49a$^+$CD49b$^-$ cells, although differential effects on trNK vs. helper ILC1s have not been assessed (64). In contrast, implantation site NK cells were strikingly absent in pregnant *Runx3*$^{-/-}$ mice, although the requirement for Runx3 in development of specific uterine NK or ILC1 subsets remains unknown (55, 83).

Thymus

The thymus harbors a unique population of Gata-3$^+$ NK cells with a CD127$^+$CD11b^{lo}CD69^{hi}CD49b$^+$CD49a$^-$ surface phenotype and low expression of Ly49 receptors (84, 85). Functionally, these thymic NK cells (tNKs) are less cytotoxic but produce more IFN-γ than splenic cNKs, and are similar to liver trNKs in their ability to produce TNF-α and GM-CSF (84). Unlike T cells, tNKs do not require Notch signaling for development and do not develop from T cell-committed progenitor cells (86, 87). They can, however, develop from NKPs in the fetal thymus (88), and from early double-negative (DN) 1 and DN2 thymocyte precursors (70, 89–91).

The molecular requirements for tNK development are unique. Unlike cNKs and many other trNK populations, tNK cells require both IL-7 and IL-15 signaling for development (84, 92). Moreover, genetic deficiencies that disrupt tetramerization of Stat5, which signals downstream of both IL-7 and IL-15, reduce overall tNK numbers (46). tNK cell development is also strictly dependent on Gata-3, mirroring the requirement for Gata-3 in T cell development past the DN2 stage (84, 93). tNK development has been reported as Nfil3-dependent in some (32, 85) but not all (70) studies, possibly due to differences in mouse strains and gating strategies across studies.

Ets1 and Id2 also play important roles in tNK development. Ets1-deficient mice (on a *Rag1*$^{-/-}$ background) harbor fewer tNKs overall, and those present have a CD11b^{hi}KLRG1^{hi}CD27^{lo} phenotype typically associated with mature cNKs (85). Conversely, Id2-deficient *Rag1*$^{-/-}$ mice have normal numbers of tNKs, but these have an abnormal CD27^{hi}CD11b$^-$ phenotype reminiscent of immature cNKs (85).

Both Mysm1 and T-bet are dispensable for tNK development, although tNK cells do express T-bet (42, 85). They also express Bcl11b, a zinc finger transcription factor that is essential for T cell development (94). Bcl11b–deficient thymocytes have been shown to acquire an NK cell-like phenotype, although whether these cells represent *bona fide* tNKs remains unclear (94–96).

Salivary Glands

The salivary glands (SG) contain several tissue-resident NK and helper ILC1 populations with unique phenotypes and functions (97–101). Among these, NK lineage cells represent ∼80–90% and helper ILC1s ∼10–20% of the total pool, based on lineage tracing studies involving PLZF–reporter/fate mapping mice and patterns of Eomes expression (101). Notably, both SG helper ILC1s and the majority of SG NKs exhibit long-term tissue-residency in parabiotic mice, and peripheral cNKs are not recruited to the SG even during viral infection, suggesting that trNKs constitute a sizeable fraction of the NK lineage compartment (97, 99).

SG NK cells have a distinct surface phenotype, with most co-expressing both CD49a and CD49b, although small populations of CD49a$^-$CD49b$^+$ cNK-like cells and CD49a$^+$CD49b$^-$ cells are also present (101). SG helper ILC1s are predominantly CD49a$^+$CD49b$^+$ or CD49a$^+$CD49b$^-$ (101). At steady state, SG NKs exhibit low or no expression of CD27, CD43, CD127, and

KLRG1, but express high levels of CD69 and CD44 (97, 98). ∼40% also express CD103 (also known as integrin alpha E), a marker often associated with tissue residency (98).

Functionally, SG NK cells are poor producers of IFN-γ and degranulate less than splenic cNKs (97, 98). However, some SG NK cells do express TRAIL. Notably, SG NKs were shown to cull activated SG $CD4^+$ T cells in a TRAIL-dependent manner during chronic viral infection (98, 101, 102). This activity was important for limiting autoimmune-like tissue destruction, suggesting that SG NK cells may be critical modulators of pathogenic T cell responses in the SG (102).

SG NK cells are critically dependent on a non-canonical Smad4-independent TGF-β signaling pathway for development or maintenance (100). Mechanistically, TGF-β is thought to act by modulating Eomes expression—$CD49a^+$ SG NKs are $Eomes^{mid}$ in contrast to the $CD49a^-$ $Eomes^{hi}$ cNK-like population in the SG—and by enhancing expression of other factors that support NK cell survival (100). In line with this, disruption of TGF-β signaling impairs both the abundance and distinct surface phenotype of SG NK cells (100).

SG NK cell development was initially reported to be Nfil3-independent (98), but later studies involving PLZF–reporter/fate-mapping mice and mixed bone marrow chimeric mice demonstrated that the majority of SG NK cells develop in an Nfil3-dependent manner, and only a minor fraction are Nfil3-independent (52, 101). Although the Nfil3-dependent and -independent populations have similar surface phenotypes and are functionally hyporesponsive when in the SG, these features are specifically reversible in the Nfil3-dependent subset following transfer into the spleen or liver (101). Thus, tissue-specific signals likely instruct the unique phenotype of SG NK cells.

All SG NK and helper ILC1 populations are $T\text{-}bet^{hi}$ (98, 100, 101). Although experiments in mixed bone marrow chimeric mice suggested that T-bet is important for SG ILC1, and to a lesser extent SG NK, development in a competitive setting (52), non-chimeric *Tbx21*$^{-/-}$ mice (T-bet is encoded by *Tbx21*) have a relatively intact SG NK compartment (100). $Eomes^-$ SG helper ILC1s do not require Eomes for development (52). However, the role of Eomes in SG NK development is surprisingly nuanced. As mentioned above, $CD49a^-$ SG cNKs are $Eomes^{hi}$ and $CD49a^+$ SG trNKs are $Eomes^{mid}$ owing to TGF-β-mediated restriction of Eomes expression in the latter subset (100). Unexpectedly, cell-specific deletion of Eomes in non-chimeric mice does not alter the overall abundance of total SG NKs, but rather reduces the fraction that expresses CD49b and actually enhances the distinctive surface phenotype of SG NKs (100). These findings highlight an unusual modulatory role for Eomes in SG NK development or maintenance, which contrasts with the generally strict requirement for Eomes in cNK development.

Other transcriptional regulators that have been evaluated for roles in SG NK and helper ILC1 development include Hobit and the lncRNA *Rroid*, as well as Runx3 and its co-factor Cbf-β. Both SG helper ILC1s and $CD49b^+$ SG NKs develop independently of Hobit, a feature that distinguishes them from liver ILC1s (52). Similarly, the SG compartment is largely unperturbed in mice lacking the lncRNA *Rroid* (43). In contrast, cell-specific deletion of Runx3 or Cbf-β results in a significant reduction in the total SG compartment, underscoring a key role for the Runx pathway in SG NK and/or helper ILC1 development (56).

Intestines

Several IFN-γ-producing NK and helper ILC1 populations have been identified in the intestinal mucosa of mice. These include $Lin^-CD160^+NK1.1^+NKp46^+$ intraepithelial lymphocytes (IEL), comprised of both $Eomes^+$ NK cells and $Eomes^-$ helper ILC1s, which have been implicated in colitis-associated inflammation (103, 104). Additionally, the small intestine lamina propria (siLP) harbors both $CD49b^+Eomes^+$ cNK-like cells and $CD49a^+Eomes^+$ trNK-like cells, in addition to a population of $CD49a^+CD49b^-Eomes^-$ helper ILC1s that contribute to defense against certain enteric pathogens (99, 104, 105). Phenotypically, siLP helper ILC1s are $CD127^+CD62L^{lo}CD69^{hi}CD44^{hi}$ cells that exhibit low or no expression of CD11b and most Ly49 receptors (104), but variously express CCR9, CXCR3, and CXCR6, chemokine receptors associated with lymphocyte homing to tissues (104, 105). Functionally, siLP helper ILC1s produce more IFN-γ, TNF-α, and GM-CSF than cNKs, a phenotype that is reminiscent of liver ILC1s, but have low expression of cytotoxicity-associated molecules such as granzyme B, perforin, and CD107a (104, 105). Parabiosis studies demonstrated that siLP helper ILC1s, and possibly a portion of siLP NK cells, are genuine tissue-resident cells (99, 105).

With respect to developmental requirements, both siLP NKs and siLP helper ILC1s are significantly reduced in *Il15*$^{-/-}$, but not *Il7Ra*$^{-/-}$, mice, indicating that IL-15 signaling is critical for the development and/or maintenance of both populations (104). In contrast to findings in the siLP, the overall IEL compartment is only modestly reduced in *Il15Rα*$^{-/-}$ mice, although the differential impact on NK cells vs. helper ILC1s was not evaluated (103). Nevertheless, partial gene deficiencies in *Stat5a* and/or *Stat5b* result in fewer helper ILC1s in both the IEL and siLP compartments, as well as fewer IEL NK cells (45).

In addition to Stat5, several other transcriptional regulators are known to impact intestinal NK and/or helper ILC1 development. For example, siLP helper ILC1s require T-bet and Gata-3 for development or maintenance, but not Eomes, the lncRNA *Rroid*, or Retinoid-related orphan receptor gamma t (RORγt) (43, 104, 105). In contrast, siLP NKs require Eomes but not T-bet (104, 105). The bulk IEL population is significantly reduced in mice lacking T-bet, Runx3, or Cbf-β, although the extent to which these factors differentially impact the $Eomes^+$ NK vs. $Eomes^-$ ILC1 fractions remains unclear (56, 103). In contrast, both AhR and RORγt are dispensable for IEL NK cell and/or helper ILC1 development (103). And finally, Nfil3 appears to be required for all described intestinal populations: siLP NK cells, siLP helper ILC1s, and the IEL population(s) (103–105).

Adipose Tissue

IFN-γ-producing NK and helper ILC1 populations in the adipose tissue (AT) have been shown to contribute to obesity-related metabolic dysfunction, in part by promoting the differentiation of inflammatory M1 macrophages (106–110). The AT NK compartment includes sizeable populations of

$CD49a^{-}CD49b^{+}Eomes^{+}$ cNKs, and $CD49a^{-}CD49b^{-}Eomes^{+}$ and $CD49a^{+}CD49b^{-}Eomes^{+}$ NKs that have been called immature NK cells by some groups (109, 110). $Eomes^{-}$ helper ILC1s are also abundant in the AT, the majority of which are $CD49a^{+}CD49b^{-}$ (109, 110). Parabiosis studies indicate that AT helper ILC1s and many of the $CD49b^{-}$ NKs are genuine tissue-resident cells, whereas mature cNKs in the AT are non-resident (109, 110).

The $CD49b^{-}$ trNK populations in the AT have unique surface phenotypes. Most are $CD90^{hi}CD69^{hi}$ and variably express many cNK-associated molecules (e.g., CD11b, KLRG1, Ly49D, and Ly49H) but not CD127 (109, 110). All NK and helper ILC1 populations in the AT are $TRAIL^{-}$. $CD49b^{-}$ trNKs and helper ILC1s express lower levels of granzyme B than mature cNKs in the AT (110). In addition, AT helper ILC1s produce more IFN-γ than AT cNK cells, possibly reflecting a central role in AT macrophage polarization and/or metabolic dysfunction (107, 109, 110).

Important transcriptional regulators of AT NK cells and ILC1s include Nfil3, which is critical for development of all cNKs, trNKs, and helper ILC1s in the AT (52, 109, 110). The roles of T-bet and Eomes, on the other hand, appear to be subset-specific. T-bet-deficiency selectively affects AT cNKs and helper ILC1s, particularly in mixed bone marrow chimeras and in *Rag2*$^{-/-}$ mice, but has little impact on the $CD49b^{-}$ trNK compartment (52, 109, 110). In contrast, Eomes is strictly required for development of cNKs but not helper ILC1s in the AT (its role in $CD49b^{-}$ trNK development remains unclear) (52). And lastly, both AT cNKs and helper ILC1s develop in a Hobit-independent manner (52).

Skin

In addition to $CD49a^{-}CD49b^{+}Eomes^{+}$ cNKs, mouse skin harbors a distinct population of $CD49a^{+}CD49b^{-}Eomes^{lo/-}$ cells that are $CD69^{hi}CD127^{-}$ and are largely tissue-resident in parabiotic mice (64). This tissue-resident population has been referred to as a trNK subset, although its lack of Eomes expression suggests a helper ILC1 identity. Both skin cNKs and the skin-resident ILC1s require Runx3 and its co-factor Cbf-β for development (56). The skin-resident ILC1s are similar to liver-resident cells in requiring T-bet and IL-15, but not Nfil3, for development (64). Notably, liver- and skin-resident populations not only share many developmental requirements, but they also appear to cooperate in inter-organ immune responses involving the skin and liver. For example, liver ILC1s can mediate hapten-specific CHS memory responses in the skin, and hapten-specific memory ILC1s in the skin-draining lymph node were recently shown to migrate to and reside in the liver (59–61, 64, 111, 112). Altogether these findings point to a unique relationship between the liver- and skin-resident populations that remains to be fully defined.

Peritoneum

The peritoneal cavity (PC) contains both $CD49a^{-}CD49b^{+}Eomes^{+}$ cNKs and PC-resident $CD49a^{+}CD49b^{-}Eomes^{-}$ helper ILC1s (52, 64, 113). At steady state, PC helper ILC1s are $CD200r1^{+}CD61^{+}CD27^{+}$ cells that lack expression of Ly49H, CD11b, CD69, and CD103 (52, 113). In response to viral challenge in the peritoneum, PC helper ILC1s produce more IFN-γ than PC cNKs (52). Transcription factors known to regulate the development of PC cNKs include Nfil3, Eomes, and to a lesser extent, T-bet (52). In contrast, PC helper ILC1 development is critically dependent on T-bet, but not Eomes or Nfil3 (52). Additionally, both PC cNKs and helper ILC1s develop in a Hobit-independent manner, unlike liver helper ILC1s (52).

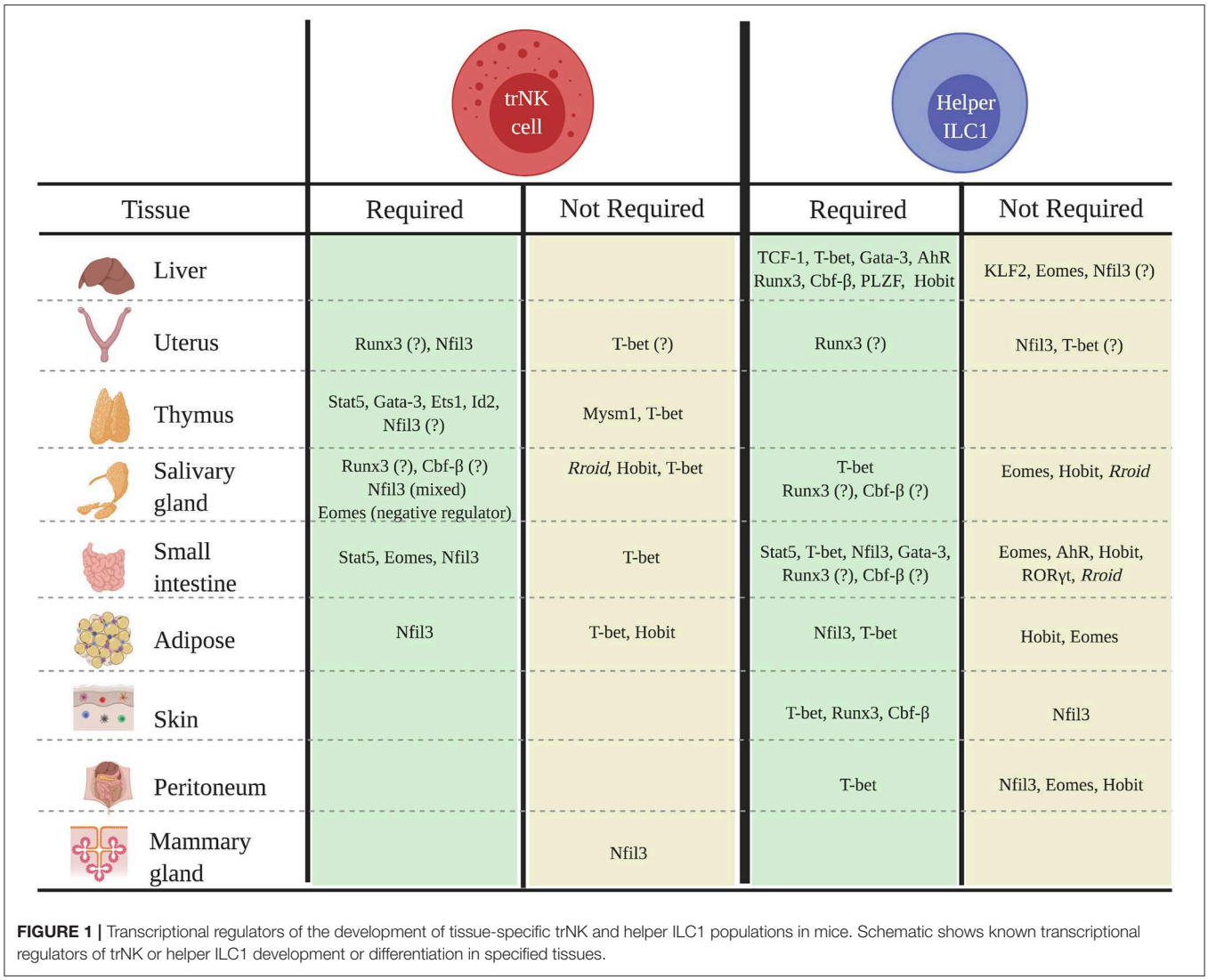
Other Tissues

In addition to the populations described above, increasing evidence suggests that tissue-resident NK and/or helper ILC1s do or may exist in many other tissues. For example, in addition to $CD49b^{+}CD49a^{-}$ cNK-like cells, the kidney contains a minor subset (15–20%) of $CD49a^{+}CD49b^{-}$ cells that are tissue-resident (114). These trNK and/or helper ILC1s are $TRAIL^{+}CD44^{hi}$, but express little CD62L or KLRG1. Importantly, this tissue-resident population(s) was specifically associated with tissue damage following ischemic acute kidney injury, suggesting a unique pathogenic role for these cells (114).

$CD49a^{-}CD49b^{+}$ cNK and $CD49a^{+}CD49b^{-}$ trNK (called ILC1-like in the study) populations have also been described in the mammary glands (MG) (115). Both subsets are $CD127^{-}T\text{-}bet^{+}$, but MG cNK cells are $Eomes^{hi}$, whereas MG trNKs are $Eomes^{lo}$ (115). In addition, a portion of the MG trNKs, but few or none of the MG cNKs, express CD103, Ly49E, and TRAIL (115). Functionally, both MG populations were poor producers of IFN-γ and TNF-α, but remain capable of killing tumor cells through a perforin-dependent pathway (115).

The lacrimal gland (LG) also contains several distinct populations of $Eomes^{+}$ NK and $Eomes^{-}$ helper ILCs with still undefined tissue-residency properties (116). $CD49b^{+}CD49a^{-}$ cells comprise the majority of the LG compartment, with $CD49b^{+}CD49a^{+}$ and $CD49b^{-}CD49a^{+}$ cells representing minority populations. Similarly, the majority of cells in the LG compartment are $CD27^{+}KLRG1^{low}TRAIL^{-}CD127^{-}$ and are functionally hyporesponsive, producing less IFN-γ than splenic cNKs during viral infection (116). However, LG cells that have been adoptively transferred into lymphocyte-deficient hosts and then recovered from the spleen and liver, are no longer hyporesponsive (116). These findings suggest that the altered functionality of LG NKs and/or helper ILC1s may be instructed by the LG tissue environment, analogous to the effect of the SG environment on SG NKs.

Additionally, a distinct population of $NKp46^{+}CD3^{-}$ cells has been shown to localize to the exocrine pancreas in young non-obese diabetic (NOD) mice and to infiltrate the endocrine pancreas in adult NOD mice, possibly reflecting a role in diabetes-related autoimmunity (117). In NOD mice, this pancreatic population is generally hyporesponsive to stimulation, exhibiting reduced IFN-γ production and CD107a upregulation following receptor crosslinking, but displays higher spontaneous production of IFN-γ *ex vivo*, as compared to splenic cNKs (117). Phenotypically, these cells are $CD69^{hi}CD27^{hi}KLRG1^{+}$,



Tissue	trNK cell: Required	trNK cell: Not Required	Helper ILC1: Required	Helper ILC1: Not Required
Liver			TCF-1, T-bet, Gata-3, AhR Runx3, Cbf-β, PLZF, Hobit	KLF2, Eomes, Nfil3 (?)
Uterus	Runx3 (?), Nfil3	T-bet (?)	Runx3 (?)	Nfil3, T-bet (?)
Thymus	Stat5, Gata-3, Ets1, Id2, Nfil3 (?)	Mysm1, T-bet		
Salivary gland	Runx3 (?), Cbf-β (?) Nfil3 (mixed) Eomes (negative regulator)	*Rroid*, Hobit, T-bet	T-bet Runx3 (?), Cbf-β (?)	Eomes, Hobit, *Rroid*
Small intestine	Stat5, Eomes, Nfil3	T-bet	Stat5, T-bet, Nfil3, Gata-3, Runx3 (?), Cbf-β (?)	Eomes, AhR, Hobit, RORγt, *Rroid*
Adipose	Nfil3	T-bet, Hobit	Nfil3, T-bet	Hobit, Eomes
Skin			T-bet, Runx3, Cbf-β	Nfil3
Peritoneum			T-bet	Nfil3, Eomes, Hobit
Mammary gland		Nfil3		

FIGURE 1 | Transcriptional regulators of the development of tissue-specific trNK and helper ILC1 populations in mice. Schematic shows known transcriptional regulators of trNK or helper ILC1 development or differentiation in specified tissues.

but express little or no CD62L and CD127 (117). Later studies established that most are CD49b$^+$, although a small number are CD49a$^+$ (64). Whether the CD49b$^+$ or CD49a$^+$ subsets are comprised of *bona fide* tissue-resident NKs and/or helper ILC1s remains to be determined.

Overall, relatively little is known about the specific developmental requirements of kidney, MG, LG, and pancreatic NK cells, with a few exceptions. cNKs in the MG, bulk CD3$^-$NK1.1$^+$ cells in the LG, and the non-tissue-resident CD49b$^+$CD49$^-$ cNK-like subset in the kidney are all significantly reduced in Nfil3-mice, suggesting that development of these populations is fully or partially Nfil3-dependent (114–116). In contrast, MG trNKs are not significantly reduced in mice lacking Nfil3, and the tissue-resident CD49a$^+$CD49b$^-$ NK and/or ILC1 subset in the kidney is actually more abundant (115, 116). Notably, T-bet-deficiency does not significantly alter the number of CD49a$^+$CD49b$^-$ or CD49a$^-$CD49b$^+$ cells in the kidney (114).

CONCLUDING REMARKS

Recent studies have demonstrated that, like many other immune cell lineages, unique tissue-resident NK and helper ILC1 populations exist in a broad array of tissues and organs. These populations exhibit tissue-specific phenotypes and functions, and have important roles in diverse biological processes with both positive and negative consequences for organismal health. Examples include the immunomodulatory function of SG trNKs in dampening T cell-mediated tissue damage in the SG during viral infection, and the negative impact of IFN-γ-producing NK and helper ILC1 populations in the adipose tissue on obesity-related metabolic dysfunction (102, 106–110). As discussed above, some but not all trNKs and helper ILC1s share expression of markers associated with tissue-residency, e.g., CD49a, CD103, CD200r1, and CD69, although the phenotype and functional properties of these populations are often unique to the tissue environment in which they exist.

While our understanding of the transcriptional networks that regulate tissue-specific trNKs and helper ILC1s is still very limited, the balance of evidence suggests that these populations often exhibit unique developmental requirements that can differ not only across tissues, but also among the cNK, trNK, and helper ILC1 populations in a specific tissue (**Figure 1**). Indeed, findings that some trNKs and helper ILC1s in the liver, uterus, skin, kidney, salivary gland, and mammary glands do not strictly require Nfil3, and that trNKs and/or helper ILC1s in the kidney, uterus, and adipose tissue do not require T-bet for development, are particularly notable, given the key roles of these factors in the development of cNKs and other ILC subsets. Understanding why some, but not all, trNK and helper ILC1 populations bypass developmental requirements for otherwise "lineage-defining" factors such Nfil3 and/or T-bet is an important topic for future studies.

Although this review has focused principally on studies involving tissue-resident NKs and helper ILC1s in mice, it is important to note that similar tissue-associated populations have now been described in diverse human tissues, including the uterus, adipose, tonsils, intestines, liver, kidney, and lung (118, 119). Most of these tissue-associated populations are $CD56^{bright}$, distinguishing them from highly cytotoxic $CD56^{dim}$ cNKs in peripheral blood, although accurate discrimination of human trNKs and helper ILC1s is currently limited by the lack of faithful lineage-tracking markers and tools (120). Nevertheless, these tissue-associated $CD56^{bright}$ populations as a whole share many similarities with mouse trNKs and helper ILC1s, including high expression of tissue residency-associated surface markers (e.g., CD69, CD49a, and CD103) and chemokine receptors (e.g., CXCR6 and CCR5), and low or no expression of CD62L and CCR7 (121). For example, the human liver harbors a population of $CD56^{bright}$ cells that are $Eomes^{high}$ and express CD49a, CXCR6, and CD69 (122). These $CD56^{bright}$ cells are long-lived (up to 13 years) and persist in the liver without recirculation, suggesting they likely represent *bona fide* liver trNKs/ILC1s (122). Notably, these cells also express Hobit, raising the possibility that Hobit may regulate liver trNK/ILC1 development in humans, similar to its role in mice (123). Although, little is currently known about transcriptional regulation of human trNK/ILC1 development, a better understanding of these pathways could inform the development of novel therapies to treat or prevent human disease.

It is now appreciated that NK cells are capable of mediating adaptive immune responses in certain settings. Indeed, memory or memory-like responses have been described for tissue-resident NKs and helper ILC1s in the liver, skin, and uterus (60, 62, 112, 124). Whether trNKs in other tissues are also capable of immunological memory, and the impact such responses might have on tissue homeostasis and chronic inflammatory diseases, remains to be elucidated. Other important and outstanding questions in the field include: how do tissue-resident vs. bone marrow-derived progenitors contribute to the replenishment of tissue-specific trNKs at steady state and during inflammation? How do tissue-specific signals shape the phenotype and function of trNKs? And finally, will the use of single-cell technologies—e.g., scRNA-seq—reveal new and previously unappreciated heterogeneity in the trNK populations that exist within various tissues? Addressing these questions will critically shape our understanding of the unique biological processes that regulate trNK and helper ILC1 biology at steady state and in settings of tissue-specific inflammatory diseases.

AUTHOR CONTRIBUTIONS

NV-P and AB wrote the manuscript and designed the figure.

FUNDING

This work was supported by funding provided from NIH/NIAID K22AI116802 (AB), NIH/NIAID R01AI148695 (AB), NIH/NHLBI R01HL139818 (AB), the MCJ Amelior Foundation (AB), and the Rutgers University Chancellor Scholar Fund (AB). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

We thank Marc Brillantes for helpful discussions and review of the manuscript. **Figure 1** created with Biorender.com.

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

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CORRECTION
published: 08 July 2020
doi: 10.3389/fimmu.2020.01355

Corrigendum: Transcriptional Regulation of Mouse Tissue-Resident Natural Killer Cell Development

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Keywords: natural killer cells, tissue-resident NK cells, transcriptional regulation, transcription factors, group 1 innate lymphoid cells

A Corrigendum on

Transcriptional Regulation of Mouse Tissue-Resident Natural Killer Cell Development
by Valero-Pacheco, N., and Beaulieu, A. M. (2020). Front. Immunol. 11:309. doi: 10.3389/fimmu.2020.00309

In the original article, there was an error. Several words were omitted in the first sentence of a paragraph which altered the meaning of a sentence. A correction has been made to the section the **Development of Tissue-Specific or Tissue-Resident NK Cells and Helper ILC1s**, subsection **Liver**, paragraph 2. The corrected paragraph appears below:

"Phenotypically, liver ILC1s resemble immature cNKs in having low or no expression of killer cell lectin-like receptor G1 (KLRG1), CD11b, CD122, and Ly49 receptors such as Ly49A, Ly49D, Ly49G2, and Ly49H (50, 51, 63, 64). However, liver ILC1s are transcriptomically distinct from both immature and mature cNKs and exhibit an activated phenotype at steady state, characterized by high expression of CD69, CD44, and CD160, and low expression of CD62L (also known as L-selectin) (51, 59, 64, 65). They also express high levels of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and CD127, as well as chemokine receptors such as CXCR3 and CXCR6 that support residence in the liver sinusoids (50, 51, 59, 64, 66, 67). Although activated liver ILC1s retain cytotoxic functionality against target cells, they differ from cNKs in their higher production of TNF-α, IL-2, and granulocyte-macrophage colony-stimulating factor (GM-CSF), their preferential expression of granzyme C instead of granzyme B, and their reduced expression of perforin (51, 59, 64, 65). Liver ILC1s also express molecules involved in immune regulation, including PD-L1, LAG3, CD39, and CD73, and were recently shown to inhibit T cell function via the PD-1–PD-L1 axis (68)."

The authors apologize for this error and state that this does not change the key scientific conclusions of the article in any way. The original article has been updated.

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Approved by:
Frontiers Editorial Office, Frontiers Media SA, Switzerland

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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: *26 May 2020*
Accepted: *27 May 2020*
Published: *08 July 2020*

Citation:
Valero-Pacheco N and Beaulieu AM (2020) Corrigendum: Transcriptional Regulation of Mouse Tissue-Resident Natural Killer Cell Development. Front. Immunol. 11:1355. doi: 10.3389/fimmu.2020.01355

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 April 2020
doi: 10.3389/fimmu.2020.00714

Characterization of the Impact of Daclizumab Beta on Circulating Natural Killer Cells by Mass Cytometry

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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: 21 November 2019
Accepted: 30 March 2020
Published: 24 April 2020

Citation:
Ranganath T, Simpson LJ, Ferreira A-M, Seiler C, Vendrame E, Zhao N, Fontenot JD, Holmes S and Blish CA (2020) Characterization of the Impact of Daclizumab Beta on Circulating Natural Killer Cells by Mass Cytometry. Front. Immunol. 11:714. doi: 10.3389/fimmu.2020.00714

Daclizumab beta is a humanized monoclonal antibody that binds to CD25 and selectively inhibits high-affinity IL-2 receptor signaling. As a former treatment for relapsing forms of multiple sclerosis (RMS), daclizumab beta induces robust expansion of the CD56^{bright} subpopulation of NK cells that is correlated with the drug's therapeutic effects. As NK cells represent a heterogeneous population of lymphocytes with a range of phenotypes and functions, the goal of this study was to better understand how daclizumab beta altered the NK cell repertoire to provide further insight into the possible mechanism(s) of action in RMS. We used mass cytometry to evaluate expression patterns of NK cell markers and provide a comprehensive assessment of the NK cell repertoire in individuals with RMS treated with daclizumab beta or placebo over the course of 1 year. Treatment with daclizumab beta significantly altered the NK cell repertoire compared to placebo treatment. As previously reported, daclizumab beta significantly increased expression of CD56 on total NK cells. Within the CD56^{bright} NK cells, treatment was associated with multiple phenotypic changes, including increased expression of NKG2A and NKp44, and diminished expression of CD244, CD57, and NKp46. These alterations occurred broadly across the CD56^{bright} population, and were not associated with a specific subset of CD56^{bright} NK cells. While the changes were less dramatic, CD56^{dim} NK cells responded distinctly to daclizumab beta treatment, with higher expression of CD2 and NKG2A, and lower expression of FAS-L, HLA-DR, NTB-A, NKp30, and Perforin. Together, these data indicate that the expanded CD56^{bright} NK cells share features of both immature and mature NK cells. These findings show that daclizumab beta treatment is associated with unique changes in NK cells that may enhance their ability to kill autoreactive T cells or to exert immunomodulatory functions.

Keywords: natural killer cell, daclizumab beta, multiple sclerosis, immune profiling, CyTOF/ mass cytometry, uniform manifold approximation and projection, CytoGLMM, clustering

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes that are best known for the eponymous function: that of killing other cells. Yet NK cells also play a critical role in immune regulation by secreting cytokines that influence the character of the immune response. NK cell function is controlled by signals received through activating, inhibitory and cytokine receptors (1). Activating receptors, examples of which include the natural cytotoxicity receptors NKp30, NKp44, and NKp46, the C-type lectin receptors NKG2D and NKG2C, and certain classes of activating Killer-cell Immunoglobulin-like Receptors (KIRs), generally sense stress on the target cell and promote NK cell activation. Inhibitory receptors, including most KIRs, NKG2A, and LILRB1 (CD85j), generally recognize Major Histocompatibility Class (MHC) I receptors and dampen NK cell responses to normal healthy cells. Broadly, NK cells are divided into two major classes. In the blood, the mature $CD56^{dim}$ NK cells are the predominant subset and have potent cytotoxic activity, while the relatively immature $CD56^{bright}$ NK cells are generally present at <10% and primarily secrete cytokines. Recent studies have demonstrated significant heterogeneity in the human NK cell repertoire, with a wide range of NK cell subsets expressing different combinations of these activating and inhibitory receptors (2–6).

Natural killer cells also express a wide range of cytokine receptors making them extremely responsive to cytokine stimulation. NK cells undergo dramatic shifts in phenotype and function in the presence of cytokines such as IL-2, IL-12, IL-15, and IL-18, singly and in combination (7–9). IL-2 plays a particularly critical role in activating NK cells by binding to the low affinity IL-2 receptor, a heterodimer of CD122 (IL-2Rβ) and CD132 (IL-2Rγ, otherwise known as the common gamma chain). In general, NK cells do not express the high affinity IL-2 receptor, CD25 (IL-2Rγ). The $CD56^{bright}$ NK cell subset expresses much higher levels of CD122 than the $CD56^{dim}$ subset (10–12).

Daclizumab is a humanized monoclonal antibody that irreversibly blocks CD25, preventing signaling through the high affinity IL-2R while increasing IL-2 bioavailability to bind to the low affinity receptor [reviewed in (13, 14)]. Due to the complex roles of IL-2 *in vivo*, daclizumab induces several immunological changes, including inhibition of T cell activation, reduction in the frequency and survival of regulatory T cells, and expansion of $CD56^{bright}$ NK cells (13, 14). It was originally developed as an intravenous treatment for several disease indications, including the prevention of transplant rejection and the treatment of severe uveitis and T cell leukemia (13, 14). Later a subcutaneous form (daclizumab beta) was developed and approved for the treatment of relapsing forms of multiple sclerosis (RMS) due to its beneficial effects including reduction in lesion size and slowed disease progression (10, 15–18); (19). In these initial trials, daclizumab beta treatment was associated with adverse events including cutaneous reactions, malignancies, infections, and transaminase elevations, though these were not sufficiently severe to preclude approval. In 2018, daclizumab beta was voluntarily withdrawn from the market due to the nature and complexity of adverse events associated with the drug and limited number of patients treated, which presented challenges in further characterizing its evolving benefit/risk profile. Subsequently, cases of immune-mediated encephalitis were confirmed as adverse drug reactions that can be related to treatment with daclizumab beta.

While no longer used therapeutically, a better understanding of the effects of daclizumab beta may provide insight into the pleiotropic effects of IL-2 in the setting of RMS. Surprisingly, the beneficial effects of daclizumab beta treatment were linked not to changes in T cell function, but instead were strongly correlated with expansion of $CD56^{bright}$ NK cells (10, 13, 14, 16). Although $CD56^{bright}$ NK cells generally have poor cytotoxic activity, the daclizumab beta-expanded $CD56^{bright}$ NK cells could kill activated, autologous $CD4^{+}$ T cells, potentially driving the therapeutic effect by eliminating autoreactive T cells (10, 20). This study was undertaken to provide a better understanding of the effects of daclizumab beta on circulating NK cells *in vivo*.

MATERIALS AND METHODS

Study Subjects

Cryopreserved peripheral blood mononuclear cells (PBMCs) from daclizumab beta-treated and placebo-treated individuals living with RMS were chosen from the Biogen SELECT (NCT00390221) and DECIDE (NCT01064401) studies (19, 21). Subjects were treated subcutaneously with 150 mg daclizumab beta every 4 weeks for 52 weeks. For the placebo and the treatment cohort, we received de-identified PBMCs at 3 timepoints: Baseline, Week 24 and Week 52. For the healthy donor cohort, leukoreduction system chambers from anonymous donors were purchased from the Stanford Blood Bank. PBMCs were isolated by Ficoll density gradient centrifugation and then cryopreserved in fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO). We had 16 healthy donors, 17 placebo and 30 daclizumab beta treated individuals. As part of their initial enrollment, all subjects provided written informed consent. The studies were approved by the relevant central and local ethics committees and were conducted in accordance with the International Conference on Harmonization guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki.

Antibody Conjugation, Mass Cytometry Staining and Data Acquisition

Antibodies for mass cytometry were conjugated to heavy metals using MaxPar® × 8 labeling kits (Fluidigm) as described (22). To ensure antibody stability over time, the antibody panel was lyophilized into single-use pellets prior to use (Biolyph). PBMCs were thawed at 37°C in RPMI-1640 media (supplemented with 10% FBS, L-glutamine, and Penicillin-Streoptomycin-Amphotericin) with benzonase. NK cells were purified by magnetic bead isolation via negative selection (Miltenyi, cat. 130-092-657) and stained with the NK cell antibody panel (**Supplementary Table S1**) as previously described (4, 23, 24). Cells were resuspended in 1x EQ Beads (Fluidigm) for normalization before acquisition on a Helios mass cytometer (Fluidigm).

Data Analysis

The open source statistical software R[1] was used for all statistical analyses (25). Signal intensities were transformed using the hyperbolic sine transformation (asinh function) prior to statistical analysis, with cofactor equal to 5, to account for heteroskedasticity. We used the custom-made package *CytoGLMM* (26, 27) to identify markers predictive of a given sample type while taking into account the subject effect. To this end, this package uses a generalized linear mixed model with paired comparison (used for analyses of the same individual over time) and generalized linear model with bootstrap resampling (for cross-sectional comparisons between daclizumab beta- and placebo-treated individuals). Using the empirical marker distribution, the model generates the log-odds that the expression of a given marker is predictive of the sample type (for example, drug-treated vs. placebo-treated) with the 95% confidence intervals. For paired comparisons, we computed *p*-values using the asymptotic theory implemented in R package *mbest* (28). For unpaired comparisons, we computed *p*-values by inverting the percentile bootstrap confidence intervals and assuming two-sided intervals with equal tails (29). To correct for multiple comparisons, the Benjamini-Hochberg method controlling the False Discovery Rate (FDR) at level 0.05 was used, which is conservative as it assumes independence of markers. For paired analysis on daclizumab beta-treated individuals over time, where the response variable was timepoint, we used all cells from each donor. For unpaired analysis using the bootstrap, where the response variable was daclizumab beta or placebo treatment, we used 1,000 cells from each sample for the total NK cell and $CD56^{dim}$ analyses, and used all cells from each sample for $CD56^{bright}$. There were fewer than 1,000 $CD56^{bright}$ NK cells in most samples except for daclizumab beta treated individuals. The number of subjects used for each analysis is specified in the figure legends.

UMAP Visualizations

The Uniform Manifold Approximation and Projection (UMAP) algorithm was used as a visualization and dimensionality reduction technique for our CyTOF data (30, 31). The *uwot* R package provides an implementation of UMAP and was used with a minimum distance set to 0.1 and nearest neighbors set to 20. The UMAP loadings were visualized using Cytobank. Separate analyses were performed on total NK cells and $CD56^{bright}$ NK cells, including both placebo and drug treatment at three different timepoints. All markers in **Supplementary Table S1** were used excluding markers used for gating (CD3, CD19, CD33, CD14, CD56, CD4), and markers with extremely low or non-specific staining (FcRγ, Ki-67, KIR2DS2, CXCR6, PD1).

Clustering and Differential Abundance Tests

We used a clustering method to identify subsets of cells in the NK and $CD56^{bright}$ cell populations in the placebo and daclizumab beta treated individuals. The clustering analysis was performed using the CATALYST package version 1.10.0 [Crowell et al. (32) CATALYST: Cytometry dATa anALYSis Tools] from Bioconductor. The clustering method provided by the package combines two algorithms. The first step uses the FlowSOM clustering algorithm (33) to cluster the data into 100 high-resolution clusters. The second step regroups these clusters into metaclusters using the ConsensusClusterPlus metaclustering algorithm (34). The default parameters of the cluster function were used except for the maximum of metaclusters which was defined to 30. The delta area plot provided by the package was used to select the optimal number of metaclusters (9 for the $CD56^{bright}$ cell population; 5 for the NK cell population).

We performed differential abundance tests to highlight differences in cell clusters due to the Daclizumab beta treatment. The differential abundance tests were performed with the diffcyt package version 1.6.0 (35). The diffcyt-DA-edgeR method uses the edgeR package (36) which fits a negative bionomial generalized linear model to identify populations that are present at different frequencies. For each test, we filtered the data to the comparison of interest. We created the design matrix corresponding to the experimental design and contrast matrix specifying the comparison of interest. The differential abundance test reports adjusted *p*-values (FDR).

Data Availability

The dataset generated and analyzed for this study can be found in FlowRepository ID FR-FCM Z2D6.

RESULTS

Characteristics of Study Population

For this study, individuals living with RMS received 150 mg daclizumab beta or placebo subcutaneously every 4 weeks for 52 weeks. The demographics of the healthy controls, placebo-treated, and daclizumab beta-treated groups are given in **Table 1**. As expected with RMS, we had a high frequency of females in the trial, with 90% in the daclizumab beta-treated group and 70% in the placebo group. Peripheral blood samples for our research study were taken from both the SELECT and DECIDE trials (19, 21).

Characterization of the NK Repertoire

Frozen peripheral blood samples from baseline (pre-treatment), 24 weeks post-treatment initiation, and 52 weeks post-treatment initiation were obtained for this study. Purified NK cells from each sample were stained for mass cytometry using a panel of 41 antibodies conjugated to heavy metals (**Supplementary Table S1**

TABLE 1 | Demographics of the study population.

Group	Total *N*	Pct. Female	Age, years: median (range)
Healthy	16	50%	52.5 (27–82)
Placebo	17	70.5%	34 (21–45)
Daclizumab beta	30	90%	32 (19–52)

[1]https://www.r-project.org/

and **Supplementary Figure S1A**). Total NK cells, CD56^{bright} and CD56^{dim} NK cells were analyzed by gating in FlowJo (**Supplementary Figure S1B**). Example staining of each of the 31 NK markers used in this analysis are shown in **Supplementary Figure S2**.

Daclizumab Beta Induces Higher Frequency of CD56^{bright} NK Cells

The *CytoGLMM* R package was used to identify which NK markers predicted daclizumab beta treatment compared to placebo. This generalized linear model with bootstrap resampling allows for identification of markers that predict a given outcome, while controlling for inter-individual variability. The model takes into account the full distribution of the marker measurements (rather than a single summary measure such as mean signal intensity) and yields the log-odds with which that marker predicts the outcome, with 95% confidence intervals. Among total NK cells at 24 weeks, NKp30, NTB-A, and CD2 expression predicted daclizumab beta treatment, while NKG2D, CD244, TIGIT, FAS-L, and KIR2DL5 predicted placebo treatment (**Figure 1A**). After controlling for multiple comparisons, these changes were not statistically significant. At 52 weeks, among the total NK cell population, CD56, NKp30, TACTILE, NKp44, and NTB-A predicted daclizumab beta treatment, while CD244, CD69, TIGIT, NKp46, and CD57 predicted placebo treatment (**Figure 1A**), but these changes were not significant after correction for multiple comparisons. There were no markers that significantly predicted placebo or daclizumab treatment at baseline, indicating that the groups were relatively well matched, and changes we observe at later time points were not due to baseline differences between the two groups (**Supplementary Figure S3A**). There were only two markers with altered expression over the course of 52 weeks in the placebo group: CD16 and KIR2DS4 (**Supplementary Figure S3B**), which did not change in the daclizumab beta treated group. This suggests that the expression changes in 10 markers observed after 52 weeks of daclizumab beta treatment were due to the treatment rather than normal drift in marker expression over a year.

Within the daclizumab beta-treated group, most protein expression changes that occurred in total NK cells by 24 weeks were preserved at 52 weeks. CD56, NKp44, NKG2A, and CD2 predicted 24 weeks of treatment, while HLA-DR and FAS-L predicted baseline samples (**Figure 1B**). With the increased power from these paired comparisons, the changes in CD56, NKp44, NKG2A, CD2, and HLA-DR were significant after correction for multiple comparisons. When comparing baseline and 52 weeks, CD56, NKp44, NKG2A, and CD2 significantly predicted 52 weeks of treatment, while CD244, FAS-L, HLA-DR, NTB-A, and NKG2C significantly predicted baseline samples (**Figure 1B**).

CD56 was the most significant predictor of daclizumab beta treatment, and predicted both 24- and 52-week samples compared to baseline. Using UMAP visualization, we found an increase in frequency of CD56^{bright} NK cells by 24 weeks of daclizumab beta treatment, which continued to increase at 52 weeks (**Figure 1C**). This increase in CD56^{bright} NK cells was confirmed by gating in FlowJo (**Figure 1D** and **Supplementary Figure S1B**), which showed that the frequency of CD56^{bright} NK cells was increased in daclizumab beta-treated subjects compared to placebo-treated, and increased from baseline to 24 weeks and 52 weeks. There was no significant difference in frequency of CD56^{bright} NK cells in placebo-treated individuals over time, or in individuals with MS before treatment compared to healthy controls (**Figure 1D**).

Both the *CytoGLMM* analysis and UMAP visualizations revealed an increase in the CD56^{bright} population of NK cells upon daclizumab beta treatment. In order to test whether the CD56^{bright} population clustered distinctly from other NK cells, we used the CATALYST package to perform clustering of the NK cells. We determined that there were 5 metaclusters of NK cells in our data (**Supplementary Figure S4A**). These clusters varied in frequencies and marker expression (**Supplementary Figure S4B**). We performed differential abundance tests between the clusters to determine which clusters were more or less abundant at 52 weeks of daclizumab beta treatment compared to placebo (**Supplementary Figure S4C**). Two clusters showed differential abundance; cluster 5, representing 19.2% of NK cells, was less abundant upon 52 weeks of daclizumab beta treatment, and had high expression of CD16 and CD57. Cluster 2, representing 46.0% of total NK cells, had significantly higher frequency upon 52 weeks of daclizumab beta treatment, and had high expression of CD56 and CD2, and low expression of CD57 and HLA-DR. This analysis confirms that the CD56^{bright} population is the most significantly altered subset of NK cells upon daclizumab beta treatment.

Daclizumab Beta Alters Expression of NK Receptors on the CD56^{bright} Population

As the CD56^{bright} and CD56^{dim} NK cell subsets are distinct, we next focused solely on the CD56^{bright} NK cells (**Supplementary Figure S1B** for gating strategy). The *CytoGLMM* package was used to identify which markers predicted daclizumab beta treatment compared to placebo within the CD56^{bright} population at 52 weeks. NKp30, Perforin, NKp44, TACTILE, Siglec-7, KIR2DL3, and CD16 predicted daclizumab beta treatment compared to placebo after 52 weeks of treatment in the unadjusted comparison, but were not significant following adjustment for multiple comparisons (**Figure 2A**).

In order to determine what changes in NK receptor expression occurred in response to daclizumab beta in treated individuals over time, the predictors of baseline or 52 weeks of treatment were determined using *CytoGLMM* (**Figure 2B**). Many NK receptors significantly predicted 52 weeks of daclizumab beta treatment: NKG2A, NKp44, CD38, CD8, KIR2DL3, Siglec-7, TACTILE, KIR3DL1, Perforin, NKp30, and CD16 (**Figure 2B**). CD244, CD57, NKp46, CD69, TIGIT, DNAM-1, Syk, KIR2DL5, and NKG2C significantly predicted baseline samples compared to 52 weeks.

Expression of each of the top three predictors of 52 weeks of daclizumab beta treatment and the top three predictors of placebo treatment was visualized using UMAP in both placebo and daclizumab beta treated groups at baseline and

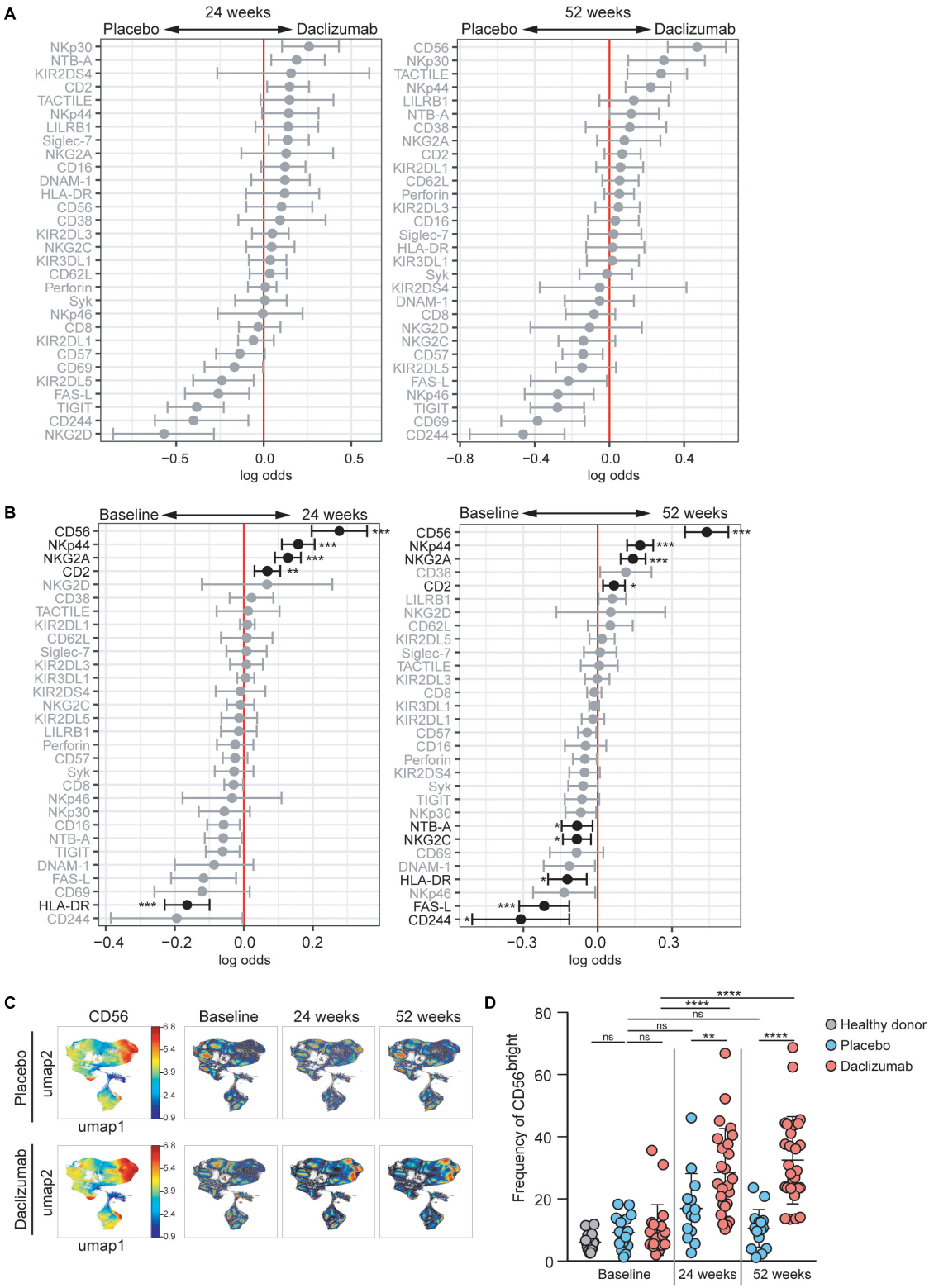

FIGURE 1 | Daclizumab beta induces CD56^{bright} NK cells with a distinct phenotype**. (A)** A generalized linear model with bootstrap resampling was used to identify NK markers predictive of daclizumab beta- and placebo-treated individuals at 24 (left) and 52 (right) weeks of treatment. Log-odds are logarithm of ratios of the probability that a cell belongs to each treatment group. An increase in the parameter coefficient corresponds to the strength of the classification power, with the 95% confidence interval represented by the line surrounding the point estimate. Gray lines indicate markers with adjusted *p*-values > 0.05. Black lines indicate markers

(Continued)

FIGURE 1 | Continued
with adjusted *p*-values < 0.05. Total NK cells were used, with subsampling to 1000 cells per individual. Placebo: 24 weeks, *n* = 14; 52 weeks, *n* = 16. Daclizumab beta: 24 weeks, *n* = 25; 52 weeks, *n* = 27. **(B)** A generalized linear mixed model with paired comparison was used for analyses of the same individual over time, comparing baseline and 24 weeks of daclizumab beta treatment (left), and baseline and 52 weeks of daclizumab beta treatment (right). Total NK cells were used with no subsampling. Daclizumab beta baseline vs. 24 weeks, *n* = 21. Daclizumab beta baseline vs. 52 weeks, *n* = 22. **(C)** UMAP visualization of all NK cells from placebo and daclizumab beta treatment groups. Panels on the left are colored by CD56 expression. Baseline, 24 weeks, and 52 weeks samples are shown colored by density. **(D)** The frequency of CD56^{bright} NK cells for each individual are shown as a percentage of total NK cells. Healthy donors (*n* = 16, gray), placebo treatment (baseline, *n* = 16; 24 weeks, *n* = 14; 52 weeks, *n* = 16; blue), daclizumab beta treatment (baseline, *n* = 22; 24 weeks, *n* = 25, 52 weeks, *n* = 27; pink). *Adjusted *p*-value < 0.05, **adjusted *p*-value < 0.01, ***adjusted *p*-value < 0.001, ****adjusted *p*-value < 0.0001. Adjusted *p*-values calculated on generalized linear mixed model in **(B)** using Benjamini-Hochberg method with FDR = 0.05. Adjusted *p*-values in **(D)** calculated using one-way ANOVA with Sidak's multiple comparisons test.

52 weeks (**Figure 2C**). In the UMAP projections, the plots show an overall increase in density of CD56^{bright} NK cells at 52 weeks of daclizumab beta treatment (**Figure 2C**). NKG2A has higher expression in the CD56^{bright} population of the daclizumab beta treated group, and particularly higher expression in the areas of high cell density. Conversely, CD244 predicted placebo treatment, and had higher expression across the CD56^{bright} population of placebo-treated individuals compared to daclizumab beta-treated individuals. The UMAP plots reveal broad changes in expression of each of these markers across the CD56^{bright} population, as opposed to small subsets of CD56^{bright} cells with altered NK marker expression.

The mean signal intensity was calculated for each of the top six predictors of 52 weeks of daclizumab beta treatment and the top six predictors of baseline samples in each individual (**Supplementary Figure S5**). NKG2A and NKp44 expression increased in almost every individual by 24 weeks of daclizumab beta treatment, and tended to stay elevated at 52 weeks. CD38, CD8, KIR2DL3, and Siglec-7 expression increased in some but not all subjects, suggesting that the daclizumab beta-induced CD56^{bright} population is not equivalent in all subjects receiving daclizumab beta. CD244, CD57, NKp46, CD69, and TIGIT expression were significantly decreased by 24 weeks of daclizumab beta treatment in nearly all subjects, while NKG2D expression varied between individuals. These graphs highlight the fact that most of the changes in NK receptor expression observed with daclizumab beta treatment occur within the first 6 months of treatment, and are maintained throughout the course of treatment.

Daclizumab Beta Treatment Does Not Alter a Specific Subset of CD56^{bright} NK Cells

In order to test whether the NK marker expression changes observed upon daclizumab beta treatment were due to changes in a particular subset of CD56^{bright} NK cells, we performed clustering analysis using the CATALYST package. We determined that there were 9 metaclusters of CD56^{bright} NK cells (**Figure 3A**) that varied in metacluster frequency and marker expression (**Figure 3B**). We performed a differential abundance test between clusters in the daclizumab beta and placebo groups at the 52-week time point (**Figure 3C**). This test revealed one cluster that was significantly less abundant in daclizumab beta-treated individuals; cluster 2, only representing 1.0% of CD56^{bright} NK cells, had high expression of CD16, CD57, and LILRB1, and low expression of Syk and Perforin. We performed a differential abundance test between clusters in the daclizumab beta-treated group comparing baseline and 52 weeks of treatment, and found two clusters with significantly altered frequency; both cluster 2, again representing only 1.0% of NK cells, and cluster 4, representing only 0.7% of NK cells, showed lower abundance at 52 weeks than at baseline (**Figure 3D**). Cluster 4 had high expression of CD57 and Perforin. While these analyses do reveal significant differences in two subsets of CD56^{bright} NK cells upon daclizumab beta treatment, the clusters that have differential abundance represent such a small portion of NK cells that we conclude that the alterations we observed in NK receptor expression occur broadly across the CD56^{bright} population, rather than as a result of a significant shift from one CD56^{bright} subset to another.

Daclizumab Beta Also Alters NK Receptor Expression in the CD56^{dim} Population

While the focus of this study was to determine NK receptor expression in the daclizumab beta-induced CD56^{bright} population, it was interesting to find that there were some distinct changes in NK receptor expression observed in the CD56^{dim} population as well. Using *CytoGLMM*, we identified several weak (low log-odds) predictors of daclizumab beta treatment compared to placebo in the CD56^{dim} population in the unadjusted analysis, including NTB-A and CD2 at 24 weeks, and TACTILE, NTB-A, and CD2 at 52 weeks; these findings were not significant after correcting for multiple comparisons (**Figure 4A**). There were stronger (higher log-odds) predictors of placebo treatment at both 24 and 52 weeks, including NKG2D, TIGIT, FAS-L, KIR2DL5, CD69, CD244, and NKp46 in the unadjusted analysis (**Figure 4A**). This suggests that daclizumab beta more strongly decreased expression of several NK receptors in the CD56^{dim} population rather than increased.

When comparing baseline and 24 or 52 weeks of treatment in daclizumab beta-treated individuals, we identified two significant predictors of treatment: CD2 and NKG2A (**Figure 4B**). Conversely, FAS-L, HLA-DR, CD16, CD8, NTB-A, NKp30, and Perforin predicted baseline samples compared to 24 or 52 weeks. Interestingly, several of the markers that predicted baseline samples in the CD56^{dim} population actually predicted daclizumab beta treatment in the CD56^{bright} population, suggesting that distinct responses to daclizumab beta treatment occur in the CD56^{bright} and CD56^{dim} NK cells.

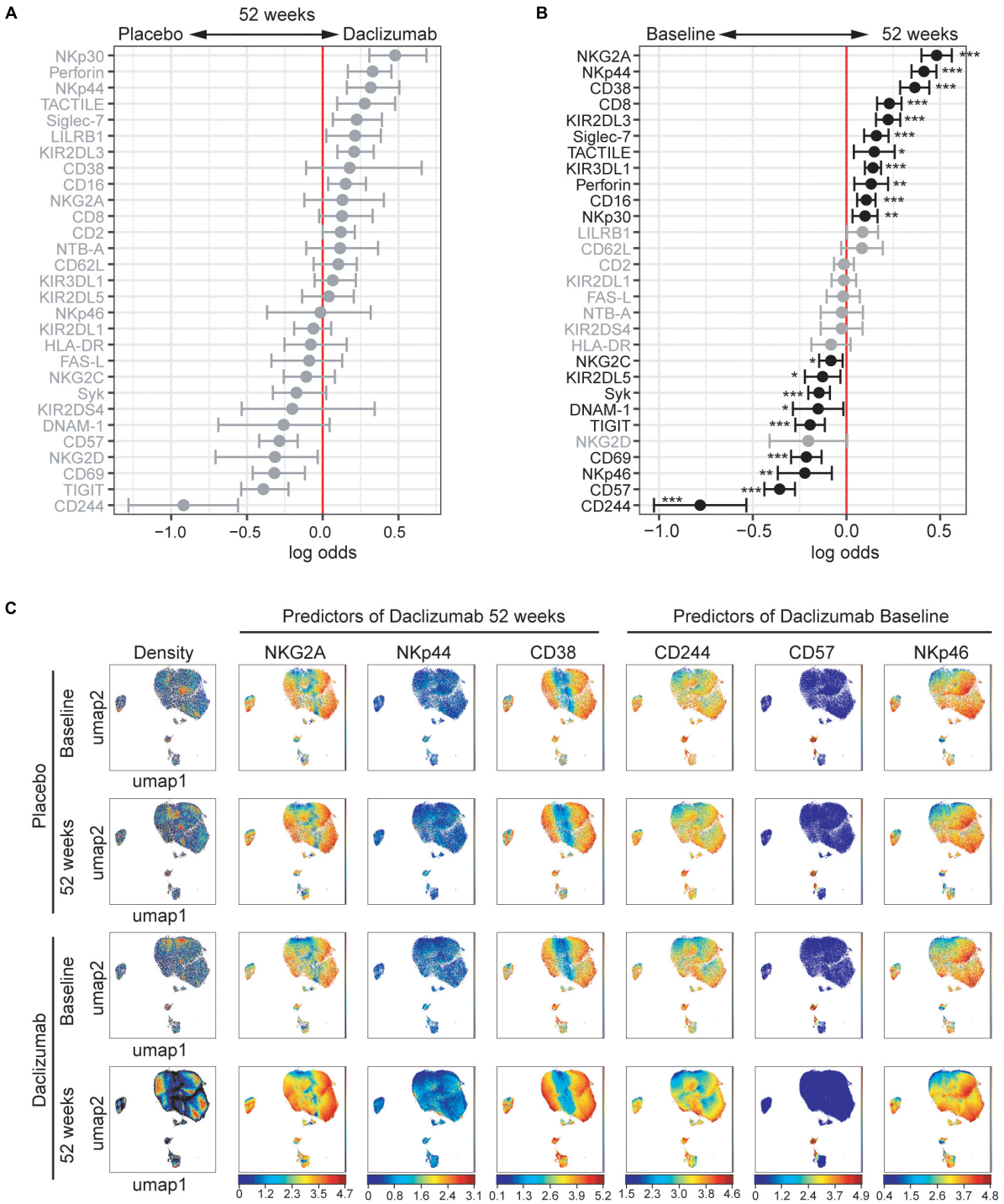

FIGURE 2 | Daclizumab beta alters the CD56^{bright} population. **(A)** A generalized linear model with bootstrap resampling was used to identify NK markers on CD56^{bright} NK cells predictive of daclizumab beta- and placebo-treated individuals at 52 weeks of treatment. CD56^{bright} NK cells were used with no subsampling. Placebo: 24 weeks, $n = 14$; 52 weeks, $n = 16$. Daclizumab beta: 24 weeks, $n = 25$; 52 weeks, $n = 27$. Gray lines indicate markers with adjusted p-values > 0.05. Black lines indicate markers with adjusted p-values < 0.05. **(B)** A generalized linear mixed model with paired comparison was used for analyses of the same individual over time, comparing baseline and 52 weeks of daclizumab beta treatment. CD56^{bright} NK cells were used with no subsampling. Daclizumab beta baseline vs. 52 weeks, $n = 22$. Gray lines indicate markers with adjusted p-values > 0.05. Black lines indicate markers with adjusted p-values < 0.05. **(C)** UMAP visualizations of CD56^{bright} NK cells from the placebo at baseline (top row) and 52 weeks (second row), or the daclizumab beta-treated at baseline (third row) and 52 weeks (bottom row) groups. Leftmost panels are colored by cell density. NKG2A, NKp44, and CD38 were predictors of 52 weeks of daclizumab beta treatment. CD244, CD57, and NKp46 were predictors of baseline samples. Each plot is colored by marker expression, with color scale consistent between groups but specific for each marker. *Adjusted p-value < 0.05, **adjusted p-value < 0.01, ***adjusted p-value < 0.001. Adjusted p-values calculated using Benjamini-Hochberg method with FDR = 0.05.

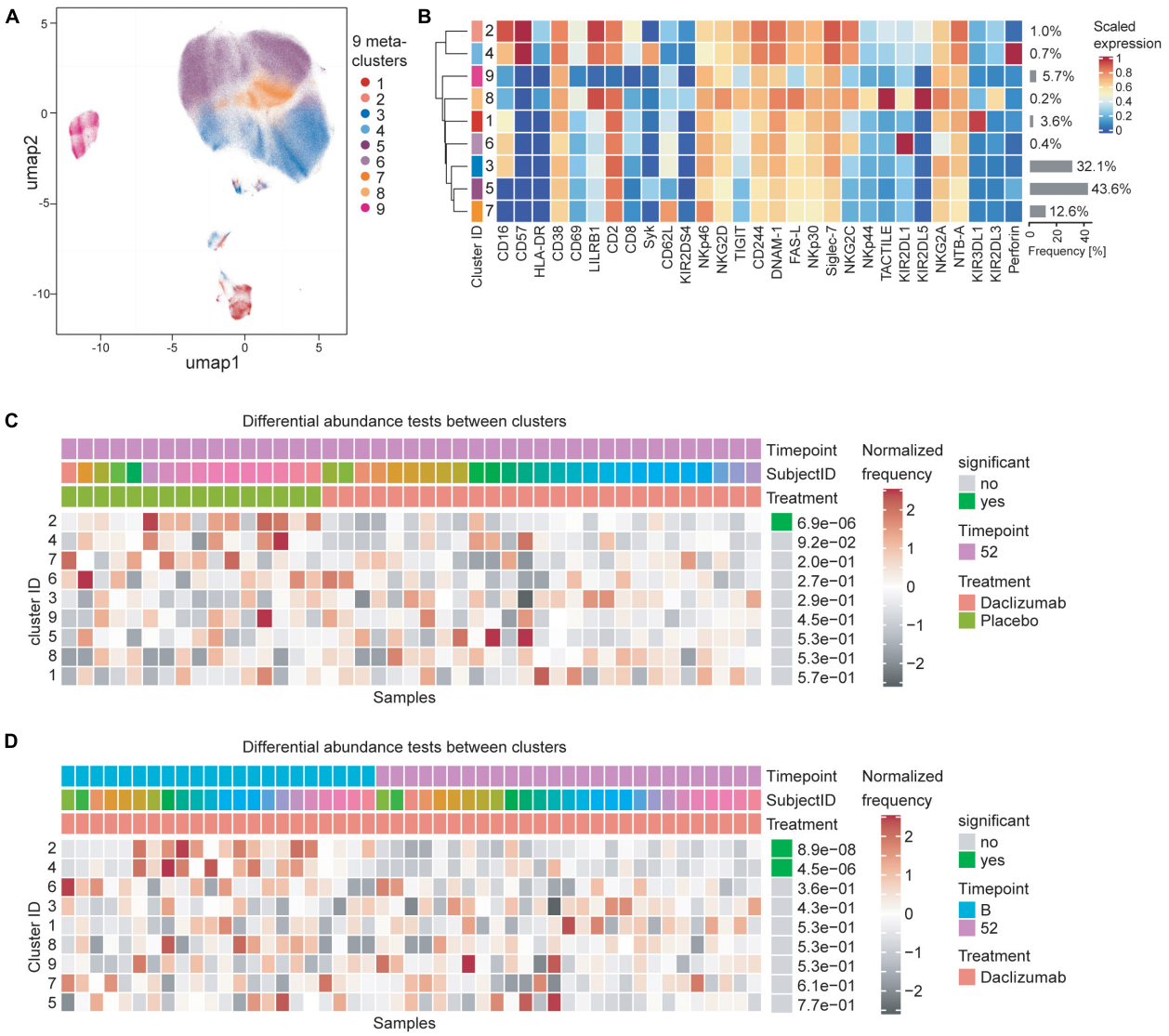

FIGURE 3 | Daclizumab beta treatment does not alter a specific subset of CD56^{bright} NK cells. **(A)** The CATALYST package was used to perform clustering on all CD56^{bright} NK cells from both placebo and daclizumab beta treated groups at all time points. Using the delta area plot provided by the clustering analysis, 9 metaclusters were retained. The metaclusters are displayed in the UMAP space, and colored by metacluster. **(B)** The heatmap shows the cluster IDs (leftmost column) hierarchically ordered by similarity (dendrogram calculated using Euclidean distance as a metric and average as a linkage). Markers are labeled at the bottom of the heatmap. The marker expressions are scaled to values between 0 and 1. Along the right side of the heatmap, the frequency of each cluster among the total CD56^{bright} NK population is shown in gray bars, with the frequency printed next to it. **(C)** Differential abundance test between clusters at 52 weeks of treatment comparing placebo and daclizumab beta treatment. Across the top, Timepoint, Subject ID, and Treatment are shown for each sample (columns). The heatmap represents the proportion of each metacluster in each sample, with gray showing under-representation and red showing over-representation. The proportions are first scaled with arcsine-square-root transformation and then z-score normalized in each cluster. The cluster ID is labeled along the left side of the heatmap, ordered by significance, which is shown along the right side of the heatmap. The differential abundance test reports adjusted *p*-values (FDR). **(D)** Differential abundance test between clusters within the daclizumab beta treated group, comparing baseline and 52 weeks of treatment. Across the top, Timepoint, Subject ID, and Treatment are shown for each sample (columns). The cluster ID is labeled along the left side of the heatmap, ordered by significance, which is shown along the right side of the heatmap. The differential abundance test reports adjusted *p*-values (FDR).

DISCUSSION

While daclizumab beta has been voluntarily withdrawn from the market, understanding the effects of daclizumab beta may provide insight into the role of NK cells in the pathogenesis of RMS and in the potential of other NK cell-based therapeutic strategies for RMS. Here, we used mass cytometry to profile the NK cells expanded in the setting of daclizumab beta treatment of RMS. As observed previously (10), daclizumab beta led to a dramatic shift in the NK cell repertoire with an increase in the frequency of CD56^{bright} NK cells and enhanced expression of NKG2A and CD2. Here, we extended the characterization of

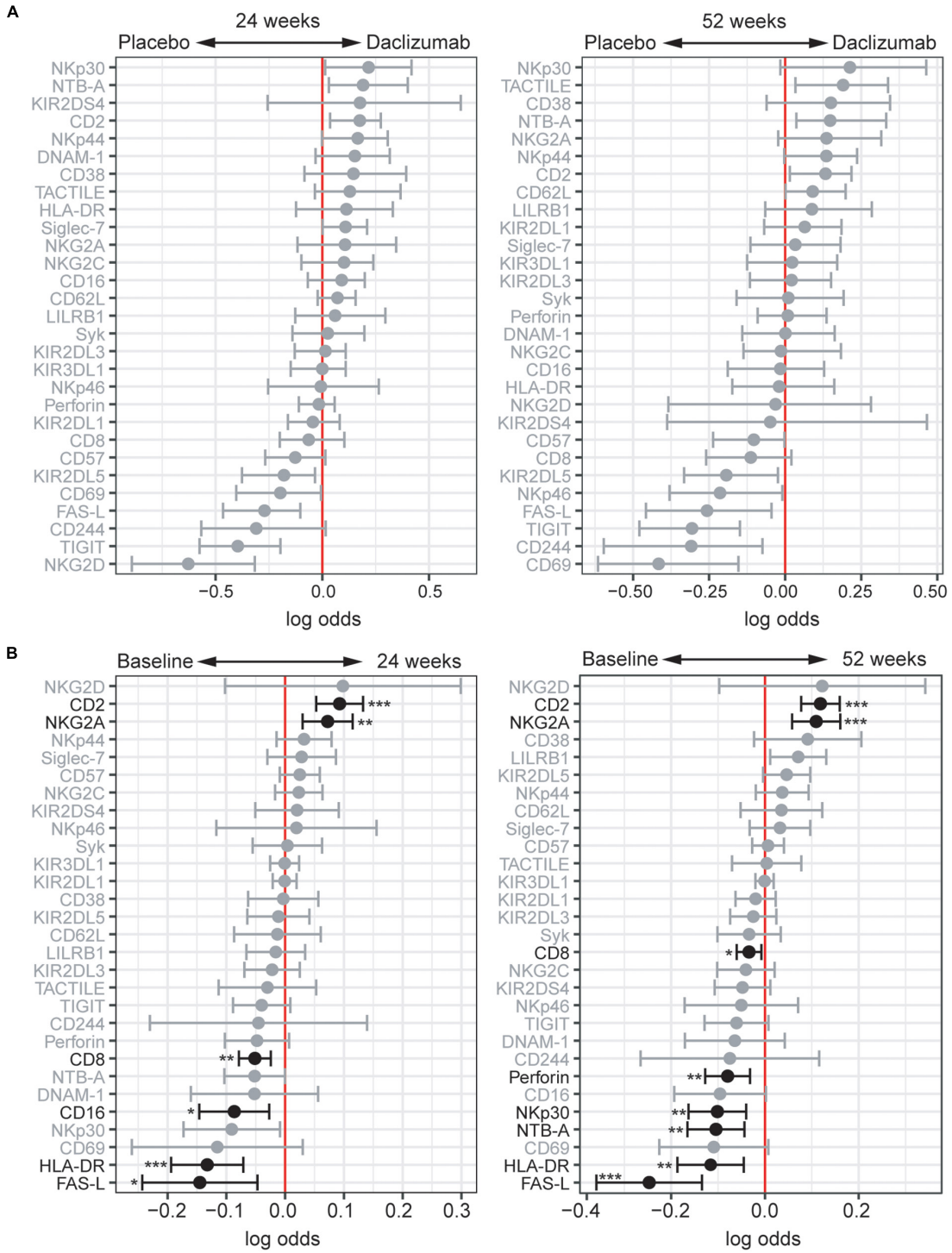

FIGURE 4 | Daclizumab beta changes NK receptor expression on CD56^{dim} cells distinctly from CD56^{bright} cells. **(A)** A generalized linear model with bootstrap resampling was used to identify NK markers on CD56^{dim} NK cells predictive of daclizumab beta- and placebo-treated individuals at 24 (left) and 52 (right) weeks of treatment. CD56^{dim} NK cells were used with subsampling to 1000 cells per individual. Placebo: 24 weeks, $n = 14$; 52 weeks, $n = 16$. Daclizumab beta: 24 weeks, $n = 25$; 52 weeks, $n = 27$. Gray lines indicate markers with adjusted p-values > 0.05. Black lines indicate markers with adjusted p-values < 0.05. **(B)** A generalized linear mixed model with paired comparison was used for analyses of the same individual over time, comparing baseline and 24 weeks of daclizumab beta treatment (left), and baseline and 52 weeks of daclizumab beta treatment (right). CD56^{dim} NK cells were used with no subsampling. Daclizumab beta baseline vs. 24 weeks, $n = 21$. Daclizumab beta baseline vs. 52 weeks, $n = 22$. *Adjusted p-value < 0.05, **adjusted p-value < 0.01, ***adjusted p-value < 0.001. Adjusted p-values calculated using Benjamini-Hochberg method with FDR = 0.05.

the phenotype, demonstrating that the expanded CD56^{bright} NK cells had a unique phenotype that was present by 24 weeks of treatment and remained at 52 weeks of treatment.

Daclizumab beta treatment was associated with enhanced expression of the activation markers CD38 and Perforin, the activating receptors NKp44, TACTILE (CD96), and CD16, and the inhibitory receptors NKG2A, KIR2DL3, and Siglec-7 within the CD56^{bright} population. The enhanced expression of a number of markers of cellular activation and receptors that mediate NK cell activation suggests that the CD56^{bright} NK cells that emerge upon daclizumab beta treatment are primed for responsiveness. At the same time, it is difficult to fully predict the effects of these alterations in receptor expression profiles. While CD56^{bright} NK cells normally express NKG2A, the inhibitory receptor that binds HLA-E, this expression is even higher following daclizumab beta treatment (**Figure 2**). While enhanced expression of this inhibitory receptor could diminish NK cell responsiveness, it is also possible that these NK cells are "educated" through NKG2A, leading to their enhanced ability to detect "altered self" (37, 38). Along similar lines, the increased expression of KIR2DL3 on the CD56^{bright} NK cells in daclizumab beta-treated subjects indicates that at least a subset of these CD56^{bright} NK cells have a more mature phenotype, and could be educated through this KIR to enhance their ability to respond to missing self. Thus, daclizumab beta treatment drives several phenotypic changes in NK cells that could contribute to enhanced NK cell responsiveness.

Daclizumab beta treatment had less effect on the mature CD56^{dim} subset that is generally associated with higher cytolytic activity, yet phenotypic changes still occurred. Similar to CD56^{bright} NK cells, expression of NKG2A and CD2 on CD56^{dim} cells was predictive of daclizumab beta treatment. Interestingly, several markers associated with cellular cytotoxicity, including FAS-L and Perforin, predicted baseline samples among CD56^{dim} NK cells, suggesting that the CD56^{dim} NK cells from daclizumab beta treated subjects may be less efficient at killing. This could partially explain the killing of autoreactive T cells by CD56^{bright} NK in the setting of daclizumab beta treatment (10).

In general, CD56^{bright} NK cells are thought of as the immature subset of NK cells that do not express KIRs, have limited cytolytic activity, and secrete cytokines. Their expansion following daclizumab beta treatment could therefore improve outcomes in RMS through either immunomodulation by cytokine secretion or by killing autoreactive T cells. Surprisingly, prior work demonstrated that the daclizumab beta-expanded CD56^{bright} NK cells can kill activated autologous CD4^{+} T cells in a granzyme K dependent manner *in vitro* (10, 20), which may well explain the therapeutic effect of daclizumab beta. The fact that there was a strong correlation between CD56^{bright} NK cell expansion and T cell contraction following daclizumab beta treatment supports the idea that the CD56^{bright} NK cells could be limiting disease by eliminating T cells *in vivo* (10). One potential explanation for this surprising cytotoxicity mediated by CD56^{bright} NK cells is that they are not "conventional" CD56^{bright} NK cells, but instead fully mature CD56^{dim} NK cells that upregulated CD56 expression to the point where they were re-classified as CD56^{bright} NK cells. In fact, prior work demonstrates that IL-2 increases CD56 expression on NK cells (7). However, as visualized by UMAP, the expanded NK cells in daclizumab beta-treated individuals cluster in the same region as CD56^{bright} NK cells from placebo-treated individuals, not with CD56^{dim} NK cells (**Supplementary Figure S4**). Instead, the CD56^{bright} NK cells appear to have acquired key characteristics that could enhance their cytolytic activity with daclizumab beta treatment, including enhanced expression of Perforin and CD16. Further, the daclizumab beta-induced CD56^{bright} NK cells express other markers of maturity, including KIR2DL3, Siglec-7, CD38, and CD8. Together, these data are consistent with the idea that the CD56^{bright} NK cells induced by daclizumab beta treatment are activated and have acquired sufficient maturity to be cytolytic.

Prior studies have indicated that NK cells may play a role in MS pathogenesis in the absence of drug treatment. Two groups have reported that CD56^{bright} NK cells are present in the CNS in MS lesions (39, 40), suggesting they may play a role in eliminating activated CD4^{+} T cells in brain lesions. Martinez-Rodriguez et al. reported that CMV infection, which drives an expansion of mature, "adaptive" NKG2C-expressing NK cells, is associated with a lower risk of disease progression in MS (41). The CD56^{bright} NK cells observed after daclizumab beta do not resemble the mature NKG2C-expressing NK cells seen after CMV infection, but nonetheless could contribute to protection within the CNS.

Gross et al. report that CD56^{bright} NK cells are present in CNS lesions in MS, but that NK cells from MS patients are deficient in killing autologous, activated CD4 + T cells (40). This defect is attributed to poor expression of DNAM-1 on NK cells and its ligand, CD155 on CD4^{+} T cells in the setting of MS (40), and is consistent with studies revealing that DNAM-1 polymorphisms may play a role in susceptibility to MS (42). Independently, CD56^{bright} NK cells have been reported to mediate killing through NKG2D, TRAIL, and LFA-1 expression (43). In our study of peripheral blood NK cells, DNAM-1 expression was predictive of baseline samples, indicating that DNAM-1 expression is not increased by daclizumab beta treatment. However, it is important to note that our study characterized peripheral blood NK cells, and the prior studies showing DNAM-1-mediated killing were all focused on NK cells in the CNS.

Several studies suggest that NK cells may be deficient in the setting of MS (44–50), providing hope that enhancing their frequency and/or function could improve outcomes. However, some studies have not demonstrated a defect in NK cell numbers in MS subjects (40, 51). Another study suggests that 'regulatory' NK cells may be more important in disease pathogenesis (52, 54). A limitation of our study was that our control group of healthy controls was collected independently of the SELECT and DECIDE trials. For comparison of CD56^{bright} frequencies, we used local healthy blood bank control subjects, but the potential for batch effects based on collection of blood samples at different times and with different methods (e.g., CPT tubes vs. heparin tubes), precludes our ability to compare NK cells between RMS subjects and healthy controls without concern for batch effects.

There are several limitations to our study. The first is that the sample size is quite modest, leading to reduced power to find differences in this high-dimensional analysis, particularly in

the cross-sectional comparison between daclizumab beta-treated and placebo-treated individuals. Second, as discussed above, we did not have a healthy control group to which we could directly compare NK cells in RMS patients without concern for batch effects. Third, we did not perform functional assessments, so cannot confirm that the enhanced expression of killing and activation markers on $CD56^{bright}$ NK cells in fact drives better killing of autologous, activated $CD4^+$ T cells. It is important to note that enhanced killing of $CD4^+$ T cells was demonstrated previously in daclizumab beta treated subjects (10, 20).

Overall, these data demonstrate that significant changes occur in NK cells in response to the increased IL-2 availability induced by daclizumab beta treatment. These data extend prior findings indicating that daclizumab beta treatment increases the frequency of the $CD56^{bright}$ population, which is not due to an increase of a specific subset of $CD56^{bright}$ NK cells. NK receptor expression is broadly altered across $CD56^{bright}$ NK cells, highlighting the unique phenotypic features of these expanded NK cells. The high expression of activation markers, activating receptors, and perforin could enhance the ability of these $CD56^{bright}$ NK cells to control RMS through cytolytic activity or other immunoregulatory functions. The deep profiling of NK cells performed here, including in the placebo group, can also serve as a reference for future studies of NK cell phenotype in the setting of RMS. While daclizumab beta was voluntarily withdrawn from the market due to serious adverse events, it is notable that several other medications used to treat RMS, including natalizumab, fingolimod, glatiramer acetate, or beta interferon, are also associated with expanded $CD56^{bright}$ NK cells in the setting of clinical response (53). Thus, in order to improve treatment for RMS, it will be critical in future studies to determine whether specific features of NK cells are associated with clinical response or serious adverse events.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author. Our data is publicly available in flow repository (http://flowrepository.org/id/FR-FCM-Z2D6).

ETHICS STATEMENT

This study used samples from two clinical trials: NCT00390221 and NCT01064401. We received de-identified patient data and blood samples. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CB and JF conceptualized this study. TR and CB designed the experiments. TR and EV conducted the experiments. TR, LS, and NZ analyzed the data with statistical analysis input from CS, A-MF, and SH. LS, TR, and CB wrote the manuscript. All authors contributed to the revision of the manuscript.

FUNDING

This study was funded by the Biogen Idec.

ACKNOWLEDGMENTS

We thank Holden Maecker and Michael Leipold at the Human Immune Monitoring Core (HIMC) at Stanford University for use of their Helios machines and for their technical expertise. We also thank Wanda Castro-Borrero and Timothy Zheng at Biogen for their help compiling patient data and selecting samples for our study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00714/full#supplementary-material

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Conflict of Interest: CB received funding from Biogen to perform this study. JF was employed by Biogen Idec when the study was initiated and is currently employed by Sangamo Therapeutics. Biogen played no role in the analysis or interpretation of data.

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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REVIEW
published: 12 May 2020
doi: 10.3389/fimmu.2020.00812

Deciphering Natural Killer Cell Homeostasis

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Natural killer (NK) cells have a central role within the innate immune system, eliminating virally infected, foreign and transformed cells through their natural cytotoxic capacity. Release of their cytotoxic granules is tightly controlled through the balance of a large repertoire of inhibitory and activating receptors, and it is the unique combination of these receptors expressed by individual cells that confers immense diversity both in phenotype and functionality. The diverse, yet unique, NK cell repertoire within an individual is surprisingly stable over time considering the constant renewal of these cells at steady state. Here we give an overview of NK cell differentiation and discuss metabolic requirements, intra-lineage plasticity and transcriptional reprogramming during IL-15-driven homeostatic proliferation. New insights into the regulation of NK cell differentiation and homeostasis could pave the way for the successful implementation of NK cell-based immunotherapy against cancer.

Keywords: natural killer cells (NK cells), IL-15, mTOR, homeostasis, NK cell differentiation

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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: *28 January 2020*
Accepted: *08 April 2020*
Published: *12 May 2020*

Citation:
Pfefferle A, Jacobs B, Haroun-Izquierdo A, Kveberg L, Sohlberg E and Malmberg K-J (2020) Deciphering Natural Killer Cell Homeostasis. Front. Immunol. 11:812. doi: 10.3389/fimmu.2020.00812

NK CELL DEVELOPMENT

Natural killer (NK) cells are granular lymphocytes able to unleash stored cytotoxic potential to kill foreign, transformed or infected cells. Compared to other cytotoxic cells, NK cells are not restricted by the need for prior sensitization and can further orchestrate the early phase of the adaptive immune response. NK cells are found in significant numbers in blood, bone marrow, liver, lymphoid organs, lung, and uterus (1) and develop from common lymphoid progenitors in the bone marrow (2). Identification of NK cell precursors outside the bone marrow, namely fetal thymocytes ($CD34^+CD3^-CD4^-CD8^-$) and fetal liver cells ($CD34^+CD38^+$) suggests that NK development is not restricted to the bone marrow (3–5). Commitment to the NK cell lineage requires the transcription factors ID2 and E4BP4 along with IL-15 signaling (6–11). The search for an NK-restricted precursor identified $CD34^+CD38^+CD45RA^+CD7^+CD10^+CD123^-CD127^-$ cells which can give rise to T-bet$^+$ and Eomes$^+$ NK cells, two transcription factors central for NK cell maturation in mice (12, 13). Expression of T-bet and Eomes induces CD122 (encoded by *IL2RB*) expression on NK cells, a component of both the IL-2 and IL-15 receptor allowing for survival and effector function signaling to occur (12, 14). Although NK cells belong to the innate immune system, many aspects of T cell biology share a striking similarity with NK cells (15).

NK CELL DIFFERENTIATION AND FUNCTIONAL SPECIALIZATION

In humans, NK cells are characterized as $CD56^+CD3^-$ cells. They can be broadly divided into $CD56^{bright}$ and $CD56^{dim}$ subpopulations based on clear functional and phenotypic differences

(16–18). CD56^{bright} NK cells are highly responsive to cytokine priming and fulfill an immunomodulatory role. Expression of CCR7, CD62L, CXCR3, CCR5, CCR2, and CXCR4 allows CD56^{bright} cells to home to secondary lymphoid tissues, the liver, skin and bone marrow, where they represent the dominant NK cell subset (1, 19–22). Conversely, cytotoxic CD56^{dim} NK cells, which prioritize activating and inhibitory receptor input, mainly express CX3CR1 and CXCR1 (22), and account for the majority of peripheral blood NK cells (23).

CD56^{bright} NK cells have been suggested as precursors of CD56^{dim} NK cells based on combinatorial approaches including transcriptional studies (24–27). CD56^{bright} NK cells can acquire CD16 expression, effectively transitioning into CD56^{dim} NK cells (18) and CD16^{+}CD56^{bright} NK cells exist as functional intermediates (28). Furthermore, CD56^{bright} NK cells are the first lymphocyte population to reconstitute after stem cell transplantation, with CD16 acquisition, decreased surface expression of CD56 and cytotoxic effector functions following at a later time point (29–31). Conversely, in response to cytokine stimulation CD56^{dim} NK cells can adopt a "bright-like" phenotype via upregulation of CD56 (32). CD56^{bright} NK cells also have longer telomers compared to CD56^{dim} NK cells, evidence for having undergone fewer cell divisions (18), and have an increased proliferative capacity compared to CD56^{dim} NK cells (33).

Within the CD56^{dim} NK cell population, further distinctions of individual subsets based on phenotypic and functional characteristics can be made (**Figure 1**) (34). Cells expressing the inhibitory receptor NKG2A are found on the immature end of the spectrum, whereas acquisition of killer cell immunoglobulin like receptors (KIR) gives rise to more differentiated educated and uneducated NK cells with varying functional potential (35). The inhibitory signal strength between self-MHC and NKG2A and KIR fine-tunes the functional potential in a process termed education (35, 36). Expression of CD57, a carbohydrate epitope of unknown binding, is associated with terminal maturation, reduced proliferative capacity, and increased functional potential (37). At the mature end of the spectrum is a unique group of NK cells termed adaptive or memory-like NK cells (38, 39) that can be found in approximately 40% of cytomegalovirus (CMV) seropositive individuals. Adaptive NK cells are characterized by single self-KIR expression, epigenetic downregulation of intracellular signaling molecules and expression of the activating receptor NKG2C and CD57 (40–45). Functionally, adaptive NK cells exhibit increased ADCC activity compared to their non-adaptive counterpart. Although the combination of NKG2A, KIR and CD57 expression is commonly used to define NK cell subsets in humans, this is a simplified model considering that up to 100,000 unique subsets exist within healthy individuals (46).

Transcriptional Regulation of Human NK Cell Differentiation

Recently, several studies have shed light on the transcriptional regulation of NK cell differentiation. Mouse studies identified the importance of T-bet and Eomes in the differentiation step from immature CD27^{+}CD11b^{-} to mature CD27^{-}CD11b^{+} NK cells[22], as well as the role of ZBTB32, IRF2, and IKZF3 in NK cell differentiation (47–49). Bulk sequencing, combined with ChIP sequencing, of human CD56^{bright} and CD56^{dim} NK cells identified the TCF1-LEF-MYC axis within the CD56^{bright} population and the PRDM1-MAF-ZEB2 axis within CD56^{dim} NK cells (50). These transcription factor controlled regulatory schemes within effector cells (CD56^{dim} NK cells) and proliferative precursor cells (CD56^{bright} NK cells) dictated their functional programs as well as localization and trafficking. Expression of BACH2 in CD56^{bright} NK cells repressed BLIMP1 expression while ZEB2 expression in CD56^{dim} NK cells repressed TCF1 expression. The first single-cell RNA sequencing (scRNA seq) study in human NK cells was focused on characterizing the heterogeneity within peripheral blood and organs in both mice and humans (51), without detailing the gene regulatory circuit involved in NK cell differentiation. A recent study from our group (52) set out to delineate the temporal transcriptional regulation of human NK cell differentiation with the aid of scRNA seq. Confirming previous phenotypic and functional studies, we identified two main transcriptional islands, which corresponded to the CD56^{bright} and CD56^{dim} NK cell populations. Intriguingly, they were connected by a narrow bridge which, based on RNA velocity analysis (53), identified a transition from the CD56^{bright} to CD56^{dim} island. This gradual transition between the two main subsets was further corroborated by mapping a confined set of gene trends along pseudotime using Palantir (54).

Formation of the Functional Template for Education

NK cell education is the process whereby NK cells are functionally tuned via inhibitory interactions mediated between self-MHC and KIR or NKG2A. This is further fine-tuned by the signal strength determined by the number of inhibitory interactions (35, 36). As NK cells do not undergo positive or negative selection, it was initially assumed that they would express a minimum of one inhibitory receptor in order to maintain tolerance to self (55). However, the presence of NKG2A^{-}KIR^{-} cells and evidence of completely stochastic KIR repertoires in the developing immune system (56–59) suggested that alternative mechanisms are in play to ensure tolerance to self. Indeed, NK cells that lack self-specific inhibitory receptors circulate in a hypo-responsive state (56, 60, 61). Furthermore, NK cells have the ability to undergo re-education after transfer from one MHC class I environment to another, further validating the need for sustained inhibitory interactions in order to retain functionality (62, 63).

Despite education being a dynamic process that forms an important cornerstone in NK cell functionality, the intracellular mechanism underlying education remained elusive until recently. Multiple models have been proposed, including the arming, the disarming and the rheostat model without a general consensus being reached (35, 64, 65). Discriminating between educated and uneducated NK cells required a functional readout or sequencing of the HLA genes, as no phenotypic readout existed. Recent work from our lab identified granzyme

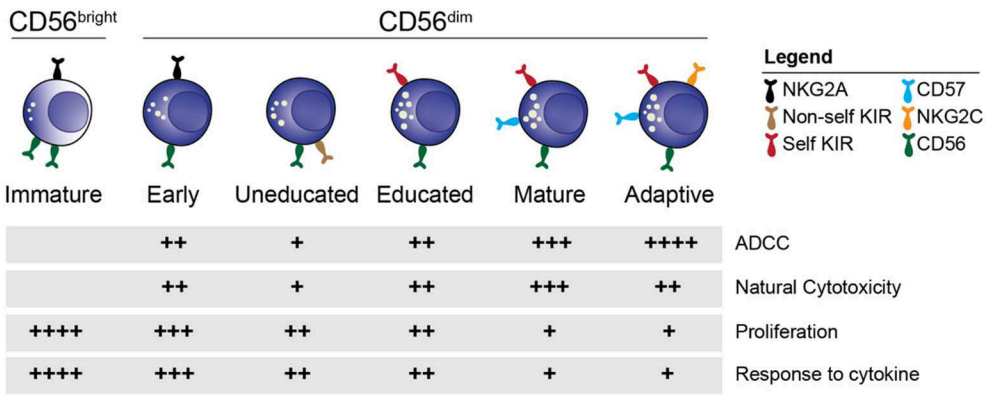

FIGURE 1 | NK cell subsets. Overview of the distinct stages of NK cell differentiation based on phenotypic and functional properties.

B retention as a sensitive and specific phenotypic readout for education in resting NK cells, putting the core cytolytic machinery itself in the spotlight in the search for an underlying mechanism behind NK cell education (66). Transcriptionally, educated NK cells were identical to uneducated NK cells, but accumulated granzyme B in dense-core secretory lysosomes located close to the centrosome. After target cell interaction, these large granules containing granzyme B were released, in line with increased cytotoxicity compared to uneducated cells lacking these particular granules. Pharmacological inhibition of the protein kinase PIKfyve and genetic silencing of its downstream target, the lysosome-specific calcium channel TRPML1, suggested a model where unopposed activating receptor input leads to remodeling of the lysosomal compartment and loss of dense-core secretory lysosomes in cells that lack self-specific receptors. Downstream of such morphological changes, signaling from acidic calcium stores may fine-tune the cell's functional potential through inter-organelle communication with the endoplasmic reticulum.

Our recent scRNA-seq study (52) identified a gradual increase in expression of effector molecules and genes involved in lysosomal function within the $CD56^{dim}$ population. Furthermore, genes important for vesicle formation and trafficking, such as *RAB27A*, were higher expressed within the $CD56^{dim}$ NK cell subset. Mutations in *RAB27A* cause Griscelli syndrome type 2, resulting in a degranulation defect (67), as Rab27a is recruited to the lytic granules by LFA-1 stimulation, aiding the granule in docking to the plasma membrane (68, 69). Hence, $CD56^{dim}$ NK cells are poised for modulation of the lysosomal compartment mediated via inhibitory and activating receptor input received at the cell surface, resulting in fine tuning of their functionality.

NK CELL HOMEOSTASIS

IL-15 is the main cytokine required for NK cell development, but also for survival, proliferation, metabolism and functionality (70). The importance of IL-15 signaling in NK cell development is best observed through mutations in the receptor components and downstream signaling molecules which, together, present as immunodeficiencies characterized by a lack of NK cells (71–74). Immune cells, including DCs, monocytes and other non-hematopoietic cells trans-present IL-15 on the IL-15Rα chain, which binds to the heterodimer consisting of IL-2Rβ (CD122) and the common γ-chain (CD132) found on the NK cell surface. Downstream signaling is mediated via JAK1/3, allowing for recruitment and activation of the transcription factor STAT5, a survival signal for NK cells (73). A downstream target of STAT5 is the cytokine induced SH2-containing protein (CIS, encoded by *CISH*), which functions as a negative feedback loop by inhibiting the upstream JAK1 (75). $Cish^{-/-}$ knockout mice presented increased anti-tumor activity and proliferative capacity as a result of being hyper-responsive to IL-15 signaling (75). Mathematical modeling has been implemented in an attempt to better understand the impact of IL-15 receptor signaling on proliferation. The model predicted that increasing IL-15Rα expression on the cell surface will accelerate the formation of IL-15/IL-15R complexes, particularly at low IL-15 concentrations, until a saturation level is reached and no further proliferative response can be achieved (76). These results highlight the broad and central role for IL-15 in NK cell development, differentiation, homeostasis and priming of effector function.

Quorum sensing, which is a form of chemical communication in bacteria whereby sensing of an autoinducer is used to synchronize group behavior, has recently been proposed to also control immune cell homeostasis (77). For example, colony stimulating factor 1 (CSF1) produced by the surrounding stromal cells is proposed to function as the autoinducer in macrophages, whereby uptake of CSF1 controls the rate of proliferation and survival to maintain a steady population density at homeostasis (78). In T cells, IL-2 replaces CSF1 as the autoinducer, which together with IL-6 has been suggested to also modulate the differentiation from an effector T cell to a central memory T cell (79, 80). The logical autoinducer counterpart in NK cells is IL-15. The threshold for IL-15 induced proliferation is subset-dependent, as observed by the onset of proliferation

across the maturation spectrum. This is in line with the concept of quorum sensing, whereby the level of IL-15 in the microenvironment dictates the degree of proliferation and overall size of the population.

The IL-15 mTOR Axis

The unique role of IL-15 in NK cell biology is largely attributed to the IL-15 mammalian target of rapamycin (mTOR) signaling axis and the metabolic regulation of NK cells. Mouse studies identified a dose-dependent downstream signaling pathway, where high dose IL-15 activated the mammalian target of rapamycin (mTOR) as well as STAT5. mTOR, a serine/threonine kinase consisting of the two complexes mTORC1 and mTORC2, is a master regulator in cells. mTORC1 senses for nutrients in the microenvironment to control metabolism while mTORC2 is involved in controlling the cytoskeletal organization of the cell (81–83). Metabolic reprogramming due to environmental cues has been identified as a key regulatory mechanism behind immune cell differentiation and function in NK cells and other immune cells (81–85). In mice, increased cytokine priming led to metabolic reprogramming, with increased metabolic activity, and a switch in energy source from oxidative phosphorylation (OXPHOS) to glycolysis. An increase in metabolism allowed for IFNγ and granzyme B production, conferring increased functionality which could be reversed through the use of rapamycin, an mTOR inhibitor (81). Viral infection can also activate mTOR leading to metabolic reprogramming, as observed in murine CMV infection[122]. It is possible that in a tumor setting, a lack of available glucose due to high glycolytic activity by the tumor cells could lead to functional inhibition due to lack of mTOR activation (81, 86).

In addition to mediating NK cell functionality via modulation of the cellular metabolism, mTOR may serve as a functional rheostat during NK cell education (82, 87). Educated NK cells exhibited higher basal mTOR activity, which was further increased upon activating receptor ligation and also correlated with the number of inhibitory receptors expressed (87). Expression of SHP-1, a phosphatase required to convert inhibitory receptor input into functional responsiveness, was required for increased mTOR activity in educated cells (88). Conversely, continuous activating receptor input in the absence of inhibitor input dampened mTOR activity. Although education is not transcriptionally regulated in human NK cells, mTOR activity is dependent on its localization to the lysosomal compartment which in turn can be negatively regulated by TRPML1 (89, 90). The connection between lysosomal remodeling during education and metabolic regulation through mTOR is an unexplored area in NK cell biology that warrants further investigation.

NK Cell Repertoire Dynamics and Intra-Lineage Plasticity

At the donor level, the NK cell repertoire is vastly diverse and unique (46). However, once the NK cell repertoire has been fully formed and in some cases further shaped by pathogens such as CMV, it is well-maintained over time considering the rather rapid turnover of the cells (44, 91). Proliferation therefore plays an important role in replenishing the NK cell pool at steady-state and in maintaining a stable repertoire. NK cell proliferation has mainly been examined in viral or disease settings, where it is associated with rapid cell turnover resulting in subset skewing toward immature NK cells with higher proliferative potential (92–94). In a recent study we asked the question of whether or not stable NK cell repertoires are maintained under homeostatic proliferation (95). We hypothesized that the observed stability was either the result of self-renewal from an immature pool of progenitor cells followed by differentiation, or the result of intra-lineage plasticity (**BOX 1**). This process has been observed in other immune cells (96, 97) but NK cell plasticity has largely remained unexplored (98).

BOX 1 | Cellular plasticity

Plasticity refers to phenotypic and functional changes occurring within populations of cells. Intra-lineage plasticity, also known as functional plasticity, refers to cells of a given lineage adapting to their surroundings in response to cytokine or receptor input which is translated into transcriptional changes resulting in an altered phenotype and modified functionality. An example of this is macrophages transition between an M1 and M2 phenotype, T cells transitioning from Th to Treg phenotype or ILC subsets transitioning between ILC1- 3 phenotypes.

We developed a simplified model that induced a linear onset of IL-15 induced proliferation with maximal retention of NK cell subsets (based on NKG2A, KIR and CD57) to mimic homeostatic conditions. We observed subset-specific proliferation kinetics, which correlated with mTOR activation. IL-15-induced mTORC1 upregulation prior to proliferation onset could predict downstream proliferation 3 days later at both the donor and subset level. Repeated sampling of the same blood donors over time confirmed stable NK cell repertoires, but also an intrinsic metabolic set point determining the level of mTOR activation in response to IL-15 stimulation.

Despite subset-specific proliferation kinetics, the actual subset frequencies at the population level remained largely stable, suggesting that the repertoires were maintained through intra-lineage plasticity. Indeed, sorting individual NK cell subsets prior to proliferation revealed a surprising degree of cellular plasticity in both immature and mature subsets. Acquisition of NKG2A in sorted KIR+ NK cells was associated with increased proliferative potential and decreased functionality, while the reverse was true for CD57 acquisition by the same subset. Surprisingly, a fraction of $CD57^+$ NK cells lost CD57 expression, acquired NKG2A and started to proliferate, suggesting they may not be terminally differentiated. Rapidly cycling educated NK cells underwent transcriptional reprogramming, resulting in a more immature signature, while slowly cycling educated NK cells acquired a more mature signature when compared with baseline subsets.

Our simplified *in vitro* homeostatic NK cell proliferation model allowed us to examine the central role IL-15 plays in maintaining NK cell homeostasis (**Figure 2**). CD57 expression was associated with a negative influence on mTOR activation and proliferation but enhanced functional potential. Although it

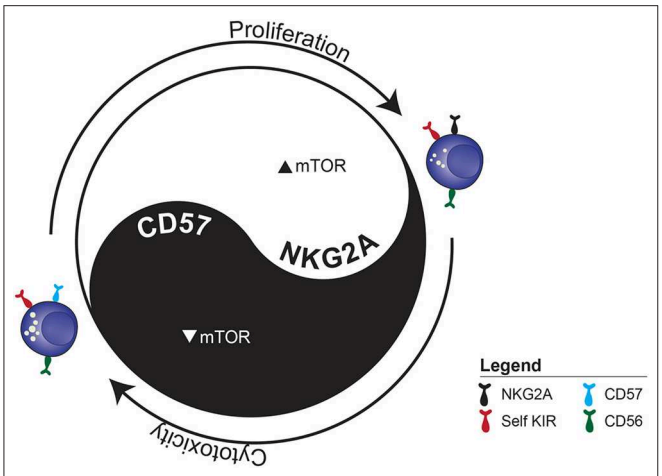

FIGURE 2 | The functional dichotomy between proliferation and cytotoxicity observed during IL-15-induced homeostatic proliferation. The example illustrates the two distinct fates of sorted $CD56^{dim}$ KIR^{+} $NKG2A^{-}CD57^{-}$ NK cell depending on whether they acquire NKG2A or CD57.

is used as a main marker for subset discrimination in NK cells, the function of CD57 remains unknown (37, 99). In neural cells, CD57 has mainly been associated with adhesion proteins, while binding to the IL-6 receptor has also been proposed (100). It would be interesting to further delineate whether CD57 plays a functional role, or if it is simply a surrogate marker for other ongoing cellular modifications.

Due to their differential mTOR activation profile, it is tempting to speculate that $NKG2A^{+}$ and $CD57^{+}$ cells display distinct metabolic profiles. Metabolic reprogramming is responsible for the differentiation of naïve T cells into active effector and later into memory T cells (101–106). The transition of naïve into effector T cells depends on the upregulation of glycolysis and the TCA cycle to provide material for *de-novo* synthesis of proteins, nucleic acids and lipids, whereas formation of memory T cells rely on OXPHOS and fatty acid oxidation (FAO) (107). Such differential use of metabolic programs has also been observed in Th cell subsets (108). In addition, T cell memory formation is influenced through the reorganization of mitochondrial content (109). Interestingly, survival of memory-like NK cells in mice upon CMV infection is dependent on sufficient degradation of dysfunctional mitochondria via mitophagy upon virus clearance (110).

Differences in terms of proliferation speed, phenotype, and functionality between homeostatic and spontaneous proliferation have been investigated in murine T cells (111–113). Spontaneous proliferation, occurring in severely lymphopenic mice, was characterized by a rapid onset of cell division that was cytokine-independent. Homeostatic proliferation, on the other hand, occurred in mildly lymphopenic mice at a slower division rate and required both cytokine and T cell receptor (TCR) stimulation. The proliferation-induced phenotype was reverted after removal of the proliferation cues and cytotoxic capacity of $CD8^{+}$ T cells was lost during the initial phase of intense proliferation (111–113).

Considering the asymmetric PI3K and mTOR activity post-cell division observed in T cells and its role in controlling differentiation fate and the functional dichotomy in proliferating vs. arrested NK cells (101–106), it would be of interest to do microscopy studies of cellular division or functional interactions with target cells. Based on the induced transcriptional signature in rapidly cycling NK cells, which included both RNA-modifying metabolic genes and actin filament organization genes (95), the loss of functionality in rapidly cycling cells may be due to underlying deficits at the immune synapse. Conjugate formation experiments combined with F-actin staining at the site of the immune synapse would further shed light on the loss of functionality observed during intense homeostatic proliferation.

NK Cell Homeostasis *in vivo*

Given the essential role of IL-15 on NK cells, stimulation of IL-15 signaling pathways has been explored in clinical settings (114–120). In this regard, three main strategies have been pursued; using recombinant-human IL-15 (116) generated by *E. coli*, an IL-15 superagonist, ALT-803 (114, 115) and transfection of an IL-15 containing CAR construct (121, 122). These have been thus far tested in Phase I and II clinical trials, with recombinant-human IL-15 and ALT-803 both showing moderate success in inducing NK cell proliferation and activation *in vivo* and in particular cases inducing disease remission. A limitation of this approach has been the induction of some minor side effects relating to an increased inflammatory environment. Subcutaneous delivery of the compounds has resulted in a partial reduction of these side effects (115). Recent pre-clinical studies have highlighted the potential of combination therapy using this IL-15 signaling stimulation and other immunotherapeutic agents such as monoclonal antibodies or check-point blockade (118, 119). Reflecting this, there are currently more than 100 registered clinical trials exploring IL-15 stimulation via either of these two methods in a series of different cancer settings (www.clinicaltrials.gov). *In vitro*, transfection with an IL-15 containing CAR construct sustained autonomous NK cell growth over 42 days and increased systemic IL-15 serum levels were observed in mouse studies (121). However, in 11 patients treated in a Phase I/II trial, the detection of infused CAR^{+} NK cells by flow cytometry was limited to the first 2 weeks post infusion (122).

In the setting of stem cell transplantation, NK cells are the first lymphocyte population that can be detected following engraftment (123). Their ability to mediate graft-vs.-leukemia (GVL) effects is vital for elimination of residual disease, as increased number of NK cells after transplantation result in better treatment outcome (124, 125). Insights into the specificity of NK cell alloreactivity, determined by specific combinations of KIR and HLA, paved the way for the ground-breaking discovery of a potential role of NK cells in mediating GVL in haploidentical HSCT against AML (126, 127). Studies aiming at harnessing NK cell alloreactivity in the context of HSCT have recently been reviewed (128, 129). The indication that NK cells may deliver a potent GVL effect in the setting of HSCT inspired the whole NK cell community to develop adoptive NK cell therapy based on transfer of "KIR ligand mismatched" NK cells across HLA barriers to promote missing

self-recognition. Whereas many studies did not find a beneficial effect of genetic KIR ligand mismatch, calculation of the functional dose of KIR ligand mismatched NK cells was associated with less relapse after NK cell therapy against AML (130–132).

A recent series of Phase III clinical trials have brought mTOR inhibition to the forefront of transplantation (117, 120). In both of these studies a series of patients received Sirolimus, an alternate name for rapamycin, as a prophylactic against *graft vs. host disease* (GVHD). Sandmaier et al. reported that inclusion of Sirolimus to the standard calcineurin inhibitor treatment showed decreased incidence of grade 2–4 acute GVHD. Similarly, increased overall survival, and progression-free survival, as well as decreased non-relapse related mortality was observed in the sirolimus treated group. Due to the clear improved benefit of Sirolimus treatment, the trial carried out by Sandmaier et al. was terminated prior to complete patient recruitment.

On the other hand, a parallel study by Gooptu et al. did not report significant differences compared to standard treatment regarding GVHD incidence, progression free survival nor overall survival (117). This discrepancy may be due to the differences in standard treatment and dosage of Sirolimus between the two studies. In the latter study, the immune reconstitution was evaluated at a series of timepoints up to 24 months. Sirolimus treatment led to a decreased lymphocyte cell count in the first 3 months of treatment, and an increased ratio of regulatory T cells to $CD8^+$ T cells throughout the first 6 months of treatment. Lower NK cell counts were observed in the first month following Sirolimus treatment, although this recovered to similar levels compared to standard treatment by the 3rd month. Given the phenotypic and functional heterogeneity of NK cell subsets and the critical role of mTOR and IL-15 signaling in driving NK cell plasticity, it would be of great interest to further evaluate the precise composition of the NK cell compartment during such therapies.

Cytokine-Based Expansion Protocols for NK Cell Therapy

There are several up to date and comprehensive reviews describing the prospects of using various preparations of NK cells in cell-based immunotherapy (133, 134). These include strategies based on autologous and allogeneic NK cells that have been stimulated by various cytokines alone or in combination with irradiated feeder cells expressing membrane bound cytokines such as IL-21 or IL-15 (121, 135–138). Therefore, we will focus on a general discussion on how these protocols may drive dramatic phenotypic and functional changes to the NK cell repertoire (34, 95). To expand large numbers of cells for multi-dosing schemes, many strategies are based on supra-physiological levels of cytokines, including any combination of IL-2, IL-15, IL-12, and IL-18 (139, 140). However, this can result in severe and acute cytokine deprivation post-infusion as the cells become "addicted" to cytokines (**BOX 2**). Severe side-effects (141, 142) prevent patients from being treated with the same cytokines and persistence is further limited through clearance of infused NK cells by host immunity.

BOX 2 | Cellular addiction

Cytokine priming results in intracellular signaling changes occurring within cells. Continuous stimulation with non-physiological cytokine levels can result in an altered cellular state, which requires further cytokine stimulation to support survival. This can be referred to as cytokine-dependence or addiction, whereby cytokine withdrawal can lead to detrimental consequences to the cell.

The Balance Between Pro- and Anti-apoptotic Molecules During IL-15 Driven Proliferation

We recently set out to characterize the mechanism behind IL-15 addiction and withdrawal in expanded NK cells. NK cells exhibited a dose-dependent IL-15 addiction, where high-doses induced rapid proliferation, skewing toward a naïve phenotype, and subsequent crash upon cytokine withdrawal (143). Timing of IL-15 dosing is crucial for NK cell survival and effector function as chronic high-dose IL-15 stimulation leads to decreased viability of NK cells with reduced respiratory spare capacity and functional activity (144).

Numerous pro- and anti-apoptotic genes make up the apoptosis network balancing the outcome of the cell during various types of stimulations (70, 145–147). Within resting NK cells, BCL-2 has been identified as an important anti-apoptotic protein which can be further upregulated through IL-15 stimulation, leading to downstream STAT5, but not mTOR activation (82, 148). In actively proliferating NK cells, MCL-1 expression is vital to maintain viability (149). BIM is a pro-apoptotic molecule and its downstream target BAX is directly inhibited by BCL-2 (147). In murine effector $CD8^+$ T cells, increased BIM levels are balanced by increased BCL-2 levels, expression of which dictates the amount of BIM that can be tolerated (150). Similarly, in murine NK cells, the BCL-2/BIM ratio was influenced by IL-15 stimulation and withdrawal, whereby changes in the ratio could render the cells sensitive to cell death (70, 150, 151). In line with these studies, we observed an IL-15 dose-dependent increase in BCL-2, MCL-1, and BIM expression. BCL-2 and MCL-1 were both crucial for survival in NK cells stimulated with high-dose IL-15 as shown through blocking experiments. Interestingly, rapidly cycling NK cells exhibited reduced BCL-2 levels compared to slowly or non-cycling NK cells during their expansion phase, in line with T cell proliferation studies (145).

After cytokine withdrawal, anti-apoptotic proteins decreased, and a potent apoptosis-inducing splice variant, BIM S, (152, 153) was preferentially upregulated in proliferating cells and remained highly expressed until 24 h after cytokine withdrawal. This severely altered the pro/anti-apoptotic ratio, exposing rapidly cycling cells to high levels of toxic BIM S within 24 h after cytokine withdrawal (**Figure 3**). The importance of these

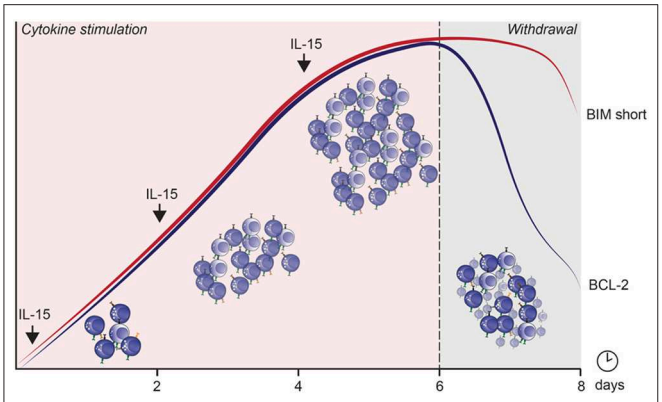

FIGURE 3 | The mechanism by which apoptosis is induced in cycling NK cells after IL-15 induced cytokine dependence and subsequent withdrawal. The curves represent expression of BIM short (red) and BCL-2 (blue) over culture time.

apoptotic proteins in IL-15 mediated survival and function has also been observed in murine studies (70). How homeostatic and induced proliferation affects NK cell cytotoxicity, and how apoptosis is induced in cycling cells upon cytokine withdrawal, has potentially important implications for current cell therapy expansion protocols.

CONCLUDING REMARKS

NK cells circulate in a pre-primed state full of effector molecules, such as granzyme B and perforin, and have a natural ability to kill cancer cells. Based on their cytotoxic capacity they hold great potential in the clinic as a cancer treatment, made evident by the number of ongoing clinical trials. However, to date most completed and ongoing clinical trials are based on the transfer of cytokine-activated polyclonal NK cell populations from donors with very variable NK cell repertoires. To fully harness the clinical potential of NK cells, future trials need to be founded on recent breakthroughs in our understanding of the vast repertoire diversity and the fundamental mechanisms that govern the intrinsic functional potential of distinct NK cell subsets at steady state and following cytokine stimulation.

Understanding how NK cells repertoires are formed and maintained over time, and what functional roles individual cell subsets perform in a homeostatic setting, are important to improve current therapies and develop future treatment strategies. Generating an "ideal" NK cell product for treatment could involve modifying existing cells to improve functionality, expanding highly cytotoxic subsets while ensuring retention of functionality or designing a "synthetic" genetically engineered killer cell from induced pluripotent stem cells.

Furthermore, we need to understand how NK cells are functionally shaped by their surroundings. The soluble factors, metabolic cues, fluctuations in oxygen levels, and pH encountered by an NK cell in the tumor microenvironment are very different from steady state and their impact on NK cell function and persistence cannot be underestimated. This is particularly difficult to study in the human setting, with mouse models only providing an approximation.

By understanding the basic biology, from development to differentiation to receptor and cytokine input, we will build up our tool kit that can then be applied to design and develop effective treatment strategies. After all, the "natural" killing capacity is there, we just need to understand how to harness it.

AUTHOR CONTRIBUTIONS

AP made the figures. AP and K-JM wrote the paper. LK, AH-I, BJ and ES contributed to the writing of the paper.

ACKNOWLEDGMENTS

This review including figures is partly based on the introduction of the PhD thesis by Dr Aline Pfefferle Dynamics of natural killer cell homeostasis: implications for cell-based cancer immunotherapy defended at the Karolinska Institute June 5, 2019.

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Conflict of Interest: K-JM is a consultant at Fate 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.

frontiers in Immunology

REVIEW
published: 08 July 2020
doi: 10.3389/fimmu.2020.00813

NK Cell Development in Times of Innate Lymphoid Cell Diversity

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After being described in the 1970s as cytotoxic cells that do not require MHC-dependent pre-activation, natural killer (NK) cells remained the sole member of innate lymphocytes for decades until lymphoid tissue-inducer cells in the 1990s and helper-like innate lymphoid lineages from 2008 onward completed the picture of innate lymphoid cell (ILC) diversity. Since some of the ILC members, such as ILC1s and CCR6^{-} ILC3s, share specific markers previously used to identify NK cells, these findings provoked the question of how to delineate the development of NK cell and helper-like ILCs and how to properly identify and genetically interfere with NK cells. The description of eomesodermin (EOMES) as a lineage-specifying transcription factor of NK cells provided a candidate that may serve as a selective marker for the genetic targeting and identification of NK cells. Unlike helper-like ILCs, NK cell activation is, to a large degree, regulated by the engagement of activating and inhibitory surface receptors. NK cell research has revealed some elegant mechanisms of immunosurveillance, coined "missing-self" and "induced-self" recognition, thus complementing "non-self recognition", which is predominantly utilized by adaptive lymphocytes and myeloid cells. Notably, the balance of activating and inhibitory signals perceived by surface receptors can be therapeutically harnessed for anti-tumor immunity mediated by NK cells. This review aims to summarize the similarities and the differences in development, function, localization, and phenotype of NK cells and helper-like ILCs, with the purpose to highlight the unique feature of NK cell development and regulation.

Keywords: NK cells, innate lymphoid cells, immune recognition, immune receptor, innate lymphocytes

OPEN ACCESS

Edited 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: *30 October 2019*
Accepted: *08 April 2020*
Published: *08 July 2020*

Citation:
Stokic-Trtica V, Diefenbach A and Klose CSN (2020) NK Cell Development in Times of Innate Lymphoid Cell Diversity. Front. Immunol. 11:813. doi: 10.3389/fimmu.2020.00813

INTRODUCTION

In the mid-70s of the last century, two groups independently reported the presence of small lymphocytes with non-MHC-restricted cytolytic activity against cells expressing tumor antigens in mice (1–4). Such "natural" killer (NK) cells, capable of cell-mediated, rapid cytotoxicity in a germline-encoded receptor-dependent fashion upon encountering of target cells, were observed in humans as well (5). NK cells remained the only subset of innate lymphocytes for two decades until an additional subset was discovered, which expressed the integrin $\alpha_4\beta_7$, lymphotoxin (LT)$\alpha_1\beta_2$, and lymphoid cytokine receptors. However, this newly described cell subset was giving rise to neither T-lymphocytes nor B-lymphocytes.

They were named lymphoid tissue-inducer (LTi) cells because they were among the first cells to infiltrate lymph node anlagen during embryogenesis and hence are instrumental for the development of most secondary lymphoid tissues (6).

Furthermore, from 2008 onwards, several groups reported the discovery of new types of non-T and non-B lymphocytes which, like NK cells and LTi cells, require the transcriptional regulator *inhibitor of DNA-binding 2* (ID2) and the common gamma chain (γ_c) of the cytokine interleukins (IL)-2, 4, 7, 9, 15, and 21 for their development and/or maintenance (7–21). These cells were termed "innate lymphoid cells" (ILCs), which constitute lineages of professional cytokine-producing cells that mirror T helper cells in the utilization of transcription factors (TFs) required to establish distinct patterns of lineage-specific cytokine production and effector functions. It became obvious that the different ILC populations resemble the functional diversity found in T helper cell subsets, thus establishing a complementary innate counterpart to T helper cells (22).

In connection with these findings of ILC diversity, a novel ILC nomenclature was proposed in 2013 and amended in 2018 (22, 23). In analogy to T cells, two principal subsets of ILCs can be distinguished: cytotoxic ILCs (i.e. conventional NK cells) and helper-like ILCs (i.e. ILC1, ILC2, and ILC3) (24, 25). The general division of NK cells and helper-like ILCs is supported by various findings. First, while there is a common progenitor to all innate lymphocytes, variably referred to as early innate lymphoid progenitor (EILP) (26) or innate lymphoid cell progenitor (ILCP) (27), a more restricted common helper-like innate lymphoid cell progenitor (CHILP) with reduced potential for helper-like ILC can only be found downstream of the bifurcation with the NK cell lineage. Second, all helper-like ILCs but not NK cells require GATA binding protein 3 (GATA-3) for their differentiation (28). Third, helper-like ILCs are remarkably tissue-resident cells, whereas NK cells are circulating cells (29–31). Finally, the use of inhibitory and activating receptors of the KIR and the Ly49 families was found in NK cells but not in ILCs. Thus, two principal lineages of innate lymphocytes exist: helper-like ILCs and cytotoxic ILCs.

In analogy to T cells, ILCs are divided into functional groups, based on TFs required for their development as well as their role in immune responses (22). NK cells are functionally important for immunity against tumors and intracellular pathogens *via* classical perforin-dependent, cell-mediated cytotoxicity and production of interferon-gamma (IFN-γ). ILC1s are an important source of IFN-γ and tumor necrosis factor (TNF) to trigger type 1 immune responses and limit intracellular infections. While NK cells and ILC1s are functionally both promoting type 1 immune responses, they are developmentally dependent on two evolutionary related T-box TFs: eomesodermin (EOMES) and T-box expressed in T cells (T-bet) (32). NK cells express both EOMES and T-bet, but their development is only strictly dependent on EOMES. NK cells develop in T-bet-deficient mice and have a relatively mild functional defect (16, 33, 34). In contrast, ILC1s express T-bet but not EOMES and do not develop in T-bet-deficient mice (21, 35, 36). ILC2s require GATA-3 and B-cell lymphoma/leukemia 11B (BCL11B) for development and produce type 2 cytokines, mostly IL-5, IL-9, and IL-13, as well as other effector molecules, such as amphiregulin, promoting worm expulsion and tissue remodeling (12–14, 17, 37–42). Group 3 ILCs include fetal LTi cells and can be further divided into two groups in adult mice based on CCR6 expression with different developmental requirements and effector mechanisms (43, 44). Both $CCR6^+$ ILC3s and $CCR6^-$ ILC3s are dependent on the TF RORγt and produce IL-22 to fortify the epithelial barrier against infections, damage, and genotoxic stress (45–51). $CCR6^+$ ILC3s also produce IL-17 and protect from fungal infections, whereas $CCR6^-$ ILC3s down-regulate RORγt and IL-22, up-regulate the TF T-bet. $CCR6^-$ ILC3s in addition acquire the capacity to produce IFN-γ and transform into ILC1-like cells (19, 44, 52–55).

Helper-like ILCs were reported as tissue-resident cells enriched at barrier surfaces and underrepresented in secondary lymphoid organs (29–31). In contrast, NK cells are patrolling lymphocytes, which express CD62L to migrate from blood to lymph nodes (21, 30, 56). As patrolling cells, immune recognition by NK cells is mediated by the interaction of immunoreceptors that scan target cells for the expression of their ligands. Therefore, the development and regulation of NK cells depend on the interaction of the immunoreceptors and their ligands. Although the expression of some immunoreceptors (e.g. KLRG1, PD-1) has been reported for helper-like ILCs as well, their activity seems to be predominantly regulated by soluble factors such as cytokines and neuronal factors (21, 35, 52, 57–59).

IMMUNE RECOGNITION STRATEGIES OF NK CELLS

The complexity of multicellular organisms demands essential immune recognition strategies to maintain their self-integrity in a hostile environment. Almost all organisms, from bacteria to higher animals, possess recognition systems that allow them to discriminate between self and non-self and possess effector mechanisms to defend themselves from an invasion of pathogens. The immune system of vertebrates consists of two arms: innate and adaptive. Recognition of non-self molecules is broadly used by both the innate and the adaptive immune system to protect the host from infections (60). However, although NK cells are capable of directly sensing non-self molecules, their development and activation are regulated to a large extent by the recognition of self molecules. Discrimination between self and non-self is mediated by an array of stimulatory and inhibitory immunoreceptors expressed by NK cells. They either recognize non-self structures directly (Ly49H, NKG2C/CD94) or indirectly *via* binding immune complexes to Fc receptors. Alternatively, they interact with self MHC I (Ly49s and KIR, "missing-self" recognition) or with ligands absent on healthy cells (NKG2D and NKp30, "induced-self" recognition). The regulation of NK cells, which relies on cell surface immunoreceptor–ligand interactions, is complemented by cytokines, such as type I interferons, IL-12, IL-15, and IL-18 (61–64).

Non-self Recognition

The recognition strategy of "microbial non-self" is approached differently from the innate and the adaptive immune system. The cellular components of the innate immune system express germline-encoded receptors, called pattern recognition receptors (PRRs), which come in two forms: transmembrane receptors and secreted receptors (60, 65). These molecules recognize conserved pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). The secreted PRRs lead to the opsonization of microbes and label them for destruction either by the complement system or by phagocytosis. PRRs expressed on the cell surface of innate immune cells, such as Toll-like receptors (TLRs), lead to the activation of immune signaling pathways, which trigger inflammatory or antimicrobial effector responses (66, 67).

Upon recognition of PAMPs and MAMPs on microbes by antigen-presenting cells (APCs), phagocytosis and processing of antigens in the lysosomal compartment of these cells are triggered. T cells express strictly antigen-specific T cell receptors (TCRs) that are generated by somatic genetic recombination, thereby providing a vast repertoire of specificities. TCRs recognize self and non-self peptides presented on APCs via MHC molecules, providing "signal 1" for T cell activation. However, TCR ligation by itself is not sufficient for efficient T cell activation. It requires a co-stimulatory signal ("signal 2"), e.g. provided by APC-expressed CD80 (B7-1) and CD86 (B7-2), ligands for the constitutively expressed CD28 receptor on T cells (68). CD80 and CD86 are not expressed by unstimulated APCs but are rapidly up-regulated following the encounter of MAMPs or PAMPs, providing an additional "quality control" for T cell responses. In addition, stimulated APCs produce cytokines and IFNs, which further enhance T cell responses ("signal 3"). In the case of naive $CD4^+$ T lymphocytes, distinct cytokines have been shown to drive the differentiation into one of three T helper (Th) subsets. IFN-γ and IL-12 are important for inducing Th1 cells, IL-4 for Th2 commitment, and TGF-β and IL-6 for Th17 cell differentiation (69–72).

TLR expression was also described on NK cells, but its contribution to NK cell activation remains unclear. While the direct activation of NK cells by TLR engagement has been reported for human NK cells (73), genetic data from mice demonstrated that TLR signaling to activate NK cells was cell-extrinsic *via* mononuclear phagocytes (62). While recognition of "non-self" *via* PRRs may not be central for NK cells, they express other families of receptors to directly recognize "non-self" molecules such as Ly49H in mice or NKG2C/CD94 in humans (74–76). Ly49H is a stimulatory receptor that recognizes the MHC-like protein m157 encoded by murine cytomegalovirus (MCMV), which is expressed in infected cells and confers host protection in C57BL/6 (B6) mice. It should be noted though that, in most inbred mouse strains other than B6, m157 binds to an inhibitory Ly49 receptor, leading to immune evasion. NK cells can also recognize non-self peptides in the context of non-classical MHC I molecules, very similar to the immune recognition strategy of T cells. For example, subsets of NK cells expressing the stimulatory receptor NKG2C/CD94 were shown to recognize the UL40 antigen of human cytomegalovirus presented in the context of the non-classical MHC-I molecule HLA-E, and this recognition activates NK cells (77). The different recognition strategies of immune cells are depicted in **Figure 1**.

Missing-Self Recognition

In 1981, Klas Kärre formulated the missing-self hypothesis (78). Missing-self recognition was conceived as the capacity of NK cells to attack cells that fail to express sufficient levels of class I MHC molecules. This concept was discovered while investigating the role of class I MHC molecules in NK cell and T cell responses to tumor cells (79, 80). Given the role that the class I MHC antigen presentation pathway plays in the revelation of virally infected cells to $CD8^+$ T cells, it is not surprising that many viruses have evolved mechanisms that interfere with this pathway, thereby binding $CD8^+$ T cells to virus-infected cells (81). The missing-self hypothesis predicted that NK cells express inhibitory class I MHC-specific receptors and that the down-regulation of MHC-I expression on virus-infected cells or tumors would unleash NK cells from inhibition. Years after postulating "missing-self recognition," various classes of inhibitory MHC class I-specific NK cell receptors were identified. Ly49 receptors in mice (82) and the structurally unrelated but functionally analogous KIR family of inhibitory receptors in humans (83, 84) directly interact with class I MHC molecules. Both human and mouse NK cells express the heterodimeric CD94/NKG2A receptor, which monitors class I MHC molecules by another mechanism. CD94/NKG2A recognizes a non-classical MHC class I molecule, HLA-E in humans and Qa-1b in mice, when loaded with peptides that are derived from the signal peptide of classical class I MHC proteins (85). The inhibitory receptors have an immune-receptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain. Upon ligand recognition, phosphorylation of the ITIM's tyrosine residue serves as a signal for recruitment of protein tyrosine phosphatases, SHP-1 and SHP-2, which inhibit cytotoxic activity by further dephosphorylating tyrosine residues that are critical for NK cell activation (86, 87).

Missing-self recognition does not require viral infection or a malignant transformation of target cells. Uninfected and untransformed cells can be lysed by NK cells, as demonstrated in NK cell-mediated rejection of F1 bone marrow grafts (88) and bone marrow of β2-microglobulin-deficient mice that do not express class I MHC on the cell surface (89). Since T cells are not capable of recognizing and killing cells that down-regulated class I MHC expression due to viral proteins that hijack their expression pathway, NK cells are able to compensate this immunological function *via* missing-self recognition of MHC-deficient target cells.

Induced-Self Recognition

Induced-self ligands of NK cell receptors are molecules that are absent or only at a very low level expressed on normal cells but up-regulated on infected cells, stressed cells, or tumor cells as a marker of "abnormal self." Induced-self ligands bind to stimulatory immunoreceptors on NK cells and mediate their activation, leading to the lysis of the target cell (63, 90). The activating NK cell receptor natural killer group 2D family

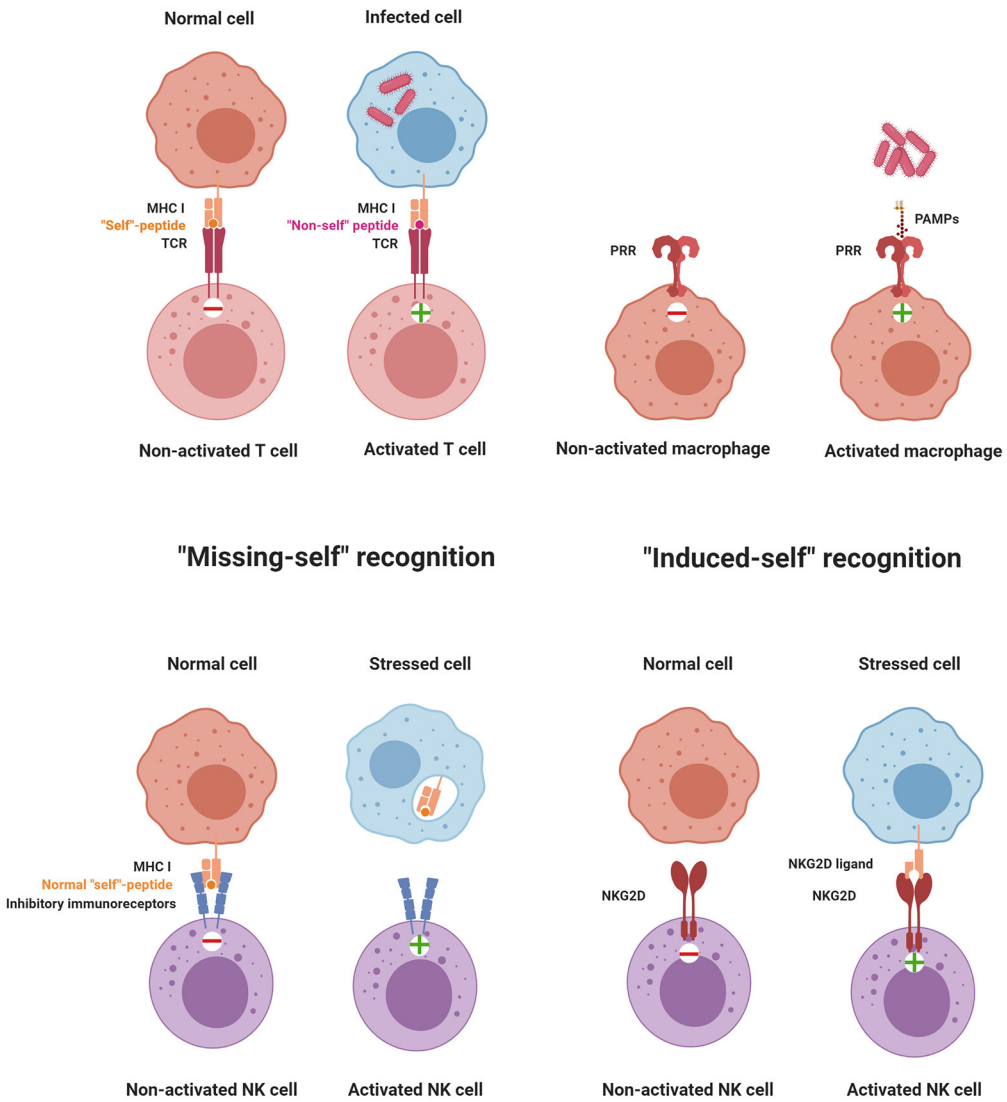


FIGURE 1 | Principles of immune recognition. The immune system constantly senses the presence or absence of "self" and "non-self" molecules by stimulatory and inhibitory receptors. Activation of immune cells is triggered by the direct recognition of microbial "non-self," "missing-self" recognition, or "induced-self" recognition (illustrations were created with BioRender.com).

(NKG2D) has served as a paradigm for understanding the recognition of induced-self antigens. NKG2D binds to several induced-self ligands. The mouse ligands include RAE1α, RAE1β, RAE1γ, RAE1δ, RAE1ε, H60 (H60a, H60b, and H60c), and MULT1 (91–96). These NKG2D ligands belong to a group of non-classical MHC I molecules and contain α1 and α2 extracellular domains with homology to class I MHC molecules. In humans, MHC class I polypeptide-related sequence A (MICA) and B (MICB) represent additional NKG2D ligands that are not present in mice (91). MICA/B possess an α3 domain homologous to class I MHC molecules, but they neither require β2-microglobulin for expression nor do they present peptides (90). Another example of "induced-self recognition" is the natural cytotoxicity receptor NKp30 which interacts with the B7-like self-ligand B7-H6, the expression of which is induced on transformed cells (97). Thus, immunosurveillance of induced-self ligands by immunoreceptors such as NKG2D and NKp30 allows the immune system to detect and eliminate cells that have undergone "stress." These receptor–ligand pairs represent interesting targets of anti-tumor therapies. In **Tables 1A** and **1B**, ligand–receptor

TABLE 1 | (A) Summary of ligand-receptor interactions and their effect on immune cell activation. (B) Summary of self and non-self effects on immune cells when they are present or absent. Illustrations created with BioRender.com.

A

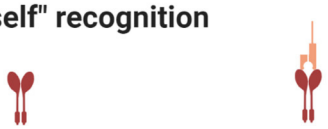
"Non-self" recognition

Ligand	MHC I -"self"- peptide	MHC I - "non-self"- peptide	PAMPs (e.g. LPS)	PAMPs (e.g. LPS)
Receptor	TCR	TCR	PRRs (e.g. TLR)	PRRs (e.g. TLR)
Receptor type	Stimulatory	Stimulatory	Stimulatory	Stimulatory
Resulting ligand - receptor interaction	absence of stimulation	presence of stimulation	absence of stimulation	presence of stimulation
Effect on immune cell activity	inhibition	activation	inhibition	activation

"Missing-self" recognition

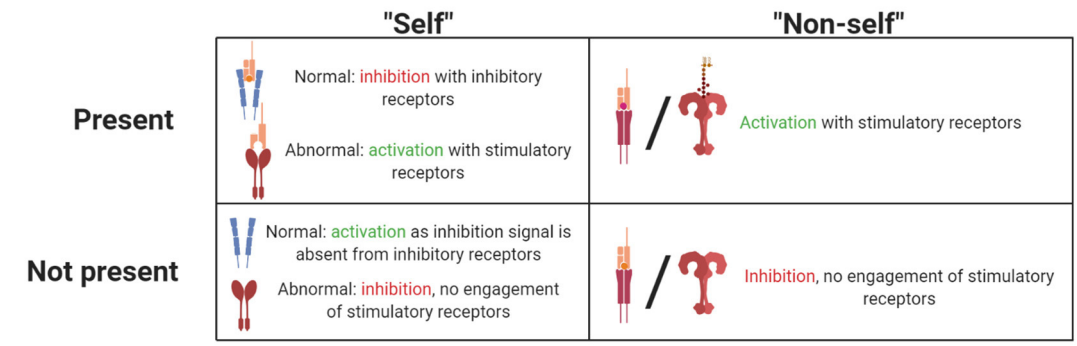
Ligand	MHC I	MHC I-absent
Receptor	Ly49, KIRs and CD94/NKG2A	Ly49, KIRs and CD94/NKG2A
Receptor type	Inhibitory	Inhibitory
Resulting ligand - receptor interaction	presence of inhibition	absence of inhibition
Effect on immune cell activity	inhibition	activation

"Induced-self" recognition



Ligand	MIC A/B, RAE1α-ε, H60a-c, ULBPs	MIC A/B, RAE1α-ε, H60a-c, ULBPs
Receptor	NKG2D	NKG2D
Receptor type	Stimulatory	Stimulatory
Resulting ligand - receptor interaction	absence of stimulation	presence of stimulation
Effect on immune cell activity	inhibition	activation

B



interactions, as well as the functional consequences for NK cell activation are summarized.

Notably, NK cell-mediated immune regulation is tightly linked to both classical and non-classical class I MHC molecules. NK cells sense the absence of classical MHC-I ("missing-self") but also recognize non-classical MHC-I molecules as non-self or induced-self ligands. In addition, NK cells require the recognition of self-MHC ligands not only for their activation but also for proper development, which will be discussed in the following chapters.

TRANSCRIPTIONAL REGULATION OF PROGENITOR COMMITMENT TO ALL INNATE LYMPHOID CELL LINEAGES

The initial steps of ILC differentiation from precursor cells take place in the fetal liver and after birth in the adult bone marrow (BM). In **Figure 2**, ILC progenitors and their differentiation stages into ILC subsets are presented.

Hematopoietic stem cells (HSCs) give rise to all blood cell progenitors, among which common lymphoid progenitors (CLPs) are precursors of all lymphocytes, belonging to both adaptive and the innate arms of the immune system. It is generally believed that the CLP differentiates into the various lymphocyte subsets by integrating environmental signals that establish characteristic transcriptional programs, usually regulated *via* several key TFs, that lead to a step-wise restriction of their precursor potential and to the instruction of lymphocyte subset-specific transcriptional circuitry (99–101).

An important conceptual advance of the last 5 years was the description of multipotent ILC progenitor cells such as α-lymphoid progenitor ($\alpha_4\beta_7^+$αLP), EILP, CHILP, and ILCP, which have the developmental potential for ILC lineages but can no longer differentiate into adaptive lymphocytes or myeloid cells (21, 26, 27, 98). Early evidence indicated that ILC progenitors may be contained within a population with phenotypical characteristics similar to CLPs. Indeed Lin^- IL-$7R\alpha^+$ $CXCR6^-$ cells, which in contrast to CLPs expressed integrin $\alpha_4\beta_7$ but were negative for FLT3, a receptor tyrosine kinase expressed by the CLPs (102), gave rise to all three groups of ILCs and T cells but had lost B cell potential (103). The subsequent acquisition of the chemokine receptor CXCR6 is indicative of the loss of T cell potential and Lin^- IL-$7R\alpha^+$ $CXCR6^+$ integrin $\alpha_4\beta_7^+$ $FLT3^-$ cells are referred to as αLPs (103, 104). It became clear, however, that αLPs are a quite heterogeneous population of innate lymphocyte progenitors, which was further explored in subsequent work.

The earliest defined subset of ILC-committed progenitors downstream of the CLP (and contained within the αLP population) was characterized by high expression of the transcriptional regulator T cell factor 1 (TCF-1, encoded by the *Tcf7* gene). Such TCF-1^{high} progenitors are referred to as EILPs or ILCPs. EILPs already show a substantial expression of nuclear factor interleukin 3-regulated (NFIL3, also known as E4BP4) and thymocyte selection-associated high mobility group box protein (TOX) known to be involved in early ILC differentiation (see below) (26). While the CLP does not express ID2, a transcriptional regulator required for the differentiation of all ILCs (105), EILPs express intermediate level of ID2. The EILP gives rise to all ILC lineages (including NK cells) but lacks T and B lymphocyte or myeloid potential (26). Unlike other ILC progenitors and CLPs, EILPs were IL-7Rα low-expressing cells and developed independently of IL-7Rα signaling. Therefore this finding provoked the question on whether EILPs might constitute an alternative route to ILC development because the upstream and downstream cells are both IL-$7R\alpha^+$. However, it was demonstrated in consecutive work that EILPs developed from CLP transiently down-regulating IL-7Rα expression and then differentiated into ILC progenitors with increased IL-7Rα expression (26, 106).

The existence of a common progenitor for ILCs was already hypothesized years before their discovery, mainly based on the phenotype of mice deficient for ID2 (107). ID2 belongs to the family of helix–loop–helix proteins, which form heterodimers with E-proteins, thus preventing their binding to DNA and antagonizing the gene regulatory function of E-proteins during cell development (108). Since $Id2^{-/-}$ mice lacked all ILCs, it was hypothesized that this phenotype might be explained by the existence of a common ILC progenitor, which expresses ID2 and is developmentally dependent on it (12, 105, 109). Indeed the analysis of ID2 reporter mice [$Id2^{Gfp/+}$ mice; (110)] revealed that both mature and immature ILCs expressed ID2 (17, 111, 112). An interrogation of the αLP population for ID2 expression revealed a population of $ID2^{high}$ cells within Lin^- IL-$7R\alpha^+$ $CD25^-$ integrin $\alpha_4\beta_7^+$ $FLT3^-$ αLP (21). Upon transfer and on a clonal level, this cellular subset gave rise to all three groups of ILCs, including $CCR6^+$ ILC3, but not to conventional NK cells and was accordingly named CHILP. While the CHILP did not express any ILC lineage-defining TFs, they expressed intermediate levels of GATA-3 (17). However, CHILPs were heterogeneous for the expression of the TF promyelocytic leukemia zinc finger protein (PLZF) (21). Interestingly, while $PLZF^+$ CHILP could generate ILC1, ILC2, and $CCR6^-$ ILC3, they lacked the potential to differentiate into $CCR6^+$ (LTi-like) ILC3s and NK cells (98). These findings might be explained by data showing that the CHILP contained subsets of $CXCR5^+$ cells, which gave rise to $CCR6^+$ ILC3s/LTi cells and were not contained in the $PLZF^+$ population (113, 114). Single-cell sequencing of ILC progenitors has confirmed the developmental stages of early ILC commitment and further contributed additional markers, such as programmed cell death protein 1 (PD-1), to define $PLZF^+$ precursors (115). Therefore, we will refer to these two populations of ILC precursors as CHILP-A ($ID2^+$ $PLZF^-$ PD-1^-) and CHILP-B ($ID2^+$ $PLZF^+$ PD-1^+). Using reporter mice for several TFs, later studies showed some degree of heterogeneity in CHILP-A and CHILP-B which could be further subdivided into cell subsets that were committed to one ILC subset and *bona fide* CHILP subsets that still maintained multi-ILC lineage potential (111–113).

A recent report attempted to challenge the view that $ID2^{high}$ CHILPs are progenitors to all helper-like ILCs but not to conventional NK cells (112). This study was based on the generation of a very bright reporter allele for ID2. Somewhat expectedly (26), the authors found that $ID2^{int}$ precursors (i.e. EILP), which could be discriminated in these new reporter mice, still have NK cell differentiation potential. These results

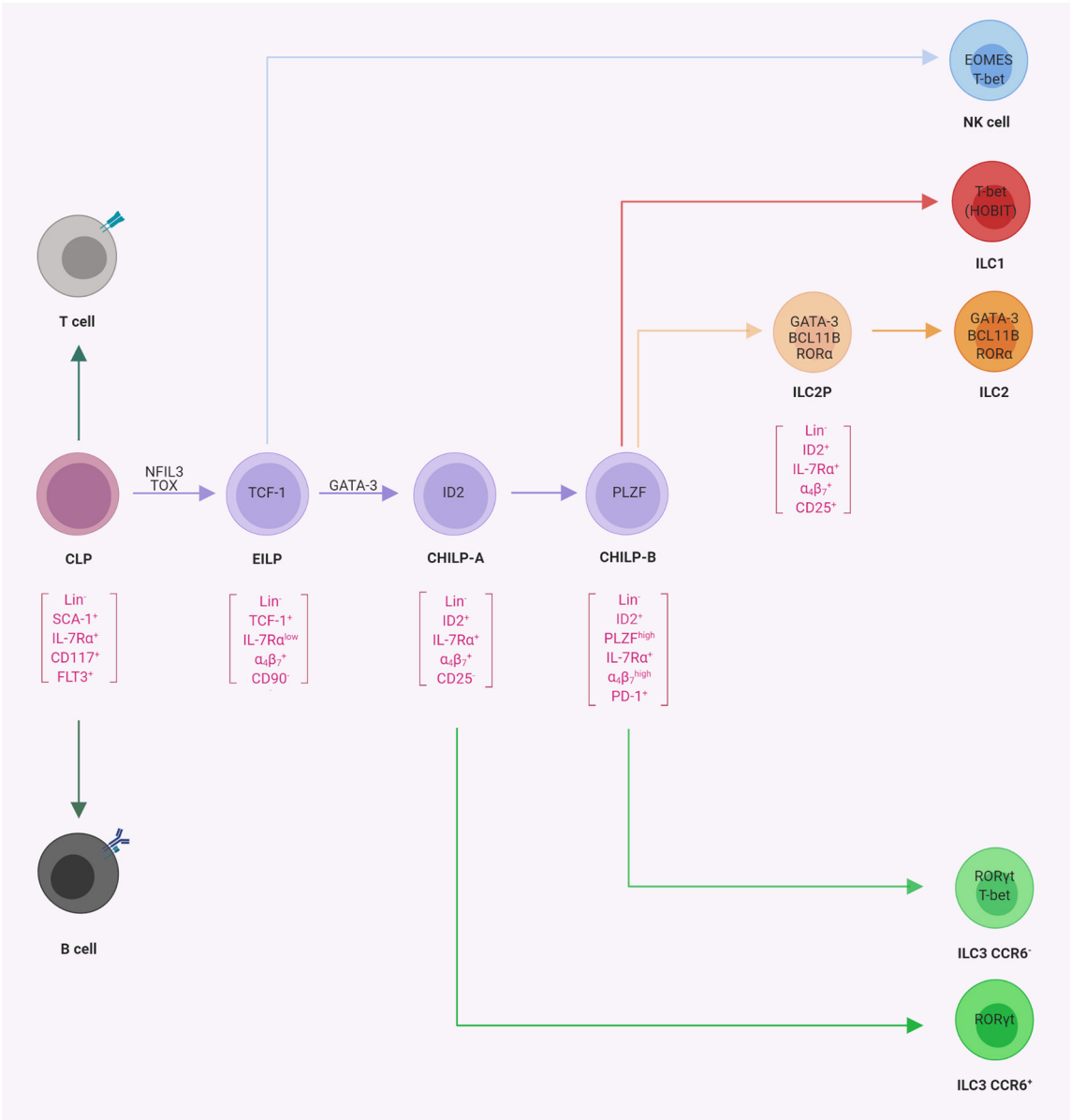


FIGURE 2 | Progenitor commitment to innate lymphoid cells (ILCs). Schematic representation of progenitor populations with various differentiation potentials toward ILCs. The common lymphoid progenitor (CLP) gives rise to B-cells, T cells, and ILCs. The early innate lymphoid progenitors (26) possess the potential for NK cells, ILC1s, ILC2s, and ILC3s, whereas CHILP-A (21) and CHILP-B (98) possess the potential for ILC1s, ILC2s, and ILC3s as indicated. In square brackets are the population-defining markers reported in the literature. The transcription factors required for the indicated lineage or transition from one population to another are indicated within the cells or on the arrows, respectively (illustrations created with BioRender.com).

are supporting previous work which show that EILP expressed intermediate levels of ID2 and can generate all ILC subsets and NK cells (26, 106, 116). It is worth noting that during cellular differentiation processes, TFs often act as gradients rather than as binary switches. Once past the NK cell bifurcation (**Figure 2**), ID2 expression increases (26, 100, 112) and ID2^{high} CHILPs have a more restricted potential. Collectively, the available data support a model of ILC differentiation downstream of CLP with three major bifurcations and consecutively restricted differentiation potential, namely, TCF1^{+} ID2^{int} EILP (or ILCP), ID2^{high} PLZF^{-} CHILP-A, and ID2^{high} PLZF^{+} CHILP-B (**Figure 2**).

Single-cell sequencing data and analysis of multi-color reporter mice have demonstrated that a further subdivision of ILC progenitors is technically possible. These findings can advance the ILC field by defining different ILC progenitors that have a more restricted differentiation potential and therefore open the perspective of finding the cues that control the

commitment and the differentiation of CHILPs into different ILC cell subsets. However, defining progenitors by clustering based on highly expressed genes that are detected using single-cell sequencing comes with the caveat that cell clustering is not necessarily of biological relevance and differences in differentiation potential remain to be demonstrated.

Several additional TFs were recognized, which play an essential role in early commitment to the ILC fate. In addition to ID2 and PLZF, these TFs include NFIL3, TOX, and GATA-3. The phenotype of the knockout mice for these TFs was characterized by a deficiency or a reduction in all or almost all ILC lineages, except NK and LTi-like cells in GATA-3-deficient mice. NFIL3 is important for the transition from CLPs to ILC progenitors, where the relative expression of this TF increased and its deletion led to a substantial decrease in ILC progenitor numbers (104, 117–122). Since the down-regulation of the transcriptional regulator TOX was described in *Nfil3*$^{-/-}$ mice in comparison to wild-type controls, it was proposed that NFIL3 is directly regulating the expression of TOX, which then acts downstream in ILC development (104). Indeed *Tox*$^{-/-}$ mice had a similar phenotype as that of *Nfil3*$^{-/-}$ mice and lacked mature ILCs and ILC progenitors (123, 124). Based on these data, it was proposed that NFIL3 and TOX orchestrate the transition from CLP to EILP (106), whereas GATA-3 (28, 125) is required later in ILC development for the transition to PLZF^{+} ILCPs. It should be noted though that NK cells and CCR6^{+} ILC3s still develop in GATA-3-deficient mice. Altogether these data indicate that the developmental potential for NK cell and CCR6^{+} ILC3s/LTi cell is consecutively lost during the transition from EILP to CHILP-B (28, 98, 114, 125, 126).

While ILC progenitors are certainly present in the primary organs of hematopoiesis, the BM and fetal liver (21, 26, 98), it should be considered that ILCs may be derived from local precursors as tissue-resident cells. In mice, fetal ILC precursors migrated to the intestine before Peyer's patch organogenesis and accumulated at the sites where intestinal lymphoid tissue organogenesis is initiated and became a localized source of ILC populations (127). While intestinal ILC precursors were identified based on arginase expression, adult BM ILC precursors lacked arginase expression, indicating tissue adaption of the ILC precursor population (127). Since fate-labeling studies suggest that the ILC pool is generated in different pre- and postnatal time windows (128) and ILC precursors were also detected in human blood (27), further research is required to investigate the relation among ILC precursor cells in different compartments and their relevance in ontogeny.

MATURATION OF LINEAGE-COMMITTED NK CELL PRECURSORS

Developmental Stages of NK Cell Maturation

NK cells develop from a committed NK cell progenitor (NKP) in the BM, which was first described in 2001 based on the expression of the IL-2/IL-15 receptor beta chain (CD122), a well-recognized T-bet target gene (129, 130). In **Figure 3A** distinct developmental and maturation stages of NK cells are described. It should be noted that the major thrust of work on NK cell development was performed before ILCs with an NK cell phenotype (ILC1, subsets of ILC3) were recognized. We will critically discuss here the conventional definition of NKPs and immature NK cells in the new framework of ILC diversity.

An NKP population, which gave rise only to mature NK cells but did not possess a potential for T or B-cell lineages, was originally identified to be within Lin^{-} NK1.1^{-} DX5^{-} CD122^{+} cells (129). However, the frequency of such NKP differentiating into NK cells was only one in 12 in limiting dilution assays, revealing a highly heterogeneous population and a requirement for additional markers to further narrow down the true NKP, also because some T cell potential was still detectable in this population (131). Technical progress in multicolor flow cytometry allowed a more accurate definition of the NKP within Lin^{-} CD27^{+} CD244^{+} CD122^{-} IL-7Rα^{+} FLT3^{-} cells, in which 50% of the cells were giving rise to NKp46^{+} NK cells (132). This NKP subset was designated as a pre-NK cell precursor (pre-NKP), suggesting to be the earliest precursor of NK cells. Pre-NKPs express natural killer cell receptor 2B4 (CD244) and lack the expression of other surface markers associated with NK cells (NKp46, NKG2D, NK1.1, or inhibitory receptors such as Ly49 and CD94/NKG2A). Under NK cell-promoting culture conditions, the expression of CD122 is up-regulated, thereby giving rise to the 'refined' NK cell precursor (rNKP), which has full NK cell potential (132). Apart from CD122, these precursors also acquire during their differentiation the IL-2 receptor γ_c chain, which makes NKPs responsive to IL-15, a cytokine essential for NK cell differentiation and survival (133–135). Both pre-NKPs and rNKPs showed the potential of differentiating into NK cell receptor-positive cells in spleen and liver, albeit with different frequencies. Since EOMES, a lineage-specifying TF for NK cells was not analyzed, these studies do not allow definite conclusions regarding a possible bipotential of NKPs for NK cells and ILC1s. The notion that the NKP population might contain committed ILC1 precursors was supported by Constantinides et al., who demonstrated heterogeneity within the pre-NKP population, with one subset expressing PLZF and other markers characteristic for ILC progenitors and another subset belonging to NKP (56). Therefore, more detailed analyses are required to delineate the separation of NK and ILC1 lineages in early precursors.

While NKG2D was already expressed by at least one subset of NKPs, the expression of NK1.1, NKp46, and CD94/NKG2A marked the immature NK cell stage (stage 2). The expression of Fc receptors and Ly49s, which provide inhibitory receptors for the NK cell education process, defines stage 3 of NK cell development (136–139). At this stage, "NK cells" display a T-bet signature, but since T-bet does not allow the distinguishing between NK cells and ILC1s, the delineation between the two lineages is difficult until EOMES and DX5 are expressed in stage 4 (16, 21, 35, 36). Furthermore, surface markers such as CD69, CD51, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which are often used to describe immature NK cells, are phenotypic markers of ILC1s but not mature NK cells in most tissues (136, 140, 141). Therefore,

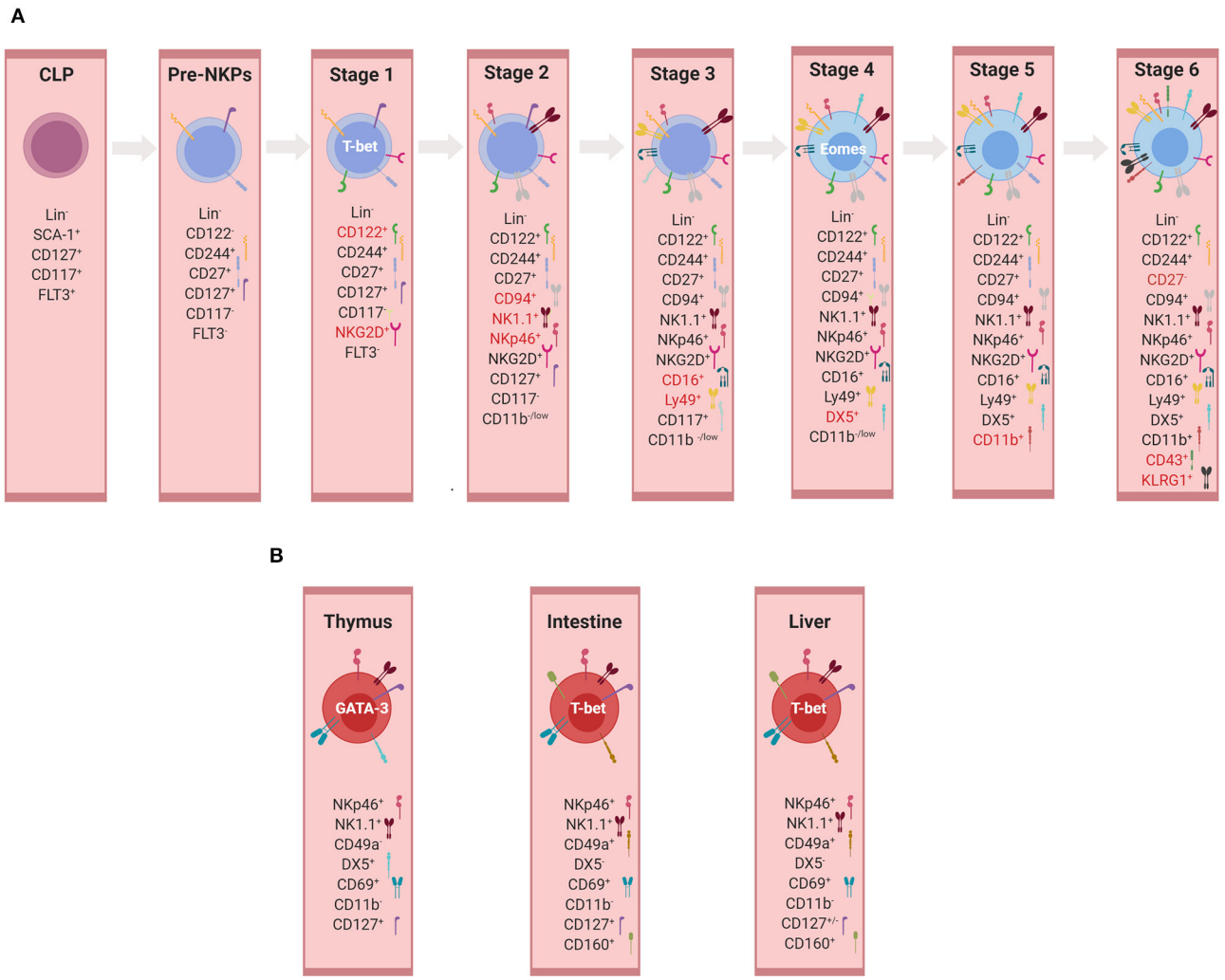


FIGURE 3 | Development of NK cells and ILC1s. **(A)** Developmental stages of murine conventional NK cells. Representation of markers used for the identification of individual developmental stages. Markers highlighted in red represent those expressed mainly in the given subset and therefore important for its identification. At each stage, the expression of the listed markers is depicted as "+", whereas the absence of expression is indicated by "–" or alternatively "low." **(B)** Tissue-specific non-conventional NK/ILC1 subsets. Representation of markers used for the identification of non-conventional/ILC1 subsets in thymus, intestine, and liver (illustrations created with BioRender.com).

the view that the T-bet$^+$ EOMES$^-$ NKp46$^+$ subset represents immature NK (iNK) cells is currently questionable (16, 142).

T-bet$^+$ EOMES$^-$ "NK cells" represent the major murine liver NK-like subpopulation during fetal and neonatal developmental stages. During aging, the ratio between these "T-bet$^+$ EOMES$^-$ immature NK cells" and mature NK cells changed, which represented another argument favoring the presumption that T-bet$^+$ EOMES$^-$ liver "NK cells" are in fact immature NK cells that can further mature. This hypothesis was supported by data demonstrating that EOMES$^-$ TRAIL$^+$ "immature" NK cells in the adult liver gave rise to EOMES$^+$ DX5$^+$ NK cells (16, 142). Contradicting results were obtained in several other publications (21, 35, 36). Daussy et al. utilized EOMES reporter allele and performed extensive phenotypic profiling of the EOMES-positive and EOMES-negative populations. Even though EOMES$^-$ "NK cells" had an "immature phenotype" and EOMES$^+$ NK cells had "mature" characteristics, *in vivo* and *in vitro* differentiation experiments did not show any transition between these two populations. PLZF fate mapping supported these results because EOMES$^+$ DX5$^+$ NK cells did not derive from PLZF-expressing progenitors, whereas EOMES$^-$ TRAIL$^+$ populations originated from PLZF$^+$ precursors. In addition, EOMES$^-$ TRAIL$^+$ ILC1s did not differentiate into EOMES$^+$ DX5$^+$ NK cells (35, 36, 56). Moreover, Constantinides et al. demonstrated that ILC1s predominate over cNK cells during development in murine liver, while cNK cell number increases during adulthood (56). Therefore, since ILC1s and NK cells have parallel progression at an early stage during development and are phenotypically similar, further analyses have to be performed to separate iNK cells from ILC1s.

Mature *bona fide* NK cell subsets are characterized by the following: 1) expression of EOMES and DX5 (stage 4); 2) acquisition of CD11b (stage 5); 3) the consecutive loss of CD27 (stage 6) and the up-regulation of CD43 and KLRG1 (136, 143). Developmental intermediates were identified among $CD11b^{hi}$ cells, designated as mature NK cell subsets (136). CD27 is a key marker of the NK cell lineage, dissecting the mature $CD11b^{+}$ NK cell pool into two functionally distinct subsets (144, 145). The $CD27^{low}$ NK cell subset possesses a higher threshold to stimulation and appears to be tightly regulated by the expression of NK cell inhibitory receptors. The preceding subset is consisting of the $CD27^{high}$ NK cells that display a greater effector function, exhibiting a distinct tissue distribution and responsiveness to chemokines and productively interacting with dendritic cells (144, 145).

Cytokine Signals Regulating NK Cell Development

Although NK cell commitment is not dependent on IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21, which are executing their function through a common cytokine receptor γ chain, early NKPs have the capacity to respond to cytokines through the co-expression of CD122 and CD127 (146). Since mice lacking IL-2, IL-4, and IL-7 developed normal numbers of phenotypically mature NK cells with a regular capacity to exert natural cytotoxicity *in vitro*, produce IFN-γ, and kill tumor cells *in vivo* (146), IL-15 was identified as the major γ_c cytokine to promote NK cell development, and it plays a dominant role in early NK cell differentiation by maintaining normal numbers of immature and mature NK cells in the BM and spleen (146, 147). Given that the close association of T-box TFs and IL-15 responsiveness *via* CD122 is a hallmark of many lymphocytes, including ILCs and unconventional tissue-resident T cells (148–152), IL-15 was also indispensable for the development of ILC1s and ex-ILC3s although they co-express CD127 (21, 35, 153). IL-15 activated NK cells by STAT5 signaling and promoted the expression of the anti-apoptotic protein MCL1 and, at the same time, restricted the expression of pro-apoptotic proteins such as BIM and NOXA (153, 154).

Transcriptional Regulation of NK Cell Development

NK cell development and function are regulated by a plethora of TFs expressed at different developmental stages, and at each stage these sets of TFs constitute regulatory networks for the establishment of distinct phenotypes. While numerous TFs that regulate pivotal steps during NK cell development were identified, the regulation of NK cell development is much less understood on a molecular level. As a consequence, the NK cell-specific target genes of TFs are insufficiently defined. Although TFs were proposed to mainly act during one stage of NK cell development, it should be considered that they might regulate NK cell development at various stages and depending on the amount of TFs being expressed (i.e. TF gradients). Additional difficulties in assembling the available data into a satisfying model are represented by the limitations in accurately recording relatively small NKP populations given the very limited availability of multicolor flow cytometry and by the separation of immature NK cells from ILC1s (as already discussed above). Therefore, although supported by data, the conclusion that certain TFs act at a certain stage of NK cell development should be taken with caution as these analyses often pre-dated the discovery of ILC1s and other ILC subsets expressing NK cell receptors such as NKp46 and NK1.1. With this in mind, we will discuss here the major TF modules that have been associated with NK cell differentiation.

Developmental defects in NK cells were reported from mice deficient for the TFs PU.1 and IKAROS that are broadly expressed early in hematopoiesis before commitment to the ILC/NK lineage and therefore affect multiple hematopoietic lineages, including but not limited to NK cells (155–158). Since it is controversial if PU.1 is expressed during NK cell development at all, it is unclear whether PU.1 or IKAROS are mediating effects during NK cell development or whether the phenotypes might be explained by the effects in upstream hematopoietic precursors (159).

Various TFs, such as TCF-1, NFIL3, and TOX, that were already introduced to regulate early commitment to the ILC lineage are indispensable for NK cell development, likely by acting on the EILP or upstream progenitors. Mice deficient for either TCF-1, NFIL3, or TOX lacked NK cells and also other ILC lineages (117, 123, 160). Since these TFs are already expressed upstream of the NKP in multipotent ILCPs such as EILP, CHILP-A, or CHILP-B, mice deficient in these TFs lacked most ILC lineages. Therefore, at least some of the effects likely occur already in multipotent precursors before commitment to the NK cell lineage (106, 124). Whereas, the mechanistic role for TOX after NK cell commitment is elusive, it was shown that TCF-1 restricts granzyme expression, thus protecting the developing NK cells from self-destruction (161). While NFIL3 was recognized as a TF important for the transition from CLPs to early ILC precursors, it was shown to be also up-regulated in the NKP cells. Further, its deficiency in mice led to decreased numbers of NK cells (121, 162). While it was proposed that NFIL3 regulates ID2 expression (117, 163), Seillet et al. showed that NFIL3 was influencing EOMES expression, whereas ID2 expression in the absence of NFIL3 remained the same. Moreover, ectopic expression of EOMES in *Nfil3*$^{-/-}$ hematopoietic progenitor cells was sufficient to rescue cNK cell development (162).

Mice deficient for the TF ETS-1 had a strong reduction in mature NK cell numbers (164). Similar to NFIL3, ETS-1 was already expressed in CHILPs and also in NKPs. ETS-1 regulated the fitness of CHILPs, but the effects on NK cell development are probably emerging later with reduction at pre-NKP and rNKP stages, where ETS-1 might regulate T-bet and ID2 (165, 166). MEF is another member of the ETS TF family, which regulated essential functions during NK and NKT cell development, whereas B and T cells developed in normal proportions (167). While MEF-deficient mice have reduced NK cells and impaired effector function, including cytotoxicity and IFN-γ production, mechanistic insights are scarce (167).

Important regulators of NK cell development include ID2 and GATA-3, known to regulate early ILC commitment (21, 125).

However, due to the phenotype of the gene-deficient mice and their expression pattern, it seems more likely that these TFs mediate their decisive effects after NK cell commitment. Mice deficient in ID2 or GATA-3 developed NKPs and immature NK cells but had a maturation defect of NK cells. In contrast, they lacked the other ILC lineages (12, 28, 105, 109, 126). While GATA-3 is required for ILC1 development (21, 28, 168, 169), it was dispensable for the development but not for the maturation of NK cells. GATA-3-deficient NK cells had an immature phenotype, were poor producers of IFN-γ, and showed defects in BM egress because they are retained in the BM due to the high CXCR4 expression (28, 126, 169). ID2 represents another transcriptional regulator that was up-regulated during NK cell development from NKPs and that was essential for the development of mature NK cells (105, 109, 162). Notably, unlike other ILC populations, NKPs and immature NK cells developed in ID2-deficient mice. However, ID2 deficiency causes loss of terminally differentiated $CD11b^+$ NK cells, indicating a persistent need for the sequestration of E-proteins during NK cell maturation (170, 171). In support of this notion, the genetic deletion of ID2 and ID3, which both bind E-proteins, resulted in the complete loss of NK cells. It was also proposed that ID2 regulates IL-15 receptor signaling *via* the suppression of SOCS3. Interestingly, both ID2 and IL-15 signaling were linked to the regulation of apoptosis in NK cells *via* either anti-apoptotic MCL1 or pro-apoptotic BIM (154, 170, 172). Therefore, ID2 could be a link between sensing of the vital cytokine IL-15 and cell survival.

Several TFs with a more restricted expression during hematopoiesis played pivotal roles during the maturation of NK cells. These include EOMES, T-bet, and ZEB2. Unlike ILC1s, which only expressed and were developmentally dependent on T-bet but not EOMES, mature NK cells co-expressed both T-bet and EOMES. While mice with a conditional deletion of EOMES lacked NK cells, these cells normally differentiated in T-bet-deficient mice where they were accumulating in the BM and the lymph nodes due to the altered expression of S1P5R and CXCR3. They displayed an immature phenotype characterized by the persistent expression of CD27 and the reduced CD11b, CD43, and KLRG1 levels (33, 34, 173, 174). A similar NK cell maturation phenotype was reported from mice deficient in the Zinc finger-containing protein (ZEB2) (175). The notion that ZEB2 and T-bet might cooperatively regulate NK cell maturation is also supported by data showing that the overexpression of ZEB2 can partially rescue the phenotype of T-bet-deficient NK cells (175).

Although EOMES is also expressed by non-hematopoietic cells as well as in $CD8^+$ T cells, where the TF regulates CD8 memory formation, among ILCs, EOMES represents a specific TF for NK cells (16, 176, 177). Moreover, mice harboring a conditional deletion of EOMES using $NKp46^{Cre}$ completely lacked NK cells but still contain other ILC lineages (178, 179). Therefore, EOMES represents an attractive candidate for the specific targeting of NK cells by using, for example, $NKp46^{Cre}$ $Eomes^{fl/fl}$ mice to exclude effects on T cells. While epigenetic studies provide evidence that the EOMES and the T-bet promoters are both in an open chromatin configuration in NK cells, downstream targets of the T-box TFs are not well-defined in NK cells and were largely extrapolated from studies that have investigated other cell types (180). However, the importance of EOMES in NK cell fate and in the expression of prototypic markers of NK cells is also illustrated by data showing that the overexpression of EOMES under the *Tbx21* regulatory elements reprogrammed ILC1s to adopt phenotypical hallmarks of NK cells (178). Since the down-regulation of EOMES in NK cells mediated by TGF-β drove the NK cells to adopt an ILC1 phenotype, EOMES appears as a major signaling hub that dictate NK cell identity (181, 182).

Numerous TFs including AIOLOS, PRDM1 (BLIMP1), FOXO1, IRF2, RUNX3, and KLF2 regulate the late developmental stages of NK cells with main effects on terminal maturation and effector functions. PRDM1 (encoding BLIMP1) was shown to be regulated by T-bet and IL-15. Further, BLIMP1-deficient mice had fewer $KLRG1^+$ mature NK cells. Although granzyme B expression was altered in PRDM1-deficient NK cells, effector functions, including cytotoxicity, remained normal (183). A similar phenotype was reported for mice deficient in the IKAROS zinc finger TF member AIOLOS. NK cells developed in AIOLOS-deficient mice but terminally differentiated $CD11b^+$ NK cells were reduced. While NK cell effector functions were largely maintained, $Aiolos^{-/-}$ NK cells were hyper-responsive to tumor cells, resulting in superior tumor surveillance (184).

The conditional deletion of the Krüppel-like TF KLF2 in hematopoietic cells using Vav^{Cre} resulted in the ablation of mature $CD11b^+$ NK cells and consequently reduced the cytotoxicity toward target cells (185). It was proposed that KLF2 regulates the survival of NK cells *via* the regulation of IL-15 sensing and the expression of homing receptors. Reduced NK cells were also reported from mice deficient in the Th1 regulator interferon regulatory factor 2 (IRF-2) (186). IRF-2 deficiency disturbed mainly mature splenic NK cells, whereas NK cell development in the BM was only mildly affected. IRF-2 NK cells were more prone to undergo apoptosis during development independently from IL-15 (187).

TFs regulating NK cell development involves FOXO proteins as well. However, the precise role is hard to evaluate because of data that are difficult to reconcile with a model. While Wang et al. found decreased numbers of NK cells in mice with conditional deletion of FOXO1 using $NKp46^{Cre}$ deleter mice (188), Deng and colleagues reported increased numbers of mature, hyper-reactive NK cells using $NKp46^{Cre}$ and also Vav^{Cre} deleter mice to genetically ablate FOXO1 (189, 190).

Runt-related TFs (RUNX) are important regulators of lymphocyte development, including T cells and several ILC lineages. RUNX members 1–3 form heterodimers with the TF core-binding factor beta (CBF-β) in order to bind to regulatory DNA sequences and mediate gene transcription (191, 192). The RUNX3 isoform is highly expressed in NK cells. Different strategies were used to genetically interfere with RUNX to investigate the function *in vivo*, including the overexpression of dominant-negative RUNX3 or the conditional deletion of RUNX3 or CBF-β. While RUNX3 regulated the development of ILC1s and ILC3s by different mechanisms, ILC2 development remained intact and RUNX proteins protected ILC2s from

an exhaustion-like phenotype (192–194). Concerning NK cell development, the deletion of either RUNX3 or CBF-β altered NK cell development *via* the regulation of CD122 and IL-15 responsiveness. This was accompanied by reduced numbers of $CD11b^+$ and $CD43^+$ mature NK cells and enhanced cytokine production (195–197). Consistent with a role later in development, RUNX and CBF-β were also crucial for NK cell memory formation following MCMV infection (198).

Finally, TFs that constitute regulatory network during NK cell development represent a nice example of how these proteins act as a part of a complex context that dictates their function and how compensatory mechanisms in their absence could, in some situations, buffer the entire system.

DEVELOPMENT OF ILC1s/TISSUE-RESIDENT NK CELLS

NK cells and ILC1s share many phenotypical and functional properties that make the differentiation between these two innate lymphocyte subsets, especially in humans, very challenging (59). In addition, ILC1s comprise several subsets of lymphocytes previously referred to as "immature," "tissue-resident," or "unusual" NK cells before the revised nomenclature in 2013. These include $TRAIL^+$ NK cells (142) and thymic NK cells (168) or (after the revised nomenclature) ILC1s in the BM, the lamina propria (21), the epithelium of the intestine [intraepithelial (ie)ILC1s] (20), the salivary glands (199), the adipose tissue (200), or the uterus (201). ILC1 subsets differ in terms of dependency on TFs during development, e.g. EOMES and NFIL3, and cytokines, e.g. IL-7 and IL-15 (21–23, 36, 120, 168, 202). Although these subsets are often all referred to as ILC1s, it is very difficult to conclude whether different developmental requirements reflect the tissue adaption of one cell lineage or different cell lineages of phenotypically similar cells. Besides the heterogeneity and the tissue adaptation of ILC1s, differences between mouse and human ILC1s add an additional layer of complexity to the topic. For example, it is well-established that murine liver $TRAIL^+$ NK cells express and are developmentally dependent on T-bet but not EOMES. However, the human liver contains a population of $CD56^{bright}$ lymphocytes, which phenotypically resembled ILC1s but expressed high levels of EOMES and only low levels of T-bet (203). Nevertheless, the functional and the phenotypical characterizations of different subsets of ILC1s are contributing to a better understanding of their biology and diversity as well as enabling their separation in a more comprehensive way. While being recognized as tissue-resident cells, ILC1s have been residing in various tissues, expressing specific markers that are represented in **Figure 3B** and which will be discussed below.

The characteristic feature of thymic non-conventional NK/ILC1 is that they express CD127 and developmentally depend on IL-7 signaling (168). This is in contrast to splenic and BM cNK cells, the phenotype and the function of which were not perturbed in the absence of IL-7. To a lesser extent, thymic NK cells required IL-15 for their development similar to NK cells (204). Moreover, thymic non-conventional NK cells depended on GATA-3 for their development and showed an elevated expression of this TF in comparison to splenic cNK cells (168). Phenotypically, thymic NK cells resembled ILC1s rather than NK cells because of the lack of CD11b and Ly49 receptors and their expression of CD69. However, whether thymic NK cells belong to the same lineage as ILC1s and $TRAIL^+$ liver NK cells requires further clarification, especially because they express EOMES and DX5, which are usually not found on ILC1s (202). Thymic NK cells were reduced in *Foxn1*$^{-/-}$ mice, which do not develop a functional thymus, suggesting that the thymus is an organ required for the generation of this ILC1 subset. Data obtained in reporter mice for TCR-δ germ-line transcription suggest that thymic NK cells might be derived from lymphocytes with T cell potential (205). This is in line with data showing that primitive, double-negative T cell progenitors still possess the potential to differentiate into cells that phenotypically resemble NK cells (206). Further, it was proposed that thymic NK cells might be the counterpart of $CD56^{bright}$ NK cells, which are potent IFN-γ producers but have weak cytotoxic potential (168).

Based on CD56 expression, a unique subset of ILC1s was also described in the intestinal epithelium of humans. $NKp44^+$ $CD103^+$ and $NKp44^-$ $CD103^-$ ieILC1s were discriminated with similar functional properties, such as strong IFN-γ production. In addition, ieILC1s showed signs of TGF-β imprinting, such as CD103 expression, and were phenotypically different from cNK cells as illustrated by the expression of CD160, CD49a, CXCR6, CD69, and CD39, which were also found on ILC1s in other organs (141). Unlike thymic NK cells, ieILC1s lacked the expression of CD127 (IL-7Rα) but did express IL-2Rβ chain. The murine counterpart of human ieILC1s localizing within the gut epithelium co-expressed CD160, NKp46, and NK1.1 (20). Examining the developmental pathway of ieILC1s in mice, Fuchs et al. demonstrated the requirement of NFIL3 and T-bet. These ieILC1s were in part independent of IL-15Rα, indicating that intraepithelial ILC1s are developmentally distinct from cNK cells (20). Functionally, and similar to ex-ILC3s, ieILC1s were linked to the immunopathology in the αCD40 model of colitis due to their IFN-γ production (20, 52, 207). Further, ILC1s were enriched in patients with Crohn's disease and may, therefore, contribute to the development of inflammatory bowel disease similar to lamina propria ILC1s (19, 20, 208).

In the lamina propria of the intestine, it was challenging to identify ILC1s because of the sizeable populations of NK cells and ex-ILC3s, which all expressed the prototypic makers of ILC1s, such as NKp46 and NK1.1. Using double-reporter mice for EOMES (labeling NK cells) and fate-labeling for RORγt (labeling all ILC3s independent of their RORγt expression), a subset of lymphocytes within NKp46 and NK1.1 lymphocytes was defined, which expressed T-bet. This population within $NKp46^+$ $NK1.1^+$ lymphocytes lacked EOMES and RORγt expression and did not have a history of RORγt expression either. Further, such ILC1s were developmentally dependent on T-bet, NFIL3, and GATA-3, but not EOMES or RORγt. Phenotypically, intestinal ILC1s expressed markers associated with ILC1s in different tissues such as CD127, CD160, or CD49a, lacked markers of cNK cells such as CD11b and CD62L, and showed low Ly49 receptor expression. Despite expressing both CD127 and CD122, ILC1s were strictly IL-15-dependent and did not require IL-7. Upon

transfer into alymphoid mice, ILC1s were a stable lineage without differentiation potential into cNK cells or ex-ILC3s and could also be found in the BM (35). BM ILC1s phenotypically overlap with the previously described immature NK cells based on markers such as CD69 (140). However, markers often connected to immature NK cells, such as CD69, TRAIL, or CD51, are rather found on ILC1s, and it should also be noted that they are not expressed before or after that developmental stage during NK cell development. In addition, CD69 is considered to be a marker for cell activation or tissue residency, which is associated with activated rather than with immature lymphocytes (136, 140). Therefore, additional studies have to address the potential heterogeneity within EOMES$^-$ NK1.1$^+$ cells, previously termed "immature NK cells" in the BM.

Although cytokine IL-12 was first described as a NK cell-stimulating factor (209), IL-12 elicited stronger effects on ILC1s than on NK cells, consistent with higher expression levels of the components of IL-12 receptor on ILC1s (21, 62, 210). While ILC1s were potent producers of IFN-γ and TNF, they expressed less perforin, indicating that they are less cytotoxic and rather mediate the cytotoxic effect by TNF receptors such as TRAIL. Functionally, a lack of perforin-mediated cytotoxicity or a loss of NK cell identity resulted in decreased immunosurveillance of tumors (181, 182, 211). However, data from different infection models suggest that there is a spatial and a temporal division of labor between NK cells and ILC1s. ILC1s protected the digestive tract from *Toxoplasma gondii*, *Clostridium difficile*, or MCMV infections, which are controlled to a large degree by IFN-γ secreted by ILC1s (21, 210, 212).

ILC1s, also referred to as tissue-resident NK (trNK) or TRAIL$^+$ NK cells in the liver (142), differed from conventional NK cells since they expressed only T-bet as the key TF in mice, and this expression is favored in the liver microenvironment (16, 29, 35, 36). On the contrary, the BM provides a microenvironment that promotes lower expression levels of T-bet in NK cells, enabling the subsequent expression of EOMES (35). Another remarkable difference between cNK cells and ILC1s was the expression of the "homolog of BLIMP1 in T cells" (HOBIT) in ILC1s (213). This TF is specifically up-regulated in tissue-resident cells and controlled the expression of molecules associated with tissue residency, such as CD49a and CD69. Interestingly, HOBIT was essential for liver ILC1s but not for ILC1s in other organs investigated (210, 213). In addition, the development of ILC1s in the liver was demonstrated to be dependent on PLZF expression and independent of NFIL3, contrary to NK cells (36, 98).

TRAIL represents a prototypic marker of liver ILC1s as it is constitutively expressed on both mouse and human ILC1s, and together with CD49a and CD69, it has been used for separating liver ILC1s from NK cells. This type II transmembrane protein causes apoptosis primarily in tumor cells by binding to certain death receptors. Recent findings are suggesting that TRAIL expression is regulated by the activation of the NKp46 receptor in ILC1s since NKp46-deficient mice lack this effector protein (214–216).

Another important functional hallmark of liver pro-inflammatory ILC1s is that they are activated *via* IL-12, which are produced by conventional dendritic cells upon infection. After activation with IL-12, ILC1s respond with IFN-γ secretion to limit viral load and thereby contribute to early antiviral immunity at sites of primary viral infection (210). The genetic ablation of liver ILC1s is leading to increased MCMV load in mice; hence, NK cell responses are not the only early antiviral response in mice. In addition to rapidly responding to IL-12, "memory-like" qualities have been reported for ILC1s in models of contact hypersensitivity and MCMV infection. This is remarkable because these cells were originally considered as "immature NK" cells due to the lack of surface markers characteristic of mature NK cells (142). ILC1s were described to mediate tissue-resident memory responses to MCMV depending on glycoprotein m12 (217). Furthermore, previous reports have already linked liver ILC1s to memory responses during contact hypersensitivity reactions (29, 218). However, the mechanism underlying recognition of haptens by ILC1s following memory responses remains elusive.

Taken together, the experimental evidence obtained from knockout mice suggests that ILC1s constitute a separate tissue-resident lineage distinct from cNK cells. Further investigation is required to answer questions of ILC1 diversity.

EPIGENETIC AND microRNA-MEDIATED REGULATION OF NK CELL AND ILC1 DEVELOPMENT

Among epigenetic modifications, the deubiquitination of histone H2A by MYSM1 is important for NK cell generation as the deletion of this enzyme is causing maturation defects in NK cells (219). The MYSM1 histone H2A deubiquitinase also contributed to the development of ILC1s in other organs. In addition to modifying histones, MYSM1 also functions as a transcriptional regulator of ID2 expression during the maturation of NK cells by recruiting NFIL3 to the *Id2* gene locus. MYSM1 was involved in maintaining an active chromatin configuration at the *Id2* locus (219), further promoting its expression. Another epigenetic mechanism that regulates NK cell development involves repressive histone marks such as the tri-methylation of lysine residue 27 of Histone 3 protein during early NK cell differentiation (220). In the absence of this marker through the repression of EZH2 enzymatic activity (enhancer of zeste homolog 2), ILC1 and NK cell lineage commitment was enhanced, together with increased NK cell survival and NKG2D-mediated cytotoxicity (220).

Apart from the regulation of gene expression on the transcriptional level, another epigenetic mechanism is required for the proper development of ILC1s and the adequate maturation of NK cells. Available data implicate small non-coding RNA molecules (221–226), such as microRNAs (miRs), to regulate posttranscriptional gene expression by binding to the 3′ untranslated region (UTR) of mRNAs and inducing either suppression or mRNA translation or its degradation (227). Deletion of the RNase III enzyme Dicer-1, an enzyme required for the generation of single-stranded 20–25 bp long non-coding

RNA molecules, in NKp46-expressing cells revealed the role of miRs in murine NK cells and ILC1s (223). The number of NK1.1$^+$ cells in the organs of Dicer-1 mutant mice was affected, along with the impaired maturation of NK cells. NK cells without miR showed a diminished function, including reduced target cell cytotoxicity and IFN-γ production. Additionally, in Dicer-1-deficient mice, the IL-15 receptor signaling in NK cells was impaired. This finding explains, at least in part, the decreased survival of NK cells and the observed perturbations in NK cell maturation.

The effects of single miRs, such as miR142, miR155, miR150, and miR15/16, revealed specific effects and potential target genes. The conserved *miR142* sequence encodes two highly expressed mature miRNAs, 142-3p and 142-5p, which have different mRNA targets (221). The target of the miR142-3p is the 3′ UTR of *Itgav* gene that encodes integrin-α_V. In the absence of miR142-3p, this integrin was up-regulated in ILC1s and promoted their survival. The other product of the *miR142* sequence, miR142-5p, was targeting the 3′ UTR *suppressor of cytokine signaling 1* (*Socs1*) gene, a negative regulator of IL-15 signaling. Thus, in the absence of miR142-5p, SOCS1 un-antagonized, leading to impaired IL-15 signaling (221).

In humans, miR155 was shown to down-regulate SH2 containing 5′ inositol phosphatase (SHIP1), which in part contributes to the regulation of IFN-γ production following stimulation (225). In mice, miR155 targeted the 3′ UTR of *Noxa* transcripts during homeostasis and of *Socs1* transcripts during the activation of NK cells (226). The direct functional target of miR-150 and miR15/16 was the TF c-Myb, through which the maturation program was controlled (222, 224).

PLASTICITY TOWARD GROUP 1 ILCs

Although ILCs comprise separate lineages of innate lymphocytes defined by distinct lineage-specifying TFs, a considerable amount of plasticity after fate commitment was reported for most ILC lineages in mice and humans, often connected to a certain tissue microenvironment or in the context of inflammation (228). Plasticity is characterized by the down-regulation of lineage-specifying TFs, such as RORγt for ILC3s or GATA-3 for ILC2s, and acquisition of master TFs of alternative cell fates, acquisition of phenotypic characteristics of other ILC lineages (e.g. up-regulation of NK cell receptors), and production of cytokines not associated with the original lineage. The plasticity of ILCs was first described for ILC3s (52, 229). Fate-labeling for RORγt expression revealed that ILC3s were able to differentiate into cells phenotypically resembling ILC1s (referred to as ex-RORγt$^+$ ILC3s or ex-ILC3s) (52, 229–231). This process was accompanied by the gradual up-regulation of ILC1 signature genes such as T-bet, NK receptors (NKp46, NK1.1, and NKG2D), and cytokine receptors (IL12Rβ2), as well as effector functions (19, 21, 44, 53, 55). During this process, ex-ILC3s became IFN-γ-producing lymphocytes, which were responsive to several cytokines, including IL-12 and IL-23, and promoted inflammation and immunopathology in experimental models of colitis and *Salmonella enterica* infection (44, 52, 207, 229, 232, 233). T-bet deficiency was *vice versa* reported to promote colitis in response to *Helicobacter typhlonius* that was mediated by IL-17A-producing ILC3s (234, 235). In humans, differentiation of ILC3s toward CD127$^+$ ILC1s was described in the intestine of patients with Crohn's disease and was promoted by cytokines IL-2 and IL-12 and CD14$^+$ DCs. Interestingly, this process was found to be reversible and stimulated by IL-1β, IL-23, retinoic acid, and CD14$^-$ DCs (19, 229, 230). Data obtained in fate-labeling studies in mice using either *RORγt*^{Cre} (230) or *NKp46*^{Cre} (236) also support the model that the plasticity of ILC3s is a reversible process. Signals regulating NKp46 expression on CCR6$^-$ ILC3s included the Notch-T-bet axis as a positive regulator and TGF-β signaling as a negative regulator (236). Altogether these studies provide evidence for the reversible plasticity of CCR6$^-$ ILC3s toward ILC1s, mediated by signals that regulate RORγt and T-bet.

While the down-regulation of RORγt and the up-regulation of T-bet occurs at steady state in CCR6$^-$ ILC3s, the plasticity of NK cells or ILC2s might require a trigger, such as chronic inflammation. The conversion of ILC2s to an ILC1-like phenotype is triggered by cytokines, such as IL-1, IL-12, and IL-18, and was described in the context of chronic obstructive pulmonary disease (237–240). This process is connected to the up-regulation of T-bet, and the genetic deletion of T-bet using *NKp46*^{Cre} resulted in enhanced ILC2 responses, suggesting that the balance of the lineage-specifying TFs GATA-3 and T-bet determines ILC2 plasticity (241).

Whether the conversion of NK cells to ILC1-like cells is occurring at a steady state is difficult to evaluate because of the lack of fate-labeling studies for the NK lineage-specifying TF EOMES. However, fate-labeling was carried out using Cre under the NKp46 promoter, which is expressed in NK cells, ILC1s, CCR6$^-$ ILC3s, and subsets of γδ T cells (138). While the down-regulation of NKp46 was described for ILC3s (236), the results did not provide evidence that NK cells down-regulate NKp46 at steady state (138). It should be considered though that conversion in other ILC lineages, which also express NKp46, would not be detected using this fate-labeling strategy. The first evidence for the potential conversion of NK cells into ILC1-like cells came from studies that investigated the unusual subsets of ILC1s in the salivary gland. Unlike ILC1s in other organs, the salivary gland ILC1s co-expressed EOMES and T-bet but did not developmentally depend on either of these TFs and also not on NFIL3, suggesting that they have different developmental requirements (199, 242). In addition, the salivary gland ILC1s depicted hallmarks of tissue-resident cells, such as TGF-β imprinting, that was also reported for ILC1s in different organs, for instance, the intestine (20). ILC1s in the salivary gland were reduced in the absence of TGF-β signaling, and the phenotypical markers of ILC1s, such as CD49a and TRAIL were down-regulated, whereas EOMES was up-regulated (199). Furthermore, NK cells that were hyper-responsive to TGF-β, due to the genetic manipulation of TGF-βRI or deletion of SMAD4, developed an ILC1-like phenotype in the salivary gland or within tumor tissue. As a consequence, these ILC1-like NK cells failed to control tumor growth or viral infection with

cytomegalovirus (181, 182). Some of the effects that TGF-β has on the NK/ILC1 fate decisions are mediated *via* the balance of the master TFs T-bet and EOMES. Notably, it was reported that the forced expression of EOMES driven by the T-bet promoter turned ILC1s into cells with NK cell properties (178). However, it remains unclear whether this occurs *in vivo*, and if yes, under which conditions.

While ILC plasticity after lineage commitment is now well-established to occur, additional investigation is required to elucidate how the plastic behavior of ILCs could be therapeutically harnessed.

SPECIFIC TARGETING TO UNCOVER FUNCTIONAL SPECIALIZATION OF GROUP 1 ILC SUBSETS

Despite progress in the generation of genetically modified mice, specific targeting of ILCs remains a major challenge in the field because of the large overlap in gene expression between ILCs and T cells as well as other immune cells. Since a systematic review of genetic models for the investigation of ILC function was recently published (243), we aim to focus the discussion on NK cell receptor $(NKR)^+$ ILCs that comprise conventional NK cells, ILC1s, and CCR6$^-$ ILC3s. Concerning NKR^+ ILCs, specific targeting of each subset is further complicated by shared receptors such as NKG2D, NKp46, and NK1.1 and TFs such as T-bet, NFIL3, or TOX within NKR^+ ILCs, making them alone not a suitable target (141). While antibody-mediated depletion strategies using αNK1.1 or αThy1 were effective, more specific depletion strategies were developed using genetic models based on *NKp46*^{Cre} mice (138, 153, 244). While NKp46 is fairly specific to group 1 ILCs, a second allele is required to ensure specificity among group 1 ILCs, which is often a floxed mouse for an essential TF such as EOMES or RORγt. Following the targeting strategy, the generation of *NKp46*^{Cre} *Eomes*$^{fl/fl}$ resulted in the selective ablation of NK cells, thus allowing a definitive conclusion about the contribution of NK cells in an experimental autoimmune encephalomyelitis model (178, 179). *NKp46*^{Cre} *Rorc*(γt)$^{fl/fl}$ mice were likewise generated to investigate redundant and non-redundant functions of ILC3s during *Citrobacter rodentium* infection and in colitis models (233, 245). While these two strains provide specific targeting for NK cells and CCR6$^-$ ILC3s, respectively, the genetic mouse models for ILC1s are even more difficult to develop. *NKp46*^{Cre} *Tbx21*$^{fl/fl}$ mice lacked ILC1s (179), but a contribution of NK cells or CCR6$^-$ ILC3s in these mice could not be excluded because NK cells and CCR6$^-$ ILC3s have a migration or maturation defect in T-bet-deficient mice. It should be also considered that the phenotype might be dependent on which line of NKp46^{Cre} deleter mice is used (179, 241). Furthermore, mice deficient for the TF HOBIT were used to investigate ILC1 function in the liver because ILC1s, but not NK cells, are reduced in the liver of these mice. However, the use of this mouse line is limited to $TRAIL^+$ NK cells and not ILC1s in other organs (210, 213). Therefore, the goal for *NKp46*^{Cre} to delete a selective TF important for ILC1 subsets in many organs is still not achieved.

REGULATION OF NK CELL DEVELOPMENT AND FUNCTION BY RECEPTOR–LIGAND INTERACTION

The activation of NK cells is mediated to a large degree by the integration of stimulatory and inhibitory signals as measured by the engagement of NK receptors by its cognate ligands. NK cells need to be calibrated during development to become activated if a defined threshold of stimulatory to inhibitory signals is exceeded, a process coined "NK cell education" or "licensing." Classical NK cell education is linked to self recognition and mediated by inhibitory receptors for class I MHC, such as Ly49 receptors or KIR (82, 246–248). Thus, this process requires the timely expression of the corresponding ligands for the receptors involved in the education process. Moreover, besides the type of MHC molecule expressed and the type of receptors on NK cells, the strength of class I MHC–Ly49 receptor interaction also defines the quality and the quantity of NK cell education (249). In connection with this, it was observed that the absence of MHC I on the surface of cells, by the genetic deletion of β_2-microglobulin, TAP, or K^bD^b, resulted in the hypo-responsiveness of NK cells (250–252). In line with these findings, NK cells with mutations in ITIMs required for inhibitory signaling were functionally impaired. Further, the deletion of intracellular downstream signaling molecules, SH-2-domain-containing protein tyrosine phosphatase 1 (SHP1) and SH-2-domain-containing inositol-5-phosphatase (SHIP), resulted in the hypo-responsiveness of NK cells (253–255). On a molecular level, the MHC I education process was linked to the reorganization of the nanostructure of immunoreceptors and confinement in domains, thus generating the basis for different activation thresholds (251, 252, 256).

Interestingly, Ly49s were strongly underrepresented but not totally absent on ILC1s, suggesting differences between NK cell and ILC1 education. However, with respect to ILC1s, fundamental questions remain unanswered. These questions include whether ILC1s require an education process at all and, if so, whether the education is regulated by cell-bound immunoreceptor–ligand interaction. If this is true, how much of education is regulated by inhibitory receptors such as CD94/NKG2A expressed by ILC1s? Due to the lack of data for ILC1 education, we focus on the regulation of NK cell development and activation (21, 141).

NK cell education not only is limited to self recognition of MHC I molecules but also involves self recognition of non-MHC ligands such as those provided by the receptor–ligand pairs CD155-TIGIT, CD48-2B4, and CLR-b-NKRP1-B (252). Moreover, it became apparent that stimulatory receptors mediating induced-self recognition are involved in the NK cell education process as well. NKG2D is a stimulatory receptor, which recognizes induced-self ligands and which regulates NK cell education (90). NKG2D is already expressed early on from the NKP stage. For induced-self ligands, it is however incompletely understood when these ligands are expressed under homeostatic conditions and which cell types would be involved in this process (132, 257, 258). Parallel to the finding that

NK cells from MHC I-deficient mice were hypo-responsive, a similar paradoxical phenotype was uncovered for NKG2D-deficient mice having hyper-responsive NK cells despite the lack of an important stimulatory NK cell receptor. Notably, mice deficient for NKG2D (*Klrk1*$^{-/-}$) were hyper-responsive, resulting in the superior control of MCMV infection and tumor growth (257, 258). However, immunosurveillance of tumors expressing NKG2D ligands was impaired in NKG2D-deficient mice (259). Mechanistically, NKG2D regulated signaling *via* the natural cytotoxicity receptor NKp46 and the signaling molecule CD3ζ (258). While the precise timing of NK cell education is not well-defined, some studies suggested that NK cell education is not limited to a time window during development but represents a continuous process. This is supported by studies that used adoptive transfer of uneducated NK cells in MHC I-sufficient hosts that could restore NK cell functionality (260, 261). In addition, data obtained in models that overexpressed ligands for stimulatory receptors such as m157 or NKG2D ligands, in which the NK cells were persistently exposed to a receptor engagement, revealed that NK cells adapted to this stimuli, for instance by the down-regulation of the stimulatory receptor (262–264). Although it remains elusive if the adaptation of NK cell reactivity to sustained activation by stimulatory ligands due to overexpression is the underlying similar mechanism described for NK cell education, the findings become relevant in the context of anti-tumor immunity where, for instance, NKG2D ligands might be continuously expressed or shedded from the tumor cells, thus saturating their receptors. Although it is controversial if the chronic expression or shedding of NKG2D ligands should be regarded as a tumor escape mechanism or if it is promoting tumor immunosurveillance, these findings indicate the importance of the regulation of NK cell activity by receptor–ligand interaction (265–267). Apart from the chronic expression of induced-self ligands on tumor cells, blocking antibodies for inhibitory receptors targeting KIR or NKG2A are evaluated in clinical trials to promote anti-tumor immunity (267). The blockade of inhibitory receptors on NK cells has the potential to complement T cell immunotherapy because the efficiency of T cell checkpoint blockade correlated with the production of neoantigens by tumor cells present on MHC I. However, the tumor cells that did not produce neoantigens or escaped MHC I-peptide recognition by CD8$^+$ T cells (268) could be recognized and lysed by NK cells expressing inhibitory receptors to detect the presence of MHC I. However, data available so far might indicate that there is a narrow therapeutic window defined by the blocking of the inhibitory receptor and the effects on NK cell education, rendering the cells hypo-responsive (267).

In summary, NK cells need education mediated by the engagement of inhibitory and stimulatory receptors during development. NK cell education is required for both adequate reactivity and tolerance toward self. Blocking of inhibitory NK cell receptors during anti-tumor therapy can complement checkpoint blockade and illustrates the transfer of basic knowledge for human therapy.

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 from the German Research Foundation (DFG; KL 2963/1-1 and KL 2963/2-1 to CK) and the European Research Council (ENTRI to CK).

ACKNOWLEDGMENTS

We thank Dr. Divija Deshpande, Dr. Katja Jarick, Caroline Tizian, and Akriti Kanth for critically reading the manuscript. The figures were created with BioRender.com.

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