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Diversification of human NK cells: lessons from deep profiling

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Abstract

Natural killer (NK) cells are innate lymphocytes with important roles in immunoregulation, immunosurveillance, and cytokine production. Originally defined on the functional basis of their 'natural' ability to lyse tumor targets and thought to be a relatively homogeneous group of lymphocytes, NK cells possess a remarkable degree of phenotypic and functional diversity due to the combinatorial expression of an array of activating and inhibitory receptors. Diversification of NK cells is multifaceted: mechanisms of NK cell education that promote self-tolerance result in a heterogeneous repertoire that further diversifies upon encounters with viral pathogens. Here, we review the genetic, developmental, and environmental sources of NK cell diversity with a particular focus on deep profiling and single-cell technologies that will enable a more thorough and accurate dissection of this intricate and poorly understood lymphocyte lineage.

Summary:

The contexts that influence human NK repertoire diversification and their implications for human health and disease are reviewed.

Keywords

Natural killer cell or NK cell; lymphocyte diversity; innate lymphoid cell; host-pathogen interaction; mass cytometry; single-cell technology

Introduction

Natural killer (NK) cells are a diverse group of innate lymphoid cells (ILCs) that can coordinate and execute the rapid elimination of neoplastic and virus-infected cells [1,2]. ILCs represent a group of largely tissue-resident common lymphoid progenitor-derived cells that do not express somatically-recombined antigen-specific receptors [3–7]. ILCs are now

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Contributions

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recognized to play significant roles in immune homeostasis, and their characteristics and functions have been thoroughly reviewed elsewhere [4,5,7]. Here, we focus on "conventional" human NK cells, a subset of type I ILCs which have been primarily evaluated in the blood.

NK cells were first identified in both mice and humans in the 1970s on the basis of their ability to kill tumor cells without the requirement of prior priming [8,9]. Primary functions of NK cells include production of IFN-γ and other cytokines, immunoregulation through the perforin-dependent elimination of activated leukocytes, and immunosurveillance through cytotoxicity against tumors or virus-infected cells [2,10,11]. These functions have intriguing clinical implications. For example, NK cells may have protective roles in several autoimmune diseases by eliminating activated autoreactive lymphocytes [12–15]. Further, higher NK cell cytotoxicity is protective against the development of cancer [16]. This latter finding, considered along with the ability of NK cells to eliminate their targets in rapid succession, has led to much interest in the development of NK cell-based cancer therapies, including adoptive NK cell therapy and pharmaceuticals that modulate the activity of NK receptors (NKRs) [17,18]. That NK cells play an important role in the control of early viral infection, particularly by herpesviruses, is evident from severe herpesvirus infections in exceedingly rare cases of primary NK cell deficiency [19], as well as evidence of NK cell dysfunction in immunodeficiencies like X-linked lymphoproliferative disorder [20–22]. Evidence of strong antiviral responses by NK cells, combined with recent findings of memory-like responses by NK cells, has bolstered the argument that future vaccine development should seek to provide long-lasting NK cell immunity [1,23].

In a sense, NK cells can be considered immune intermediaries, with both lifetimes and receptor-to-pathogen ratios between their adaptive lymphocyte and common myeloid progenitor-derived counterparts [24]. NK cells combine diversity, on the order of 10^4 subpopulations per person, with repertoire flexibility, being capable of altering receptor expression on the timescale of cellular processes [25]. Neither the clinical consequences of this diversity nor the functional characteristics of individual NK cell subsets have been fully elucidated. A better understanding of how this diversity influences NK cell responses and how it can be modulated is necessary for the development of therapeutic strategies employing NK cells. Here, we review the various contexts of NK cell diversification, as well as recent findings and future directions in the deep profiling strategies needed to dissect the functions of this complex group of ILCs.

Single-cell diversity of NK cells

Diversity is an essential characteristic of the immune system, as it must be prepared to respond to innumerable unknown pathogens. Leukocyte diversity is most often attributed to adaptive lymphocytes, which express somatically rearranged antigen-specific receptors, generating on the order of 10^6-10^8 distinct specificities per person [26]. NK cell diversity, on the other hand, is determined by the combinatorial expression of an array of germline-encoded inhibitory and activating receptors. While self-tolerance of individual T and B cells is ensured through selection on a single cell surface receptor, the differential expression of NKRs necessitates a complex developmental process to ensure a tunable and self-tolerant

NK cell repertoire. These processes are referred to as NK cell education, arming, or licensing and are reviewed extensively elsewhere [27–31].

On a population level, NK cell diversity at the protein expression level arises in three main fashions: genetic diversity of killer immunoglobulin-like receptors (KIRs), stochastic expression of KIRs, and differential expression of other NKRs [32]. Early studies of NK cell diversity relied on multi-color flow cytometry to profile NK cell KIR repertoires [33–40], and a more comprehensive view has emerged in a recent study that employed cytometry by time-of-flight (CyTOF) [41]. CyTOF is a flow cytometry-based platform where, instead of labeling cells with fluorophore-conjugated antibodies, cells are labeled with heavy metal isotope-conjugated antibodies. This allows for the simultaneous assessment of ~40 parameters with a mass cytometry readout without the need for compensation of spectral overlap [42,43]. While this technology vastly improves the number of markers evaluated, downsides include the inefficient capture of events, such that only ~20% of live cells are captured as singlet events, and its slow speed [42–44]. Currently, inroads are being made to add a similar number of parameters to conventional flow cytometry platforms, but any high dimensional analysis involves analytic complexity, often involving multiple parallel analytic techniques to feel confident in results obtained [44].

Recently, our group used CyTOF to evaluate 28 NKRs by CyTOF, identifying between 6,000 and 30,000 distinct NK cell subsets per donor and >100,000 distinct subsets in the 22 donors studied based on a Boolean analysis of subsets based on yes/no expression patterns of the different receptors [41]. Additionally, our study utilized twin donors to demonstrate that inhibitory NKR diversity was more genetically controlled than activating NKR repertoires, which were under more environmental influence [41]. This diversity indicates that some subsets could be better tuned to detect certain types of infections or malignancies, based on NKR expression levels relative to ligand expression patterns during infection or malignancy. While this raises interesting possibilities, there are several important caveats when interpreting these data. There are more NKRs that can be added to this marker panel, and increases in the number of markers evaluated could increase the number of distinct NK cell subsets detected. Further, while this study reveals a high degree of phenotypic diversity among human NK cells, this may not reflect the same degree of functional diversity. It will be important for future studies to evaluate functional markers as well to assess the relationship between phenotypic and functional diversity. Lastly, by interrogating proteomic diversity, this study only analyzes one metric of NK cell diversity. Other methods, including scRNA-seq and scATAC-seq, should be utilized to gain a more comprehensive understanding of NK cell diversity. This study, nonetheless, presents significant insight into the magnitude of NK cell repertoire diversity and provides a methodological foundation for future deep functional profiling of individual NK cell subsets.

Mechanisms of NK cell diversification: genetic and environmental determinants

Development and functional specialization of NK cell subsets

NK cells develop in the bone marrow and secondary lymphoid tissues in discrete stages that have been well characterized [45–51]. After several precursor stages, immature NK cells gain expression of CD56, CD94/NKG2A, and several activating receptors including NKp46, NKp30, and NKG2D. These cells, called $CD56^{bright}$ NK cells, are highly proliferative, capable of producing high amounts of IFN-γ, and express IL-7Rα and KIT, two surface markers associated with non-NK ILCs [3,52,53]. In humans, $CD56^{bright}$ NK cells are hypothesized to give rise to $CD56^{dim}$ NK cells during the process of education; this involves a decrease in CD56 expression coupled with increased expression of CD16 and acquisition of KIRs [45,48,54]. $CD56^{dim}$ NK cells are potently cytotoxic, but have substantially lower proliferative and cytokine-producing capacity than their $CD56^{bright}$ counterparts. Further maturation of $CD56^{dim}$ NK cells is generally indicated by the loss of CD62L and acquisition of CD57.

That $CD56^{bright}$ NK cells are precursors to $CD56^{dim}$ NK cells in humans remains unclear. This hypothesis, originally advanced in 1986 [55], is supported both by the observations that $CD56^{bright}$ NK cells have longer telomeres than $CD56^{dim}$ NK cells [52], and that *in vitro* stimulation of $CD56^{bright}$ NK cells with IL-2 results in acquisition of CD16 and KIR expression and loss of IL-7Rα and KIT [52]. However, a recent study used genetic barcoding of hematopoietic stem cells in macaques and demonstrated that $CD56^{bright}$ and $CD56^{dim}$ NK cells have distinct developmental precursors [56]. Considered along with new findings of NK/ILC1 plasticity [57–59], these data suggest that the ontological relationships between human NK cells and ILCs may be more nuanced than originally appreciated.

Outside of these major conventional NK cell subsets, two other classes of NK cells bear mention. First, some $NKG2C^{hi}CD57^{hi}$ NK cells that are expanded by HCMV infection display enhanced IFN-γ production and cytotoxic capacity upon secondary challenge by the homologous pathogen and are called ‘adaptive’ NK cells due to their memory-like properties [1,33,60–63]. Understanding the development and function of adaptive NK cells is of considerable interest as their memory-like properties could be harnessed for therapeutic benefit in vaccines or cancer immunotherapy. Secondly, while we have only discussed the properties of circulating NK cells, there are numerous subsets of NK cells resident in various human tissues. These largely tissue-resident NK cells have diverse functions in reproduction and tissue remodeling, and their ontogeny and diversity have been reviewed elsewhere [64,65].

Below, we discuss the genetic and environmental factors that influence the combinatorial expression of various NKRs. These findings are summarized in Figure 1.

KIR diversity and NK cell education

The KIRs, encoded by 14 genes and 2 pseudogenes clustered on chromosome 19, represent an important family of NKRs. KIRs are HLA class I binding receptors and are the second

most polymorphic gene family in the human genome, after HLA [66]. KIRs are named according to the relative length of their cytoplasmic tail, with ‘S’ denoting a short tail that generally confers activating activity, and ‘L’ denoting a long tail that corresponds to inhibitory activity. Importantly, KIRs are expressed stochastically by NK cells and this, combined with the polygenic and polymorphic nature of KIRs, contributes significantly to NK cell diversity [33,35,67].

KIR-HLA interactions represent the main mechanism by which NK cells are tolerized to and sense perturbations in the self-HLA environment [68,69]. Although the majority of NK cells express at least one inhibitory KIR for a self-HLA molecule [31], significant proportions of NK cells do not express any self-specific inhibitory KIR [27,70]. To compensate for this, most KIR-negative NK cells express CD94/NKG2A, an inhibitory receptor that binds to the ubiquitously expressed HLA-E [36,71–73]. Therefore, the vast majority of NK cells retain mechanisms to recognize cells that have downregulated HLA class I due to pathogen infection. Nonetheless, approximately 10% of NK cells in a given individual express neither CD94/NKG2A nor self-specific KIR [35,74]. Some of these inhibitory NKR-deficient NK cells are capable of promoting autoimmunity due to their expression of granzyme and perforin. [75,76]. The process that renders NK cells responsive to ‘missing self’ involves functional calibration to self-HLA, and is called education, licensing, or arming [27–31]. Interestingly, recent data suggest that NK cells can also be educated through CD94/NKG2A, and that this form of education results in higher phenotypic diversity, IFN-γ production, and cytotoxic capacity [77].

Despite the apparently stochastic nature of KIR expression during NK cell development, certain KIR-expressing NK cells are favored for survival or expansion based on their ability to interact with their cognate HLA molecule. For instance, there is a preponderance of human NK cell repertoires with a bias for expression of the KIRs that are capable of binding to the HLA molecules expressed in that individual (for instance, KIR2DL1 recognizes HLA-C2, and in individuals homozygous for HLA-C2, there is an increase in the frequency of KIR2DL1-expressing NK cells) [33,78–80]. This biased repertoire is dependent on human cytomegalovirus (HCMV) infection, suggesting that the NK cells expressing KIRs specific for the HLA expressed in that individual are selected for during the response to HCMV infection [33,78–80].

In addition to HCMV infection inducing expansions of NK cells expressing self-specific KIRs, other viral infections have been associated with skewing of the KIR repertoire. In the setting of HCMV seropositivity, chronic Epstein-Barr virus (EBV) infection results in a NK repertoire more dramatically skewed towards self-specific KIRs [81]. In the absence of HCMV infection, infectious mononucleosis during primary EBV infection causes transient fluctuations in the KIR repertoire, but does not result in stable KIR skewing [82,83]. Additionally, Chikungunya virus (CHIKV) selectively modulates the NK KIR repertoire by preferentially expanding those cells expressing HLA-C-specific KIRs, such as KIR2DL1, specific for HLA-C2 alleles, and KIR2DL2/DL3, specific for HLA-C1 alleles [84].

Notably, HIV-1 infection results in the expansion of KIR3DS1- and KIR3DL1-expressing NK cells in individuals expressing the cognate HLA-Bw4–80I epitope [85,86]. In these

individuals, KIR3DS1/DL1 is associated with slower progression to AIDS and KIR3DS1/DL1 copy number is inversely correlated with the set point viral load [86–92]. It remains unclear how an activating and an inhibitory NKR with virtually identical extracellular domains both correlate with better clinical outcomes, but recent evidence suggests that this paradox may be explained by heretofore unrecognized KIR3DS1 ligands, the involvement of HIV peptides in modulating the KIR3DS1/HLA-Bw4–80I interaction, and the level of education through KIR3DL1 [32,93–95].

CD57 and CD62L: markers of maturation

CD57 is a carbohydrate epitope created by the enzymatic activity of beta-1,3-glucuronyltransferase and has long been appreciated as a marker of differentiation, maturation, and activation on NK and T cells [96–99]. On NK cells, CD57 expression is limited to $CD56^{dim}CD16^{+}$ NK cells, is correlated with lower proliferative capacity upon exposure to cytokines or neoplastic cells, and is associated with increased cytolysis and IFN-γ production [97,100–102]. CD62L, on the other hand, is inversely associated with NK cell maturation. CD62L (also known as L-selectin) is a homing receptor important in lymphocyte-endothelial cell interactions. During NK cell development, CD62L is first expressed by $CD56^{bright}$ NK cells and is gradually lost during the processes of education and differentiation [103–106]. This process, perhaps analogous to the shedding of CD62L by T cells following activation, could prime NK cells to leave secondary lymphoid tissues and enter the circulation to mediate innate immune responses.

As markers of differentiation, CD57 and CD62L expression by NK cells is modulated by various viruses. Chronic infection with HCMV, EBV, hepatitis B virus (HBV), and hepatitis C virus (HCV) have all been reported to result in increased expression of CD57 by NK cells [82,83,107,108]. Notably, primary EBV infection induces a transient decrease in CD57 expression on $NKG2A^{+}$ NK cells, likely due to expansion of $CD57^{-}$ NK cells which either contract or acquire CD57 over time [82]. Although CD57 expression is generally considered to correlate with greater functional capacity, a $CD57^{-}$ NK cell subset was identified as the predominant population that degranulates and produces IFN-γ after co-culture with latent EBV-infected lymphoblastoid cell lines (LCLs) [109]. Additionally, loss of CD62L expression is characteristic of a $CD56^{bright}$ subset of tonsilar NK cells that accumulates during chronic EBV infection and restricts EBV-induced B cell transformation [110]. These findings collectively suggest that viral infections and NK cell maturity have a nuanced relationship at various stages of NK cell development.

C-type lectin receptors

NK cells express several members of the NKG2 C-type lectin receptor family. NKG2A and NKG2C form heterodimers with CD94 and bind the non-classical HLA molecule HLA-E [72,111]. NKG2A is an inhibitory receptor, whereas NKG2C is an activating receptor; similar to the role of inhibitory and activating KIR binding the same HLA ligand, the role of HLA-E inducing two ostensibly contradictory signaling pathways remains unclear [112–116]. However, NKG2C and NKG2A are rarely co-expressed on $CD56^{dim}$ NK cells, suggesting that the contradictory functions of NKG2A- and NKG2C-expressing NK populations may promote immune balance [60,117]. Recognition of HLA-E expression

through NKG2A/C is thought to be a secondary mechanism by which NK cells can monitor expression of classical HLA class I molecules, as HLA-E binds peptides derived from the leader sequence of classical HLA class I molecules [118]. NKG2A is expressed on all $CD56^{bright}$ NK cells and is gradually lost during ostensible differentiation to $CD56^{dim}$ NK cells [117,119].

NKG2D is an activating receptor that is more distantly related to the NKG2 family, forms a homodimer, and is reported to bind several ligands, including MICA, MICB, and ULBP1–6 [120–122]. As expression of these and other putative NKG2D ligands is associated with DNA damage, cellular stress, and malignant transformation [123–126], NKG2D is a critical NKR in the control of tumorigenesis [127–129].

Among the most dramatic and well-studied virus-induced NK cell expansions is that of $NKG2C^+$ NK cells in HCMV infection. First identified by Gumá and colleagues in 2004, up to 25% of NK cells in HCMV seropositive individuals express NKG2C, compared to ~2% in HCMV seronegative donors [60]. Expanded $NKG2C^+$ NK cells also tend to be $CD57^+$ [62], mediate superior ADCC, and produce higher levels of IFN-γ and TNF. Notably, in $NKG2C^+$ NK cells in HCMV seropositive individuals, IFN-γ and TNF promoters are stably hypomethylated [61,130–133]. This epigenetic reprogramming may provide a mechanistic explanation for the memory-like phenotypes observed in these $NKG2C^+$ NK cells.

Expansions of $NKG2C^+$ NK cells have also been reported in the context of immunodeficiency [134], organ transplantation [63,135–137], hantavirus infection [138], CHIKV infection [84], HIV-1 infection [139], and chronic HBV and HCV infection [107,140,141]. It remains unclear, however, if $NKG2C^+$ NK cell expansions induced by viruses other than HCMV are the result of a direct impact of the virus on NKG2C expression. Unfortunately, most studies that demonstrated expansion of $NKG2C^+$ NK cells during infection by viruses other than HCMV did not stratify donors based on HCMV seropositivity. It is conceivable, therefore, that the observed $NKG2C^+$ expansions are actually the result of a subclinical HCMV reactivation [142]. Primary EBV infection does not alter NKG2C expression in the NK cell repertoire, thus supporting the hypothesis that NKG2C expansions are an HCMV-specific phenomenon [82,83,143].

$NKG2A^+$ NK cells have also been reported to expand in the context of viral infection, particularly by EBV. Primary EBV infection expands a population of early-differentiated $CD56^{dim}NKG2A^+KIR^-$ NK cells that do not contract and gradually gain CD57 expression and lose CD62L expression over time [82,83,109,144,145]. Accumulation of this subset inversely correlates with EBV DNA levels in peripheral blood mononuclear cells (PBMCs), and is endowed with increased IFN-γ production and cytotoxic degranulation against autologous LCLs [82,109]. EBV-induced $NKG2A^+$ expansions are not limited to $CD56^{dim}$ NK cells, as a distinct $CD56^{bright}IFN\text{-}\gamma^{hi}NKG2A^+$ NK cell subset accumulates in the tonsils of EBV seropositive individuals [110]. Although it is unknown whether other viruses cause $NKG2A^+$ NK cell expansions, $NKG2A^+$ NK cells are implicated in better control of HIV-1 infection in *in vitro* models [146], due to an HLA-E-presented HIV peptide that prevents inhibitory interaction with NKG2A [147].

Several viral infections have been associated with changes in NKG2D expression by NK cells. There is an increase in NKG2D$^+$ NK cell frequency in EBV seropositive individuals, and such an increase is apparent as soon as 10 days after first EBV exposure in an *in vitro* model of infection [109,143,148]. NKG2D, along with NKG2A, is one marker represented on the predominant NK cell subset that produces IFN-γ and degranulates in response to coculture with EBV-infected LCLs [109]. HCV, on the other hand, appears to have a more nuanced impact on NKG2D expression by NK cells. Acute HCV infection induces an increase in NKG2D expression by both CD56^{bright} and CD56^{dim} NK cells [149]. However, patients with chronic HCV infection have lower frequencies of NKG2D$^+$ NK cells which have impaired cytotoxic potential and lower IFN-γ production; this may be due to increased levels of circulating IL-10 and TGF-β during chronic HCV infection [150]. Importantly, HCV-induced NKG2D downregulation can be reversed by addition of IL-15 *in vitro*, representing a potential way to bolster current treatment of chronic HCV infection.

Natural cytotoxicity receptors

Natural cytotoxicity receptors (NCRs) are members of the Ig-superfamily and represent a set of recently evolved NKRs [151]. Some NCRs, including NKp30, NKp46, and NKp80, are expressed on both activated and resting NK cells, whereas others, including NKp44, are upregulated after stimulation [152,153]. NCRs have been reported to be critical both in the control of viral infection and tumors. For example, higher NCR expression correlates with enhanced control of and delayed progression during HIV infection [154,155], and deletion of single NCRs impairs NK cell cytotoxic capacity against tumor cells [156,157]. There are many putative NCR ligands that have not been confirmed by independent research groups, and several conflicting reports on the role of particular NCRs in a given viral infection [120,158–162]. Refined studies of NCR signaling and structural analysis of NCR-ligand interactions will be necessary to dissect the complex roles of this receptor family in NK cell biology.

The impact of particular viruses on NK cell expression of NCRs remains poorly studied. During acute HCV infection, both NKp30 and NKp46 are upregulated exclusively on CD56^{bright} NK cells [149]. Additionally, NK cells stimulated with EBV-infected LCLs *ex vivo* have increased frequencies of NKp30$^+$ cells [143,148]. NCR expression is also altered on CD56^{neg} NK cells in the setting of chronic infection. CD56^{neg} NK cells are an aberrant subset of NK cells that are found in low frequencies in healthy individuals, but expand during chronic HIV and HCV infection. These cells are likely derived from CD56^{dim} NK cells, as they express CD16 and KIRs, but have impaired capacity for cytokine production, cytolysis, and proliferation [163]. Although early studies on CD56^{neg} NK cells may be unreliable as a result of incomplete exclusion of monocytes during flow cytometric gating analysis, more recent studies have confirmed that CD56^{neg} NK cells express lower levels of NKp30 and NKp46 in chronic HIV infection [164–167].

Other NKRs

Expression of several other NKRs have been reported to be influenced by different viral infections. 2B4 (also called CD244 or SLAMF4) is an NKR that interacts with CD48, which is expressed by all hematopoietic cells [168–170]. Although murine 2B4 has predominantly

inhibitory function [171,172], human 2B4 is conventionally considered to be an activating receptor that may function as a co-receptor for other activating NKRs like NKp46 [151,173]. Exposure of human NK cells to EBV-infected LCLs or the EBV-producing Akata cell line result in higher frequencies of $2B4^+$ NK cells [109,143], and a $2B4^+$ subset is responsible for greater IFN-γ production and cytotoxicity in this setting [109]. Additionally, acute HCV infection induces upregulation of 2B4 on both $CD56^{bright}$ and $CD56^{dim}$ NK cells [149].

Parallel to the Fas/FasL system, TNF-related apoptosis-inducing ligand (TRAIL) is a ligand for five death receptors that mediates apoptosis [174]. As such, TRAIL represents one effector mechanism for NK cells. TRAIL is required for IFN-γ-dependent suppression of tumor growth *in vivo* [175], and blockade of TRAIL activity in encephalomyocarditis virus (ECMV)-infected mice resulted in higher viral titers and earlier death [176]. TRAIL is upregulated by human NK cells during acute HCMV, EBV, and HCV infection, perhaps representing one mechanism by which NK cells provide early control of infection by these viruses [143,149,177].

DNAM-1 (also called CD226) is an activating NKR and adhesin that binds Nectin-2 (CD112) and Necl-5 (PVR), which are upregulated on several tumors [178-180]. DNAM-1 is also involved in the NK cell response to HCMV, HIV, and HCV infection [149,181–183]. Primary EBV infection has recently been reported to upregulate DNAM-1 on NK cells, although the functional consequences of this upregulation are unknown [143,148].

LILRB1 (also known as LIR1 or ILT2) is an HLA class I-binding inhibitory NKR, although KIRs and CD94/NKG2A are thought to dominate HLA class I-mediated inhibition of NK cells [184–186]. LILRB1 expression correlates with CD57 expression, suggesting that LILRB1 may be a marker of NK cell differentiation [107]. Despite its role as an inhibitory receptor, $LILRB1^+$ NK cells are implicated in better control of HIV-1 infection [187]. However, this may simply reflect the higher cytotoxic potential of more mature NK cells. LILRB1 is also upregulated on NK cells during infection with HBV, HCV, and EBV [107,145].

NK diversification and epigenetic reprogramming

While the NK cell repertoire is remarkably malleable and influenced by viral infections, it remains stable in the absence of environmental perturbations [188]. This suggests that epigenomic modifications may be involved in the processes of NK cell maturation and diversification. Although this ostensible epigenomic reprogramming remains poorly understood in conventional NK cells, there is evidence that epigenetic modifications could represent one mechanism that accounts for the rapidity of NK cell responses. During development, murine NK cells have a long-range acetylation profile across the *Ifng* locus that is rapidly modifiable by cytokine stimulation [189]. Such hyperacetylation should presumably result in rapid and robust IFN-γ production in appropriate activating contexts. Additionally, HCMV-expanded $NKG2C^{hi}$ NK cells display complete demethylation of the *IFNG* promoter, which is required to induce stable IFN-γ production after engagement of NKG2C or 2B4 [61]. Clues to epigenetic regulation of NK cell effector function may also be present in studies of T cells. For example, H3K9 acetylation of the *GZMB* (encoding granzyme B) and *PRF1* (encoding perforin) promoters increases granzyme B and perforin

expression and enhances $CD8^+$ T cell-mediated cytotoxicity [190]. Stat4-dependent recruitment of Brahma-related gene 1 (Brg1), a chromatin remodeling complex [191], remodels nucleosomes in the *IFNG* promoter of Th1 cells [192]. IFN-γ expression by $CD56^{dim}$ NK cells may be regulated by a similar mechanism, as $CD56^{dim}$ NK cells express high levels of Stat4 and Brg1 [105]. How $CD56^{bright}$ and $CD56^{dim}$ NK cells tightly and differentially regulate IFN-γ expression would provide valuable understanding of the mechanisms governing functional specialization of NK cell subsets.

Epigenetic reprogramming of adaptive NK cells, on the other hand, has been more thoroughly characterized. A recent pair of studies has demonstrated that HCMV drives the differentiation and diversification of adaptive human NK cells through epigenetic silencing of several signaling proteins and transcription factors [131,132]. These studies have provided a model for adaptive NK cell diversification whereby engagement of DAP12-coupled activating receptors results in epigenetic imprinting of the *ZBTB16* locus, resulting in loss of PLZF expression. PLZF-deficient NK cells also display stochastic hypermethylation of promoters encoding signaling proteins SYK, EAT-2, DAB2, and FcεRγ, resulting in diversification of the adaptive NK cell repertoire. Lower expression of these signaling proteins results in lower expression of NCRs NKp30 and NKp46, as well as decreased signaling through SLAM family receptors, including 2B4 [133]. The transcriptional profiles and epigenomic changes induced by other viruses remain unknown, and could represent valuable insights into the diversification and maintenance of the conventional NK cell repertoire.

How does immune experience diversify NK cells?

Although the virus-induced shifts in expression of particular NKRs have been fairly well-characterized, the impact on NK repertoire diversity and the nature of the NK diversification process remain poorly understood. While there are many methods to assess diversity, the Inverse Simpson Index is commonly used to quantify the diversity of leukocytes as well as the microbiome, in part because it does not require normally distributed data [41]. It measures two metrics of a sample to quantify diversity: richness, the number of individual subpopulations per sample, and evenness, the degree to which each subpopulation is represented in the sample. In theory, education, differentiation, and maturation of NK cells should all serve to increase NK repertoire diversity, because all three processes result in the expression of previously un-expressed NKRs (including KIRs, CD57, and CD94/NKG2C), thereby increasing repertoire richness. However, if a lymphocyte undergoes a clonal expansion in response to a particular pathogen, the diversity of that lymphocyte class should decrease by the Inverse Simpson Index, because evenness is decreasing with no apparent change in richness. There is evidence that viruses other than HCMV, like EBV, HIV, and CHIKV, induce clonal-like expansions of NK cells from a well-differentiated NK cell repertoire [81,84–86,193]. Thus, exposure of a mature NK cell repertoire to these viruses might be expected to decrease NK cell diversity [65].

There are suggestions that this is not the case. First, NK cell diversity increases with age. Although differences in the structure of the human NK cell repertoire between cord blood and adult peripheral blood are largely limited to maturity-related markers like CD57 and

NKG2A [194], NK cell diversity is higher in adult peripheral blood than in cord blood [188]. NK cell diversification with age could be a physiologic process, or represent the accumulation of diversity induced by exposures to new pathogens. The latter hypothesis is supported by the recent observation that NK cell diversity increases after short-term exposure to cells infected with HIV-1 or West Nile Virus [188]. As it does not seem likely that interacting with virus-infected cells should increase the evenness of NK cell subpopulations, it is reasonable to suspect that these viruses cause a process akin to differentiation where NK cell repertoire richness increases. While this could represent an adaptation to generate a variety of NK cell subsets, some of which might be better tuned to respond to the viral threat, there are several noteworthy considerations when interpreting these data. The viral exposures were performed *in vitro*, and whether viral exposure *in vivo* has a similar impact on NK cell diversity remains an open question. Additionally, the Inverse Simpson index represents only one method to measure diversity, may be sensitive to the particular NKRs used in the diversity calculation, and may not reflect different functional phenotypes of samples [32]. Nonetheless, the receptor-ligand interactions and signaling modalities that govern rapid virus-induced NK cell diversification *in vitro* or maturity-related diversification *in vivo* could represent important factors influencing NK cell repertoire stability and function, as well as potential viral evasion strategies. The existing data on immune experience largely reflect studies of the effects of viral infection. It will be of interest in future studies to evaluate the impact of malignancy on the phenotypic and functional diversity of the NK cell repertoire.

Importance of NK cell diversity in health and disease: lessons learned from deep profiling

It is appreciated that NK cells have critical roles in the control of early viral infection and malignant transformation [1,2,10,24,195], and the importance of functional specialization of the major NK cell subsets in human health and disease is also well characterized [132,196]. To our knowledge, only one study to date has examined how NK cell repertoire diversity impacts viral susceptibility. This study, performed by our research group, used CyTOF to demonstrate, surprisingly, that NK cell diversity correlated positively with risk of HIV-1 acquisition in a cohort of Kenyan women [188]. Risk of HIV-1 acquisition was correlated neither with CD4 or CD8 T cell diversity nor with expression of specific NK cell receptors. These results are counterintuitive, as lymphocyte diversity is conventionally considered to be beneficial by allowing lymphocytes to recognize and respond to a greater breadth of potential novel pathogens. There are two notable caveats to this study: its data are generated from a small cohort (HIV-1 acquisition, n = 13; matched controls, n = 23) and diversity may be a confounder that correlates with some unidentified metric that itself represents the link to increased viral susceptibility.

The caveats notwithstanding, one way to explain the paradox presented by this study is to posit that NK repertoire diversity is inversely related to repertoire flexibility. Therefore, greater NK cell diversification could impair the ability of the repertoire to respond to and control a novel pathogen, thereby increasing viral susceptibility. Though this hypothesis remains unconfirmed, there are suggestions in the literature that corroborate this assertion. In

particular, it appears that murine cytomegalovirus (MCMV)-induced adaptive murine NK cells have decreased capacity to control heterologous infections by influenza virus or *Listeria monocytogenes* compared to their naive counterparts [197]. This underscores the antigen-specificity of adaptive NK cell responses and supports the notion that virus-induced repertoire diversification may be akin to lineage commitment, where differentiated NK cells are unable to respond to *de novo* pathogens and thus impair the functional capacity of the repertoire as a whole.

Conclusion: future directions in single-cell technologies

Despite the progress that has been made in deep profiling NK cells to understand the genetic and environmental determinants of their diversification, much uncertainty still remains as to the mechanisms of NK diversification and its role in human health and disease. What epigenetic mechanisms promote NK cell repertoire stability? What are the receptor-ligand interactions and signaling pathways that govern virus-induced NK cell diversification? Which NK cell subsets are signatures of certain viral infections, and do these signatures represent possible viral evasion strategies? Can we identify specific subsets of NK cells that are best adapted to promoting immune homeostasis or controlling particular viral infections or cancers? Does NK cell diversity decrease repertoire flexibility and promote viral susceptibility?

The advent of single-cell technologies for deep profiling of NK cells has made answering such questions imminently possible. Our group and others have recently pioneered the use of mass cytometry to better dissect NK cell diversity [41,109,143,188,198,199]. Single-cell resolution deep sequencing and proteomics methods will also facilitate further complex interrogation [200]. These methods should provide a deeply needed understanding of the links between NK cell phenotype and functional capacity that will be essential for future NK cell-based therapeutic developments.

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Abbreviations

NK	natural killer
ILC	innate lymphoid cell
IFN-γ	interferon gamma
NKR	natural killer cell receptor
KIR	killer immunoglobulin-like receptor
CyTOF	cytometry by time-of-flight

CD	cluster of differentiation
IL	interleukin
HLA	human leukocyte antigen
HCMV	human cytomegalovirus
EBV	Epstein-Barr virus
CHIKV	Chikungunya virus
AIDS	acquired immunodeficiency syndrome
HIV	human immunodeficiency virus
HBV	hepatitis B virus
HCV	hepatitis C virus
LCL	lymphoblastoid cell line
MIC	MHC class I-related protein
ULBP	UL16 binding protein
ADCC	antibody-dependent cellular cytotoxicity
TNF	tumor necrosis factor
TGF	transforming growth factor
PBMC	peripheral blood mononuclear cell
NCR	natural cytotoxicity receptor
TRAIL	TNF-related apoptosis-inducing ligand
DNAM	DNAX accessory molecule
LILRB1	leukocyte immunoglobulin-like receptor B1
PLZF	promyelocytic leukemia zinc finger protein
MCMV	murine cytomegalovirus

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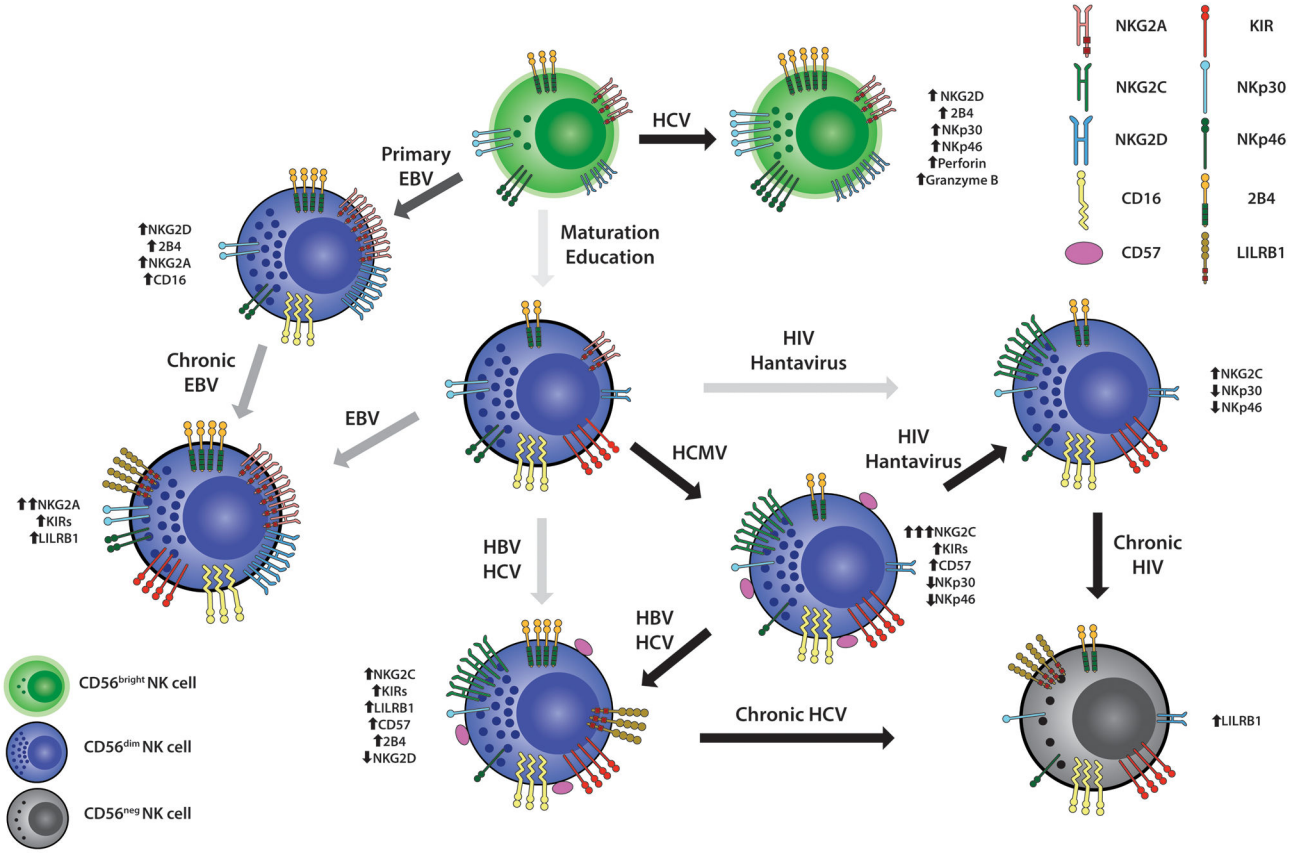

Figure 1:

Human NK cell repertoire shifts and expansions induced by particular viral infections. The inexperienced NK cell repertoire begins as a collection of relatively homogenous cytokine-producing $CD56^{bright}$ NK cells, and $CD16^{+}KIR^{+}$ cytotoxic $CD56^{dim}$ NK cells. These subsets differentiate and diversify in response to various viral infections. EBV expands a population of early-differentiated $CD16^{+}KIR^{-}NKG2A^{+}$ $CD56^{dim}$ NK cells which gradually acquire KIRs through education. HCMV, on the other hand, induces expansion of late-differentiated $CD57^{+}NKG2C^{+}$ NK cells. Chronic infection, in particular by HIV or HCV, can induce the formation of anergic $CD56^{neg}$ NK cells. It is important to note that, while specific expansions or subsets are depicted as single cells, virus-induced changes in NKR expression typically correspond to repertoire-wide changes and not necessarily specific populations. Here, a darker arrow corresponds to a more conclusive relationship between the two NK cell subsets. For example, it remains unclear if $CD56^{bright}$ NK cells represent $CD56^{dim}$ precursors *in vivo*.