NK cell memory and help in HIV infection

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While natural killer (NK) cells are lauded for their killing effector function, they also play a critical role as immune helper cells, responding with great flexibility to environmental cues to provide subsequent alarm signals crucial for shaping and regulating the adaptive immune response. Most recently, the concept of immunological memory has been extended to NK cells, with the emergence of an inflated population of NK cells deficient in FcRγ (FcRγ$^-$) described in the setting of HIV-1 infection. The physiological relevance of FcRγ$^-$ NK cells, including their relationship with HIV-1-associated chronic inflammatory events, and their potential to be targeted to improve HIV-1 control, are poorly understood. Here, I present an in-depth analysis of the impact of chronic HIV-1 infection and long-term antiretroviral therapy (ART) on the phenotypic and functional character of NK cells, with particular attention directed toward characterizing FcRγ$^-$ NK cells and exploring the reciprocal crosstalk between NK cells and dendritic cells (DCs) to enhance cellular-mediated immunity to HIV-1. Although NK cells are capable of providing immune help in response to innate stimuli during ART-mediated viral suppression, HIV-1 infection accelerates the expansion of highly differentiated FcRγ$^-$ NK 'effector memory' cells with limited function. By specializing in antibody-specific responses, FcRγ$^-$ NK cells compromise their responsiveness to DC-derived innate stimuli, leading to qualitative differences in their helper function and crosstalk with DCs. However, the potential exists for exploiting the inflated populations of FcRγ$^-$ NK cells in chronic HIV-1 infection to facilitate the induction of DC-mediated cellular immunity to HIV-1 via broadly neutralizing HIV-1-specific monoclonal antibodies (bNAbs) due to their superior

antibody-dependent reactivity. Improving our understanding of the specialized nature of FcRγ^{-} memory-like NK cells will be imperative for optimizing interventions to improve health outcomes and the effectiveness of HIV-1 therapies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

To those currently living with and to those whose lives have been lost to HIV/AIDS: Your activism, optimism, and persistence in challenging conventional research boundaries have inspired untold progress in the fight for an HIV cure. I especially express my sincere gratitude and appreciation to William Buchanan, Jeffrey Toth, and the Pittsburgh MACS participants. This study would not have been possible without your tireless dedication and magnanimity.

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1.0 INTRODUCTION

1.1 Basic biology of NK cells

Natural killer (NK) cells are uniquely positioned to positively impact immune responses due to the pleiotropic nature of their effector functions. As innate lymphocytes, NK cells serve on the front line of immunity, mounting immediate and powerful defenses against cells showing generic signals of stress, transformation, or infection (1-4). Their critical role in early control of viral infections is underscored by a heightened susceptibility to viral infections, particularly herpesviruses, in individuals with deficiencies in NK cell numbers or functionality (5, 6).

The term *natural killer cell* was rooted in the discovery that they could 'naturally' kill a target cell. That is, the killing occurred in a spontaneous fashion in the absence of any priming (1, 2, 7). Subsequent experiments in mouse models of bone marrow graft rejection prompted the development of the 'missing-self' hypothesis, a notion that NK cells would kill any cell lacking self-major histocompatibility complex (MHC) class I molecules (8-10). This laid the framework for our current, although nascent, understanding that NK cell activation is dictated by the integration of multiple activating and inhibitory signals, with the response potential and activation threshold of each individual NK cell determined by the unique combination of receptors expressed on its surface. The interplay between activating and inhibitory processes is not only complex but also highly dynamic (11, 12). In spite of this inherent complexity, NK cells are classically divided into two basic subsets based on relative surface expression of CD56 (the 140-kDa isoform of neural cell adhesion molecule) (13, 14) and CD16 (or FcγRIIIA, the low-affinity receptor for IgG) (15-

17). These populations are easily distinguished by flow cytometry, as well as their distinct phenotypic and functional properties.

1.1.1 The classic NK cell subsets

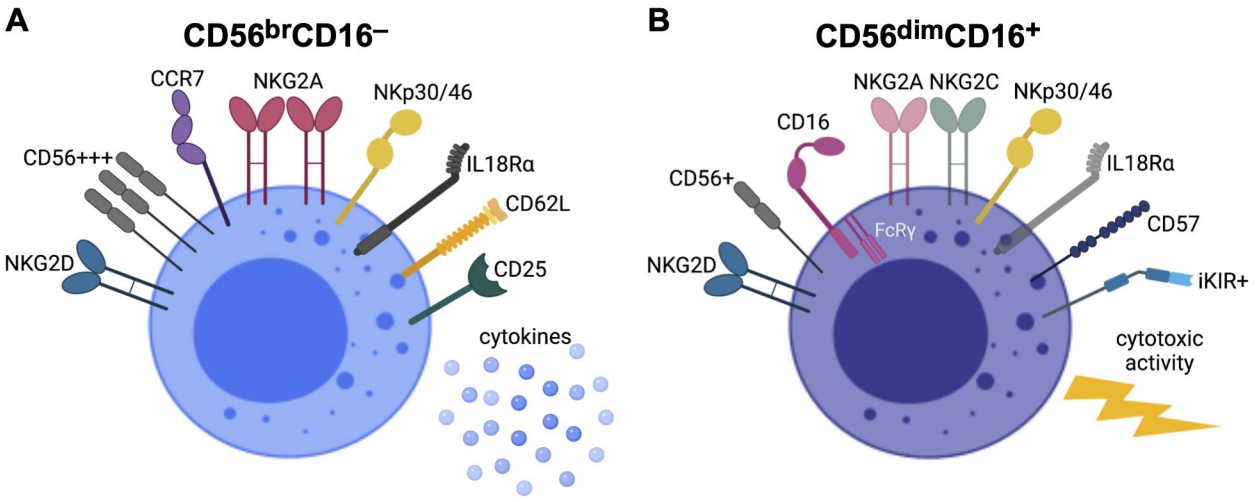
Representing approximately 90% of all NK cells in the peripheral blood, $CD56^{dim}CD16^{+}$ NK cells are the major circulating subset (15, 18). These cells are widely recognized as potent mediators of cytotoxicity, carrying perforin and homing markers for inflamed peripheral sites, both of which allow for rapid deployment of this effector function. $CD56^{dim}CD16^{+}$ NK cells are also unique in their ability to efficiently lyse antibody-opsonized cells via their low-affinity FcγRIIIA molecule (16, 19-23). By contrast, the $CD56^{bright}$ subset typically lacks expression of CD16 and accounts for a maximum of 10% of circulating NK cells. Resting expression of CCR7, coupled with high-density expression of CD62L, facilitates their preferential migration to and enrichment in secondary lymphoid tissue (SLT) (18, 19, 24). In addition to constitutively expressing the high-affinity receptor for IL-2 and displaying higher levels of other cytokine receptors, including IL18R and IL1RI (18, 25, 26), $CD56^{bright}$ NK cells are the most abundant producers of cytokines and chemokines. However, they have a limited ability to spontaneously kill tumor cell targets (27) (Fig. 1).

The variegated expression of activating and inhibitory receptors contributes remarkably to the phenotypic and functional heterogeneity observed within the total NK cell population. Most inhibitory NK receptors (iNKRs), which include killer cell immunoglobulin-like receptors (KIRs) and NKG2A, recognize MHC class I molecules as their cognate ligands (28-30). Found to varying degrees on subsets of $CD56^{dim}CD16^{+}$ but notably absent from $CD56^{bright}$ NK cells (18, 20), KIRs engage classical MHC class I, with an ability to distinguish polymorphic HLA-B and HLA-C

molecules (20). On the other hand, the heterodimeric C-type lectin inhibitory receptor CD94/NKG2A (NKG2A) binds to the nonclassical MHC class Ib molecule HLA-E, which presents peptides derived from signal sequences of classical MHC class I molecules (15, 18, 30, 31). Whereas the $CD56^{bright}$ population uniformly expresses NKG2A, this receptor is present on only a fraction of $CD56^{dim}$ NK cells (18). The main activating receptors constitutively found on all peripheral blood NK cells are NKG2D and the natural cytotoxicity receptors (NCRs) NKp30 and NKp46 (32-35) (Fig. 1). Phenotypic differences between blood and lymph node (LN)/tonsil NK cells include absence of CD16 and KIRs on the $CD56^{dim}$ subset (36), as well as no or very low expression of the constitutive NCRs (24, 36) (Table 1).

Table 1. Characterization of resting human peripheral blood and lymph node NK cell subsets

	Blood		Lymph Nodes	
	$CD56^{bright}$	$CD56^{dim}$	$CD56^{bright}$	$CD56^{dim}$
CD56	+++	+	+++	+
CCR7	++	–	–	–
CD62L	+	–	–	–
Perforin	–	+	–	–
Activating Receptors				
CD16	–	+	–	–
NKp30	+	+	–	–
NKp44	–	–	–	–
NKp46	+	+	+/–	–
NKG2D	+	+	+	+
Inhibitory Receptors				
NKG2A	++	+/–	+	+/–
KIR	–	+/–	–	–



	CD56^{bright}	CD56^{dim}
CD56	+++	+
CD57	–	+
Activating Receptors		
CD16	–	++
NKp30	+	+
NKp44	–	–
NKp46	+	+
NKG2C	–	+/–
NKG2D	+	+
Inhibitory Receptors		
NKG2A	++	+/–
KIR	–	+/–
Cytokine Receptors		
IL2Rαβγ	+	–
IL2βγ	++	+
IL1R1	++	+
IL18R	++	+
Chemokine Receptors		
CCR7	++	–
CXCR1	–	++
CXCR3	+	+/–
CX3CR1	–	++
Adhesion Molecules		
CD2	++	+/–
CD62L	+	–

Figure 1. The classic NK cell subsets are distinguished by expression of CD56 and CD16

(A) Displaying higher levels of cytokine receptors, including constitutive expression of the high-affinity IL-2 receptor CD25, CD56^{bright}CD16^{-} NK cells are extremely responsive to cytokine signaling. They are also recognized as potent producers of immune modulating cytokines that preferentially reside in SLT due to resting expression of CCR7 and high-density expression of CD62L. **(B)** As CD56^{dim}CD16^{+} NK cells progress through the differentiation process, they lose expression of NKG2A and sequentially acquire inhibitory KIRs and CD57. Functionally, fully mature CD56^{dim}CD16^{+} NK cells are less responsive to innate cytokine signaling, instead serving as efficient mediators of antibody-dependent and natural cytotoxic activity. All circulating NK cells constitutively express the activating receptors NKp30, NKp46, and NKG2D. *Figure created with BioRender.com.*

1.1.2 NK cell development

1.1.2.1 Linear model

Based on the selective enrichment of $CD34^+CD45RA^+$ pre-NK cells within SLT relative to bone marrow or blood, Freud and Caligiuri have proposed SLT as a site for human NK cell development and maturation *in vivo* (15, 37, 38). In this stepwise model, bone marrow–derived $CD34^+$ hematopoietic progenitor cells circulate in the blood and enter the parafollicular space through LN high endothelial venules. These cells progress to $CD10^+$ pro-NK cells (stage 1 progenitors) and subsequently transition to stage 2 pre-NK cells marked by loss of CD10 and expression of CD117. In stage 3 of development, iNK cells lose CD34 expression and are presumed to be restricted to the NK cell lineage. Acquisition of NKG2A signals the commitment to $CD56^{bright}$ NK cells (stage 4), and further differentiation leads to stage 5 $CD56^{dim}$ NK cells that notably gain expression of CD16 (37). Whereas maturing $CD56^{dim}$ NK cells return to the circulation via the efferent lymph (39), the majority of the $CD56^{bright}$ subset remains within SLT (24, 36, 40, 41). Circulating $CD56^{dim}$ NK cells are a rather heterogenous population due to a continuous process of differentiation, during which they lose expression of NKG2A and sequentially acquire inhibitory KIRs and CD57 (42). These phenotypic changes are accompanied by a gradual, but increasing, decline in proliferative potential and responsiveness to cytokine stimulation. However, they retain their cytotoxic capacity (42, 43) (Fig. 2).

The phenotypic and functional properties of both the $CD56^{bright}$ and $CD56^{dim}$ subsets lend credence to the linear model of differentiation in which $CD56^{bright}$ NK cells are the immediate immature precursors of the $CD56^{dim}$ population. This model is further supported by studies involving *in vitro* stimulation of purified $CD56^{bright}$ NK cells. First, purified $CD56^{bright}$ NK cells cultured in the presence of synovial or skin fibroblasts exhibit CD56 downregulation, as well as

phenotypic and functional features of peripheral blood CD56^{dim} NK cells (44). A subsequent study noted that activation of SLT CD56^{bright} NK cells with IL-2 induces expression of CD16 and KIRs, as well as efficient cytotoxic activity, resulting in a population akin to the circulating CD56^{dim}CD16^{+} subset. The ability of NK cells in SLT to produce cytokines before maturing into cytolytic effector cells demonstrates their remarkable functional plasticity (36, 39). Furthermore, CD56^{dim} NK cells have consistently been shown to display shorter telomeres than their CD56^{bright} counterparts, indicative of a higher degree of maturation (39, 44).

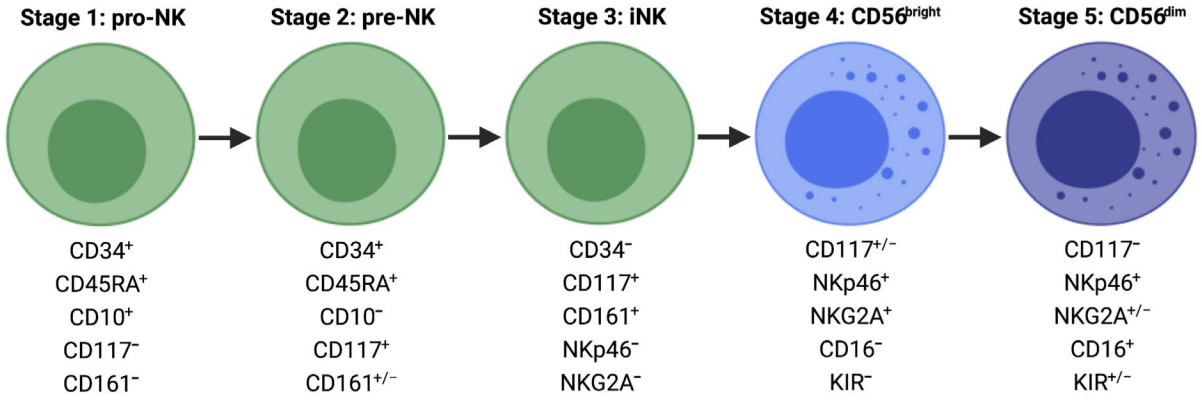


Figure 2. Schematic representation of the linear model of NK cell development

Circulating CD34^{+}CD45RA^{+} progenitor cells enter the parafollicular space through LN high endothelial venules. Here, they progress from CD10^{+} pro-NK cells to CD117^{+} pre-NK cells. Stage 3 marks the loss of CD34 and restriction to the NK cell lineage. Acquisition of NKG2A and NKp46 signifies the differentiation of iNK cells into CD56^{bright} NK cells, with the majority of this subset remaining in SLT. As NK cells continue along the spectrum of maturation, they gain expression of CD16 and inhibitory KIRs while decreasing expression intensity of CD56. Maturing CD56^{dim} NK cells return to the circulation via efferent lymphatics. *Figure created with BioRender.com.*

1.1.2.2 Nonlinear model

Despite evidence substantiating the notion that differentiation occurs along a continuum, with developmental progression from CD56^{bright} to CD56^{dim} NK cells (22, 39, 44-46), challengers of this prevailing theory contend that a more expansive model is required for a comprehensive and nuanced understanding of NK cell heterogeneity. Advances in technology have allowed for a deeper appreciation of the magnitude of phenotypic diversity within the NK cell population. As an

example, use of a Boolean gating strategy to analyze mass cytometry data of peripheral blood NK cells from five sets of monozygotic twins and 12 unrelated donors has spawned estimates of 6,000 to 30,000 phenotypic populations within an individual (47). Hierarchical clustering additionally has identified major distinguishing receptors, leading to the formation of two distinct clusters. Interestingly, the clusters, characterized as less mature ($CD94^{+}NKG2A^{+}$) and mature ($CD16^{+}CD57^{+}$), resemble the classic $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets, respectively (47). Unlike the linear model, though, in which $CD56^{dim}$ NK cells differentiate from the $CD56^{bright}$ population and phenotypic diversity reflects a spectrum of maturational states, the nonlinear model hypothesizes that NK cell diversity is determined at the precursor level. Proponents of this model cite *in vivo* rhesus macaque studies, in which distinct clonal patterns are observed for the $CD16^{+}CD56^{-}$ and $CD16^{-}CD56^{+}$ NK cell populations following autologous transplantation with barcode-labeled $CD34^{+}$ cells (48). Subsequent analysis of the same rhesus macaques revealed that the differential clonal patterns of $CD16^{+}CD56^{-}$ and $CD16^{-}CD56^{+}$ NK cells are still evident up to four years post-transplant and are maintained after short-term *in vivo* depletion with an anti-CD16 antibody (49). Assuming the $CD16^{+}CD56^{-}$ and $CD16^{-}CD56^{+}$ subsets are analogous to $CD56^{dim}$ and $CD56^{bright}$ human NK cells, respectively, these results suggest that $CD56^{dim}$ and $CD56^{bright}$ NK cells are distinct lineages (48, 49). No definitive evidence has emerged yet to unequivocally prove either the linear or nonlinear model of differentiation; however, the current paradigm favors the former, identifying $CD56^{bright}$ NK cells as precursors of the mature $CD56^{dim}$ subset.

1.1.3 Functional characterization of NK cells

Similar to T and B lymphocytes, NK cells acquire self-tolerance to normal cells. This process is also commonly referred to as *education* or *licensing*. NK cell self-tolerance can be

understood simply through missing-self recognition. Normal cells are protected from NK cell–mediated lysis when iNKRs engage with self-MHC class I molecules, thereby neutralizing stimulatory signals received by the NK cell (11, 12, 28) (Fig. 3A). Loss of self-MHC class I expression, as a result of infection or transformation, triggers NK cell–mediated lysis of the target cell via missing-self recognition (9). In other words, the NK cell is activated by stimulatory interactions with the target cell in the absence of inhibition (Fig. 3B). Recognition of MHC class I molecules by NK cells only partially explains their self-tolerance, as NK cells display an array of activating and inhibitory receptors that bind to various other ligands, with their activation determined by the balance of signaling through stimulatory versus inhibitory receptors (11, 12, 28, 50) (Fig. 3C). To account for this, several non-mutually exclusive mechanisms leading to NK cell self-tolerance have been proposed:

1. Mature NK cells express at least one iNKR specific for a self-MHC class I molecule (29, 51, 52).
2. Mature NK cells express at least one iNKR specific for non-MHC class I molecules (53-56).
3. NK cells lacking iNKRs for self-MHC class I molecules reduce expression of stimulatory receptors (50).
4. NK cells failing to interact with self-MHC class I–expressing targets are disarmed, i.e., rendered hyporesponsive due to chronic stimulatory events (57-59).
5. iNKRs specific for self-MHC class I molecules induce functional competence in developing NK cells (armed); those lacking iNKRs for self-MHC class I molecules remain immature and hyporesponsive (unarmed) (60).
6. Autoreactive NK cells are inhibited by putative regulatory cells (50).

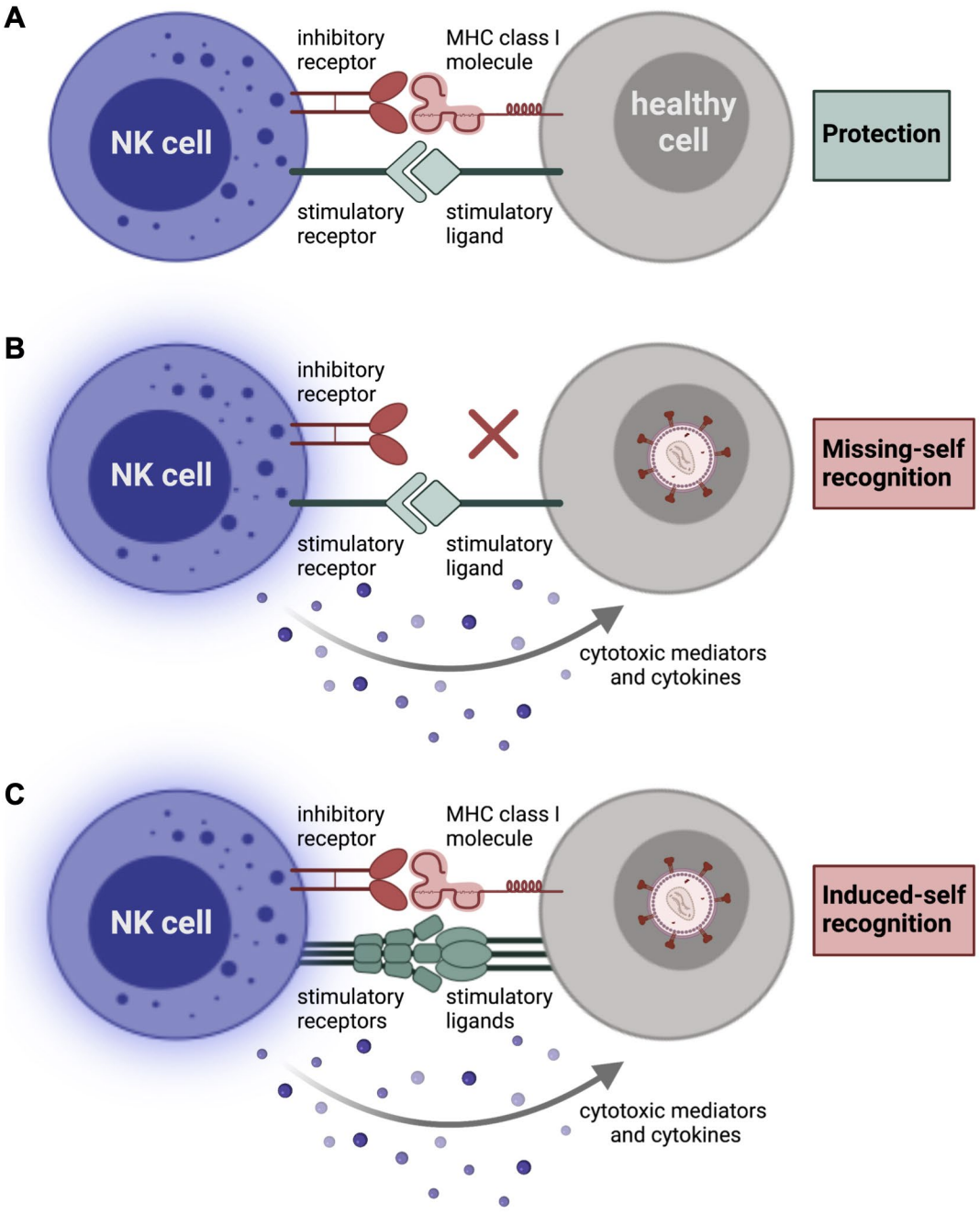


Figure 3. NK cell activation depends on the balance of activating and inhibitory signals

(A) A healthy cell expressing an activating ligand is protected from NK cell–mediated killing due to interactions between iNKRs and MHC class I molecules. This inhibitory signal neutralizes the activating signal received through the stimulatory receptor. **(B)** In the absence of inhibition, an NK cell is activated to secrete cytokines and cytotoxic mediators through stimulatory interactions with a target cell. Missing-self recognition occurs as a consequence of infection- or transformation-mediated downregulation of MHC class I molecules. **(C)** Infection and transformation can also promote upregulation of stress-induced stimulatory ligands on a target cell, overcoming the constitutive inhibition delivered through inhibitory receptors. *Figure created with BioRender.com.*

1.1.3.1 Natural cytotoxic activity

When the balance shifts to net positive signaling, NK cells are released from inhibition, resulting in lysis of engaged target cells. This interaction depends equally on the receptors expressed by NK cells and the ligands displayed by target cells. In addition to missing-self recognition, the activation threshold for NK cell cytolytic activity can be achieved through induced-self recognition. In this case, infection or transformation upregulates the expression of stimulatory ligands such that constitutive inhibitory signaling is overcome (50). Two crucial surface molecules for triggering NK cell cytotoxicity are the NCRs NKp30 and NKp46 (32-34). Both receptors, expressed on all NK cells in the peripheral blood, are type I transmembrane glycoproteins belonging to the Ig superfamily that associate with the intracellular signal-transducing molecules CD3ζ and FcεRIγ (32, 33, 61, 62). These adaptor proteins form as disulfide-linked homodimers or heterodimers and contain immune tyrosine-based activating motifs (ITAMs) that become tyrosine phosphorylated upon receptor engagement (63-65). Another NCR belonging to the Ig superfamily is NKp44. Unlike the other NCRs, expression of NKp44 is restricted to activated NK cells (66, 67), with signal transduction occurring through DAP12 homodimers containing a single ITAM in the cytoplasmic portion (66-68). The ligands for NCRs have yet to be identified; however, they are presumed to be related to cellular stress (69, 70). Apart from NCRs, NKG2D is an important activating receptor expressed as a homodimer on all NK cells. A type II transmembrane glycoprotein belonging to the C-type lectin superfamily, NKG2D signals through the adaptor protein DAP10 after binding stress-induced ligands, which include MHC class I chain-related proteins A/B (MICA/MICB) and UL16 binding proteins (ULBPs) (71-74). NKG2D and NCRs can work independently or in a concerted fashion to activate NK cells. For example, while spontaneous cytotoxicity is directly correlated to the surface densities of NCRs

(75), NKG2D can compensate for the poor cytolytic activity of NCR^{dull} effector NK cells upon engagement with complementary target cells (35). In either case, these receptors play major roles in NK cell−mediated antiviral and antitumor immunity (66, 76-89).

1.1.3.2 Antibody-dependent cytotoxic activity

As the first identified activating NK cell receptor (90, 91), CD16 (FcγRIIIA) has been characterized the most extensively. This transmembrane glycoprotein of the Ig superfamily is expressed at a high surface density on circulating $CD56^{dim}CD16^{+}$ NK cells and binds the constant (Fc) region of IgG with low affinity (16, 22, 92, 93), thereby linking antibody recognition of infected or transformed cells with effector cells. Similar to NKp30 and NKp46, CD16 signals via the ITAM-bearing adaptor proteins CD3ζ and/or FcεRIγ (94-97). Crosslinking of the ligand-binding extracellular domain leads to tyrosine phosphorylation of the ITAMs (98), triggering a signaling cascade that activates NK cells to mediate antibody-dependent cellular cytotoxicity (ADCC) and to secrete cytokines, namely IFNγ (99-101). In addition to acting on neighboring immune cells to promote antigen presentation and adaptive immune responses (102), IFNγ promotes upregulation of TRAIL on NK cells, suggestive of a synergistic enhancement of tumor killing (103, 104). Under certain conditions, CD16-mediated signaling may even cause NK cells to undergo apoptosis as a consequence of Fas ligand−induced cell death (105-107). Interestingly, the signal transduction pathways and effector functions resulting from CD16-induced activation of NK cells highly resemble those associated with TCR engagement on T cells (30), and NK cell−mediated ADCC has emerged as a significant mechanism in protecting against and controlling viral infections (108-115), including HIV-1 (116-119), as well as in effective antitumor immunity (120-129).

1.1.3.3 NK-DC crosstalk and immune modulation

NK cells were initially described as cytotoxic effectors, characterized by their ability to lyse tumor targets without prior sensitization; however, they also function as helper cells, linking innate and antigen-specific adaptive immunity. Their supportive role in shaping adaptive immune responses is highlighted through their reciprocal crosstalk with dendritic cells (DCs). DCs surveying peripheral tissues encounter pathogens and become activated via engagement of pattern recognition receptors (PRRs), e.g., TLR3, resulting in the secretion of pro-inflammatory cytokines that include IL-18, IL-12, and IL-15 (41, 130-133). $CD56^{dim}CD16^{+}$ NK cells home to sites of inflammation on the basis of their expression of the chemokine receptors CXCR1 and CX3CR1 (18, 19). Here, type I IFNs and DC-derived cytokines, such as IL-12, contribute to their activation and enhance their cytolytic activity against virus-infected or transformed cells (16-18, 79). This NK cell−mediated killing provides immature DCs (iDCs) with antigenic material for ingestion, processing, and subsequent presentation to T cells in SLT (41, 134, 135). Although $CD56^{bright}CD16^{-}$ NK cells predominantly reside in SLT due to their expression of CCR7, they are also capable of trafficking to inflamed peripheral tissues through the chemokine receptor CXCR3 (19, 136). Relative to the $CD56^{dim}CD16^{+}$ subset, peripheral blood $CD56^{bright}CD16^{-}$ NK cells produce significantly higher levels of TNFα and IFNγ in response to DC-derived cytokines (27, 137-141), inducing DC maturation and polarization (139, 140, 142). Throughout the maturation process, DCs upregulate MHC class I/II and co-stimulatory molecules, in addition to acquiring expression of CCR7 (143-145). These phenotypic changes are crucial because mature DCs (mDCs), carrying antigen and environmental cues from the periphery, migrate to parafollicular regions of SLT, where they interact not only with T cells but also $CD56^{bright}CD16^{-}$ NK cells (70, 146, 147). The functional consequences of these interactions depend on IL-12 and IFNγ (148-150),

with mDCs activating LN NK cells in an IL-12-depenedent manner to rapidly release IFNγ (147), an essential factor for driving type 1-biased immune responses (148-156). Thus, the reciprocal crosstalk between NK cells and DCs in the periphery and SLT ultimately leads to enhanced DC-mediated priming of $CD4^+$ Th1 and cytotoxic T lymphocyte (CTL) responses.

As immune modulators, NK cells are not limited to the production of type 1-polarizing cytokines, functioning simultaneously as regulators of inflammation and immune homeostasis. In fact, co-stimulation of NK cells with IL-15 and IL-18 leads to release of IL-13, associated with type 2 immune responses, while the combination of IL-15 and IL-12 promotes IL-10 production (27). Recognized as a pleiotropic regulatory cytokine, IL-10 inhibits the CD28 co-stimulatory pathway, secretion of IL-12 and expression of MHC class II molecules by DCs, and proliferative responses of Th1 cells (157-163). The consequences of IL-10 signaling are dichotomous, though, as the downstream effects can impede pathogen clearance while also safeguarding against immunopathologies (157, 158). Both NK cell subsets are capable of responding in this manner, but the $CD56^{bright}$ population, again, proves superior (27). Moreover, the ability of activated NK cells to selectively kill iDCs, a process referred to as *DC editing*, points to a regulatory role that presumably enhances adaptive immune responses. The killing of autologous iDCs is mediated by NKG2A-expressing NK cells through the NCR NKp30 (137, 139, 140, 164-166). By contrast, mDCs are spared from this grim fate due to increased surface levels of HLA-E as a consequence of maturation (164). It is hypothesized that DC editing by NK cells in the periphery results in the preferential selection of DCs that will mediate optimal antigen presentation and T cell priming (41). Furthermore, as mentioned previously, $CD4^+$ T cell–derived IL-2 confers *de novo* cytolytic activity on $CD56^{bright}CD16^-$ NK cells (36, 39, 167), suggesting an acquired ability to kill incompletely matured DCs in STL. Therefore, LN $CD56^{bright}CD16^-$ NK cells may prevent

tolerance during T cell priming by eliminating iDCs or semi-matured DCs that would otherwise induce T cell anergy (145, 168). Together, these data indicate that interactions between NK cells and DCs remarkably influence the quality and character of ensuing adaptive immune responses.

1.2 Interplay between NK cells and HIV-1

1.2.1 NK cell control of HIV-1 infection

Epidemiologic and functional studies have revealed the impact of NK cells on HIV-1 infection, with particular HLA/KIR combinations heavily influencing their effectiveness in protecting against acquisition of infection (169, 170) and in delaying disease progression (171, 172). The epistatic interaction between the activating KIR allele *KIR3DS1* and the *HLA-B Bw4-80I* allele in the setting of chronic HIV-1 infection is associated with slower depletion of $CD4^+$ T cells and delayed progression to AIDS (171). This epidemiological association may be explained by the ability of $KIR3DS1^+$ NK cells to strongly inhibit *in vitro* HIV-1 replication in target cells expressing HLA-B Bw4-80I (173). Moreover, a combined genotype of inhibitory *KIR3DL1* high-expressing alleles and *HLA-B*57* confers protection against disease progression and lowers the risk of HIV-1 infection in exposed uninfected individuals (169, 172). These data suggest that binding of KIRs with their cognate ligands impacts the natural course of HIV-1 disease by defining the activation threshold and protective efficacy of NK cell responses.

Additionally, downregulation of MHC class I molecules by HIV-1 accessory proteins to avoid recognition by CTLs simultaneously enhances the susceptibility of infected cells to NK cell−mediated killing (174, 175). HIV-1 Nef downmodulates HLA-A and HLA-B molecules

(176), while the viral Vpu protein downregulates expression of HLA-C (177), theoretically offering the missing-self trigger for NK cell activation. The ability of NK cells to subsequently kill these infected cells depends on the strength of the HLA/KIR interaction, as well as the extent of virus-mediated MHC class I downregulation (178). Another HIV-1 accessory protein, Vpr, upregulates cell-surface expression of ULBPs on infected cells, thereby promoting NK cell−mediated killing through the activating NKG2D receptor (179, 180). This NKG2D-dependent cytotoxicity is further enhanced by priming NK cells with IFNα (79). On a similar note, CD16 engagement strongly activates NK cell effector functions, leading to lysis of the target cell and release of polarizing cytokines. The potency of this response is highlighted by the fact that NK cell−mediated ADCC activity has been implicated in vaccine-induced protective immunity against acquisition of infection, phenotypes of viral control, and slower disease progression (116-119). Finally, NK cells participate in HIV-1 control by releasing the chemokines CCL3, CCL4, and CCL5, which are the ligands for the HIV-1 coreceptor CCR5. These β-chemokines presumably inhibit viral entry in target cells by blocking the binding of HIV-1 envelope protein to CCR5 (181).

1.2.2 Impact of HIV-1 infection on NK cells

With such a diverse array of effector functions, from cytotoxicity to immune modulation, the importance of NK cells in the anti-HIV-1 immune response is clear; however, NK cells are defective at controlling the virus due to the profound impact of HIV-1 on NK cell phenotype and function, including the pathologic redistribution of dysfunctional NK cell subsets (182-185). The sequential deregulation of NK cell subset distribution begins during acute HIV-1 infection. Initially, the absolute number of circulating NK cells increases, accompanied by a relative expansion of the $CD56^{dim}CD16^{+}$ subset and early depletion of $CD56^{bright}CD16^{-}$ NK cells (184).

Ongoing viral replication induces the expansion of a dysfunctional $CD56^{-}CD16^{+}$ ($CD56^{-}$) NK cell subset at the expense of cytotoxic $CD56^{dim}CD16^{+}$ NK cells (182-184, 186). Importantly, $CD56^{-}$ NK cells do not appear in chronic HIV-1-infected individuals with undetectable plasma viremia or in acute HIV-1-infected individuals with high viral loads, underscoring the role of chronic HIV-1 viremia in driving the expansion of this aberrant population (184, 186, 187). Chronic HIV-1 viremia is also characterized by significantly reduced expression of all three NCRs—NKp30, NKp44, and NKp46—yet heightened levels of iNKRs (185, 188-190). NKG2A, conversely, is the only iNKR marked by decreased expression on NK cells from chronic viremic HIV-1^{+} individuals (183, 185), which results in an inversion of the NKG2A/NKG2C ratio (191). These phenotypic abnormalities are especially pronounced within the $CD56^{-}$ population (183). By contrast, Siglec-7, an iNKR constitutively expressed on the majority of NK cells, represents a marker that is highly sensitive to high levels of HIV-1 viremia in the earliest stages of infection and precedes CD56 in its downmodulation. The differential kinetics of Siglec-7 and CD56 downregulation allow for the detection of two sequential pathologic NK cell subsets: Siglec-$7^{-}CD56^{+}$ NK cells, which preferentially expand during the initial phases of infection, and Siglec-$7^{-}CD56^{-}$ NK cells, which only become detectable in the setting of chronic HIV-1 viremia (186).

Perturbations in NK cell compartments resulting from chronic HIV-1 viremia profoundly hinder NK cell antiviral properties in a progressive fashion until the expansion of the Siglec-7^{-} $CD56^{-}$ subset signals a maximal loss of NK cell function. Concomitant with the dysregulated expression of activating and inhibitory receptors are functional abnormalities that severely limit cytotoxicity (175, 183-185). Of note, NK cells from individuals with chronic HIV-1 viremia display poor cytolytic activity against autologous endogenously HIV-1-infected $CD4^{+}$ T cells despite downregulated expression of MHC class I molecules, and this aberrant effector function is

closely linked to defective surface expression and engagement of NCRs and to the high frequency of dysfunctional $CD56^-$ NK cells (175). The increased proportion of $CD56^-$ NK cells in chronic viremic HIV-1 infection is also associated with weak ADCC responses (183, 192), and the degree of impairment in cytotoxic function correlates inversely with levels of HIV-1 plasma viremia (193). Anergic $CD56^-$ NK cells further contribute to a dysregulated immune response by disrupting the mutually beneficial interactions between NK cells and DCs. First, their failure to secrete IFNγ, TNFα, and GM-CSF interferes with the ability of NK cells to promote optimal DC maturation (183-185, 194). These abnormally matured DCs are then substantially defective at secreting IL-12 and priming neighboring NK cells. Second, the expanded $CD56^-$ population is responsible for deficiencies in NK cell–mediated editing of immature or improperly matured DCs among HIV-1-infected individuals with persistent viremia. This phenomenon, attributed to compromised signaling through NKp30 (195), likely underlies downstream ineffective T cell priming against HIV-1.

The pathologic redistribution of NK cell subsets is only one mechanism by which HIV-1 subverts NK cell functional responses. Countermeasures employed by HIV-1 to facilitate immune evasion abound, thereby affecting NK cell–mediated control of infection and the overall quality of the immune response (192, 196, 197). Given the protective immunity conferred by specific HLA/KIR combinations, HIV-1 circumvents this NK cell–mediated immune pressure by selecting for sequence polymorphisms that enhance the binding of inhibitory KIRs to infected $CD4^+$ T cells. Thus, NK cells directly contribute to viral evolution but to their own detriment, with the outcome being reduced antiviral activity of KIR-expressing NK cells (198). To counteract the Vpr-dependent upregulation of NKG2D ligands (179, 180), HIV-1 Nef protein reduces surface expression of MICA and ULBPs, dampening NKG2D-mediated killing of infected cells (199).

HIV-1 accessory proteins, likewise, block the expression on infected cells of ligands important for enhancing NK cell activation, including those that bind to the NKRs DNAM-1 and NTB-A (200, 201). The NCR^{dull} phenotype characteristic of chronic HIV-1 infection (185, 188), together with defective NKG2D-mediated killing (199) and an incomplete pattern of activation (202), negatively impacts NK cell function and immune surveillance, allowing for HIV-1 disease progression and development of opportunistic infections and malignancies (181, 203, 204). Furthermore, matrix metalloproteinases (MMPs), which are highly expressed in chronic HIV-1 infection (205), cleave NKG2D ligands from the surface of infected $CD4^+$ T cells, resulting in an accumulation of the soluble form of NKG2D ligands in the plasma of viremic HIV-1-infected individuals (206, 207). This proteolytic activity allows HIV-1-infected cells to evade NKG2D-mediated antiviral responses both by decreasing surface expression of NKG2D ligands on infected cells and by inactivating NKG2D on circulating effector cells via the soluble ligands (206, 208). MMPs also release CD16 from the surface of NK cells, consequently limiting HIV-1-specific ADCC activity (209, 210). Moreover, HIV-1-induced IL-10 promotes aberrant elimination of iDCs by NK cells but renders mDCs more susceptible to NKG2D-mediated lysis, leading to the accumulation of poorly immunogenic iDCs in LNs of infected individuals (211). This, again, interferes with the development of an effective T cell–centric immune response.

It is unclear whether the aforementioned NK cell phenotypic and functional abnormalities occur as a result of direct interactions between NK cells and HIV-1 or the establishment of chronic inflammation through persistent antigenic exposure. Fresh NK cells purified *ex vivo* from human peripheral blood mononuclear cells (PBMCs) express the HIV-1 chemokine coreceptors CXCR4 and CCR5 (189, 212); however, they lack surface expression of CD4 (185, 213), which translates into NK cells being unlikely targets of productive HIV-1 infection. Supporting this notion, $CD4^-$

NK cells purified *ex vivo* from the peripheral blood of HIV-1-infected individuals have been shown to not harbor HIV-1 proviral DNA, even in the case of active HIV-1 viremia (185). In a conflicting report, though, Valentin *et al.* have identified a subset of circulating NK cells that express CD4, as well as CCR5 and CXCR4, and that remain persistently infected in HIV-1^+ individuals even after one to two years of suppressive antiretroviral therapy (ART). Based on these data, direct infection of NK cells may account, at least partially, for the NK cell dysfunction observed in HIV-1 infection, and infected NK cells may serve as an *in vivo* reservoir of HIV-1 (212). This discrepancy could potentially be explained by differences in the activation status of the purified NK cells, as peripheral blood–derived NK cells, subjected to maximal activating conditions *in vitro*, acquire *de novo* expression of CD4 and become susceptible to HIV-1 infection (213, 214). By contrast, NK cells freshly isolated from SLT express higher levels of CD4 on their surface relative to NK cells in the periphery (215), raising the possibility that NK cells residing in SLT are susceptible to HIV-1 infection. Regardless of the mechanism, NK cells do not escape unscathed and suffer debilitating deficiencies as a consequence of HIV-1 infection that impair both their direct and indirect antiviral effector functions.

1.2.3 Does ART rescue NK cell phenotype and function?

Following consistent viral suppression with effective ART, NK cells experience, to varying degrees, a recovery of phenotype and function. Just as chronic HIV-1 viremia induces the sequential loss of Siglec-7 and CD56, ART-mediated viral suppression gradually restores surface expression of these molecules. Within the first 18 months of therapy, NK cells regain expression of Siglec-7, resulting in a shift from Siglec-7^-CD56^- to Siglec-7^+CD56^- NK cells (186), and a complete recovery of CD56 expression occurs only after 24 months of suppressive ART (183,

186). Normalization of the NKG2A/NKG2C ratio to values greater than one similarly requires a minimum of 24 months of suppressive ART. This phenomenon occurs despite enduring elevated frequencies of $NKG2C^+$ NK cells, therefore reflecting a return of surface expression of NKG2A (191). Given their sensitivity to HIV-1 viremia, normalization of the NKG2A/NKG2C ratio and NK cell subset distribution have been proposed as biomarkers for gauging the effectiveness of chronic suppression of HIV-1 replication by ART (196). Importantly, expression of iNKRs and activating receptors on NK cells derived from aviremic HIV-1-infected individuals compares to that of uninfected donors, and this reversal of phenotypic abnormalities through ART-mediated viral suppression correlates with a recovery of NK cell cytolytic function (185). In addition to rescuing NK cell–mediated cytotoxicity against iDCs and tumor cell targets (185, 195, 216), control of HIV-1 viremia by ART improves ADCC activity, the extent of which corresponds to the timing of ART initiation, with the most pronounced responses observed in individuals beginning treatment either prior to seroconversion or their $CD4^+$ T cell counts dipping below 350 cells/μl (217, 218). NK cells, indeed, benefit phenotypically and functionally from ART-mediated viral suppression, but maximal restoration of NK cell effector functions also requires properly functioning DCs. As an example, since HIV-1 interferes with DC functional maturation (219, 220), mDCs from viremic HIV-1-infected individuals fail to activate NK cells to secrete adequate amounts of IFNγ (195), accentuating the dependence of both cell types on each other for optimal activation and functionality.

Notwithstanding the significant benefits associated with adherence to ART regimens, including undetectable levels of plasma viremia, a reduction in AIDS-associated mortality, and an increase in circulating $CD4^+$ T cells, aviremic HIV-1^+ individuals show signs of residual immune dysfunction, particularly within the innate immune system (221-223). Whereas ART successfully

reverses HIV-1-related activation of T cells and monocytes, NK cell activation persists, demonstrated by elevated proportions of $CD38^{+}HLA\text{-}DR^{+}$ NK cells and heightened spontaneous degranulation in virologically suppressed HIV-1-infected individuals (224, 225). Although the duration of this effect and its impact on comorbid disease remain unknown (224), chronic immune activation is the hallmark of HIV-1 infection leading to premature aging and apoptosis of immune cells (226). It is highly plausible, therefore, that persistence of inflammation and NK cell activation contribute to a higher prevalence of comorbidities with an inflammatory etiology in treated HIV-1^{+} individuals; more specifically, markers of innate immune activation are associated with cardiovascular disease, non-AIDS cancers, and neurocognitive disorders (227-231). Additionally, ART enhances the terminal differentiation of $CD56^{dim}CD16^{+}$ NK cells, as evidenced by increased expression of CD57 (232). NK cells expressing CD57 represent a mature and stable subset with retained cytolytic capacity, whose frequency increases with age (42, 233, 234); however, expression of CD57 also signifies terminal differentiation and less functional activity (235), correlating with abrogated proliferative and IFNγ responses to cytokine stimulation (42). These data indicate an enduring effect of HIV-1 infection on NK cells despite effective ART. The clinical consequences of this persistent NK cell dysfunction, including an acceleration toward terminal differentiation, are poorly understood.

1.3 Adaptive and memory-like features of NK cells

Although NK cells are restricted to the expression of germline-encoded receptors, the combinatorial expression patterns of activating and inhibitory receptors give rise to a diverse NK cell repertoire (47, 236, 237). Reprogramming events in response to local inflammatory milieu, as

well as genetic and environmental factors, further contribute to their phenotypic and functional heterogeneity (47, 237-239). Mounting evidence also indicates that cumulative pathogen exposures elicit dynamic shifts in the repertoire of NKRs that potentially impact the quality of NK cell responses to subsequent infections (236), with recent studies even challenging the notion that memory is uniquely confined to adaptive immunity (240). In fact, NK cells, defying their classification as innate lymphoid cells, exhibit features of traditional immunological memory, including antigen-specific clonal-like expansions, heritable cell-intrinsic modifications, and the generation of long-lived populations capable of heightened recall responses. As detailed in the sections to follow, these 'memory' NK cells can develop in either a cytokine- or antigen-dependent manner (241-248).

1.3.1 Cytokine-induced memory-like NK cells

While $CD56^{dim}$ NK cells are widely recognized for their cytolytic abilities, they also play a critical role as immune helper cells, providing innate alarm signals that shape and regulate the adaptive immune response. IL-18, in particular, has been shown to have a unique ability to induce a helper pathway of differentiation in the $CD56^{dim}$ population, promoting upregulation of CD25 and transient expression of CD83 (153, 249). IL-18 also programs $CD56^{dim}$ NK helper cells to express CCR7, allowing them to migrate in response to CCL21 and to home to SLT, where they are primed to immediately produce IFNγ upon subsequent exposure to secondary stimuli, including IL-12, IL-2, or IFNα (153). This suggests that IL-18-primed $CD56^{dim}$ NK helper cells act not only on DCs in the periphery but also migrate to lymph nodes to modulate adaptive immune responses in a manner similar to the $CD56^{bright}$ NK cell subset and $CD4^{+}$ T helper cells.

Subsequent studies in mice and humans have described a similar NK cell population, arising following a brief *in vitro* exposure to IL-12, IL-15, and IL-18. Murine cytokine-induced memory-like NK cells are detectable up to three weeks post-transfer into naïve hosts; despite having a resting phenotype similar to that of naïve cells, they produce significantly more IFNγ upon restimulation with cytokines or tumor cell lines. Notably, these amplified responses are heritable across multiple generations, highlighting the ability of NK cells to retain an intrinsic memory of prior activation. By contrast, cytokine-induced memory-like NK cells do not demonstrate enhanced cytotoxic activity as compared with control-treated NK cells (241). The programming of murine NK cells into a helper population with robust IFNγ production in the presence of IL-18 corroborates the finding in human NK cells that the helper pathway of differentiation is not associated with enhancement of cytolytic activity. Whereas IL-2 selectively promotes the cytotoxic effector functions of NK cells, IL-18-primed NK helper cells support DC-mediated induction of Th1 responses via IFNγ (153). Human NK cells display analogous functional properties, with cytokine exposure inducing a memory-like pool characterized by an enhanced ability to rapidly produce IFNγ in response to restimulation with cytokines or tumor targets. Phenotypically, cytokine-induced memory-like NK cells feature increased levels of NKG2A and NKp46, as well as weak expression of NKG2C (242) (Fig. 4A).

The mechanisms underlying the programming of memory-like NK cells through this combination of cytokines are complex and incompletely understood. However, epigenetic remodeling of the conserved noncoding sequence (CNS) at the *IFNG* locus, induced by *in vitro* cytokine priming, coincides with increased transcription of *IFNG* (250, 251). Furthermore, IL-18 and IL-12 synergize to upregulate expression of CD25 (252), resulting in high responsiveness to IL-2 receptor stimulation (249). IL-18, together with IL-15, also activates the mammalian target

rapamycin complex 1 (mTORC1) pathway, which promotes glycolytic reprogramming and upregulation of glycolytic enzymes in NK cells (253, 254), presumably supporting the energy demands of elevated production of IFNγ (255). Importantly, cytokine-induced memory-like responses can be distinguished from priming and arming based on response length, heritability, and activation status. Whereas priming and arming depend on direct signaling and conceptually represent events that occur just prior to NK cell effector functions, cytokine-induced memory-like responses are heritable and detectable weeks after initial activation. Additionally, cytokine-induced memory-like NK cells do not constitutively express high levels of activating receptors, granzyme B, or cytokines, suggesting priming and arming are the initial, complementary components of long-term memory-like responses (240).

1.3.2 Adaptive NK cells in CMV

In the case of antigen-driven memory, cytomegalovirus (CMV) engineers a lasting imprint on NK cells, inducing the preferential expansion of a population with attributes of traditional immunological memory (243-245). Antigen-specific NK cells were first described in a murine model, whereby naïve NK cells expressing Ly49H clonally expand in response to the murine CMV (MCMV) antigen m157 and subsequently contract, forming a large pool of long-lived cells that reside in lymphoid and non-lymphoid organs for several months. In naïve neonatal hosts, adoptively transferred $Ly49H^+$ NK cells confer a more efficient defense against subsequent infection, whereas adoptive transfer of the same quantity of naïve NK cells results in only 25% survival (245). Paralleling these findings, human NK cells adapt in response to human CMV (HCMV) infection and reactivation following solid organ and hematopoietic stem cell transplantation, resulting in the preferential expansion and accumulation of an NK cell subset

marked by high-density surface expression of NKG2C (243, 244, 256-258). Adaptive NKG2C^{+} NK cells in HCMV-seropositive individuals display a skewed phenotype that includes elevated expression of CD57 and CD2, together with low expression of NKG2A (243, 244, 259-262). Moreover, the narrow expression patterns of otherwise stochastically distributed inhibitory KIRs suggest HCMV drives an oligoclonal or clonal-like expansion of NK cells (259, 260) (Fig. 4B; Table 2).

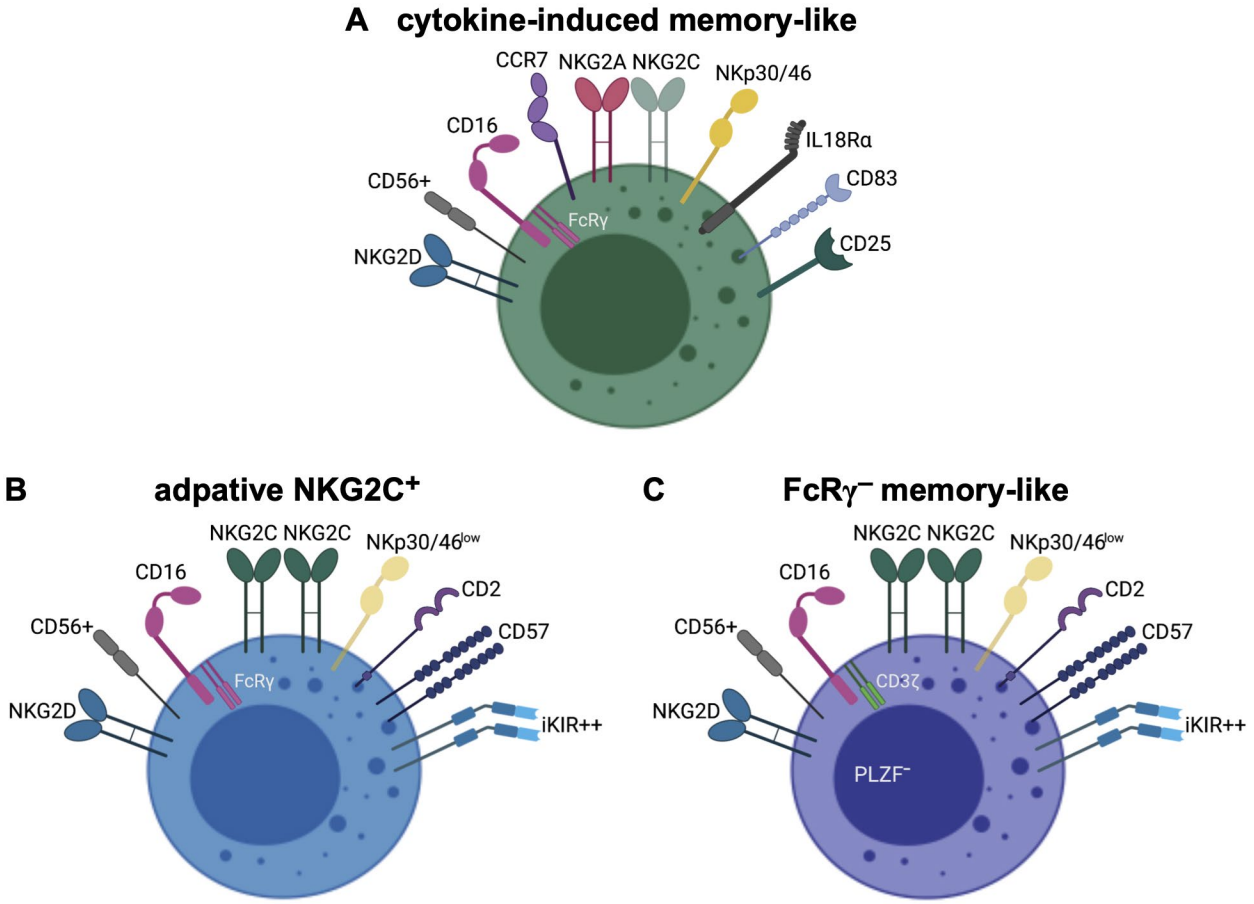


Figure 4. NK cell 'memory' populations are distinct in phenotype and function

(A) Cytokine-induced memory-like NK cells, with high surface expression of cytokine receptors and CCR7, efficiently integrate immunostimulatory cytokines to drive their differentiation and subsequent recall responses. **(B, C)** Adaptive NKG2C^{+} and FcRγ^{-} memory-like NK cells share numerous features, including high-density surface expression of CD57 and inhibitory KIRs and potent ADCC responses; however, initial programming and expansion of adaptive NKG2C^{+} NK cells result from interactions between NKG2C and specific HLA-E–peptide complexes (B). For FcRγ^{-} NK cells, preferential expansion occurs upon encounter with HCMV-infected cells in the presence of HCMV-specific antibodies. They are notably deficient for FcRγ and the transcription factor PLZF and are not restricted by the expression of NKG2C. Epigenetic modifications underlie the unique attributes of 'memory' observed in all three of these NK cell populations. *Figure created with BioRender.com.*

Belonging to the same C-type lectin family as NKG2A, NKG2C likewise forms a heterodimer with CD94 but conversely signals through the ITAM-bearing adaptor protein DAP12, thereby functioning in a stimulatory capacity (263, 264). NKG2A and NKG2C share ligand specificity for HLA-E (265-267), whose cell-surface stabilization requires bound peptides derived from host proteins, including leader sequences of classical MHC class I molecules and the heat-shock protein HSP60 (31, 268). These stabilizing nonameric peptides share conserved amino acid residues at positions two and nine (31), facilitating their loading within HLA-E molecules. By contrast, subtle changes in the HLA-E peptide ligands, particularly substitutions at positions five and eight, influence the binding affinities of NKG2A and NKG2C (269-275). As the inhibitory receptor, though, NKG2A generally displays a higher affinity for HLA-E (275, 276). Accordingly, HCMV stabilizes expression of HLA-E by providing UL40-derived peptides that mimic HLA leader sequences, thereby protecting infected cells against lysis by NKG2A-expressing NK cells despite a downregulation of classical MHC class I molecules (277-280). Adaptive NK cells, typically lacking NKG2A, circumvent this evasion strategy, preferentially expanding in response to HCMV infection through NKG2C (261). Beyond interacting with HLA-E to drive the expansion of an adaptive subset (281, 282), NKG2C demonstrates exquisite peptide specificity, with adaptive NK cells differentially recognizing HCMV strains. Interestingly, UL40 sequence variations, specifically a single amino acid substitution at position eight, control the degree of activation and proliferation of $NKG2C^+$ NK cells (261, 283), e.g., peptides with a low potency elicit suboptimal activation of adaptive NK cells and rely on co-stimulatory signaling via CD2 to trigger polyfunctional responses. Furthermore, the combination of IL-18 and IL-12 synergizes with high potency peptides to drive the accumulation and differentiation of adaptive $NKG2C^+$ NK cells from HCMV-seronegative donors (261). NKG2C, in other words, is not merely a defining feature of

this population; rather, its interactions with HLA-E–peptide complexes are critical for the function and expansion of adaptive NK cells.

A defining component of immunological memory is the ability to mount a quantitatively and/or qualitatively greater recall response (284). In this regard, the adaptive NK cell subset is characterized by superior effector functions in response to antibody-dependent and NKG2C-mediated signaling (244, 259, 260, 283, 285). Aside from persisting over time (257, 260), $NKG2C^+$ NK cells expand *in vivo* following clinical HCMV reactivation and demonstrate an increased capacity for target cell–induced cytokine production. Furthermore, $NKG2C^+$ NK cells transplanted from HCMV-seropositive donors show more potent functional activity following a secondary HCMV event compared to $NKG2C^+$ NK cells from HCMV-seronegative donors (244, 256, 257), indicative of secondary effector responses upon antigen re-exposure. By contrast, adaptive $NKG2C^+$ NK cells respond poorly to stimulation with IL-18 and IL-12 alone (259, 260, 286), but they are capable of integrating IL-18 during target cell encounter (286). Moreover, IL-18 and IL-12 work in concert with specific HCMV peptides to strengthen the establishment of an adaptive NK cell population (261).

Epigenetic imprinting underlies the adaptive features of NK cells, including reprogramming of the receptor repertoire and enhanced effector responses. Specifically, an open configuration at the *IFNG* CNS1, mirroring that of $CD4^+$ Th1 and memory $CD8^+$ T cells, promotes stable IFNγ competence (250, 261). $NKG2C^+$ NK cells also exhibit metabolic hallmarks of lymphocyte memory, including increased oxidative mitochondrial respiration, mitochondrial membrane potential, and spare respiratory capacity, as well as higher expression of genes related to the electron transport chain. These metabolic alterations involve epigenetic modifications and poise adaptive NK cells for survival and robust recall responses (287). In a further parallel to T

cells, a three-signal mechanism—NKG2C engagement with specific HLA-E–peptide complexes, CD2 co-stimulation, and pro-inflammatory signaling via IL-18 and IL-12—is necessary to drive maximal expansion and functional activity of adaptive NKG2C^{+} NK cells through broad imprinting of the transcriptional landscape (250, 261, 262, 288). Peptide recognition is crucial, though, as pro-inflammatory cytokines alone do not lead to the development of adaptive NKG2C^{+} NK cells, but instead to the generation of cytokine-induced memory-like NK cells (242, 261), underscoring the potential for discrete, yet complementary, mechanisms to contribute to the differentiation of 'memory' NK cell populations.

Table 2. Phenotypic comparision of mature CD56^{dim}, adaptive NKG2C^{+}, and FcRγ^{-} memory-like NK cells

	Mature CD56^{dim}	Adaptive NKG2C^{+}	FcRγ^{-} memory-like
CD56	+	+	+
CD57	+	++	++
PLZF	+	+	–
Activating Receptors			
CD16	++	++	+
NKp30	+	low	low
NKp46	+	low	low
NKG2C	+/–	++	++*
NKG2D	+	+	+
Inhibitory Receptors			
NKG2A	–	–	–
KIR	+	++	++
Siglec-7	+	–	–
Co-stimulatory Molecules			
CD2	+/–	+	+
CD7	+	low	low
Signaling Molecules			
FcRγ	+	+	–
CD3ζ	+	+	+
Syk	+	+	–
DAB2	+	+	–
EAT-2	+	–	–

*When present on FcRγ^{-} NK cells, NKG2C is expressed at a high surface density but is not a defining feature of this memory-like population.

1.3.3 FcRγ⁻ memory-like NK cells

Another subpopulation strongly associated with HCMV seropositivity requires antibodies to grant antigen specificity. These $CD56^{dim}$ memory-like NK cells are notably deficient in the adaptor protein FcRγ ($FcR\gamma^{-}$), which is otherwise known as FcεRIγ (246, 247). As noted previously, FcRγ is an intracellular signaling protein that associates with NKp46, NKp30, and CD16 as a homodimer or as a heterodimer with CD3ζ. Phosphorylation of the ITAMs leads to the recruitment of Zap70 or Syk, which prompts a downstream signaling cascade, resulting ultimately in cytokine secretion, cytotoxicity, and/or calcium release (62). All mature NK cells were once believed to constitutively express FcRγ and CD3ζ (289), but a distinct subset of $CD56^{dim}$ NK cells deficient for FcRγ expression is readily detectable in approximately one-third of healthy individuals, correlating with HCMV seropositivity (246, 247). $FcR\gamma^{-}$ NK cells are stably maintained, albeit at highly variable frequencies between individuals that range from 3% to 85% of the $CD56^{dim}$ population (246), through promoter DNA hypermethylation that silences FcRγ mRNA and protein expression (246, 290, 291). By contrast, they express CD3ζ at normal levels (246, 291). The phenotypic profile of $FcR\gamma^{-}$ NK cells markedly resembles that of adaptive NK cells, notably increased levels of CD57, CD2, and inhibitory KIRs, with reduced NKG2A expression (247, 290, 291); however, although expressed at high frequencies, NKG2C is not a defining feature of this subset (247, 290). In comparison with conventional ($CD56^{dim}FcR\gamma^{+}$) NK cells, the $FcR\gamma^{-}$ population differs in the expression of FcRγ-associated receptors, including NKp30, NKp46, and CD16. FcRγ deficiency appears to dramatically affect cell-surface levels of the NCRs while having only a limited effect on CD16 expression, suggesting that FcRγ is required

for the expression of both NKp30 and NKp46. By contrast, CD16 depends less on FcRγ, as its cell-surface expression is supported, to an extent, by CD3ζ (246).

Epigenetic modifications further shape the FcRγ$^-$ population. On one hand, promoter DNA hypermethylation is associated with additional deficiencies for the signaling molecules Syk, DAB2, and EAT-2, as well as the transcription factor PLZF (Fig. 4C; Table 2), and reduced expression of this transcription factor is linked to minimal cytokine responsiveness in FcRγ$^-$ NK cells (290, 291). On the other hand, FcRγ$^-$ NK cells display robust effector functions, particularly cytokine production, in response to CD16 engagement, including HCMV-infected cells in the presence of HCMV-specific antibodies (246, 247, 290). The enhanced functional capabilities of this memory-like subset compared with conventional NK cells can be explained by exclusive association of CD16 with CD3ζ in the absence of FcRγ, presumably delivering a stronger signal given that CD3ζ bears three ITAMs, whereas FcRγ only contains one such domain (246, 289), and by hypomethylation of the *IFNG* and *TNF* regulatory regions. Interestingly, genome-wide DNA methylation patterns are strikingly similar between memory-like NK cells and CTLs but differ from those of conventional mature NK cells (291). Additionally, FcRγ$^-$ NK cells undergo preferential expansion upon encounter with HCMV-infected cells in the presence, but not in the absence, of seropositive plasma, and these expanded FcRγ$^-$ NK cells maintain superior CD16 responsiveness (290). Based on these data, Lee et al. have proposed a model, whereby HCMV infection directs stochastic epigenetic modifications, leading to FcRγ-deficiency, with this particular pool of memory-like NK cells further selected and preferentially expanded in an antibody-dependent manner during HCMV reactivation (290). Although FcRγ$^-$ NK cells are a memory-like effector population specialized for antibody-dependent reactivity, their precise role in controlling HCMV reactivation or heterologous infections is not clearly defined.

Adaptive and memory-like NK cells relate in that they persist as stable populations and demonstrate enhanced recall responses due to remarkable remodeling of the epigenetic landscape. Furthermore, their intrinsic qualities are heritable across generations (250, 251, 261, 290, 291). 'Memory' NK cell subsets are, however, distinct in phenotype and function. For example, each population is characterized by a differential degree of responsiveness to IL-18. The reduced ability of adaptive NKG2C^{+} and FcRγ^{-} memory-like NK cells to produce IFNγ following cytokine stimulation suggests a level of distinction from cytokine-induced memory-like NK cells (286, 291), for whom IL-18 is a key component of their identity (153, 242). In spite of this, IL-18 does optimize the expansion and functionality of adaptive NKG2C^{+} NK cells (261). Moreover, Syk-deficiency is largely confined to FcRγ^{-}, but not NKG2C^{+}, NK cells, and the FcRγ^{-} population displays superior antibody-dependent responsiveness, regardless of NKG2C expression, compared with their conventional NK cell counterparts (290). These data reveal key differences between adaptive NKG2C^{+} and FcRγ^{-} memory-like NK cells. Taken together, multiple pathways contribute to the differentiation and accumulation of distinct subsets of 'memory' NK cells, and a deeper understanding of these mechanisms will unlock enormous opportunities to exploit the intrinsic features of adaptive and memory-like NK cells for future immunotherapies.

1.4 NK cell 'memory' in HIV-1

1.4.1 Properties of NK cell 'memory' populations in HIV-1

Antigen specificity is not exclusive to CMV, as NK cells from mice and nonhuman primates have been documented to demonstrate antigen-driven memory responses to HIV-1. In the

first of such studies, following transfer into naïve *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, liver NK cells from *Rag1*$^{-/-}$ mice immunized with viral-like particles containing HIV antigens mediate recall responses that are antigen-specific, occurring only in recipients challenged with HIV antigens, but not other viral antigens (292). More recently, Nikzad et al. discovered that human NK cells, isolated from livers of humanized mice previously vaccinated with HIV-encoded envelope protein, display vaccination-dependent, antigen-specific memory responses (293), both of which are hallmarks of adaptive immunity (294, 295). Building on the concept of antigen specificity in the context of HIV, splenic and hepatic NK cells from rhesus macaques infected with or vaccinated against simian immunodeficiency virus (SIV) and simian HIV (SHIV) specifically lyse Gag- and Env-pulsed DCs in an NKG2A/NKG2C-dependent fashion. Furthermore, vaccination elicits antigen-specific memory NK cell responses that are both durable and potent, with NK cells from vaccinated macaques efficiently killing only the antigen-matched targets five years post-vaccination (296). These data, especially, point to the possibility of inducing long-lived, antigen-specific NK cell 'memory' in humans after infection and vaccination. In fact, cytotoxic NK cells with a tissue-resident phenotype infiltrate sites of varicella-zoster virus (VZV) skin test antigen challenge in VZV-experienced human volunteers decades after initial VZV exposure (293). Identification of human 'memory' NK cells specific for HIV-1, together with a detailed understanding of their molecular signature, may help to inform efforts to develop preventative and therapeutic vaccines against HIV-1.

Despite known direct interactions between NK cells and HIV-1 peptides (198), clear evidence for HIV-1-specific NK cells in humans is lacking; nevertheless, adaptive NKG2C^{+} and memory-like FcRγ^{-} NK cells are present at elevated frequencies in HIV-1-infected individuals (233, 248, 297-299). Loss of NKG2A^{+} NK cells in the setting of chronic HIV-1 viremia is

accompanied by a dramatic expansion of NKG2C$^+$ NK cells, whose frequency remains elevated even after prolonged ART-mediated viral suppression (191, 300). The expansion of NKG2C$^+$ NK cells occurs independently of HIV-1 viral load (300), and their inflated presence appears to positively contribute to viral dynamics during primary HIV-1 infection and early responses to ART (297). Among newly infected HIV-1$^+$ individuals, a higher frequency of NKG2C$^+$CD57$^+$ NK cells correlates with a lower viral set point and immune activation, along with better responses to ART, as those with high frequencies of NKG2C$^+$CD57$^+$ NK cells more rapidly reach undetectable levels of HIV-1 viremia (297, 301). Furthermore, homozygous deletion of the NKG2C gene is associated with increased risk for HIV-1 infection and disease progression (302, 303). Conversely, during antiviral responses, NK cell repertoire diversity increases, resulting in terminal differentiation, i.e., expression of CD57, and existing high repertoire diversity has been associated with increased risk for HIV-1 acquisition in African women with a high HIV-1 exposure risk (236). This suggests that diversity within the human NK cell receptor repertoire represents reduced plasticity to new challenging pathogens.

An expanded population of FcRγ-deficient NK cells has also been identified in viremic HIV-1$^+$ individuals, and this population persists following virologic suppression with ART (224, 225, 248, 299). In agreement with the findings in the context of HCMV seropositivity, FcRγ$^-$ NK cells in HIV-1$^+$ individuals display altered phenotypic and functional properties, including superior ADCC activity and drastically reduced expression of NKG2A, NKp30, and NKp46 (248). Using a rhesus macaque model, it has also been demonstrated that FcRγ$^-$ NK cells are distributed systemically but are inclined to migrate to mucosal sites (304). The response potential of FcRγ$^-$ NK cells in different phases of HIV-1 infection has yet to be thoroughly evaluated, but their enhanced ADCC activity and preferential homing to mucosal sites have potentially important

implications for designing strategies aimed at generating immune responses that mediate early control of HIV-1 infection.

1.4.2 The confounding effect of HCMV

Given the high prevalence of HCMV seropositivity among HIV-1$^+$ individuals (>90%), the profound skewing and adaptation of the NK cell repertoire following HIV-1 infection are likely confounded by co-infection with HCMV (224, 248, 305, 306). In fact, changes in NKG2C expression on NK cells among HIV-1$^+$ individuals are related to concomitant comorbidity with HCMV rather than HIV-1 infection alone, as inclusion of HCMV serological status in a multivariate regression model abolishes the positive correlation between high levels of NKG2C$^+$ NK cells and HIV-1 infection (298). Subsequent reports have confirmed that HCMV co-infection is responsible for the expansion of NKG2C$^+$ NK cells during the course of HIV-1 infection, with the proportion of NKG2C$^+$CD57$^+$ NK cells being extremely low or undetectable in HCMV-seronegative individuals, regardless of their HIV-1 status (191, 233). Moreover, in HIV-1 infection, an inverse relationship exists between the extent of NKG2C$^+$CD57$^+$ NK cell expansion and the fraction of HCMV-specific CD8$^+$ T cells expressing CD28 (233). Loss of CD28 expression on T cells signifies effector memory status and progression toward senescence, which is thought to be driven by persistent exposure to antigen (307-309), so this relationship may, in fact, reflect the common cumulative effect of chronic HCMV infection and periodic reactivation on responding NK and T cells in the setting of HIV-1 infection (233). Although the expansion of NKG2C$^+$ NK cells has also been observed in response to other viral infections, including hepatitis B, hepatitis C, chikungunya, and hantavirus, this phenomenon is largely restricted to HCMV-seropositive individuals (259, 310, 311), suggesting infection with other viruses may reactivate HCMV from

latency and trigger the proliferation of pre-existing NKG2C$^+$ NK cells. However, as there is no evidence of HCMV reactivation during primary HIV-1 infection among HCMV-seropositive individuals (297), HCMV-primed NK cell subsets appear to expand in response to secondary viral infection alone.

On a related note, the enhanced antibody-dependent effector functions of FcRγ$^-$ NK cells are not limited to HCMV. Cultures with HSV-1- and influenza-infected target cells and virus-specific antibodies produce similarly superior responses (247, 290), indicating that while these memory-like NK cells require initial programming by HCMV, their specialization for antibody-dependent reactivity is, importantly, not restricted to a particular pathogen, as antigen specificity is conferred through the antibody. Despite elevated frequencies of the FcRγ$^-$ subset in HIV-1 infection, reports suggest that HCMV exposure has a greater impact on inducing this population (224, 248). Moreover, HCMV/HIV-1 co-infection is associated with higher HCMV antibody titers (224, 248, 306), and the proportion of FcRγ$^-$ NK cells correlates with levels of CXCL10 (248). It is plausible, then, that the expansion of FcRγ$^-$ NK cells is inflated in HIV-1$^+$ individuals as a result of ongoing immune activation and higher infectious burdens, including HCMV. The expanded NKG2C$^+$ and FcRγ$^-$ NK cell 'memory' populations found in HIV-1$^+$ individuals share phenotypic and functional properties with those identified in association with HCMV infection, but they have not been characterized nearly as extensively, including comprehensive analyses of their transcriptional signatures and epigenetic modifications. Therefore, open questions remain related to the mechanism by which HIV-1 contributes to the expansion of 'memory' NK cells, their role in protecting against HIV-1 acquisition and disease progression, and the clinical implications of their inflated expansions in chronic HIV-1 infection. Increased knowledge of the specialized nature

of ‘memory’ NK cells will provide opportunities for the creation of novel HIV-1 therapeutic modalities.

1.5 Harnessing the unique features of NK cells for a functional cure

Although effective at suppressing HIV-1 viremia, antiretrovirals as a combination therapy do not eradicate the virus. Consequently, a major barrier to curing HIV-1 is persistence of the latent viral reservoir in long-lived resting $CD4^+$ T cells, which serve as a source of plasma viral rebound following treatment interruption. Successful management of HIV-1 as a chronic infection, therefore, requires life-long adherence to a daily ART regimen, which has potential associations with long-term toxicity (312-317). Moreover, regardless of treatment, HCMV lingers as a significant cofactor in HIV-1 disease progression (318-320), strongly correlating with systemic inflammation (321-323), reduced immune resilience (324), and immune senescence (233, 321, 324), and individuals infected with HIV-1 continue to experience an increased burden of comorbidities such as cardiovascular disease and cancer (230, 231, 323, 325). For these reasons, the development of therapeutic strategies aimed at combating HIV-1 and simultaneously improving long-term health outcomes remains a global priority. While interventions capable of achieving a sterilizing cure represent the ideal, the challenges associated with total elimination of replication-competent virus have prompted a shift in focus to designing therapeutic strategies that effect a functional cure, whereby the virus is not eradicated but rather silenced to undetectable levels in the absence of ongoing ART (326, 327). One approach, referred to as the *shock and kill* or *kick and kill*, centers on inducing HIV-1 latency reversal during ART to expose infected cells, rendering them susceptible to immune-mediated clearance. However, latency reversal is not

sufficient, as administration of latency-reversing agents increases cellular HIV-1 RNA but fails to decrease reservoir size (328-330). Furthermore, the CTL responses of ART-treated, HIV-1-infected individuals are incapable of efficiently clearing infected cells that exit latency due to the scarcity and/or dysfunction of HIV-1-specific CTLs (330, 331), as well as the presence of CTL escape mutants in latent viral genomes (332). These shortcomings highlight the need to optimize the kill arm of the kick and kill strategy. NK cells are an untapped resource with enormous potential for boosting HIV-1 therapeutic modalities, given their importance in antiviral immunity and their ability to circumvent the limitations inherent to T cell approaches. In fact, with their distinct features and heightened response potentials, 'memory' NK cell populations are ideal candidates for immunotherapies to improve HIV-1 control.

1.5.1 Chimeric antigen receptor (CAR)–engineered NK cells

Since the seminal work by Medawar and colleagues (333), adoptive cell therapy has emerged as a powerful treatment for advanced cancers resistant to conventional agents. More specifically, chimeric antigen receptor (CAR)–engineered T cells have produced unprecedented clinical results in patients receiving autologous CD19-directed T cells for the treatment of relapsed or refractory B cell malignancies (334-341). CAR T cells are engineered to express an extracellular single-chain variable fragment (scFv) antibody, coupled to an intracellular signaling domain (e.g., CD3ζ) and one or more co-stimulatory domains (e.g., CD28 or 4-1BB), thus conferring antigen specificity in an MHC-independent fashion (342-344). Despite their resounding successes, CAR T cells have several limitations, including the feasibility and cost associated with generating clinically relevant doses of autologous product from heavily pre-treated lymphopenic patients and the risk of prolonged toxic effects (340, 345-348). On the other hand, NK cells offer an attractive

alternative to T cells for CAR engineering. Allogeneic NK cells, for one, show little to no risk of inducing graft-versus-host disease, allowing for scalability and a low-cost, off-the-shelf cellular immunotherapeutic agent (349-351). Additionally, the administration of CAR NK cells is not associated with the development of neurotoxicity or cytokine release syndrome (352), and CAR NK cells retain their intrinsic capacity to recognize and kill target cells through their germline-encoded receptors, reducing the risk of immune escape even if acquired mutations and alternative splicing were to render targets resistant to the engineered CAR (347, 353). Therefore, CAR-engineered NK cells are a promising immunotherapeutic tool in the HIV-1 field. To direct the selective targeting of HIV-1-infected $CD4^+$ T cells, a CAR NK cell was created by fusing CD4 to CD3ζ, thereby enabling binding of HIV-1 gp120 through CD4 and signaling through CD3ζ. Although these CD4ζCAR NK cells effectively inhibit HIV-1 replication *in vitro*, they fail to enhance suppression of HIV-1 infection *in vivo* (354-356), with a potential explanation being a lack of adequate co-stimulation (357). Indeed, including the co-stimulatory molecule 4-1BB in the chimeric anti-CD19–CD3ζ receptor markedly enhances NK cell–mediated killing of leukemic cells, and the cytotoxicity of NK cells expressing this construct uniformly exceeds that of NK cells lacking 4-1BB (358). More recently, Lim et al. have developed a universal CAR NK cell that recognizes 2,4-dinitrophenyl (DNP) and can subsequently be redirected to target various epitopes of HIV-1 gp160 (a complex between gp120 and gp41) using DNP-conjugated antibodies as adaptor molecules (345). One of the limitations of their universal approach, though, is that a small proportion (up to 1%) of naturally occurring human antibodies recognize DNP (359, 360), which can compete with anti-DNP CAR NK cells in binding to the DNP-conjugated adaptor molecules. A possibility for addressing this problem is the design of higher affinity anti-DNP CARs to strengthen their interactions with DNP-conjugated adaptor molecules (345). Based on their

findings, future research is warranted to validate the *in vivo* efficacy and toxicity of their construct and to optimize the potency of universal CAR NK cells through co-stimulatory and/or NK cell–specific signaling domains.

1.5.2 Boosting NK cell endogenous responses

1.5.2.1 Release of inhibition

Allogeneic haplo-mismatched stem cell transplantation studies in patients with acute myeloid leukemia (AML) have provided the first line of evidence for the clinical relevance of KIR inhibition, with mismatches between KIRs on donor NK cells and recipient MHC class I molecules promoting NK cell activation and correlating with improved relapse-free and overall survival. These results not only suggested that, in the absence of inhibitory KIR interactions, NK cells mediate more effective responses against leukemia (361), but also sparked the development of monoclonal antibodies (mAbs) to sterically block KIR-MHC interactions. The anti-KIR mAb IPH2101 binds with high affinity to KIR2D receptors and augments NK cell–mediated elimination of autologous HLA-C–expressing leukemic cells *in vitro* in a dose-dependent manner (362). Targeting of mAbs against iNKRs also represents a strategy for improving anti-HIV-1 responses of NK cells. Although most primary isolates of HIV-1 are capable of inducing HLA-C downmodulation (177), the functional potency of HLA-C–licensed NK cells depends on the extent to which HLA-C is downregulated, and residual KIR-mediated inhibitory signaling is associated with reduced antiviral activity (178). Heightened HLA-E levels, as a consequence of elevated HLA-A expression, similarly impair HIV-1 control through inhibition of NKG2A-expressing cells (363). Interestingly, monalizumab, a mAb that binds to NKG2A to prevent inhibitory signaling, has already been shown to improve NK cell cytotoxicity and viral clearance in both mice and

patients with chronic hepatitis B infection (364). The potential also exists for mAbs against NKG2A and KIR to boost CTL responses in a complementary fashion (365, 366). Therefore, therapeutic blockade of NKG2A and inhibitory KIRs could improve HIV-1 control by enhancing both CTL and NK cell antiviral activity. However, given the potential for blockade of iNKRs to lower the activation threshold of NK cells against normal cells expressing low levels of stimulatory ligands, significant concerns have been raised related to autoreactivity and bystander killing of activated uninfected $CD4^+$ T cells. In spite of this, IPH2101 and lirilumab, a recombinant anti-KIR mAb that recognizes the same epitope as IPH2101 (367), have demonstrated good clinical tolerability in hematological malignancies and solid tumors (367-369). *In vitro* experiments and *in vivo* tumor rejection models further indicate that full KIR occupancy is necessary for optimal enhancement of NK cell activity, but prolonged KIR inhibition may negatively impact NK cell education/licensing (367), a process that involves the acquisition of functional competence through interactions between KIRs and their cognate ligands (60, 370-372). As such, a dosing schedule that supports transient, rather than continuous, full KIR occupancy may allow for optimal clinical effectiveness without impeding the development of new, fully competent NK cells.

1.5.2.2 TLR agonists

Belonging to the family of PRRs, TLRs are potent enhancers of innate antiviral immunity and efficiently activate NK cells (373), highlighting the potential application of TLR agonists to enhance the effector functions of NK cells for HIV-1 immunotherapies. The selective TLR7 agonist GS-9620 potently inhibits HIV-1 replication in human PBMCs at a step coincident with or prior to the early stages of reverse transcription, with IFNα playing a dominant role (374). Triggering of TLR8 or TLR7/8 similarly interferes with HIV-1 replication after virus-cell fusion but before integration, and this anti-HIV-1 response is attributed to NK and $CD8^+$ T cells (375,

376). Moreover, a study involving HIV-1 serodiscordant couples showed that TLR3 activation stimulates more pronounced polyfunctional responses in NK cells from exposed seronegative individuals compared with unexposed controls (377). Combined knowledge of the natural mechanisms promoting resistance to HIV-1 infection, along with the tools available to inhibit acute HIV-1 infection, has important clinical implications for developing preventative strategies.

In addition to blocking HIV-1 replication, TLR agonists support antiviral immunity and function as latency-reversing agents. *Ex vivo* studies have demonstrated that GS-9620 and the TLR9 agonist MGN1703 activate HIV-1 from latency, thereby enhancing viral transcription in PBMCs from HIV-1^+ individuals on suppressive ART (378, 379). MGN1703 also induces strong innate immune responses, including copious production of IFNα, and boosts NK cell–mediated suppression of HIV-1 infection in autologous $CD4^+$ T cells (379), presumably through increased expression of NKp46 (79). Confirming these data, treatment of virally suppressed HIV-1-infected individuals with TLR9 agonists increases HIV-1 transcription, enhances activation of cytotoxic NK cells (380), and reduces the HIV-1 proviral reservoir (381). It is conceivable, based on these *ex vivo* and *in vivo* studies, that TLR agonists pose as a dual threat in the kick and kill strategy, reactivating expression of the latent HIV-1 reservoir while simultaneously supporting the elimination of newly exposed, latently infected cells via stimulation of effector NK cells.

An important challenge related to the kick and kill strategy as a therapeutic intervention is the identification of latency-reversing agents that induce HIV-1 expression without causing serious adverse events or compromising the effector functions of cells targeting activated reservoirs. While PKC agonists such as bryostatin are characterized by stronger latency-reversing activity in comparison with TLR agonists (378, 382, 383), bryostatin is highly toxic and has led to serious adverse events in phase II oncology clinical trials (384-386). Furthermore, some HDAC inhibitors

have been shown to reduce the killing capacity of NK cells (387) and to negatively impact antigen-specific $CD8^+$ T cell functions, with transient exposure inducing the selective death of activated T cells (386, 388, 389). In contrast, TLR agonists have proven their safety and tolerability in clinical trials (390-393), making them an appealing alternative not only due to their safety profile but also for their ability to improve the killing of HIV-1-infected cells.

1.5.2.3 Bi-specific and Tri-specific Killer cell Engagers (BiKEs and TriKEs)

Another approach for improving NK cell functionality is through the use of immunomodulators termed Bi-specific and Tri-specific Killer cell Engagers (BiKEs and TriKEs) (394). These small molecules consist of a scFv from the heavy and light variable chains of an antibody, which is connected via a short, flexible polypeptide linker to another scFv or single-domain antibody (VHH) of a differing specificity (395-397). One component is directed against an antigen expressed on the surface of the target cell of interest, and the other engages CD16 on NK cells, thus inducing NK cell–mediated directed cytotoxicity against the target cell through the creation of a synapse between the two cell types (395-400). Although BiKEs circumvent previous challenges associated with NK cell–based immunotherapies, including lack of specificity (394), TriKE molecules were later developed to also ensure maximal NK cell activation and survival *in vivo* by accommodating an IL-15 moiety as a functional linker between two scFv segments (396). As an example, a BiKE against CD16 and CD33, termed 1633 BiKE, was initially created to promote NK cell engagement with $CD33^+$ AML tumor targets (398, 400), and subsequent integration of a novel modified human IL-15 crosslinker led to the generation of the 161533 TriKE. In comparison with the 1633 BiKE, the 161533 TriKE mediates superior NK cell cytotoxicity against $CD33^+$ targets and primary AML blasts and promotes enhanced antitumor function in immunodeficient mice by improving the *in vivo* expansion and persistence of human NK cells

(396). Initial findings from a phase I/II clinical trial (NCT03214666), indicate that the 161533 TriKE (GTB-3550) drives robust NK cell proliferation without causing adverse toxicity, suggesting the design of the TriKE encourages direct delivery of IL-15 to $CD16^+$ NK cells (401). To date, BiKEs and TriKEs have been created against CD19/CD22 on B cell non-Hodgkin's lymphomas (395, 402); CD33 and CLEC12A on AML, myelodysplastic syndrome, and neoplastic mast cells (396, 400, 403-405); and EpCAM, CD133, and B7-H3 on various solid tumor types (399, 406-410). In the context of HIV-1 therapies, Li et al. have reported a BiKE consisting of CD16A-binding human antibody domains fused through a linker to an engineered one-domain soluble human CD4 that mediates specific, activating interactions with CD16 to effectively kill primary HIV-1-infected T cells (411). Importantly, the relatively small size of this anti-HIV-1 BiKE facilitates superior biodistribution, including diffusion within lymphoid tissue, where the majority of HIV-1 replication occurs (411-413). Although BiKEs and TriKEs show incredible promise as therapeutic interventions, their *in vivo* efficacy in HIV-1^+ individuals remains to be determined.

1.5.2.4 Broadly neutralizing antibodies (bNAbs)

The natural development of therapeutically effective anti-HIV-1 broadly neutralizing antibodies (bNAbs) occurs in a fraction of HIV-1-infected individuals after years of infection and viral diversification (414-418). Although first-generation antibodies were safe and well tolerated, they showed limited breadth and activity, producing little, if any, measurable effects in viremic individuals and often leading to the rapid emergence of resistant viral variants (419-421). Advances in single cell cloning technologies have allowed for the isolation, expansion, and characterization of more potent next-generation bNAbs (422-427), which have gained prominence recently in HIV-1 research aiming to elicit a functional cure for their capacity to inhibit viral entry,

block viral cell-cell transmission, and suppress HIV-1 replication (428-431), as well as their potential to promote sustained virologic remission in the absence of ART (432-436). These bNAbs bind to relatively conserved regions on the HIV-1 Env trimer, including the CD4 binding site, the V1/V2 and V3 glycan-dependent loops, the membrane-proximal external region (MPER), the Env silent face, the gp41-gp120 interface, and the fusion domain (416, 418, 425, 437). The most commonly used bNAbs to treat humanized mice, macaques, and human patients belong to the classes targeting the CD4 binding site (VRC01 (438) and 3BNC117 (439)) and the V3 glycan-dependent loop (PGT121 (440) and 10-1074 (441)). Overall, bNAb monotherapy leads to transient suppression of viremia, followed by viral rebound reaching pre-treatment levels and the emergence of antibody-resistant viral strains (434, 436, 442-445). There is one notable exception: in PGT121-treated, SHIV SF162P3-infected macaques, mutations conferring resistance were not detected, and suppression of viremia lasted 35 to greater than 100 days, with three of the 18 treated animals experiencing no viral rebound (435). In contrast to monotherapy, combination bNAb treatment promotes a longer duration of virus suppression and antibody-resistant populations emerge less frequently and generally only to one component of the administered bNAb mixture (432-434, 442, 446). To date, combination bNAb treatment studies have only been conducted in humanized mouse and macaque models (420); however, analogous to the current standard of care with ART in HIV-1-infected individuals, combination bNAb therapy, as opposed to monotherapy, will likely be required for complete viremic control and to prevent the emergence of resistant viral strains.

One key advantage of bNAbs over ART is their ability to engage host immune effector pathways, such as binding to cell-free virions to expedite clearance and prevent their entry into target cells (428, 447), forming immune complexes that activate DCs for priming of T cells (420), and attaching to HIV-1 Env on the surface of infected cells to promote killing via ADCC (448,

449). In particular, passive immunization with bNAbs capable of inducing NK cell–mediated ADCC is garnering widespread interest due, in part, to the protective effect mediated by increased ADCC activity in the RV144 human vaccine trial (116). Not only do antibodies promote *in vitro* lysis of infected cells by Fc receptor (FcR)–mediated mechanisms (449-452), but their interactions with FcRs have also proven essential in HIV-1 prevention and therapy *in vivo* (448, 453-455), with bNAbs decreasing viral rebound from latent reservoirs and accelerating clearance of infected cells in humanized mice in an FcR-dependent manner (432, 456). Indeed, significantly higher HIV-1 viremia in the absence of FcR engagement demonstrates that the therapeutic efficacy of bNAbs highly depends on the binding of the Fc domain of bNAbs to FcγRs in immune effector cells (453). Additionally, in acutely treated, SHIV SF162P3-infected rhesus macaques, co-administration of PGT121 and the TLR7 agonist GS-9620 delays viral rebound following ART discontinuation, which correlates with NK cell activation. These findings suggest that the TLR agonist–bNAb combination enables NK cells to target the viral reservoir via antibody-mediated elimination of infected $CD4^+$ T cells, prolonging the duration of virologic suppression in the absence of ART (457) (Table 3). Not all bNAbs are equal, though, in their ability to bind to heterogeneous Env epitopes from different HIV-1 strains, to induce signaling through FcγRIIIA (CD16), or to kill infected cells via ADCC (449). However, alterations to the variable and Fc domains can be employed to optimize the functionality of bNAbs, with the potential to promote highly efficacious responses upon passive administration to humans. As enhanced *in vivo* potency of anti-HIV-1 bNAbs is associated with preferential engagement of activating FcγRs, Fc domain–engineered bNAb variants with selective binding capacity for activating FcγRs can be created to augment their *in vivo* protective effect (453). It is also possible to enhance FcR binding, thereby improving antiviral activity, by shifting the Fc domain of bNAbs from a global antibody-glycosylation profile

toward agalactosylated glycoforms (452). Furthermore, combining Fc domain modifications with structure-based rational design may allow for heightened Fc effector function, as well as increased potency and breadth to different viral epitopes (458).

Table 3. Select bNAb studies with implications for NK cell–mediated ADCC

Class	bNAb	Population/Setting	Notes	Ref.
Various	Various	Human primary cells and cell lines (*in vitro*)	Identification of a subset of bNAbs that bind and kill HIV-1-infected cells through NK cell engagement.	(449)
CD4bs	b12	Human primary cells and cell lines (*in vitro*)	Variants with increased affinity for FcγRIIIa show an increase in NK cell activation and the ability to mediate ADCC.	(450)
CD4bs	A32	Human primary cells and cell lines (*in vitro*)	A32 potently mediates ADCC. An A32 Fab fragment blocks the majority of ADCC-mediating Ab activity in plasma of HIV-1^+ individuals.	(451)
N/A	Abs purified from HIV-1^+ individuals	Human primary cells (*in vitro*)	Agalactosylated Abs are associated with enhanced FcR binding and Fc-mediated reduction of viral replication.	(452)
CD4bs V3 loop V2 loop	3BNC117 10-1074 PG16	Humanized mouse model (*in vivo*)	bNAb tri-mix interferes with the establishment of a silent reservoir by Fc-FcR mediated mechanisms. bNAbs and a combination of viral inducers synergize to decrease the reservoir.	(432)
CD4bs V3 loop V2 loop	3BNC117 10-1074 PG16	Humanized mouse model (*in vivo*)	Fc domain-engineered bNAb variants with selective binding capacity for activating FcγRs display augmented protective activity.	(453)
CD4bs V3 loop	3BNC117 10-1074	Humanized mouse model (*in vivo*)	bNAbs accelerate the clearance of HIV-1-infected cells by a mechanism requiring FcγR engagement.	(456)
CD4bs	b12	Rhesus macaques (*in vivo*)	In the absence of FcγR binding, protection against SHIV challenge is dramatically decreased.	(454)
CD4bs	VRC01	Rhesus macaques (*in vivo*); human primary cells and cell lines (*in vitro*)	Variant with enhanced neonatal FcR binding improves protection against SHIV infection (*in vivo*) while retaining FcγRIIIa binding and ADCC activity (*in vitro*, human cells).	(448)
PGT121	V3 loop	Rhesus macaques (*in vivo*)	Co-administration of PGT121 and a TLR7 agonist delays viral rebound following ART discontinuation. This delayed viral rebound correlates with NK cell activation.	(457)
CD4bs	3BNC117	Humans (*in vivo*)	A single infusion reduces the viral load in HIV-1^+ individuals and viremia remains significantly reduced for 28 days.	(436)

Ab, antibody; CD4bs, CD4 binding site; FcR, Fc receptor; N/A, not applicable; Ref, reference

On a final note, early bNAb therapy following mucosal SHIV challenge in a macaque model has been implicated in the generation of $CD8^+$ T cell responses that mediate long-term control of viremia in the absence of ART, but the mechanism underlying this immune-mediated control is unclear (433). To date, no study has identified NK cells as the definitive link between bNAbs and durable CTL responses, as the aforementioned studies have merely focused on the classical killing effector function of NK cells (432, 449, 456, 457). Understanding the role of NK cells in the bNAb–CTL phenomenon will inform the design of highly effective immunotherapies against HIV-1 infection; therefore, I will investigate the helper role of NK cells in HIV-1 infection, predicting that NK cells will utilize antibodies to enhance the function of DCs, leading to the development of potent HIV-1-specific CTLs.

1.6 Conclusion

My overarching hypothesis centers on HIV-1 infection driving the expansion of highly differentiated $FcR\gamma^-$ NK 'effector memory' cells, with an impaired ability to provide proper immune help in response to innate stimuli. By contrast, the inflated population of $FcR\gamma^-$ NK cells in chronic HIV-1 infection will promote the induction of DC-mediated cellular immunity to HIV-1 in the presence of antibodies. To support my hypothesis, I will define the relationship between cytokine-induced and $FcR\gamma^-$ memory-like NK cells; expound the influence of chronic HIV-1 infection and ART on the frequency, phenotype, and functional status of NK cells, including the $FcR\gamma^-$ memory-like population; correlate the expansion of $FcR\gamma^-$ NK cells with inflammatory biomarkers and HCMV-specific adaptive immune responses; and determine the impact of NK cell ADCC activity on DC-mediated induction of durable immunity to HIV-1. Of

noteworthy significance, I predict that bNAbs can be utilized to direct FcRγ⁻ NK cells not only to target HIV-1-infected cells but also to facilitate the emergence of potent CTL responses due to their superior capacity to modulate DC function via antibody-mediated signaling. Strategies for harnessing the natural powers of NK cells to control HIV-1 infection, modulate adaptive immune responses, and promote overall health and immune homeostasis will be integral in maximizing the effectiveness of HIV-1 therapies.

2.0 SPECIFIC AIMS

2.1 AIM 1

To define the relationship between cytokine-induced and FcRγ$^-$ 'memory' NK cells in chronic HIV-1 infection.

Hypothesis: The previously described cytokine-induced NK helper and FcRγ$^-$ NK cells are mutually exclusive 'memory' populations with distinct phenotypic and functional signatures defined by differential responsiveness to IL-18.

Rationale: IL-18 induces a helper pathway of NK cell differentiation in the CD56^{dim} subset, promoting upregulation of CD25 and transient expression of CD83 and CCR7 (153, 249). Furthermore, IL-18-primed NK cells migrate in response to CCL21, a lymph node–associated cytokine, and produce an abundance of IFNγ upon subsequent exposure to secondary stimuli, particularly IL-12, suggesting CD56^{dim} NK helper cells act not only on DCs in the periphery but also migrate to lymph nodes to orchestrate adaptive immune responses in a manner similar to CD4$^+$ T helper cells (153). Subsequent studies have proven IL-18 to be necessary and sufficient for programming cytokine-induced memory-like NK cells for enhanced IFNγ responses (241, 242). Thus, NK cells strongly influence the nature of adaptive T cell responses through the production of DC-modulating cytokines, including TNFα and IFNγ (151, 153). The relationship between cytokine-induced and FcRγ$^-$ memory-like NK cells remains unclear, as does the extent to which FcRγ$^-$ NK cells are responsive to IL-18 signaling in the setting of chronic HIV-1 infection. However, the reduced IFNγ-producing capability of FcRγ$^-$ NK cells from HCMV-seropositive

individuals (291) suggests a level of distinction from the cytokine-induced memory-like population, as well as potential abnormalities in their interactions with DCs. In this aim, I seek to discriminate between cytokine-induced and FcRγ$^-$ memory-like NK cells in the context of HIV-1 infection based on their phenotypic and functional responsiveness to innate stimuli.

2.2 AIM 2

To detail the impact of chronic HIV-1 infection and long-term ART on the phenotypic subsets and functional status of 'memory' NK cells.

Hypothesis: The inflated expansion of FcRγ$^-$ NK cells in the setting of chronic HIV-1 infection will coincide with the co-expression of markers of differentiation and exhaustion; long-term ART will not significantly alter the frequency, phenotype, or functional status of FcRγ$^-$ NK cells, and will be associated with sustained levels of circulating inflammatory biomarkers.

Rationale: While the induction of FcRγ$^-$ memory-like NK cells is strongly associated with HCMV infection (247), this population of cells is further inflated in HIV-1 infection (248, 299). The mechanism by which HIV-1 contributes to this phenomenon is poorly understood. However, reports suggest that expansions of FcRγ$^-$ NK cells are not a direct consequence of HIV-1 infection but rather due to the potentially greater impact of HCMV exposure in HIV-1 infection (224, 248), as evidenced by higher HCMV antibody titers in HIV-1-infected individuals (224, 248, 306). Although ART successfully suppresses viremia and reduces AIDS-associated mortality, persistence of inflammation and innate immune activation may contribute to immune dysfunction (225, 248, 459), a notion supported by increased incidences of solid tumors and Hodgkin's lymphoma in treated HIV-1$^+$ individuals (460), as well as a higher prevalence of comorbidities

with an inflammatory etiology. In particular, markers of innate immune activation are associated with cardiovascular disease, non-AIDS cancers, and neurocognitive disorders (227-231). Whereas T cell and monocyte activation induced by HIV-1 viremia is resolved within 24 months of ART (224), activated NK cells continue to persist in those maintaining undetectable levels of plasma viremia, suggesting NK cells are more sensitive to immunologic perturbations (224, 225). We surmise that HIV-1 accelerates the expansion of FcRγ$^-$ NK cells through immune dysfunction and subclinical reactivations of HCMV. Moreover, FcRγ$^-$ NK cells may perpetuate inflammation through their enhanced ability to produce inflammatory cytokines following antibody-mediated stimulation (246-248), thereby contributing to the long-term health consequences linked to persistent innate immune activation (224). Through a longitudinal analysis of samples from HIV-1$^+$ participants, I aim to decipher the relationship between HIV-1 infection, inflammation, and expansions of FcRγ$^-$ NK cells and to determine the extent to which the phenotypic and functional defects inflicted on NK cells by HIV-1 infection are able to be reversed by ART.

2.3 AIM 3

To determine the impact of NK cell–mediated ADCC on DC induction of HIV-1-specific cellular immunity.

Hypothesis: Interactions between NK cells and DCs driven by the presence of antibodies will promote the differentiation of mature DCs with a heightened ability to produce IL-12 and to induce the development of highly functional cytotoxic T cell responses.

Rationale: FcRγ$^-$ NK cells appear to be functionally compromised or limited given their faltering responsiveness to innate cytokine stimulation. Furthermore, their physiological relevance

and *in vivo* role during chronic HIV-1 infection are not well defined. FcRγ$^-$ NK cells may be ideal targets, though, for antibody-based immunotherapies because of their inflated expansions in HIV-1$^+$ individuals and superior *in vitro* ADCC activity. In recent years, the priority of HIV-1 cure research has shifted to achieving sustained virologic remission in the absence of ART without direct eradication of the virus. A promising study demonstrated that early administration of bNAbs promotes long-term control of viremia through the induction of durable CD8$^+$ T cell responses (433), but the mechanism behind this phenomenon remains elusive. As the most efficient antigen presenting cells, DCs are particularly revered for their ability to translate environmental cues received in the periphery into polarizing signals for naïve T cells in the lymph node, thus choreographing the nature of the adaptive immune response (461-464). NK cells are important not only for the elimination of infected cells but also for the generation of antigen-specific immunity through their local interaction with DCs in infected tissues. In fact, NK cells promote the development of mature DCs with an enhanced ability to produce IL-12 and to induce type-1 immune responses (151, 156). The crosstalk between NK cells and DCs has yet to be thoroughly explored in the context of HIV-1 immunotherapies. Therefore, I seek to determine not only the impact of antibodies on FcRγ$^-$ NK cell function but also to demonstrate the importance of reciprocal NK-DC crosstalk for the induction of durable CD8$^+$ T cell–mediated control of viremia, with NK cells serving as the link between antibodies and effective CTL responses.

3.0 MATERIALS AND METHODS

3.1 Study participants

HIV-1-infected participants (n = 14), who self-identify as men who have sex with men (MSM), were randomly selected from the Pittsburgh clinical site of the Multicenter AIDS Cohort Study (MACS). All HIV-1^+ participants recorded a plasma HIV-1 load less than 20 copies/ml at the time of their study visit, with a median virally controlled treatment duration of 12.08 years (range: 1.83-22.67) (Table 4). Age-matched HIV-1-seronegative MSM (n = 14) were also selected from the MACS (median age of 58 and 57 years for HIV-1^- and HIV-1^+ men, respectively). All participants provided written informed consent prior to inclusion in this study, which was approved by the Institutional Review Board at the University of Pittsburgh.

The New Works Concept Sheet (NWCS) 486 study participants were selected from the previously analyzed AIDS Clinical Trials Group (ACTG) longitudinal study of A5321 with a total of n =101 study participants (459), which examined HIV-1 persistence and immunologic measures pre-ART (year 0), as well as year 1, 4, and years 6 through 15 on-ART. All participants had documented long-term plasma HIV-1 RNA suppression (from week 48 of ART) and no reported ART interruptions of greater than 21 days through study entry to A5321. A5321 study participants had initiated ART in ACTG ART-naïve studies and were followed in the observational study A5001 prior to enrollment in A5321.

These n = 60 NWCS 486 study participants (Table 5) were selected based on sample availability and on having cell-associated HIV-1 DNA at both year 1 and year 4 of ART (459), as well as plasma HIV-1 RNA at year 4 by single-copy assay (465). The selected samples for the

NWCS 486 testing were within 52 weeks of the previously tested on-ART timepoints of year 1 (TP1) and year 4 (TP2); if prior to ART year 1, the sample was within 16 weeks. In addition, early on-ART samples (range: 1-8 weeks on-ART; TP0) were identified for a subset of NWCS 486 participants (n = 20). Written informed consent was obtained from each participant prior to inclusion. The study was approved by ethics committees at each participating ACTG site and by the Institutional Review Board at the University of Pittsburgh.

Table 4. Characteristics of HIV-1$^+$ MACS participants

age	time to tx (yrs)	time on ART* (yrs)	CD4$^+$ T cell count$^{\#}$ (cells/mm^3)	viral load$^{\#}$ (copies/ml)	highest viral load (copies/ml)	nadir CD4$^+$ T cell count (cells/mm^3)	ART regimen	CMV status
65	14.17	20.33	529	< 20	60,909	58	TUM	SP
56	1.42	18.92	1265	< 20	68,067	392	TUM	SP
56	6.83	10.17	373	< 20	87,040	132	ATV, RAL, TUM	SP
59	4.75	8.42	486	< 20	38,580	282	ODS	SP
55	2.17	11.83	434	< 20	71,964	184	ATP	SP
83	2.75	22.67	542	< 20	94	129	DCV, DTG	SP
60	NA	16.50	1275	< 20	499,797	356	CBV, NFV	SP
53	1.42	14.75	589	< 20	400	275	RTV, DTG, DRV	SN
58	NA	16.83	1037	< 20	309,132	272	TUM	SP
69	7.67	8.83	651	< 20	460,400	210	TUM	SP
35	0.50	1.83	788	< 20	17,497	544	GNV	SP
55	3.08	12.33	421	< 20	3,524,100	148	TUM	SP
64	4.25	11.33	792	< 20	32,892	315	TUM	SP
55	1.00	5.75	707	< 20	769,565	216	TUM	SP

*suppressive ART; $^{\#}$at study visit; NA, not available due to unknown date of seroconversion; SN, seronegative; SP, seropositive; tx, treatment; yrs, years; ATP, Atripla; ATV, atazanavir; CBV, combivir; DCV, Descovy; DRV, darunavir; DTG, dolutegravir; GNV, Genvoya; NFV, nelfinavir; ODS, Odefsey; RAL, raltegravir; RTV, ritonavir; TUM, Triumeq

Table 5. Characteristics of HIV-1$^+$ NWCS 486 study participants

Age at initiation of ART, median (Q1, Q3), years	39 (34, 46)
Female	20%
Race/ethnicity:	
White, non-Hispanic	33 (55%)
Black, non-Hispanic	11 (18%)
Hispanic (regardless of race)	15 (25%)
American Indian, Alaskan Native	1 (2%)
Pre-therapy plasma HIV-1 RNA, median (Q1, Q3), $\log_{10}$copies/ml	4.6 (4.2, 4.9)
Pre-therapy CD4$^+$ T cell count, median (Q1, Q3), cells/mm^3	284 (175, 365)
Year 4 of ART CD4$^+$ T cell count, median (Q1, Q3), cells/mm^3	607 (470, 783)
Pre-therapy CD4:CD8 T cell ratio, median (Q1, Q3)	0.3 (0.2, 0.4)
Year 4 of ART CD4:CD8 T cell ratio, median (Q1, Q3)	0.8 (0.5, 1.0)
ART regimen (initial, at time of last sample collection)	
NNRTI-based	62%, 65%
PI-based	35%, 33%
INSTI-based	0%, 2%
Other	3%, 0%

INSTI, integrase strand transfer inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor

3.2 Real-time quantitative PCR for HCMV and EBV DNA

Plasma samples were first centrifuged (Thermo Scientific™ Sorvall™ ST8R) at 800 x g for 2 minutes at 4°C. Prior to extraction, 250 µl of the spun plasma was combined with 1950 µl of Guanidine thiocyanate (GuSCN)* Triton buffer (NUCLISENS® easyMAG®) and incubated at room temperature for 15 minutes. To measure proper extraction and efficiency of the quantitated PCR reaction, 10 µl of phocine herpesvirus (PhHV-1), an in-house propagated virus, was added as an internal control. A 20 µl TaqMan™ PCR was performed by mixing 5 µl of viral DNA with TaqMan™ Gene Expression Master Mix (Applied Biosystems™ by Thermo Scientific™), in addition to the appropriate forward and reverse primers (Table 6) and TaqMan™ MGB probes:

1. 5'-6-FAM-CGATCACAAACAGCG-MGB-3' (HCMV)
2. 5'-6-FAM-TAGAGGTTTTGCTAGGGAGGAGACGTGTG-MGB-3' (EBV)
3. 5'-VIC-TTTTATGTGTCCGCCACCAT-MGB-3' (PhHV-1)

Real-time PCR was performed using the ViiA 7 A&B Applied Biosystems™ instrument (Life Technologies) and the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. A no template control was included in each assay to control for PCR cross-contamination; each sample was assayed in duplicate and controls in triplicate. QuantStudio™ Real-time PCR Software (Applied Biosystems™ by Thermo Scientific™) was used for PCR data analysis, and viral copy numbers were reported using plasmid controls previously extrapolated and verified from quantitated DNA controls, with copy numbers purchased from Advanced Biotechnology, Inc.

Table 6. Real-time PCR forward and reverse primers

Target	Forward primer	Reverse primer	Reference(s)
HCMV	5'-CGATCAAGAACGCGATAACG-3'	5'-ACCGTCGATGGCAGGTCAT-3'	(466, 467)
EBV	5'-AAACCTCAGGACCTACGCTGC-3'	5'-AGACACCGTCCTCACCAC-3'	(468)
PhHV-1	5'-GGGCGAATCACAGATTGAATC-3'	5'-GCGGTTCCAAACGTACCAA-3'	(467, 469)

3.3 Quantitation of HCMV and EBV antibody titers

The HCMV and EBV antibody titers of study participants were determined by quantitative Cytomegaly and EBV EBNA-1 IgG ELISAs (GenWay), respectively, following the manufacturer's protocol. Plasma samples were diluted 1:101 with the provided sample diluent, and absorbance was read at 450 nm using the BioTek ELx800™. The ready-to-use standards and

controls of the ELISA kits were defined and expressed in arbitrary units (U/ml), resulting in an exact and reproducible quantitative evaluation. The absorptions of the standards and controls were graphed against their concentrations. From the resulting reference curve, the concentration values for each sample were then extracted in relation to their absorptions.

3.4 Isolation of monocytes and peripheral blood lymphocytes (PBLs)

PBMCs were isolated from whole blood of participants, or from buffy coat blood products (purchased from the Central Blood Bank of Pittsburgh), by standard density gradient separation utilizing lymphocyte separation medium (Corning). PBMCs were further separated into monocytes and PBLs using human CD14 MicroBeads (Miltenyi Biotec).

3.5 Generation of monocyte-derived immature DCs (iDCs)

To generate iDCs, isolated monocytes were cultured for four days in 24-well plates (Costar®) at a density of 6.25×10^5 cells/well in Iscove's Modified Dulbecco's Medium (Gibco®), containing 10% fetal bovine serum (Atlanta Biologicals) and 0.5% gentamicin (Gibco®) (cIMDM). Cultures were supplemented with GM-CSF (1000 IU/ml; Sanofi-Aventis) and IL-4 (1000 IU/ml; R&D Systems®).

3.6 Activation and polarization of DCs by NK cells

Autologous NK cells, purified from fresh or cryopreserved PBLs by magnetic bead negative selection using a human NK cell enrichment kit (EasySep™), were added directly to day-4 iDC cultures at a 1:1 ratio in the presence of innate or adaptive stimuli (NK-DC). The innate NK cell activating factors were recombinant human (rh) IL-18 (hereinafter referred to as IL-18; 1 μg/ml; MBL® International) and rhIL-12p70 (hereinafter referred to as IL-12; 100 ng/ml; BioLegend®), and the adaptive stimuli consisted of a combination of rituximab-opsonized Raji cells (NK:Raji ratio of 10:1) and rhIFNα-2b (hereinafter referred to as IFNα; 1000 IU/ml; Intron® A). Where indicated, DCs were cultured in the absence of NK cells, i.e., only with the innate (IL-18+IL-12) or adaptive (opsonized Raji cells+IFNα) stimuli (DC0), or in the absence of NK cells and additional factors (iDC). After 48h, DCs were harvested, thoroughly washed, and analyzed for their expression of maturation-associated surface markers (*see Flow cytometry*) and ability to produce IL-12. To test their IL-12-producing capacity, DCs were plated in 96-well flat bottom plates (Costar®) at $2.5x10^4$ cells/well. To mimic the interaction with CD40L-expressing Th cells, CD40L-transfected J558 cells (a gift from Dr. P. Lane, University of Birmingham, Birmingham, U.K.) were added at $5x10^4$ cells/well (151). Supernatants were collected after 24h and tested for the presence of IL-12p70 by ELISA.

To compare the ability of NK cells from HIV-1^+ MSM to impact DC production of IL-12, isolated NK cells were cultured in serum free AIM V® medium (Gibco®) in 96-well round bottom plates (Costar®) at a density of $1x10^6$ cells/ml in the presence of IL-18 (1 μg/ml; MBL® International) and rhIL-2 (hereinafter referred to as IL-2; 1000 IU/ml; Proleukin®, Prometheus Laboratories, Inc.). Supernatants from these NK cell and mock (cytokines only) cultures were

collected after 48h. To control for high donor-to-donor variability in DC IL-12 production, iDC cultures were generated from a single HIV-1^+ participant and treated for 48h with rhTNFα (20 ng/ml; R&D Systems®). The DCs were harvested, thoroughly washed, and plated in 96-well flat bottom plates (Costar®) at $2.5x10^4$ cells/well and used as assay responder cells. Supernatants from the NK cell and mock cultures were added to the responder DCs, which were subsequently stimulated with CD40L-transfected J558 cells ($5x10^4$ cells/well). Supernatants were collected after 24h and tested for IL-12p70 by ELISA.

3.7 NK cell and PBMC cultures

NK cells were purified from fresh or cryopreserved PBLs by magnetic bead negative selection using a human NK cell enrichment kit (EasySep™). Isolated NK cells were cultured in serum free AIM V® medium (Gibco®) in 96-well round bottom plates (Costar®) at a density of $1x10^5$ cells/well. PBMCs were cultured in cIMDM in 24-well plates (Costar®) at a density of $1.5x10^6$ cells/well. The phenotypic and functional profiles, as well as frequencies of FcRγ$^-$ NK cells, were determined by flow cytometry (*see Flow cytometry*) on NK cell subsets following a 24h or 48h culture in media alone or in the presence of IL-18 (1 μg/ml; MBL® International) and/or IL-12 (20 ng/ml; BioLegend®). For priming experiments, NK cell cultures were washed after a 48h exposure to a primary signal and cultured for an additional 24h in the presence of a secondary signal of either IL-18, IL-12, or IL-2 (1000 IU/ml; Proleukin®, Prometheus Laboratories, Inc.).

3.8 Flow cytometry

Cells were pre-exposed for 15 minutes to 50.0 μg/ml of unfractionated murine IgG (Sigma-Aldrich) to block nonspecific Fc receptor binding before immunostaining. The LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Life Technologies) was used for viability exclusion, and the following antibodies were used for immunostaining: CD3-APC-H7 (clone SK7, BD Pharmingen™), CD56-PE-Cy7 (clone N901, Beckman Coulter), CD57-BV421 (clone NK-1, BD Horizon™), CD16-PerCP-Cy™5.5 (clone 3G8, BD Pharmingen™), CD25-BV605 (clone M-A251, BD OptiBuild™), CD83-PE (clone HB15a, Beckman Coulter), CD86-PE (clone HA5.2B7, Beckman Coulter), CCR7-FITC (clone 150503, R&D Systems®), Siglec-1/CD169-PE (clone 7-239, BioLegend®), NKG2A-APC (clone Z199, Beckman Coulter), NKG2C-PE (clone 134591, R&D Systems®), NKp46-PE (clone BAB281, Beckman Coulter), PD-1-BV421 (clone EH12.1, BD Horizon™), KLRG1-APC (clone 13F12F2, eBioscience™), IL18Rα-Alexa Flour® 700 (clone 70625, R&D Systems®), and IL12Rβ2-APC (clone REA333, Miltenyi Biotec). Staining was done in FACS buffer consisting of 1x PBS solution (GE Life Sciences), 0.5% bovine serum albumin, and 0.1% sodium azide (Sigma). For intracellular protein expression, NK cells were fixed with BD Cytofix/Cytoperm™ (BD Biosciences), permeabilized using BD Perm/Wash™ (BD Biosciences), and labeled with anti-FcRγ-FITC (Milli-Mark®) and/or anti-IFNγ-Alexa Flour® 700 (clone B27, BD Pharmingen™). For CD107a mobilization assays, the cells were exposed to anti-CD107a-APC (clone H4A3, BD Pharmingen™) in the presence of 0.1% monensin by volume (BD GolgiStop™), followed by the viability exclusion, surface marker, and intracellular immunostaining procedures described above. Samples were stored in FACS buffer until data acquisition using a BD LSRFortessa™ flow cytometer. Data were analyzed using FlowJo version 10.5.3 (Tree Star), with

expression levels based on comparison to fluorescence minus one (FMO) samples or unstimulated controls for CD107a and intracellular cytokine staining (470). To generate the heatmap, mean fluorescence intensities (MFIs) for each marker were obtained by flow cytometry and subsequently normalized, whereby the lowest and highest MFIs of each dataset were assigned values of 0 and 100, respectively. The heatmap, with two-dimensional unbiased hierarchical clustering, was created utilizing R version 3.5.2 and the RColorBrewer package.

3.9 t-distributed Stochastic Neighbor Embedding (t-SNE)

FCS files were imported into FlowJo version 10.5.3 (Tree Star) and manual gates were applied to exclude debris, doublets, and dead cells from each sample. The number of events on each live gate population was reduced using the DownSample gate tool, followed by merging of downsampled gates with the Export/Concatenate Populations tool, during which keyword-based derived parameters were created representing the different stimulation conditions. t-SNE was then performed on the concatenated file. Different groups of samples (i.e., unstimulated vs IL-18 treatment) were gated on according to the keyword parameter. Manual gating was used to overlay color on the FcRγ^- population.

3.10 Cytokine detection

Supernatants from the DC and cytokine-stimulated NK cell cultures were collected at the end of the 24h or 48h incubation and stored at -80°C until tested. Concentrations of IL-12p70 and

IFNγ were measured by a sandwich ELISA using recombinant human protein for standards (R&D Systems®) and matched capture and detection antibody pairs (Invitrogen™) following the manufacturer's protocol. Absorbance was read at 450 nm using the BioTek ELx800™.

3.11 Flow cytometry–based ADCC assay

NK cells were plated in 96-well round bottom plates at a concentration of $1.6x10^6$ cells/ml and serially diluted two-fold until achieving a concentration of $2x10^5$ cells/ml. Raji cells were incubated for 30 minutes at 37°C with 10 μg/ml rituximab and washed two times with 1x PBS. Unopsonized Raji cells were stained with 1 μM CellTrace™ Violet dye (Invitrogen™) for 10 minutes at 37°C. After quenching the reaction with 10 ml of cIMDM, the cells were incubated for an additional 10 minutes at 37°C and washed two times with cIMDM. The CellTrace™ Violet dye-labeled Raji cells, used both as a 'cold target' inhibitor and as a control reference target, were mixed with an equal number of unstained opsonized Raji target cells. The mixture of Raji cells was subsequently resuspended in serum free AIM V® medium at a concentration of $4x10^5$ cells/ml. 100 μl of the Raji cell suspension was plated per well of NK cells, resulting in effector (NK) to target (Raji) cell ratios of 8:1, 4:1, 2:1, and 1:1. After incubating the co-cultures at 37°C for 6h, cells were stained for viability (LIVE/DEAD™ Fixable Aqua Dead Cell Stain, Life Technologies) and surface expression of CD3-APC-H7 (clone SK7, BD Pharmingen™), CD19-PerCP-Cy™5.5 (clone HIB19, BD Pharmingen™), and CD56-PE-Cy7 (clone N901, Beckman Coulter), and were subsequently fixed in 2% paraformaldehyde. The percentage of ADCC-mediated killing was calculated based on the relative proportion of viable opsonized targets to unopsonized dye-labeled Raji cells.

3.12 Induction and expansion of autologous CTLs

Total $CD8^+$ T cells were isolated from cryopreserved PBLs by magnetic bead negative selection using a human $CD8^+$ T cell enrichment kit (EasySep™). To induce CTL responses as previously described (471), $CD8^+$ T cells ($7.5x10^5$ cells/well) were co-cultured with autologous differentially matured DCs ($7.5x10^4$ cells/well) loaded with HIV-1-derived 9mer peptide epitopes (5 μg/ml; Sigma-Aldrich) (Table 7), in addition to gamma-irradiated (5000 rad) CD40L-transfected J558 cells at a concentration of $5x10^4$ cells/well. On day five and every three days thereafter, the cultures were supplemented with IL-2 (1000 IU/ml; Proleukin®, Prometheus Laboratories, Inc.) and rhIL-7 (10 ng/ml; Miltenyi Biotec). On day 14, T cell cultures were restimulated with gamma-irradiated HLA-$A2^+$ T2 cells loaded with 9mer peptides (1 μg/ml) corresponding to the viral antigens used in the initial stimulation. Antigen-specific readout assays were performed between days 24 and 26 to assess CTL activity.

3.13 IFNγ ELISPOT assay

In vitro expanded CTLs were harvested, counted, and immediately tested for reactivity to pooled HIV-1-derived 9mer peptide antigens (1 μg/ml) by ELISPOT assay using anti-human IFNγ and biotin monoclonal antibodies (clones 1-D1K and 7-B6-1, Mabtech) as previously described (472). Briefly, CTLs were plated at a concentration of $3x10^4$ cells/well in anti-IFNγ antibody-coated 96-well PVDF ELISPOT plates (MilliporeSigma). Pooled HIV-1-derived 9mer peptide antigens were added to CTL-containing wells at a final peptide concentration of 1μg/ml, and all assays included negative control wells of expanded CTLs without peptide stimulation (media

only). IFNγ responses to the peptide pools were performed in duplicate wells. Spots were enumerated by the Autoimmun Diagnostika GmbH (AID) ELISPOT reader and counting software. Recorded values, shown as IFNγ spot forming units (SFU) per 10^6 cells, were net responses compared to control wells consisting of CTLs exposed to assay medium alone.

Table 7. HIV-1-derived peptide epitopes for induction and expansion of autologous CTLs

Peptide sequence	Length (aa)	Protein	Position in sequence	Subprotein
[H]-IMMQRGNFK-[OH]	9	Gag	376-384	p2p7p1p6(13-21)
[H]-TTSTLQEQI-[OH]	9	Gag	239-247	p24(107-115)
[H]-RDYVDRFYK-[OH]	9	Gag	294-302	p24(162-170)
[H]-LSPRTLNAW-[OH]	9	Gag	147-155	p24(15-23)
[H]-FRDYVDRFY-[OH]	9	Gag	293-301	p24(161-169)
[H]-AEWDRVHPV-[OH]	9	Gag	210-218	p24(78-86)
[H]-STLQEQIGW-[OH]	9	Gag	241-249	p24(109-117)
[H]-RTLNAWVKV-[OH]	9	Gag	150-158	p24(18-26)
[H]-TSTLQEQIGWM-[OH]	11	Gag	240-250	p24(108-118)
[H]-SLYNTVATL-[OH]	9	Gag	77-85	p17(77-85)
[H]-TLNAWVKVV-[OH]	9	Gag	151-159	p24(19-27)
[H]-KLTPLCVTL-[OH]	9	gp160	121-129	gp120(121-129)
[H]-SLLNATAIAV-[OH]	10	gp160	813-822	gp41(302-311)
[H]-ILKEPVHGV-[OH]	9	Pol	464-472	RT(309-317)
[H]-AFHHVAREL-[OH]	9	Nef	190-198	
[H]-FPVRPQVPL-[OH]	9	Nef	68-76	
[H]-AIIRILQQL-[OH]	9	Vpr	59-67	
[H]-RMYSPVSIL-[OH]	9	Gag	275-283	p24(143-151)
[H]-ELKSLYNTV-[OH]	9	Gag	74-82	p17(74-82)
[H]-MLKETINEEA-[OH]	10	Gag	200-209	p24(68-77)
[H]-ELKKIIGQV-[OH]	9	Pol	872-880	Integrase(157-165)
[H]-KLVGKLNWA-[OH]	9	Pol	414-422	RT(259-267)
[H]-HLKTAVQMAV-[OH]	10	Pol	886-895	Integrase(171-180)
[H]-DLADQLIHLY-[OH]	10	Vif	101-110	

aa, amino acids; gp, glycoprotein; RT, reverse transcriptase

3.14 HIV-1-specific killing assay

CTL effector function was assessed in a modified version of the *Flow cytometry–based ADCC assay*. Briefly, autologous $CD4^+$ T cells were stained with either eBioscience™ CFSE (Invitrogen™) or CellTrace™ Violet (Invitrogen™) dyes. Target cells (CFSE) were then painted with pooled HIV-1-derived 9mer peptides at 1 μg/ml in cIMDM for 60 minutes at 37°C; excess unbound peptide was removed by washing. The CFSE and CellTrace™ Violet dye-labeled cells were mixed in equal numbers and co-incubated for 18h with autologous CTLs at various effector to target ratios. The cells were stained for surface expression of CD8-PE-Cy7 (clone SFCI21Thy2D3, Beckman Coulter), fixed in 2% paraformaldehyde, and analyzed by flow cytometry. Effector $CD8^+$ T cells were excluded from analysis gating, and the antigen-specific killing of HIV-1 peptide-loaded $CD4^+$ T cells (CFSE) was calculated based on relative changes in percentages of the remaining differentially stained target cells.

3.15 Statistical analyses

Data were analyzed using GraphPad Prism version 8.0.2. Normality was determined by the Shapiro-Wilk test, and data not following a normal distribution were analyzed by the non-parametric Mann-Whitney *U* test or Wilcoxon matched-pairs signed rank test. The paired Student's t-test was used to determine statistical significance between two related groups (e.g., TP1 vs TP2; DC0 vs NK-DC; unstimulated vs IL-18+IL-12) and for direct comparisons of the $FcR\gamma^-$ and $FcR\gamma^+$ NK cell subsets within HIV-1^+ participants with an $FcR\gamma^-$ NK cell frequency greater than 10%. The unpaired Student's t-test was used to compare the means of two independent

or unrelated groups. Where the mean of each condition was compared to a single control mean of 1.00, statistical significance was assessed by one-way ANOVA, followed by Dunnett's test for multiple comparisons. A one-way ANOVA was used to measure the significance of differences between the means of three or more groups, with correction for multiple comparisons by Tukey's HSD post-hoc test. A two-way ANOVA was used to establish the statistical significance of differences between the means of multiple groups (e.g., secondary signal) of two factors (e.g., primary signal). For analyses of repeated measures in which values were missing, data were analyzed by fitting a mixed model (i.e., mixed effects analysis), with correction for multiple comparisons by Tukey's HSD post-hoc test. The linear relationship between FcRγ^- NK cell frequency and IFNγ expression was determined by Pearson correlation analysis, and multiple linear regression analysis was used to investigate the effect of FcRγ^- NK cell frequency and HIV-1 status on IFNγ expression. Rank-based Spearman correlations examined linear associations of flow cytometry–based outcome measures with soluble markers of inflammation and antigen-specific adaptive immune responses. Measures below the assay limit were analyzed as the lowest rank. Detailed methodologies of the outcome measures related to NWCS 486 were previously described (459, 465, 473, 474). With the exception of Figures 17 and 18, community controls are denoted by triangles, HIV-1^- MSM by squares, and HIV-1^+ participants by circles.

4.0 RESULTS

4.1 NK cells require two signals to activate their helper function via IFNγ production

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ⁻ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

As the most efficient antigen presenting cells, DCs are particularly revered for their ability to translate environmental cues received in the periphery into polarizing signals for naïve T cells in the lymph node, thus directing the nature of the adaptive immune response (461-464). NK cells are important not only for the elimination of infected cells but also for shaping the character and quality of antigen-specific immunity through their local interaction with DCs in infected tissues. In fact, NK cells have the capacity to either limit DC function, through a process referred to as *DC editing* (137, 140, 164, 195), or to promote their maturation and subsequent lymph node–homing and T cell–priming functions (151, 156). Previous studies have demonstrated that active HIV-1 infection in particular leads to dysfunctional interactions between NK cells and DCs (183, 184, 195, 211, 475). Therefore, we investigated the quality of the crosstalk between NK cells and DCs from virally suppressed HIV-1-infected MACS participants. We began by assessing the basic functional status of the NK cells, confirming their requirements for the production of IFNγ, an important effector cytokine and potent DC-modulating agent (139). Consistent with previous reports (151, 156), the induction of NK cell helper activity required a second NK cell–activating signal. That is, only stimulation with two activating signals triggered production of IFNγ by NK

cells isolated from virally suppressed MACS participants with chronic HIV-1 infection (Fig. 5A, 5B), and this two-signal requirement remained true for HIV-1-uninfected blood bank donors (community controls; Fig. 5C). NK helper cells were capable of responding to a variety of secondary signals, including IFNα and IL-2, but the combination of IL-18 and IL-12 resulted in the highest levels of IFNγ secretion (Fig. 5D). Based on these data, we utilized IL-18 with IL-12, two important DC-derived cytokines for triggering NK cell activation (138, 139, 151, 153, 155), in subsequent experiments. Although NK cells from HIV-1-infected participants produced IFNγ in response to co-stimulation with IL-18 and IL-12, this production differed significantly between HIV-1-infected and uninfected individuals (Fig. 5E).

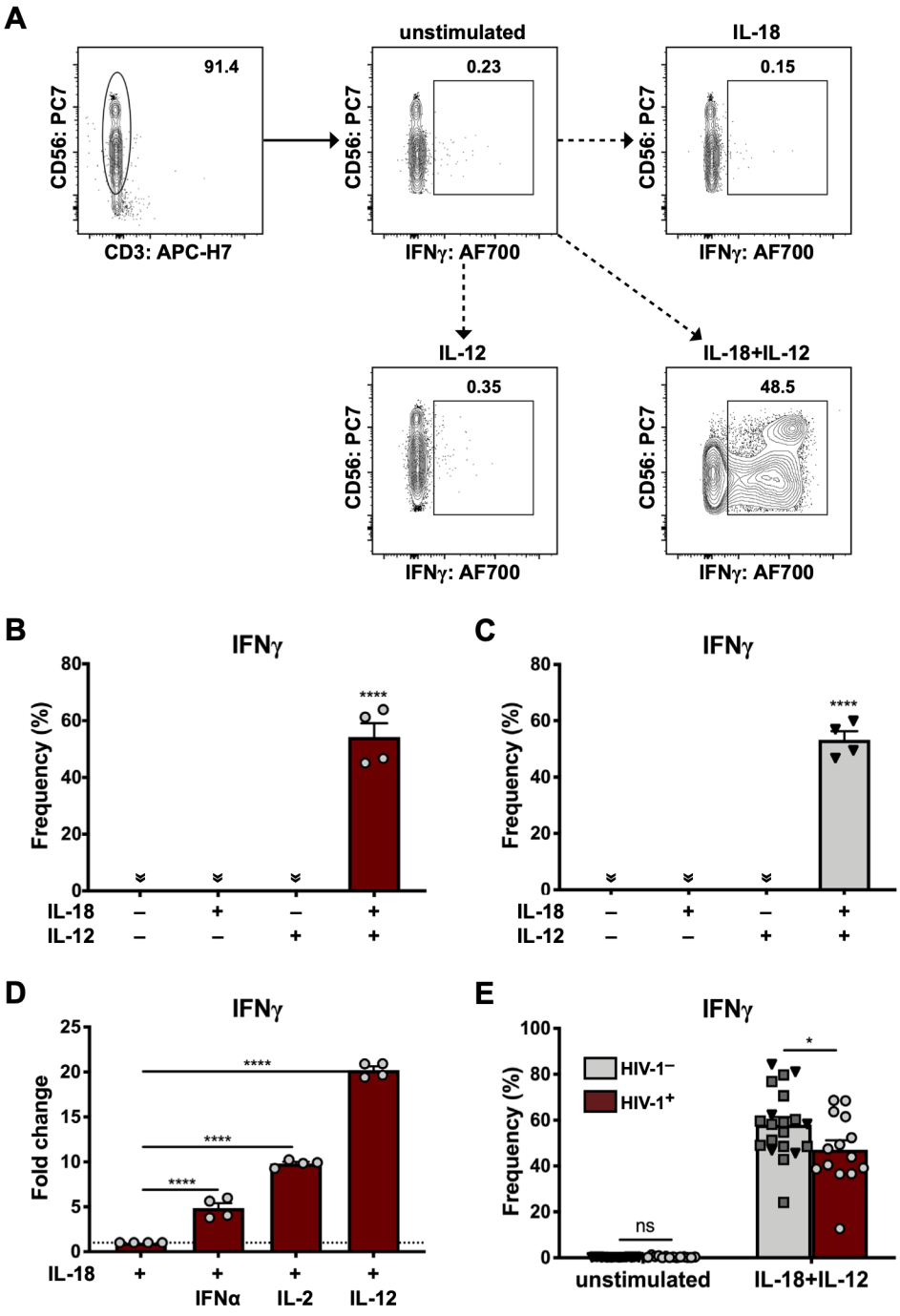


Figure 5. Two-signal requirement to activate the helper function of NK cells

(A) Representative staining of human NK cells ($CD3^{-}CD56^{+}$) for expression of IFNγ following a 48h culture in the presence of IL-18 and/or IL-12. Gating was performed on live cells by forward and side scatter areas and single cells. Gates for IFNγ were determined based on the unstimulated control. **(B)** Exposure to one signal was insufficient for activating the helper function of NK cells. Only two-signal activated NK cells produced high levels of IFNγ as determined by intracellular staining; $n = 4$ $HIV\text{-}1^{+}$ participants. **(C)** NK cells from $HIV\text{-}1^{-}$ community controls also required two activating signals for IFNγ expression; $n = 4$ $HIV\text{-}1^{-}$ community controls. **(D)** The combination of IL-18+IL-12 sparked robust NK cell secretion of IFNγ, a critical DC-polarizing agent (as measured by ELISA); $n = 2$

replicates from two HIV-1$^+$ participants. **(E)** In response to a 48h exposure to IL-18+IL-12, IFNγ expression was significantly lower among HIV-1$^+$ individuals (n = 14) compared to the HIV-1$^-$ group (n = 20), comprised of n = 6 HIV-1$^-$ community controls (black triangles) and n = 14 HIV-1$^-$ MSM (steel squares). Statistical significance was determined by one-way ANOVA (B-D) or the unpaired Student's t-test (E) (****$p < 0.0001$; *$p < 0.05$).

4.2 Two-signal activated NK cells promote type-1 polarized DCs

*Adapted from: **Anderko RR**, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ$^-$ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

To directly evaluate NK-DC crosstalk in the setting of chronic HIV-1 infection, iDCs were cultured with IL-18 and IL-12 in the presence or absence of autologous NK cells. The cells were harvested after 48h and assessed for DC maturation and polarization. Two-signal activated NK cells induced DC maturation, demonstrated by surface expression of CD83 and CCR7, as well as upregulation of CD86 and Siglec-1 (Fig. 6A), and the presence of NK cells promoted the programming of type-1 polarized DCs with an enhanced ability to produce IL-12 (Fig. 6B, 6C). Importantly, DCs matured in the presence of NK cells generated highly functional HIV-1-specific CTLs, as determined by IFNγ ELISPOT responses and the targeted killing of autologous peptide-pulsed CD4$^+$ T cells (Fig. 6D, 6E), indicating the combination of IL-18 and IL-12 activates the helper function of NK cells from participants with chronic HIV-1 infection.

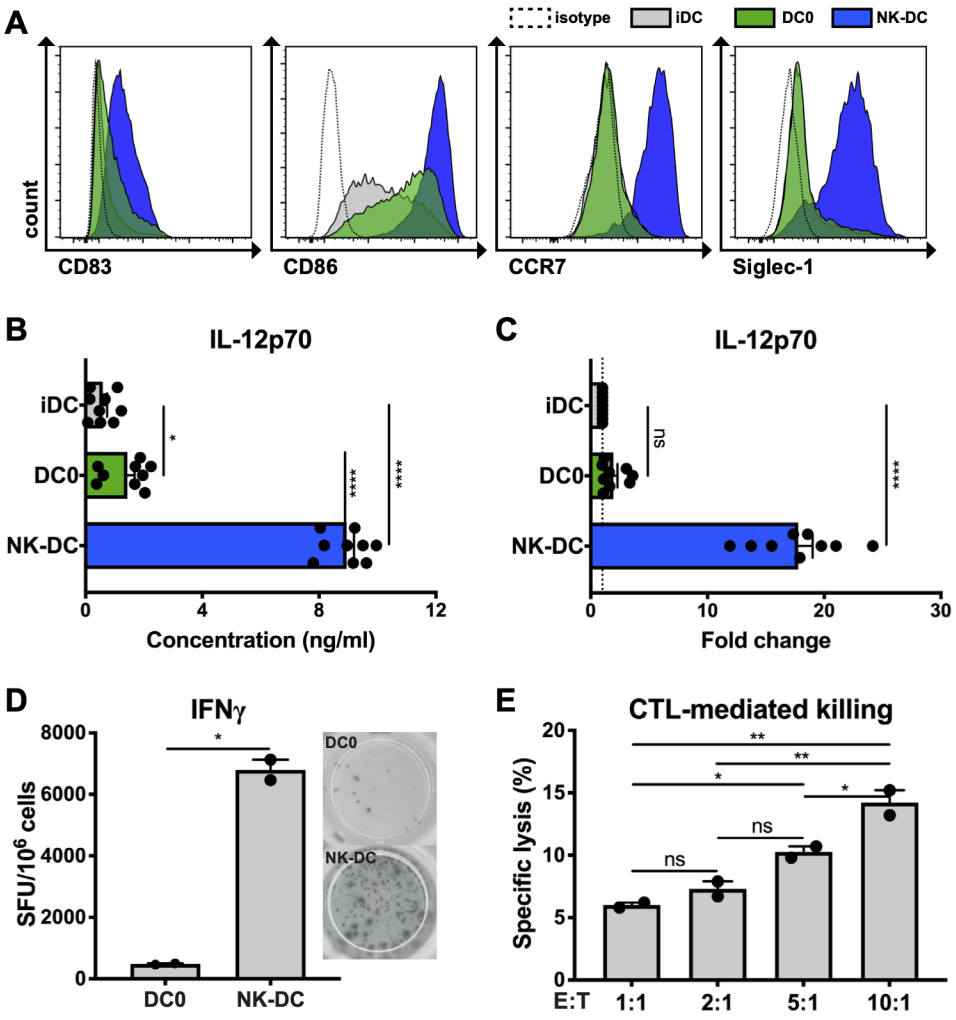


Figure 6. Two-signal activated NK cells promote DC polarization and cellular immunity to HIV-1

Day-4 iDCs were cultured for 48h either with IL-18+IL-12 in the presence of autologous NK cells (1:1), only with IL-18+IL-12 (DC0), or in the absence of NK cells and additional cytokines (iDC). **(A)** Two-signal activated NK cells induced the development of type-1 polarized mature DCs (NK-DC), demonstrated by expression of CD83 and CCR7 and upregulation of CD86 and Siglec-1; data are representative of $n = 5$ HIV-1^+ participants. **(B, C)** The presence of NK cells promoted programming of type-1 polarized DCs with enhanced IL-12p70 production following a 24h stimulation with J558-CD40L; $n = 3$ replicates from 3 HIV-1^+ participants. **(D, E)** NK cells enhanced DC-mediated induction of cellular immunity to HIV-1 as determined by $CD8^+$ T cell IFNγ responses to HIV-1-derived 9mer peptide pools (D) and the targeted killing of autologous peptide-pulsed $CD4^+$ T cells (E); $n = 2$ HIV-1^+ participants. Statistical significance was determined by one-way ANOVA (B, C, E) and the paired Student's t-test (D) (****$p < 0.0001$; **$p < 0.01$; *$p < 0.05$).

4.3 FcRγ$^-$ NK cells expand during chronic HIV-1 infection

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ$^-$ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

While there appeared to not be a defect in NK-DC crosstalk in the setting of treated chronic HIV-1 infection, we did note lower IFNγ production among HIV-1$^+$ participants (Fig. 5E), suggesting potential qualitative differences in the interactions between NK cells and DCs. Since impaired NK-DC crosstalk in HIV-1 infection has previously been attributed to the preferential expansion of CD56$^-$CD16$^+$ NK cells (195), we determined the relative distribution of NK cell subsets in HIV-1-infected and uninfected individuals. NK cells were enriched from PBLs of HIV-1-uninfected community controls (n = 8), as well as randomly selected HIV-1-infected and age-matched HIV-1-uninfected MACS participants (n = 14), who self-identify as MSM. The observed variability in IFNγ production could not be explained by an unusual CD56$^-$CD16$^+$ population as no differences in the relative proportions of CD56^{bright}CD16$^-$, CD56^{dim}CD16$^{\pm}$, or CD56$^-$CD16$^+$ NK cells were detected between the three groups (Fig. 7A-7C). However, baseline frequencies of peripheral blood–derived FcRγ$^-$ NK cells, measured by flow cytometry, varied widely, ranging from 1 to 45% (Fig. 7D, 7E). While FcRγ$^-$ NK cells comprised at least 10% of the total NK cell population in approximately 30% and 60% of HIV-1$^-$ and HIV-1$^+$ MACS participants, respectively, they accounted for less than 10% of the total NK cell population in all of the uninfected community controls (Fig. 7F). A modest increase in FcRγ$^-$ NK cell frequencies was observed in HIV-1$^-$ MSM compared to HIV-1$^-$ community controls, but this trend did not

reach statistical significance (p = 0.2119; Fig. 7F). By contrast, chronic HIV-1 infection was characterized by a significantly larger population of FcRγ$^-$ NK cells (Fig. 7G-7I). Since the induction of FcRγ$^-$ NK cells is strongly associated with HCMV infection (247), we compared FcRγ$^-$ NK cell frequencies only among HCMV-seropositive individuals. Both the HIV-1$^+$ and HIV-1$^-$ MSM experienced an HCMV seropositivity rate of 93% (n = 13 of 14; Table 4). Only 25% of the HIV-1$^-$ community controls were seropositive for HCMV (n = 2 of 8), for a combined HCMV seropositivity rate among the HIV-1$^-$ individuals of 68%. Importantly, higher proportions of FcRγ$^-$ NK cells were still present in HIV-1$^+$ participants relative to the HIV-1$^-$ group (Fig. 7J). FcRγ$^-$ NK cells, indeed, expand in the setting of chronic HIV-1 infection, but their prominence in both HIV-1$^-$ and HIV-1$^+$ MACS participants relative to community controls underscores the influence of MSM status on FcRγ$^-$ NK cell proportions, supporting the findings of Hearps *et al.* (224).

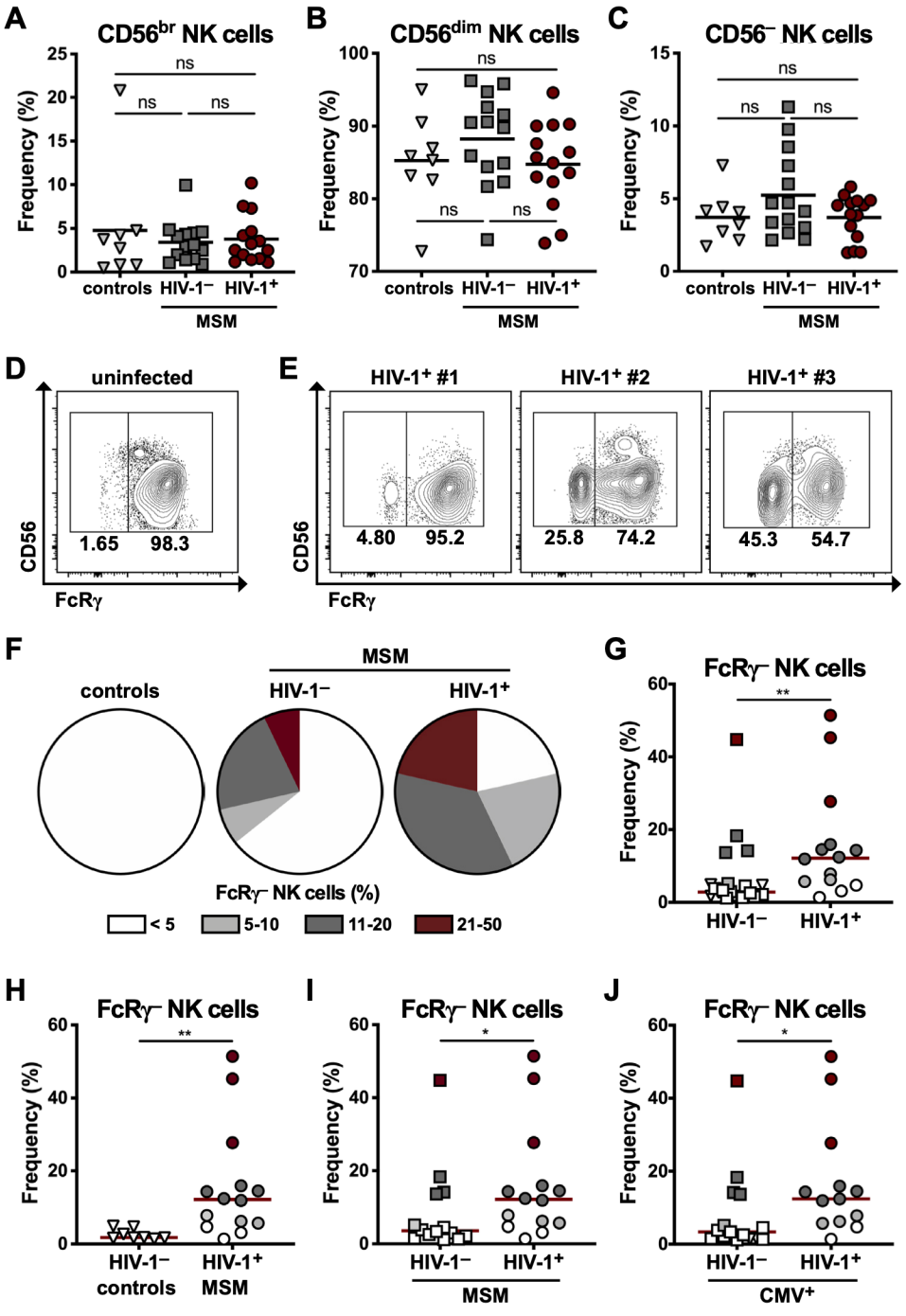


Figure 7. A notable population of NK cells deficient in FcRγ is present in chronic HIV-1 infection

(A-C) Baseline flow cytometry analyses of purified NK cells from age-matched HIV-1$^-$ and HIV-1$^+$ MSM of the MACS cohort ($n = 14$), as well as uninfected community controls ($n = 8$). The relative distribution of CD56^{bright}CD16$^-$ (A), CD56^{dim}CD16$^{\pm}$ (B), and CD56$^-$CD16$^+$ (C) NK cell subsets was comparable between the three groups. **(D)** Representative staining at baseline for FcRγ on NK cells enriched from peripheral blood, showcasing the scarcity of FcRγ$^-$ NK cells in uninfected community controls, **(E)** compared to HIV-1-infected MSM, where the frequencies varied, accounting for up to 45% of total NK cells in select participants tested. **(F)** While the proportions of FcRγ$^-$ NK cells increased among HIV-1$^-$ MSM, the highest percentages of FcRγ$^-$ NK cells emerged in HIV-1$^+$ MACS

participants, with 8 of 14 HIV-1$^+$ men having an FcRγ$^-$ NK cell frequency >10%, compared with 4 of 14 HIV-1$^-$ MSM and 0 of 8 HIV-1$^-$ community controls. **(G-I)** Chronic HIV-1 infection was characterized by an expanded population of FcRγ$^-$ NK cells, with comparisons between HIV-1$^+$ MSM (n = 14; circles) and HIV-1$^-$ individuals (n = 22), comprised of n = 8 HIV-1$^-$ community controls (triangles) and n = 14 HIV-1$^-$ MSM (squares), (G); HIV-1$^-$ community controls (n = 8; triangles) and HIV-1$^+$ MSM (n = 14; circles) (H); and HIV-1$^-$ MSM (n = 14; squares) and HIV-1$^+$ MSM (n = 14; circles) (I). **(J)** Among the HCMV seropositive individuals only, higher frequencies of FcRγ$^-$ NK cells were present in HIV-1$^+$ individuals (n = 13; circles) relative to the HIV-1$^-$ group (n = 15), comprised of n = 2 HIV-1$^-$ community controls (triangles) and n = 13 HIV-1$^-$ MSM (squares). Normally distributed data were measured by one-way ANOVA, with the solid black line representing the mean (A-C). For data not following a normal distribution, statistical significance was determined by the Mann-Whitney U test, with the solid red line representing the median (G-J) (**$p < 0.01$; *$p < 0.05$).

4.4 A mature phenotype distinguishes NK cells deficient in FcRγ

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ$^-$ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

As we were particularly interested in analyzing FcRγ$^-$ NK cells in the setting of chronic HIV-1 infection, we selected the HIV-1$^+$ MACS participants with a baseline FcRγ$^-$ NK cell frequency greater than 10% for comprehensive analyses (n = 8). An evaluation of the normalized MFIs of phenotypic and functional markers analyzed by flow cytometry illustrated the differential expression patterns of FcRγ$^-$ and conventional (FcRγ$^+$) NK cells (Fig. 8A), corroborating that FcRγ$^-$ NK cells from HIV-1$^+$ individuals adopt a unique expression profile in comparison to their FcRγ$^+$ counterparts (248).

We subsequently probed the phenotypic properties of FcRγ$^-$ NK cells from HIV-1$^+$ participants as they related to the descriptions of memory-like NK cell subsets delineated in the current literature (241, 242, 246, 247). FcRγ$^-$ NK cells from HCMV-seropositive individuals are marked by increased expression of NKG2C and CD57 but a lack of expression of NKG2A (246,

247). They also express lower levels of NKp46 and NKp30. This speaks to their reduced capacity for direct cytotoxicity of target cells (246, 247). Similarly, FcRγ$^-$ NK cells from HIV-1$^+$ MACS participants presented less NKp46 on the cell surface, both in terms of percent positivity and MFI (Fig. 8B, 8C). Compared with conventional NK cells, FcRγ$^-$ NK cells featured an enrichment of CD57 (Fig. 8B, 8C), a marker of replicative senescence (476-478), suggesting the FcRγ$^-$ subset is highly differentiated (42). Additionally, the levels of NKG2A, an inhibitory receptor, and NKG2C, an activating receptor, were reduced and elevated, respectively (Fig. 8B, 8C), resulting in an inverse of the NKG2A to NKG2C ratio typically found with conventional NK cells (Fig. 8D). These data corroborate the reported inverse correlation of CD57 expression with NKG2A and NKp46 levels on CD56^{dim} NK cells (42), and also confirm that FcRγ$^-$ NK cells from HIV-1$^+$ men resemble those reported in association with HCMV (246, 247). Notably, though, the phenotypic hallmarks observed for FcRγ$^-$ NK cells in this study starkly contrast the characteristic qualities of the previously described cytokine-induced memory-like NK cells, which are marked by increased levels of NKG2A and NKp46, decreased expression of CD57, and a weak association with NKG2C (242). Therefore, the FcRγ$^-$ memory-like NK cells that expand during chronic HIV-1 infection differ phenotypically at baseline from cytokine-induced memory-like NK cells.

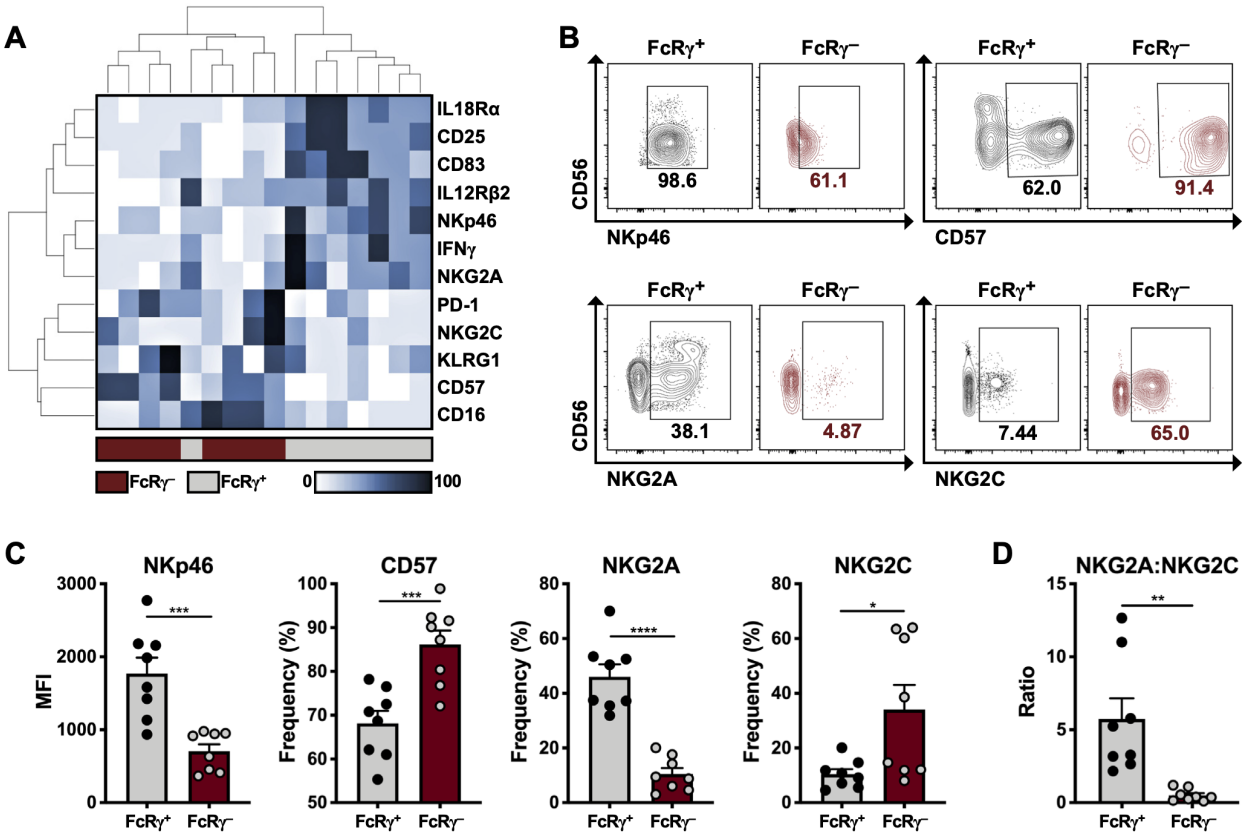


Figure 8. A differential expression signature distinguishes FcRγ⁻ NK cells from conventional NK cells

(A) An evaluation of the normalized MFIs of phenotypic and functional markers analyzed by flow cytometry emphasized the differential expression patterns of FcRγ⁻ and conventional NK cells. **(B)** Representative staining at baseline for the indicated surface markers on NK cells gated based on the expression of FcRγ. **(C)** Compared with FcRγ⁺ NK cells from the same donor, FcRγ⁻ NK cells were characterized by decreased levels of NKp46 and NKG2A but elevated expression of CD57 and NKG2C, **(D)** resulting in an inverse of the NKG2A to NKG2C ratio in the FcRγ⁻ population; $n = 8$ HIV-1⁺ participants with an FcRγ⁻ NK cell frequency >10%. Statistical significance was determined using the paired Student's t-test (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

4.5 IL-18 triggers CD16 downregulation exclusively in FcRγ^{+} NK cells

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ^{-} NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

As IL-18 plays a prominent role in programming the differentiation of CD56^{dim} NK helper cells, we were next interested in exploring the responsiveness of FcRγ^{-} NK cells to this DC-derived cytokine. NK cells were, once again, enriched from PBLs of the selected HIV-1^{+} participants having an FcRγ^{-} NK cell frequency greater than 10%, followed by a 24h culture in media alone, in the presence of IL-18, or with a combination of IL-18 and IL-12. We then assessed the phenotypic and functional impact of these cytokine treatments on the FcRγ^{-} and FcRγ^{+} subsets via flow cytometry. IL-18 alone remarkably influences NK cell differentiation (153), which was highlighted by the shift in overall protein expression patterns seen when performing t-SNE analysis of purified NK cells following a 24h exposure to the cytokine (Fig. 9A). This effect was particularly apparent within the major CD56^{dim} population of NK cells, which exclusively expressed high levels of CD16 at baseline (13, 14, 22) (Fig. 9B). The differentiation of CD56^{dim} NK cells into a helper phenotype is characterized by downregulation of CD16 expression in response to IL-18, which can be enhanced with exposure to secondary or co-activating signals (153). Indeed, short-term exposure of NK cells isolated from HIV-1-infected participants to IL-18 resulted in a decrease in CD16 expression, with the combination of IL-18 and IL-12 causing a dramatic loss of CD16 in the CD56^{dim} population (Fig. 9B). Conversely, exposure to IL-12 alone elicited enhanced surface levels of both CD56 and CD16 among CD56^{dim} NK cells (Fig. 9B).

Interestingly, the majority of NK cells downregulating CD16 in response to cytokine co-stimulation were positive for FcRγ expression, but FcRγ$^-$ NK cells predominantly resided within a distinct population that retained CD16 expression (Fig. 9C). While cytokine co-stimulated FcRγ$^-$ NK cells showed only a slight reduction in CD16 expression intensity relative to unstimulated controls, conventional NK cells exhibited a pronounced decrease in cell-surface expression of CD16 (Fig. 9D, 9E), indicating that FcRγ$^-$ NK cells are minimally responsive to IL-18.

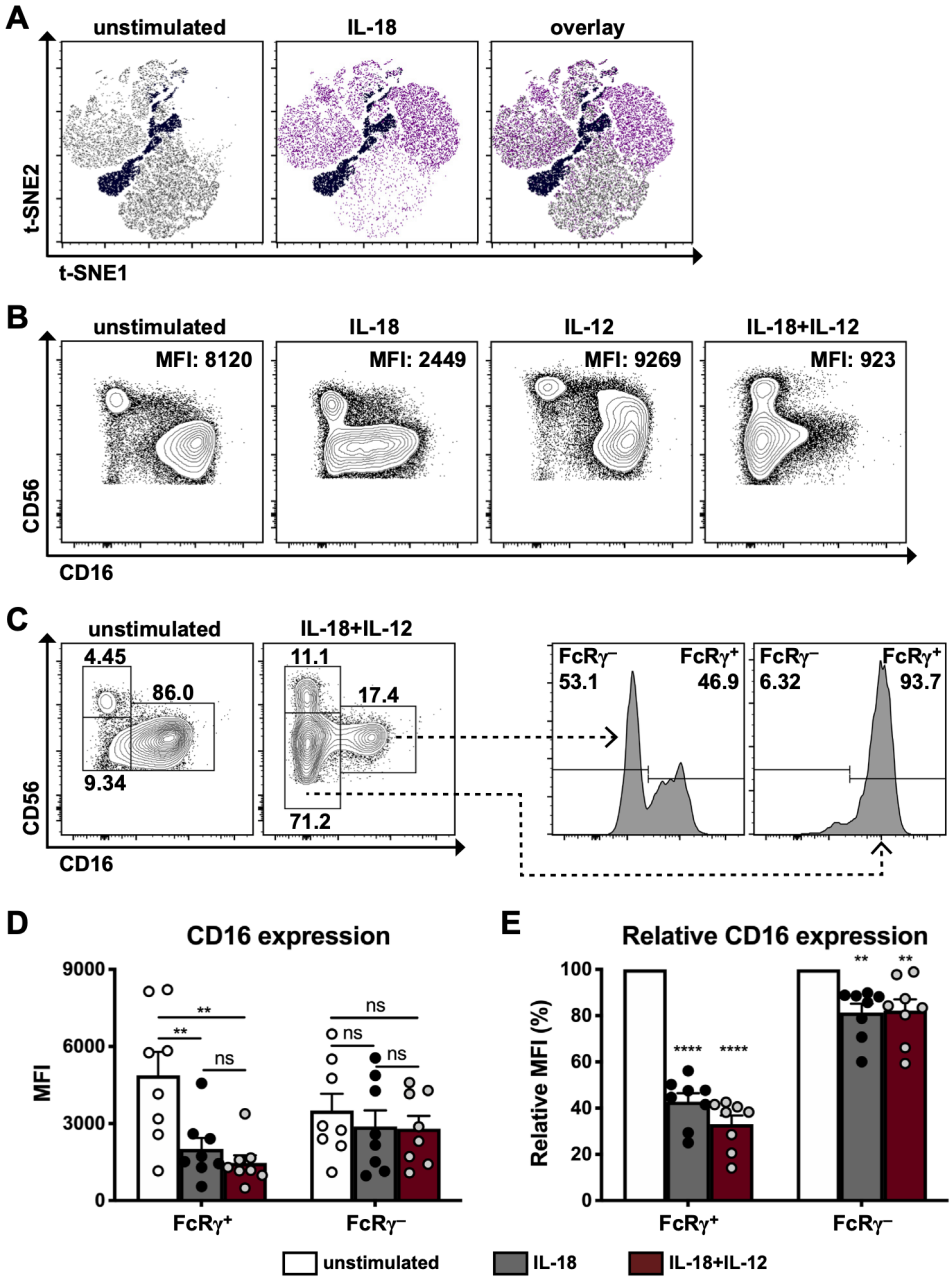


Figure 9. FcRγ⁻ NK cells resist IL-18-induced downregulation of CD16 expression

(A) Representative t-SNE plots of total NK cells cultured for 24h under the indicated conditions, highlighting the shift in protein expression patterns following treatment with IL-18. However, the FcRγ⁻ population, represented by the dark band, clustered independently and remained relatively constant post-stimulation with IL-18; n = 4 HIV-1$^+$ participants. **(B)** Representative staining for CD16 on gated total NK cells cultured for 24h under the indicated conditions, depicting IL-18-mediated downregulation of CD16. The synergy between IL-18 and IL-12 exaggerated this effect. **(C)** An analysis of the CD16$^+$ and CD16$^-$ fractions of the CD56^{dim} population indicated the majority of FcRγ⁻ NK cells failed to downregulate CD16 in response to co-stimulation with IL-18+IL-12. **(D, E)** Unlike the FcRγ$^+$ subset, cytokine co-stimulated FcRγ⁻ NK cells weakly diminished CD16 expression relative to unstimulated controls. The bar graphs depict raw MFI values (D) and relative CD16 expression, in which the CD16 MFI of cytokine-treated cells was divided by the CD16 MFI of unstimulated cells (E); n = 8 HIV-1$^+$ participants with an FcRγ⁻ NK cell frequency >10%. Statistical significance was determined by a one-way ANOVA test (****$p < 0.0001$; **$p < 0.01$).

4.6 IL-18 does not fuel the development of FcRγ⁻ NK helper cells

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ⁻ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

IL-18 primes NK cells for their helper function by causing the upregulation of various receptors. As a result, these NK cells become poised to receive and respond to a variety of potential secondary activation signals, such as IL-2, IFNα, or IL-12 (153, 479). IL-18 induced the expression of CD25 and the transient expression of CD83, both of which were further upregulated following secondary exposure to or co-stimulation with IL-12 (153, 249) (Fig. 10A). Although IL-12 synergized with IL-18 to intensify the upregulation of CD25 and CD83, IL-12 in the absence of IL-18 was powerless to incite strong expression of either CD25 or CD83, accentuating the primary role of IL-18 in mediating this effect (Fig. 10A). Importantly, the induction of both CD25 and CD83 predominated in the NK cells expressing FcRγ (Fig. 10B). Examining the $FcR\gamma^+$ and $FcR\gamma^-$ subsets separately, likewise, revealed that $FcR\gamma^-$ NK cells were grossly inferior at upregulating CD25 and CD83 in response to IL-18 (Fig. 10C, 10D). Therefore, the ability of IL-18 to drive the differentiation of $CD16^-/CD25^+/CD83^+$ NK helper cells in the $FcR\gamma^-$ population is impaired in comparison to conventional NK cells.

NK helper cells largely promote type-1 immune responses by secreting IFNγ in response to combinations of innate stimulatory factors, including IL-18, IFNα, and IL-12 (151, 153, 156) (Fig. 5D). However, $FcR\gamma^-$ NK cells were defective at producing IFNγ in response to cytokine co-stimulation, further illustrating their lack of receptivity to IL-18 (Fig. 10B-10D). Failure to produce

IFNγ upon co-activation with IL-18 and IL-12 serves as confirmation of FcRγ$^-$ NK cells not sharing the same differentiation pathway as NK helper cells. Of note, NK cells from individuals with a low FcRγ$^-$ NK cell frequency were more dynamic producers of IFNγ compared to those with an FcRγ$^-$ NK cell frequency greater than 10% (Fig. 10E), and the proportion of FcRγ$^-$ NK cells inversely correlated with the frequency of NK cells positive for IFNγ by intracellular staining (Fig. 10F). Multiple linear regression analysis of the HIV-1$^-$ and HIV-1$^+$ individuals revealed a significant relationship between the frequency of IFNγ-expressing NK cells and the proportion of FcRγ$^-$ NK cells ($|t| = 5.975$; $p < 0.0001$) but not HIV-1 status ($|t| = 0.7102$; $p = 0.4824$). Moreover, total NK cells from HIV-1$^+$ MSM with an FcRγ$^-$ NK cell frequency greater than 10% secreted lower quantities of IFNγ (Fig. 10G). The impaired release of IFNγ by cytokine-stimulated NK cells from those with an FcRγ$^-$ NK cell frequency greater than 10% translated into less effective polarization of responder DCs as determined by their IL-12-producing capacity (Fig. 10H), although NK cells from both groups promoted relative increases in DC production of IL-12 compared to the mock-treated DCs (11-fold vs 7-fold increase for FcRγ$^-$ low and FcRγ$^-$ high participants, respectively). These data support the notion that an expanded population of FcRγ$^-$ NK cells in chronic HIV-1 infection negatively impacts the quality of NK-DC crosstalk.

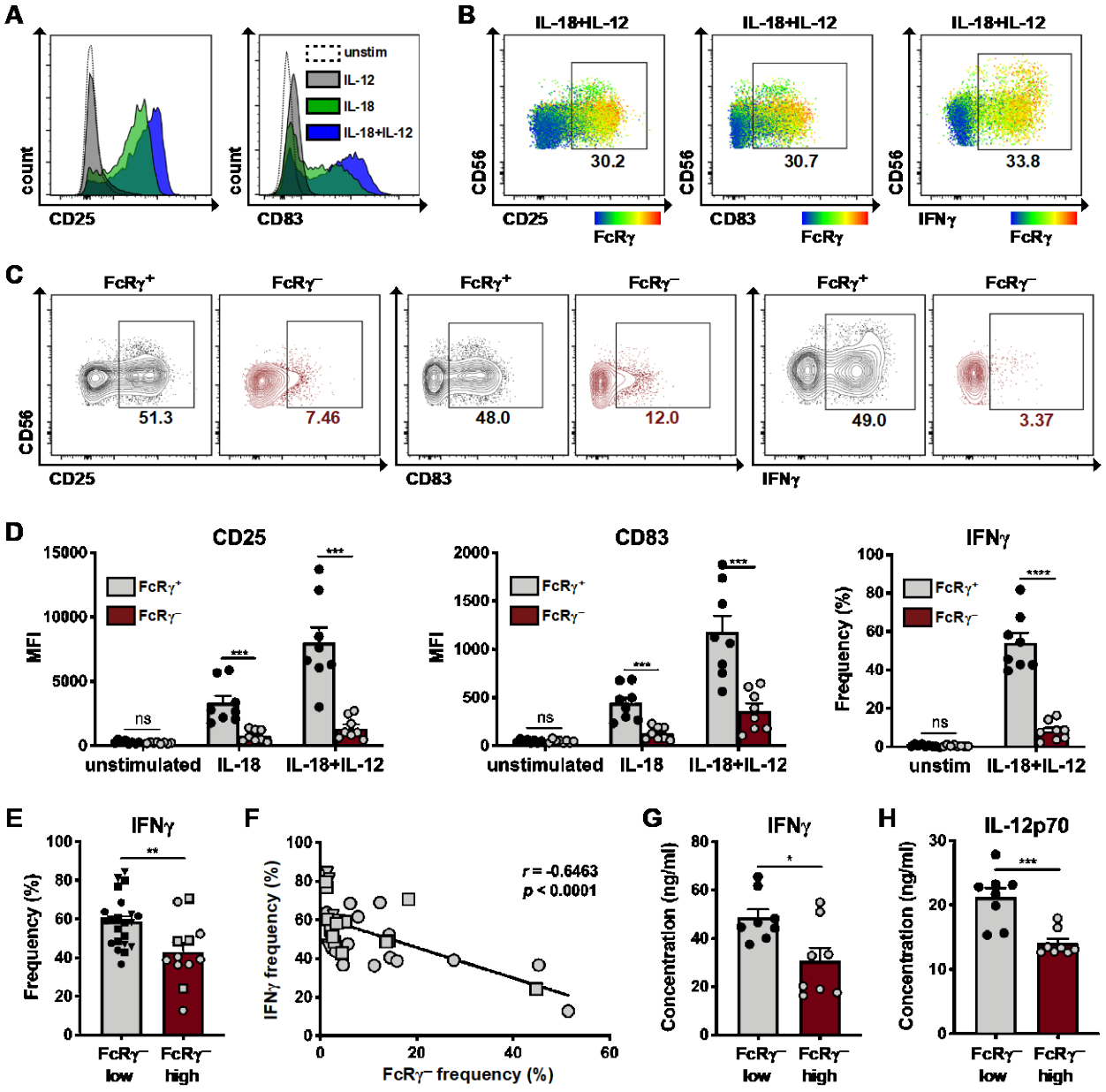


Figure 10. IL-18 does not drive the differentiation of NK helper cells in the FcRγ⁻ population

(A) Representative histogram plots of total NK cells following a 24h culture, demonstrating the upregulation of CD25 and CD83 initiated by IL-18 and augmented by co-stimulation with IL-18+IL-12. **(B)** Representative staining for CD25, CD83, and IFNγ on gated co-stimulated total NK cells, with the heatmap overlay portraying relative FcRγ expression, **(C)** and on NK cells stratified based on the expression of FcRγ. **(D)** IL-18 drove the differentiation of $CD25^{+}/CD83^{+}$ NK helper cells. By contrast, $FcR\gamma^{-}$ NK cells faltered in their responsiveness to IL-18, illustrated by limited expression of CD25 and CD83. In comparison to their $FcR\gamma^{+}$ counterparts, $FcR\gamma^{-}$ NK cells showed an impaired ability to produce IFNγ; $n = 8$ $HIV\text{-}1^{+}$ participants with an $FcR\gamma^{-}$ NK cell frequency >10%. **(E)** Total NK cells from individuals with an $FcR\gamma^{-}$ NK cell frequency >10% (high; $n = 4$ $HIV\text{-}1^{-}$ MSM (squares), $n = 8$ $HIV\text{-}1^{+}$ MSM (circles)) produced lower levels of IFNγ following a 48h exposure to IL-18+IL-12 relative to those with an $FcR\gamma^{-}$ NK cell frequency <10% (low; $n = 6$ $HIV\text{-}1^{-}$ community controls (triangles), $n = 10$ $HIV\text{-}1^{-}$ MSM (squares), $n = 6$ $HIV\text{-}1^{+}$ MSM (circles)). **(F)** The proportion of $FcR\gamma^{-}$ NK cells inversely correlated with the frequency of NK cells positive for IFNγ by intracellular staining; $n = 6$ $HIV\text{-}1^{-}$ community controls (triangles), $n = 14$ $HIV\text{-}1^{-}$ MSM (squares),

n = 14 HIV-1$^+$ MSM (circles). **(G)** Supernatants from NK cell cultures activated with IL-18 and IL-2 were collected after 48h and quantified for IFNγ by ELISA, with inferior production noted in HIV-1$^+$ participants with an FcRγ$^-$ NK cell frequency >10%. **(H)** Responder DCs from a single HIV-1$^+$ participant became differentially polarized, producing less IL-12 when exposed to cytokine-stimulated NK cell culture supernatants from HIV-1$^+$ MSM with an FcRγ$^-$ NK cell frequency >10%; *n* = 2 replicates from 4 HIV-1$^+$ participants with an FcRγ$^-$ NK cell frequency >10% (high) and from 4 HIV-1$^+$ participants with an FcRγ$^-$ NK cell frequency <10% (low) (G, H). Statistical significance was determined using the paired Student's t-test (D), unpaired Student's t-test (E, G, H), or Pearson correlation (F) (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

4.7 A diminished capacity to express IL18Rα renders FcRγ$^-$ NK cells unresponsive to IL-18

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ$^-$ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

As mentioned previously, IL-18 should increase the sensitivity of NK cells to secondary stimuli, including IL-12, by causing an upregulation of cytokine receptors. This is essential for their subsequent production of IFNγ (153). Based on this, we assessed the expression of IL12Rβ2 and found its induction to be weakened in the FcRγ$^-$ NK cell subset (Fig. 11A-11C). Since IL18R signaling plays a critical role for increased IFNγ production by NK cells (153, 479), we investigated whether the lack of responsiveness of FcRγ$^-$ NK cells expanded in HIV-1-infected individuals to IL-18 was due to a defect in the signaling pathway or a lack of expression of the IL18R itself. While conventional NK cells strongly upregulated IL18Rα in response to co-stimulation with IL-12, FcRγ$^-$ NK cells were characterized by an attenuated capacity to upregulate this receptor (Fig. 11D-11F). Even among conventional NK cells, IL-18 minimally impacted the expression of IL18Rα compared to unstimulated controls (Fig. 11F). In contrast, the enhanced levels imparted by IL-12 alone were comparable to those induced by the combination of IL-18 and

IL-12 (480) (Fig. 11G). Additionally, primary exposure of isolated NK cells for 48h to IL-12 triggered robust production of IFNγ upon secondary exposure to IL-18, but not IL-2, highlighting the importance of IL-12 in upregulating IL18Rα for heightened IFNγ responses mediated via IL18R signaling (Fig. 11H, 11I). As co-expression of the alpha and beta subunits of the IL18R is required for IL-18 responsiveness (481, 482), the anergy portrayed by FcRγ^{-} NK cells to IL-18 is likely explained by a diminished capacity to express the alpha subunit.

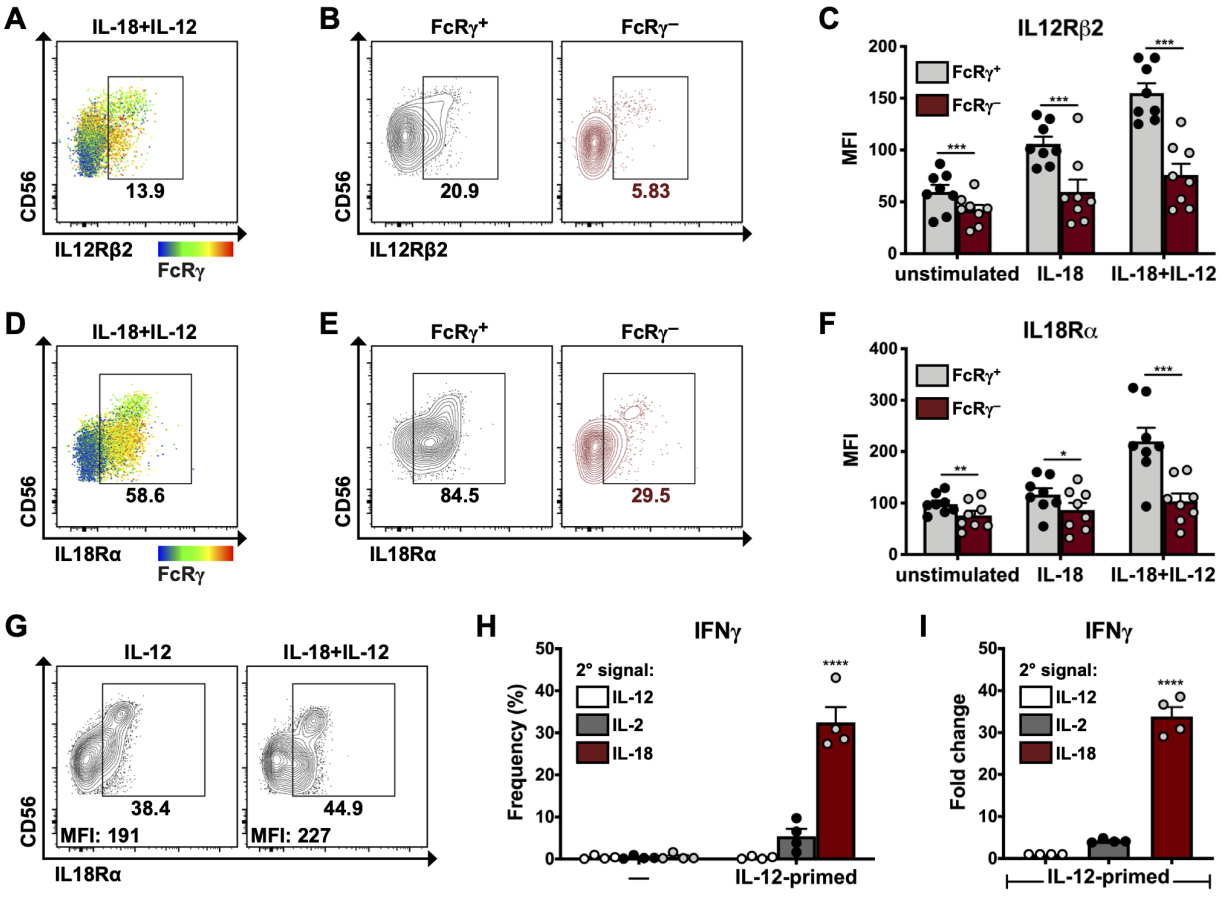


Figure 11. Aberrant inducible expression of IL18Rα limits the responses of FcRγ⁻ NK cells to innate stimuli

(A, D) Representative staining for the indicated cytokine receptors on gated co-stimulated total NK cells, with the heatmap overlay portraying relative FcRγ expression, **(B, E)** and on NK cells stratified based on the expression of FcRγ. **(C, F)** FcRγ-deficiency was characterized by an attenuated capacity to express IL12Rβ2 and IL18Rα upon IL-18+IL-12 co-activation; n = 8 HIV-1$^+$ participants with an FcRγ$^-$ NK cell frequency >10%. **(G)** Representative staining for IL18Rα on gated total NK cells. **(H, I)** Isolated NK cells were cultured in the presence or absence of IL-12 for 48h (primary signal), followed by extensive washing and a 24h culture in the presence of a secondary signal. Pre-exposure of NK cells to IL-12 promoted strong IFNγ responses in total NK cells upon secondary exposure to IL-18 as determined by intracellular staining (H; n = 4 HIV-1$^+$ participants) and ELISA (I; n = 2 replicates from 2 HIV-1$^+$ participants), where the bar graph represents fold increase in IFNγ relative to IL-12-stimulated controls. Statistical significance was determined using the paired Student's t-test (C, F), two-way ANOVA (H), or one-way ANOVA (I) (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

4.8 Time on ART is associated with increasingly differentiated NK cells

Based on the phenotypic and functional abnormalities observed in our cross-sectional study of chronic HIV-1 infection (Fig. 7-11), we expanded this work to a longitudinal setting in collaboration with the ACTG, detailing the impact of chronic HIV-1 infection and long-term ART on the phenotype and functional status of NK cell subsets. The NWCS 486 PBMC samples were selected from HIV-1^+ participants of the ACTG cohort A5321 at three timepoints: 1. ~4 weeks on-ART (TP0, n = 20); 2. 1 year on-ART (TP1, n = 60); 3. 4 years on-ART (TP2, n = 60) (Table 5). We began by drawing comparisons between total NK cells derived from age-matched HIV-1-infected and uninfected individuals. Beyond CD57 and KLRG1 (Fig. 12A, 12B), both of which signal a highly differentiated state (42, 235, 483, 484), HIV-1 infection was associated with a higher frequency of NK cells expressing PD-1 and NKG2C (Fig. 12C, 12D). As previously reported (191), following suppression of HIV-1 viremia, NKG2A was restored on the surfaced of NK cells, resulting in comparable proportions of NKG2A$^+$ NK cells in HIV-1^- and HIV-1^+ participants (Fig. 12E). Therefore, the modest reduction in the NKG2A to NKG2C ratio observed in chronic HIV-1 infection was a consequence of elevations in NKG2C expression (Fig. 12F). In concordance with total NK cells from HIV-1^+ participants moving toward a more mature phenotype, they also lost expression of the NCR NKp46 (Fig. 12G). Finally, NK cells from age-matched HIV-1-infected and uninfected individuals demonstrated comparable downmodulation of surface expression of KLRG1 after a 24h culture in the presence of IL-18 and IL-12 (Fig. 12H).

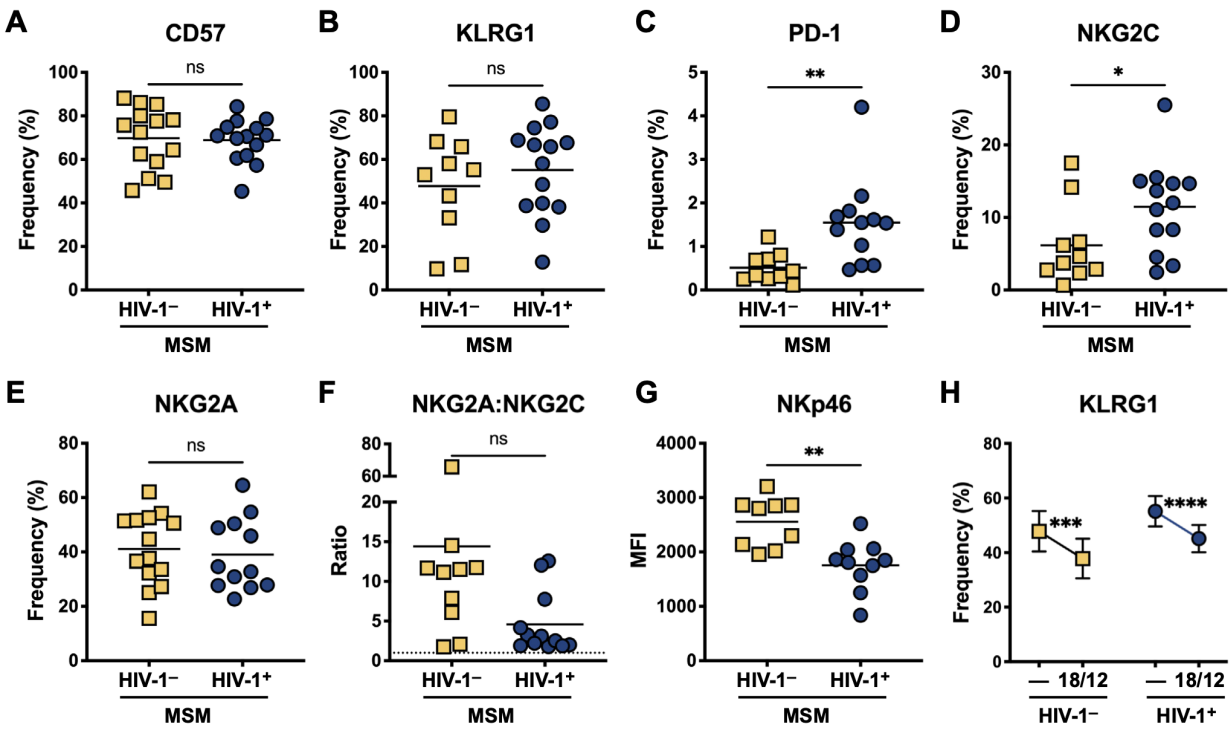


Figure 12. HIV-1 infection skews toward a differentiated NK cell phenotype

(A-G) Flow cytometry analyses of enriched NK cells at baseline from age-matched HIV-1$^-$ and HIV-1$^+$ MACS participants, highlighting the phenotypic and functional impact of HIV-1 infection on total NK cells. In particular, NK cells from HIV-1-infected individuals were more likely to express PD-1 (C) and NKG2C (D), yet they demonstrated decreased levels of NKp46 (G), together characteristic of an increasingly differentiated population of NK cells. **(H)** Comparisons of KLRG1 surface expression on NK cells, at baseline (—) and following a 24h exposure to IL-18 and IL-12 (18/12), showed that NK cells from both HIV-1$^-$ and HIV-1$^+$ individuals downmodulated expression of KLRG1 in response to cytokine activation Statistical significance was determined by the unpaired (A-G) or paired (H) Student's t-test (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

An evaluation of the phenotypic and functional changes occurring in total NK cells over the course of treatment with ART revealed that NK cells gained expression of CD57 between TP1 and TP2 (Fig. 13A); levels of KLRG1, associated with senescence in T cells (485-487), also increased with each subsequent timepoint (Fig. 13B). With the acquisition of these markers was a reduction in the expression of NKG2A (Fig. 13C), an inhibitory receptor of immature NK cells (18), and NKp46 (Fig. 13D), indicative of an increasingly differentiated phenotype. Furthermore, increasing time on ART did not alter IFNγ responses, as the expression of IFNγ by total NK cells following co-stimulation with IL-18 and IL-12 was similar across all three timepoints (Fig. 13E).

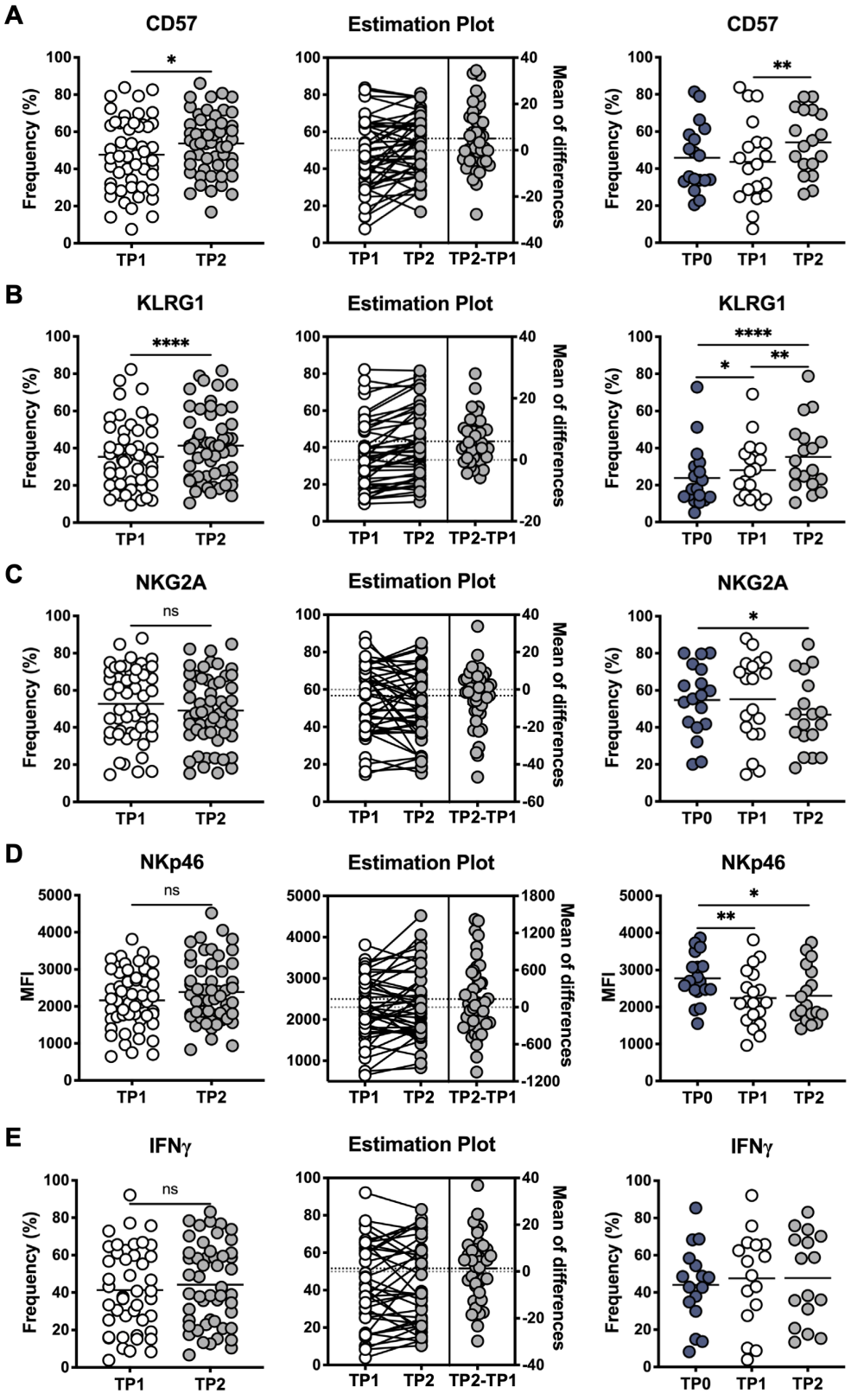


Figure 13. NK cells become more differentiated over the course of treatment with ART

Evaluation by flow cytometry of PBMCs at baseline from HIV-1$^+$ NWCS 486 study participants at ~4 weeks on-ART (TP0, $n = 20$), 1 year on-ART (TP1, $n = 60$), and 4 years on-ART (TP2, $n = 60$). Shown is a comparison between TP1 and TP2 for all participants (left), the mean of differences between TP2 and TP1, with a value greater than 0 indicating an increase in expression from TP1 to TP2 (middle), and the subanalysis performed on those with PBMCs available from all three timepoints ($n = 20$) (right). NK cells were selected from live cells based on a CD3$^-$CD56$^+$ gate. **(A, B)** NK cells gained expression of CD57 and KLRG1, both of which are markers of terminal differentiation, with the highest levels observed at TP2. **(C, D)** Concomitant with the acquisition of this differentiated phenotype was a reduction in the expression of NKG2A and NKp46. **(E)** Total NK cells responded to IL-18+IL-12 by producing IFNγ. However, they did not gain responsiveness over time. Statistical significance was determined by the paired Student's t-test (left) or mixed-effects analysis, with correction for multiple comparisons by Tukey's HSD post-hoc test (right) (****$p < 0.0001$; **$p < 0.01$; *$p < 0.05$).

4.9 Persistence of a skewed NK cell repertoire in treated HIV-1 infection

To determine if the differential expression signatures between FcRγ$^-$ and FcRγ$^+$ NK cells (noted in Fig. 8) remained consistent in a longitudinal setting of treated HIV-1 infection, we performed a detailed characterization of the two subsets in HIV-1$^+$ NWCS 486 study participants with an FcRγ$^-$ NK cell frequency greater than 10%. First and foremost, long-term ART did not affect the frequency of FcRγ$^-$ NK cells (Fig. 14A). Confirming the cross-sectional study data (Fig. 8), the FcRγ$^-$ population was enriched for cells with higher expression of CD57 (not shown), NKG2C (Fig. 14B), and PD-1 (Fig. 14C), but lower levels of NKG2A (Fig. 14D) and NKp46 (not shown). On the other hand, the frequency of KLRG1-expressing NK cells was comparable between the two subsets (Fig. 14E). Notably, the phenotypic differences between FcRγ$^-$ and FcRγ$^+$ NK cells did not diminish with increasing time on ART (Fig. 14F-14I). For example, both populations gained expression of KLRG1 (Fig. 14I). This was accompanied by decreased PD-1 expression (Fig. 14G) and slightly lower levels of NKG2A at TP2, although not statistically significant (Fig. 14H). Therefore, long-term ART does not promote a decrease in the FcRγ$^-$ population nor alterations in their phenotypic profile relative to conventional NK cells.

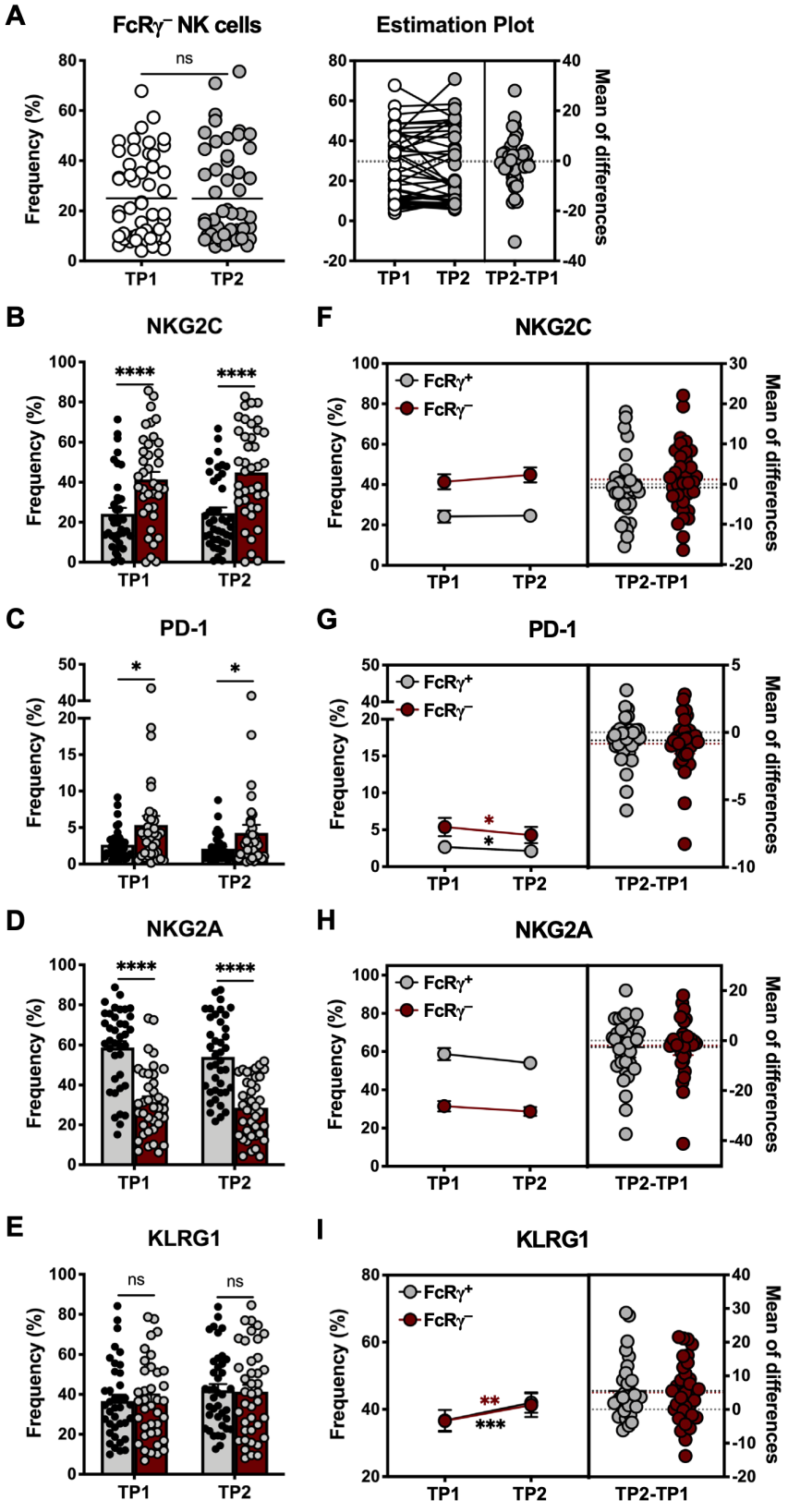


Figure 14. The phenotypic differences between FcRγ⁻ and FcRγ⁺ do not diminish in response to ART

(A) The FcRγ$^-$ and FcRγ$^+$ subsets were analyzed by flow cytometry in HIV-1$^+$ NWCS 486 study participants with an FcRγ$^-$ NK cell frequency >10%, a population that remained stable over the course of treatment with ART (n = 44). **(B-E)** Direct comparisons were drawn between FcRγ$^-$ and FcRγ$^+$ NK cells within each timepoint. The FcRγ$^-$ population was enriched for cells with higher levels of NKG2C (B) and PD-1 (C) but lower levels of NKG2A (D). FcRγ$^-$ and FcRγ$^+$ NK cells expressed comparable levels of KLRG1 (E). **(F-I)** Comparisons were drawn between TP1 and TP2 within each subset. A mean of differences value greater than 0 indicated an increase in expression from TP1 to TP2. Both populations followed the same trends over time for each of the markers analyzed, e.g., levels of PD-1 decreased (G) and KLRG1 increased (I) from TP1 to TP2 in both FcRγ$^-$ and FcRγ$^+$ NK cells. Statistical significance was determined by the paired Student's t-test (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

As expected, FcRγ$^-$ NK cells also demonstrated decreased responsiveness to IL-18 relative to conventional NK cells (Fig. 15). Although FcRγ$^-$ and FcRγ$^+$ NK cells expressed comparable levels of KLRG1 at both timepoints (Fig. 14E), conventional NK cells downregulated this marker upon stimulation with IL-18 and IL-12 (Fig. 15A, 15B), as was observed with total NK cells (Fig. 12H). By contrast, a key finding was that KLRG1 expression trended toward an increase in the FcRγ$^-$ subset at TP1 (Fig. 15A) and TP2 (Fig. 15B). FcRγ$^-$ NK cells also failed to produce IFNγ in response to innate stimuli (Fig. 15C), and the proportion of FcRγ$^-$ NK cells inversely correlated with the frequency of NK cells positive for IFNγ by intracellular staining (Fig. 15D). Similar to what was noted in total NK cells (Fig. 13E), neither subset was marked by an increased frequency of IFNγ-expressing cells at TP2 (Fig. 15E). Additionally, the differentiation of CD56^{dim} NK cells into a helper phenotype was characterized by their dramatic downmodulation of CD16 expression (Fig. 9B-9E); however, at both on-ART timepoints, cytokine co-stimulated FcRγ$^-$ NK cells maintained CD16 expression relative to unstimulated controls, which was in stark contrast to the FcRγ$^+$ subset (Fig. 15F). Together, these data confirm that FcRγ$^-$ NK cells are minimally responsive to IL-18, and this decreased responsiveness persists despite effective treatment with suppressive ART.

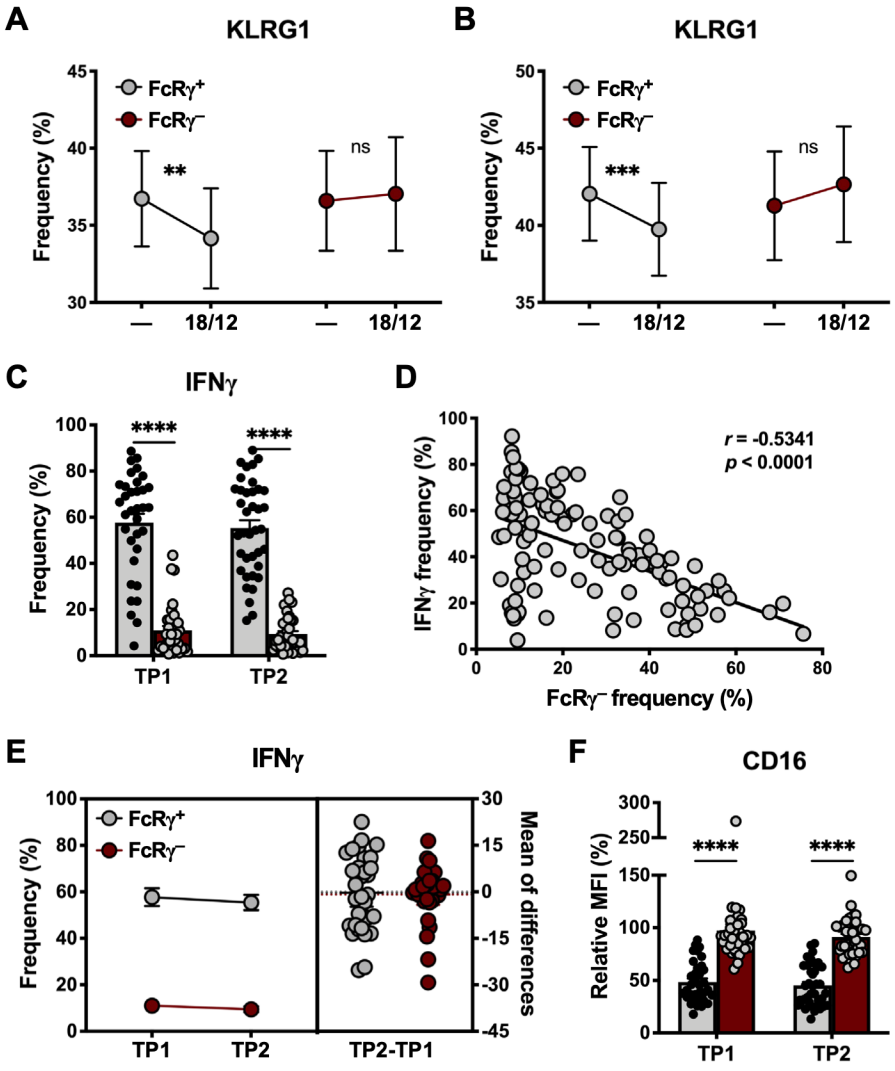


Figure 15. Persistence of reduced IL-18 responsivness in the FcRγ⁻ population

Evaluation by flow cytometry of PBMCs from HIV-1⁺ NWCS 486 study participants with an FcRγ⁻ NK cell frequency >10% ($n = 44$) at TP1 and TP2. Cultures were exposed to media alone or IL-18+IL-12 for 24h. NK cells were selected from live cells based on a $CD3^{-}CD56^{+}$ gate. **(A, B)** Comparisons of KLRG1 surface levels on NK cells, at baseline (—) and following a 24h exposure to IL-18+IL-12 (18/12). Although conventional NK cells downregulated KLRG1 following IL-18+IL-12 stimulation, its expression increased modestly within the FcRγ⁻ subset at TP1 (A) and TP2 (B). **(C)** The lack of responsiveness of the FcRγ⁻ population to innate stimuli was magnified by their dismal production of IFNγ following a 24h culture with IL-18+IL12. **(D)** The proportion of FcRγ⁻ NK cells inversely correlated with the frequency of NK cells positive for IFNγ by intracellular staining. **(E)** Expression of IFNγ was similar between TP1 and TP2 in both FcRγ⁻ and FcRγ⁺ NK cells. **(F)** Unlike the FcRγ⁺ subset, cytokine co-stimulated FcRγ⁻ NK cells maintained CD16 expression relative to unstimulated controls at both timepoints. The bar graph depicts relative CD16 expression, in which the CD16 MFI of cytokine-treated cells was divided by the CD16 MFI of unstimulated cells. Statistical significance was determined using the paired Student's t-test (A-C, E, F) or Pearson correlation (D) (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$).

4.10 Contribution of inflammation and immune dysfunction to FcRγ⁻ NK cell expansion

Although HCMV infection has been implicated in the induction of FcRγ⁻ NK cells, the mechanisms contributing to their inflated expansions in HIV-1 infection are unclear. We hypothesized that more frequent HCMV reactivations in HIV-1$^+$ individuals would promote an increase in HCMV antibody titers, in turn selectively driving expansions of FcRγ⁻ NK cells, and together supporting a sustained inflammatory state characterized by an increasing level of immune dysfunction. Indeed, active HCMV viremia was detected in 38% and 32% of NWCS 486 HIV-1$^+$ participants at years 1 and 4 of ART (Table 8), and elevated levels of markers related to innate immune activation (Fig. 16A, 16B) and inflammation (Fig. 16C, 16D) persisted despite consistent ART-mediated viral suppression. The importance of HCMV in the context of chronic HIV-1 infection was further underscored by the magnitude of HCMV-specific immunity, with HCMV antibody titers increasing between TP1 and TP2 (Fig. 16E) and T cell responses targeting one HCMV protein exceeding total T cell responses to the entire HIV-1 proteome within this cohort of HIV-1$^+$ individuals (473).

Table 8. HCMV and EBV DNA viremia

		Year 1 of ART	Year 4 of ART
HCMV DNA (copies/ml)	Detectable	38% (23)	32% (19)
If HCMV DNA detectable:	Median (Q1, Q3)	14.9 (3.5, 24.6)	15.3 (7.3, 26.5)
EBV DNA (copies/ml)	Detectable	7% (4)	5% (3)
If EBV DNA detectable:	Median (Q1, Q3)	283.1 (179.6, 385.1)	366.3 (148.8, 27,896.2)

We next tested for relationships between proportions of FcRγ⁻ NK cells and antigen-specific adaptive immune responses. Modest positive correlations were observed (albeit not

significant) with HCMV-specific IgG antibody titers (Fig. 16F) and T cell responses (Fig. 16G), whereas no relationship was identifiable between FcRγ$^-$ NK cell frequency and responses to EBV (Fig. 16H), another common herpesvirus. The lack of any significant direct correlations between frequencies of FcRγ$^-$ NK cells and HCMV-specific adaptive immune response, despite their common trigger, likely reflects complex and competing influences from a multitude of factors, with cumulative effects throughout chronic HCMV/HIV-1 co-infection obscuring relevant impacts of direct interactions. Our data do suggest, though, that immunosenescence and inflammation contribute, at least in part, to inflated proportions of FcRγ$^-$ NK cells in HIV-1$^+$ individuals. For one, long-term ART fails to reverse the inflammation induced by HIV-1 infection. This is supported by previous studies, including one by Hearps et al., in which levels of sCD163 and CXCL10 are elevated in viremic HIV-1 infection and remain elevated in virologically suppressed HIV-1$^+$ individuals relative to age-matched HIV-1$^-$ controls, instead resembling those observed in elderly controls, suggesting an acceleration of immune aging (488). Additionally, the magnitude of HCMV-specific immunity, combined with the rate of active HCMV viremia, points to an inability of the immune response to keep pace with HCMV reactivations, further feeding into the cyclic loop of immune activation, senescence, and inflammation.

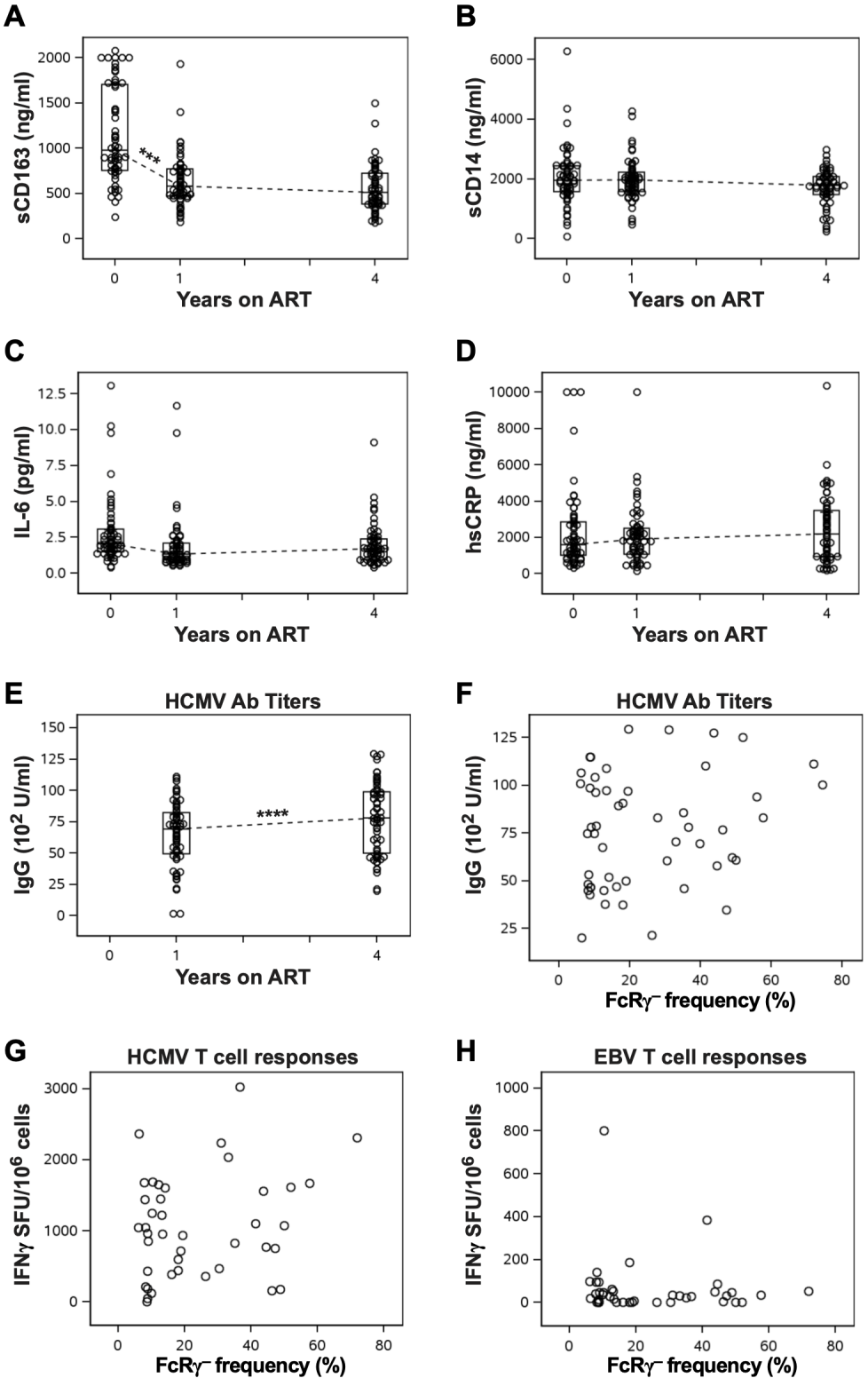


Figure 16. Sustained elevation of soluble mediators and inflammatory markers

(A-D) Plasma concentrations of sCD163, sCD14, IL-6, and hsCRP were quantified by ELISA. Following initiation of ART, levels of sCD163 dropped significantly during the first year, then stabilized (A). By contrast, sCD14 (B), IL-6 (C), and hsCRP (D) did not decline in response to ART. **(E)** HIV-1^+ NWCS 486 study participants experienced an increase in HCMV IgG antibody titers, measured by ELISA, between year 1 (TP1) and year 4 (TP2) of ART. **(F-H)** Associations were drawn between frequencies of Fc$R\gamma^-$ NK cells and HCMV IgG antibody titers (F) and antigen-specific T cell responses, as determined by ELISPOT, to HCMV (G) and EBV (H) at year 4 of ART (TP2); n = 60 HIV-1^+ participants at each timepoint. Statistical significance was determined by Wilcoxon matched-pairs signed rank test (A-E) or rank-based Spearman correlations (F-H) (****$p < 0.0001$; ***$p < 0.001$).

4.11 Abnormal NK cell subset distribution due to interrupted NK-poiesis

As described previously, NK cells are classically divided into two major populations, $CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$, with the former typically accounting for less than 10% of NK cells in the periphery (Fig. 17A (HIV-1^-), 17B). Interestingly, we noted extremely pronounced $CD56^{bright}$ populations in HIV-1^+ participants, comprising upwards of 40 to 50% of total circulating NK cells. This abnormal accumulation of the $CD56^{bright}$ subset was particularly apparent at TP1. Although the relative proportions of $CD56^{bright}$ NK cells began to normalize by TP2, they continued to account for a considerable fraction of NK cells in the peripheral blood relative to uninfected individuals (Fig. 17A). Furthermore, we observed unusually large $CD56^{bright}CD16^+$ and $CD56^{dim}CD16^-$ subsets in 24 of the 60 HIV-1^+ participants (Fig. 17C-17E). Compared with uninfected individuals, the relative proportion of the $CD56^{dim}CD16^+$ NK cell subset was significantly lower in HIV-1^+ participants as a result of elevated frequencies of the $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^-$ populations (Fig. 17D), and the most compelling perturbations in NK cell subset distribution were, again, observed at TP1. By TP2, the relative frequency of the $CD56^{dim}CD16^+$ population experienced a resurgence, which was accompanied by a decline in the relative proportions of the remaining three subsets (Fig. 17E), suggesting a positive effect of ART on the normalization of NK cell subset distribution. These observations led us to hypothesize that the abnormal NK cell subset distribution observed in HIV-1 may arise from

sustained inflammation and that direct effects exerted by the virus during initial infection contribute to interrupted NK-poiesis, arresting NK cells in transitional states and resulting in the accumulation of unconventional NK cell subsets.

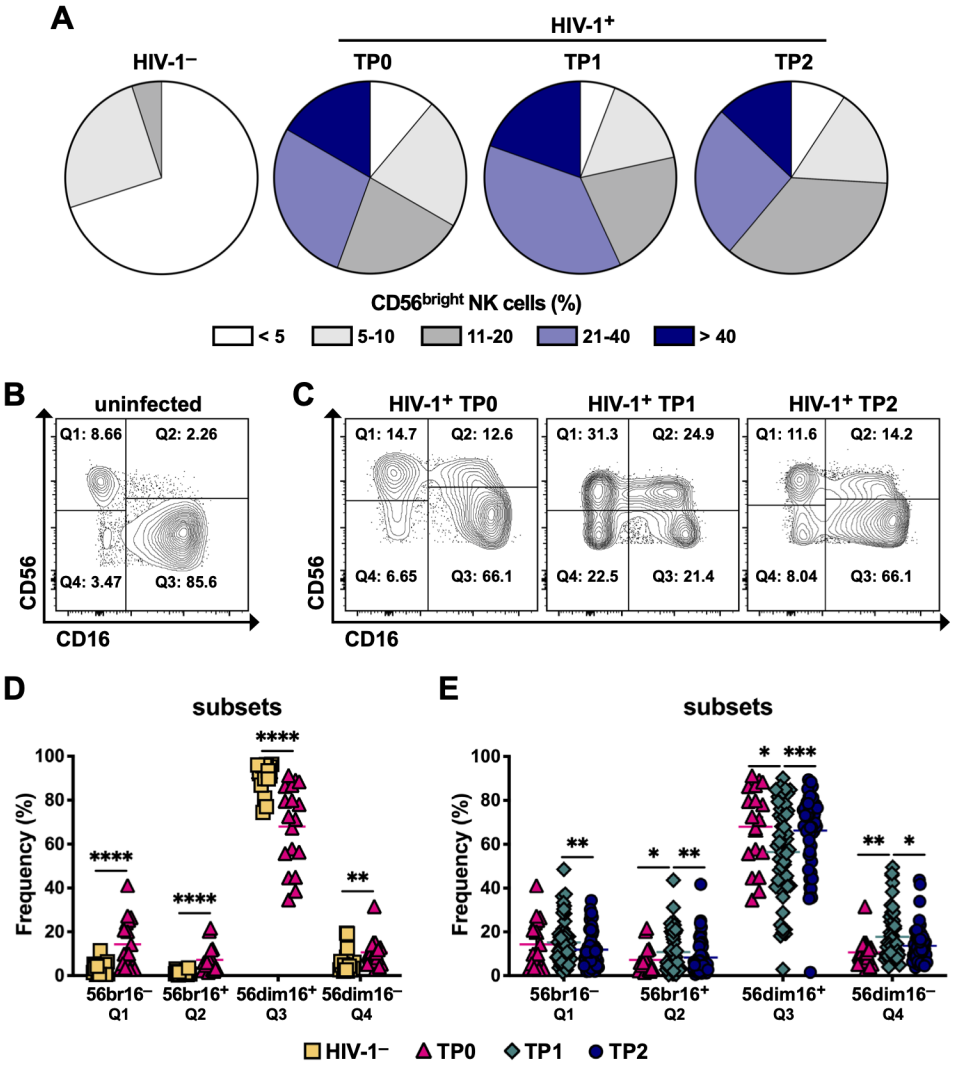


Figure 17. An abnormal NK cell subset distribution in HIV-1 infection

(A) The CD56^{bright} population comprised upwards of 50% of total circulating NK cells, whereas they normally account for <10% of NK cells in the periphery, as exemplified by the HIV-1^{-} pie graph on the left. **(B, C)** Representative FACS plots, illustrating the expected distribution at baseline of CD56^{dim} and CD56^{bright} NK cells (B) and unusually large CD56^{bright}CD16^{+} and CD56^{dim}CD16^{-} populations at the three on-ART timepoints (C). **(D, E)** Relative to uninfected individuals, HIV-1^{+} NWCS 486 study participants experienced an aberrant NK cell subset distribution, which was particularly pronounced at TP1 and began to normalize by TP2; $n = 20$ HIV-1^{-} individuals, $n = 20$ HIV-1^{+} participants at TP0, $n = 60$ HIV-1^{+} participants at TP1 and TP2. Statistical significance was determined by the unpaired Student's t-test (D) or mixed-effects analysis, with correction for multiple comparisons by Tukey's HSD post-hoc test (E) (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

To begin exploring this hypothesis, we analyzed the individual NK cell subsets for markers related to differentiation status. The four distinct subsets appeared to cluster based on surface expression of CD56, with the $CD56^{bright}CD16^{+}$ and the $CD56^{dim}CD16^{-}$ populations being phenotypically more closely related to the classic $CD56^{bright}$ and $CD56^{dim}$ subsets, respectively (Fig. 18). With KLRG1, which is understood to be marker of senescence (485-487), we saw a progressive increase in the frequency of NK cells expressing this marker from the $CD56^{bright}CD16^{-}$ through the $CD56^{dim}CD16^{+}$ subsets (Fig. 18A, 18E). The same pattern was observed for CD57 (Fig. 18B, 18F), a marker of terminal differentiation (42, 232, 235), and NKG2C (Fig. 18C, 18G), an activating receptor enriched on highly differentiated 'memory' NK cells (Fig. 14B). By contrast, the $CD56^{dim}$ subsets had the lowest expression of NKG2A (Fig. 18D, 18H), an inhibitory receptor found on immature NK cells (18). These phenotypic profiles support the notion that NK cells progress through the stages of differentiation in a stepwise fashion and that HIV-1 infection interrupts NK-poiesis, resulting in an abnormal collection of NK cells in an intermediate state. Our findings related to this concept warrant detailed investigations in the future.

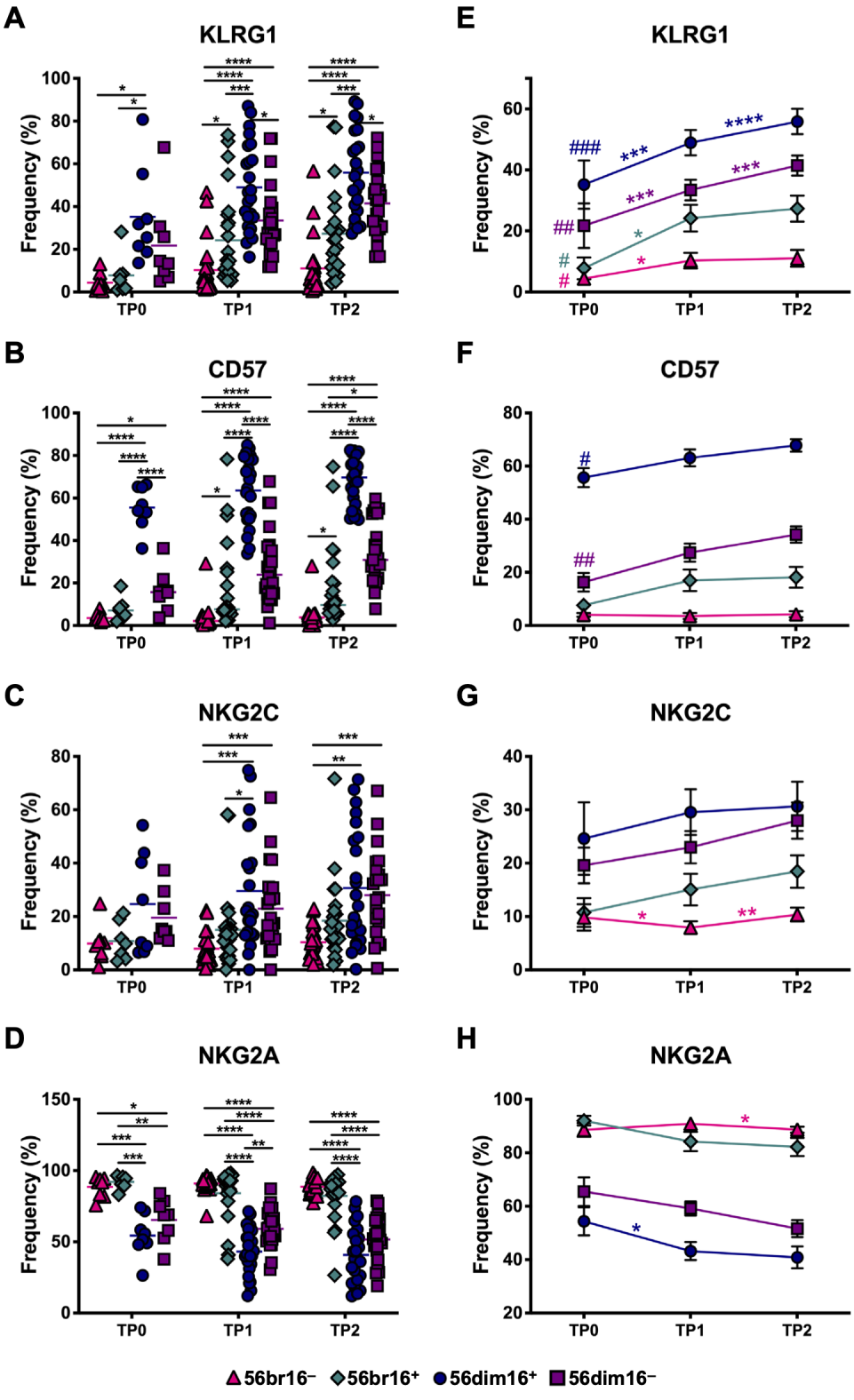


Figure 18. HIV-1 infection interrupts NK-poiesis

(A-H) Evaluation by flow cytometry of PBMCs at baseline from HIV-1^+ NWCS 486 study participants with the noted aberrant subset distributions at ~4 weeks on-ART (TP0, $n = 8$), 1 year on-ART (TP1, $n = 24$), and 4 years on-ART (TP2, $n = 24$). NK cells were selected from live cells based on a $CD3^-CD56^+$ gate. While $CD56^{dim}$ NK cells had the highest levels of KLRG1 (A, E), CD57 (B, F), and NKG2C (C, G), the $CD56^{bright}$ populations had the highest NKG2A expression (D, H), illustrating the differences in their differentiation status. Statistical significance was determined by mixed-effects analysis, with correction for multiple comparisons by Tukey's HSD post-hoc test. Comparisons were made between subsets within each TP (A-D) (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) or between TPs within each subset (E-H) (TP0 vs TP1 and TP1 vs TP2: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; TP0 vs TP2: $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$).

4.12 Functional bias of FcRγ⁻ NK cells toward antibody-dependent reactivity

Adapted from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ⁻ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

Aside from serving as mediators and effectors within the innate arm of immunity, NK cells play a supportive antigen-specific role during the adaptive immune response through engagement of CD16 by the Fc regions of IgG antibodies. These antibody-dependent effector functions include lysis of infected cells by ADCC, which has been implicated in vaccine-induced protective immunity against acquisition of infection, phenotypes of viral control, and slower disease progression (116, 118, 119, 192). Thus, we measured the ability of freshly isolated NK cells from participants with chronic HIV-1 infection to perform ADCC by exposing them to rituximab-opsonized and non-opsonized Raji cells, which are resistant to NK cell–mediated killing via natural cytotoxicity receptors, at varying ratios of effector to target cells. Based on the relative proportions of opsonized to non-opsonized target cells, we calculated the specific elimination of antibody-coated targets, with NK cells from virally suppressed HIV-1^+ MACS participants

effectively killing rituximab-opsonized Raji cells, indicated by an NK cell dose-dependent decrease of opsonized target cells (Fig. 19A).

In addition to evaluating ADCC activity through true lytic responses, we examined CD107a mobilization and intracellular IFNγ by flow cytometry to elucidate the NK cell subpopulations responding to antibody-mediated signaling through CD16. A comparison of $FcR\gamma^{+}$ and $FcR\gamma^{-}$ NK cells revealed that the latter population exhibited a functional bias toward antibody-dependent reactivity, with increased degranulation, as determined by cell-surface CD107a (Fig. 19B), and IFNγ expression in the presence of antibody-coated target cells (Fig. 19C). Interestingly, the $FcR\gamma^{-}$ subset was also marked by a higher level of spontaneous activity against non-opsonized Raji cells, suggestive of a hyper-reactive state (Fig. 19B, 19C). While conventional NK cells from HIV-1^{+} men responded with flexibility to both innate and adaptive signals, $FcR\gamma^{-}$ NK cells were highly restricted to antibody-specific responses (Fig. 19D).

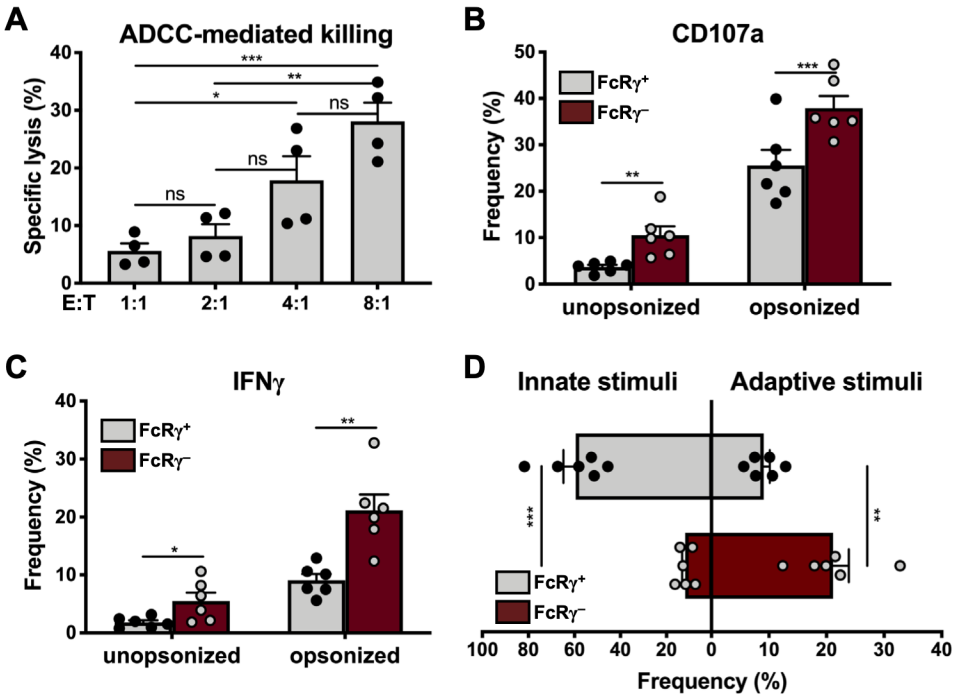


Figure 19. FcRγ⁻ NK cells specialize in antibody-dependent reactivity

(A) NK cells from virally suppressed HIV-1⁺ MACS participants effectively mediated ADCC activity against rituximab-opsonized Raji cells, with the highest level of specific lysis observed at the largest effector to target cell ratio. The percentage of ADCC-mediated killing was calculated based on the relative proportion of viable opsonized targets to unopsonized dye-labeled Raji cells; n = 4 HIV-1⁺ participants. **(B)** FcRγ⁻ NK cells showed greater degranulation in response to both non-opsonized and rituximab-opsonized Raji cells as determined by cell-surface CD107a. **(C)** Compared to their FcRγ⁺ counterparts, FcRγ⁻ NK cells produced more IFNγ in response to CD16-mediated signaling. **(D)** The functional bias of FcRγ⁻ NK cells toward antibody-dependent signaling was exemplified by their differential production of IFNγ following a 6h culture with opsonized Raji cells (adaptive) or a 48h culture in the presence of IL-18+IL-12 (innate); n = 4 HIV-1⁺ participants with an FcRγ⁻ NK cell frequency >10% from 6 independent experiments (B-D). Statistical significance was determined by one-way ANOVA (A) or the paired Student's t-test (B-D) (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

4.13 NK cell ADCC activity enhances DC-mediated cellular immunity to HIV-1

FcRγ⁻ NK cells appear to be functionally limited given their faltering responsiveness to innate cytokine stimulation (Fig. 10); however, they may be ideal targets for antibody-based immunotherapies because of their inflated expansions in HIV-1⁺ individuals and superior *in vitro* ADCC activity (Fig. 7, 19). In support of this, bNAbs were deemed responsible for the generation

of $CD8^+$ T cell responses that mediated long-term control of viremia in a macaque model (433), but the mechanism linking bNAbs to potent CTLs is unknown. It is plausible, though, that NK cells utilize antibodies not only to killed infected cells via ADCC but also to enhance the function of DCs, leading to highly functional CTLs. Therefore, we sought to demonstrate the importance of this reciprocal crosstalk for the induction of durable cytotoxic T cell responses. NK cells from virally suppressed HIV-1^+ MACS participants, indeed, mediated superior ADCC against rituximab-opsonized Raji cells, with increasing ratios of effector to target cells corresponding with more robust cytolytic activity (Fig. 20A). Additionally, rituximab-opsonized Raji target cells synergized with IFNα to promote NK cell helper activity through the production of IFNγ (Fig. 20B), resulting in the development of mature DCs with a heightened ability to produce IL-12p70 (Fig. 20C). Importantly, DCs matured in the presence of NK cells generated highly functional HIV-1-specific cytotoxic T cells, as determined by IFNγ ELISPOT responses (Fig. 20D). These data demonstrate the capacity of NK cells to enhance DC-mediated immune responses to HIV-1 via antibody and, thus, highlight the potential for harnessing the reciprocal crosstalk between NK cells and DCs in the design of novel anti-HIV-1 bNAb-based therapies.

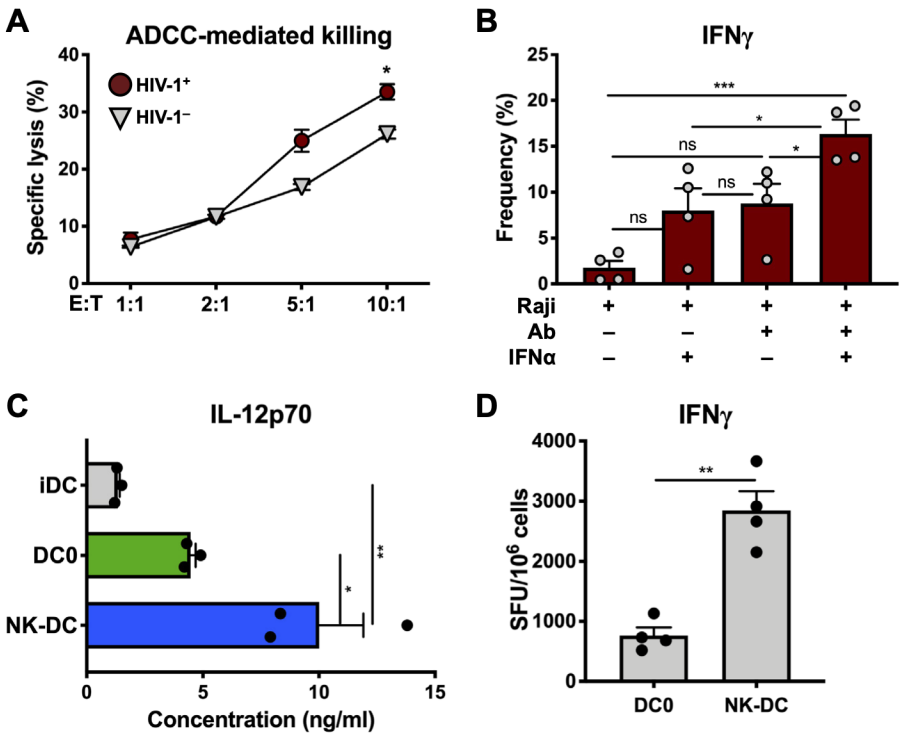


Figure 20. NK cell enhancement of DC-mediated cellular immunity to HIV-1 is driven by antibody

(A) NK cells from HIV-1$^+$ participants mediated superior ADCC against rituximab-opsonized Raji cells; n = 2 HIV-1$^+$ and n = 2 HIV-1$^-$ participants. **(B)** NK cells from HIV-1$^+$ participants required a second signal to produce IFNγ in the presence of rituximab-opsonized Raji target cells; n = 4 HIV-1$^+$ participants. **(C)** Day-4 iDCs were cultured for 48h either with opsonized Rajis+IFNα in the presence of autologous NK cells (1:1), only with opsonized Rajis+IFNα (DC0), or in the absence of NK cells and Rajis+IFNα (iDC). Two-signal activated NK cells induced the development of high IL-12-producing DCs compared with iDCs and DCs matured in the absence of NK cells (DC0); n = 3 HIV-1$^+$ participants. **(D)** NK cells enhanced DC-mediated induction of cellular immunity to HIV-1 as determined by CD8$^+$ T cell IFNγ responses to HIV-1-derived 9mer peptide pools; n = 2 HIV-1$^+$ participants from 4 independent experiments. Statistical significance was determined by the unpaired Student's t-test (A), one-way ANOVA (B, C), or the paired Student's t-test (D) (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

5.0 DISCUSSION

Expanded from: ***Anderko RR****, Rinaldo CR, Mailliard RB. IL-18 responsiveness defines limitations in immune help for specialized FcRγ⁻ NK cells. J Immunol 2020, 205(12): 3429-3442. doi: 10.4049/jimmunol.2000430.*

Our findings illustrate that HIV-1 infection alters the composition of the NK cell repertoire. First, higher proportions of NK cells from HIV-1$^+$ participants express PD-1 and NKG2C while displaying reduced surface density of the NCR NKp46 (Fig. 12), indicative of an increasingly mature phenotype. Throughout the course of treated chronic HIV-1 infection, NK cells continue to progress along the spectrum of differentiation, notably gaining expression of CD57 and KLRG1 (Fig. 13A, 13B). A previous study has reported that terminal differentiation of CD56^{dim}CD16$^+$ NK cells is enhanced after ART, as measured by CD57 expression (232). Indeed, the frequency of total NK cells expressing CD57 increases longitudinally in the context of ART-treated chronic HIV-1 infection (Fig. 13A); however, we note comparable levels of CD57 between age-matched HIV-1$^+$ and HIV-1$^-$ MSM (Fig. 12A), suggesting that HIV-1 infection is not solely responsible for the acceleration of immune aging, but rather that these immunological effects are secondary to—or heavily confounded by—HCMV, as MSM have a similar rate of HCMV seropositivity. Second, we demonstrate that HIV-1 infection has a profound effect on NK cell subset distribution, with unusually large CD56^{bright}CD16$^+$ and CD56^{dim}CD16$^-$ subsets observed in more than one-third of HIV-1$^+$ participants. These abnormal NK cell compartments are most pronounced within the first year of ART (Fig. 17), reinforcing that notion that recovery of NK cell phenotype and function occurs only after 24 months of suppressive ART (183, 186, 191). The CD56^{dim}CD16$^-$ population

is a novel NK cell subset that typically accounts for a minute fraction of fresh PBMCs (<1%). The $CD56^{bright}$ population likewise comprises less than 1% of fresh PBMCs, or less than 10% of NK cells in the periphery, and is predominantly negative for expression of CD16 (46). By contrast, in HIV-1^{+} individuals, $CD56^{bright}$ NK cells account for upwards of 50% of total circulating NK cells, with the relative frequency of the $CD56^{bright}CD16^{+}$ population reaching a staggering 44% (Fig. 17). Takahashi et al. have shown that cultures containing an inflammatory cytokine milieu induce $CD56^{bright}CD16^{-}$ NK cells into the $CD56^{bright}CD16^{+}$ population. Additionally, cytokine stimulation promotes the development of the rare $CD56^{dim}CD16^{-}$ subset into $CD56^{bright}CD16^{-}$ and further into $CD56^{bright}CD16^{+}$ NK cells (46). We also know that $CD56^{dim}CD16^{+}$ NK cells are capable of differentiating in response to IL-18 into $CD16^{-}$ NK helper cells (153). This effectively forms a circuitous clockwise loop, whereby $CD56^{bright}CD16^{-}$ NK cells first gain expression of CD16. The $CD56^{bright}CD16^{+}$ population then differentiates into $CD56^{dim}CD16^{+}$ NK cells. Under the appropriate conditions, this mature population loses expression of CD16 and further develops into the $CD56^{bright}CD16^{-}$ subset. Given that these fluctuations in NK cell compartments are driven by combinations of inflammatory cytokines, it is conceivable that the aberrant NK cell subset distribution observed in select HIV-1^{+} participants may arise from undue systemic inflammation. We also posit that HIV-1 infection interrupts NK-poiesis, arresting NK cells in transitional states and, thereby, resulting in the accumulation of unconventional NK cell subsets. Together, our data indicate that ART partially reverses HIV-1-induced NK cell abnormalities, but treated HIV-1 infection is characterized overall by persistence of a skewed NK cell repertoire, with direct implications for NK cell functionality.

In further support of this, $Fc R\gamma^{-}$ NK cells embody a distinct phenotypic and functional signature defined by aberrant IL-18 responsiveness. Our results suggest chronic HIV-1 infection

skews the differentiation of NK cells away from the helper pathway, leading to the accumulation of an unyielding population of FcRγ⁻ NK cells with limited flexibility to respond to innate stimuli. Epigenetic remodeling of the *IFNG* locus in FcRγ⁻ NK cells favors increased production of IFNγ (250, 290, 291), but this display of superiority is misleading as FcRγ⁻ NK cells are marked by limited versatility, with responsiveness highly restricted to adaptive cues mediated via CD16 signaling (246-248) (Fig. 19). Although total NK cells from individuals with chronic HIV-1 infection appear capable of inducing type-1 polarized mature DCs (Fig. 6A-6C), we demonstrate that production of IFNγ, a potent inducer of DC differentiation (139), is highly impaired when FcRγ⁻ NK cells are exposed to DC-associated innate cytokine signals (Fig. 10D-10G; Fig. 15C-15E). Furthermore, these data substantiate our claim that persistence of an expanded population of FcRγ⁻ NK cells in chronic HIV-1 infection dilutes the capacity for NK-DC crosstalk (Fig. 10H). The defective ability of FcRγ⁻ NK cells to respond to innate cytokine stimulation appears to stem from diminished expression of IL12Rβ2 and IL18Rα (Fig. 11A-11F); however, formal proof would require a genetic rescue experiment. Additionally, the FcRγ⁻ subset acquires and maintains a mature phenotype, including increased expression of CD57 and NKG2C, coupled with reduced expression of NKG2A (Fig. 8; Fig. 14B, 14D), supporting the reported reduced expression of IL12Rβ2 and IL18Rα with increasing status of NK cell maturation (42, 43, 291). The dysfunctional responsiveness of FcRγ⁻ NK cells to IL-18 extends beyond impaired production of IFNγ (291) (Fig. 10B-10D), with this population of NK cells resisting IL-18-mediated downregulation of CD16 (Fig. 9) and expression of CD25 and CD83 (Fig. 10B-10D). In other words, FcRγ⁻ NK cells poorly differentiate into NK helper cells, providing further evidence of their phenotypic and functional divergence from conventional NK cells. This concept is validated by genome-wide DNA methylation analyses revealing epigenetic commonalities between FcRγ⁻ NK and $CD8^+$

effector T cells. By contrast, the methylation profile of FcRγ$^-$ NK cells deviates substantially from that of mature canonical NK cells (291).

Our finding that FcRγ$^-$ NK cells maintain expression of KLRG1 following co-stimulation with IL-18 and IL-12 (Fig. 15A, 15B) also suggests that metabolic dysfunction, induced by HIV-1 infection, contributes to their limited responsiveness to innate stimuli (489), as accumulating evidence links impaired cellular metabolism to NK cell dysfunction (490, 491). This notion is further supported by reports in which hepatitis C virus infection provokes NK cells to increase surface expression of KLRG1 (483), which is associated with defective phosphorylation of Akt at Ser473 and impaired functionality (483). Akt phosphorylation is also inhibited in KLRG1^{lo} cells yet unaltered in KLRG1^{hi} NK cells in response to KLRG1 stimulation, but ligation of KLRG1 does enhance pre-existing AMPK activity in KLRG1^{hi} NK cells by preventing its inhibitory dephosphorylation. Moreover, silencing of either KLRG1 or AMPK restores NK cell functional responsiveness in the KLRG1^{hi} population (492). Engagement of KLRG1, thus, appears to curb the effector functions of KLRG1^{hi} NK cells by trapping them in a quiescent state. Similarly, persistent antigen stimulation in chronic viral infections leads to an increase in KLRG1 expression of virus-specific CD8$^+$ T cells, negatively influencing their function and proliferative capacity (485). However, during the contraction phase, a small percentage of these KLRG1$^+$ effector CD8$^+$ T cells are capable of downregulating KLRG1, leading to the formation of a pool of 'exKLRG1' memory T cells that retain high cytotoxic and proliferative capacity. Importantly, the developmental plasticity of KLRG1$^+$ effector CD8$^+$ T cells promotes a functionally versatile population with an ability to mediate long-term protective immunity (493). Together, these studies provide the scientific premise for our hypothesis that the metabolism of FcRγ$^-$ NK cells from HIV-1$^+$ individuals is negatively skewed, resulting in an exhausted phenotype marked by

decreased functional plasticity. A paucity of information exists on the metabolic changes in human NK cells during viral infections, including the influence of cellular metabolism on their memory-like functions; however, targeting NK cell metabolism may be key to restoring proper NK cell function in HIV-1 infection.

While the induction of FcRγ^- NK cells is strongly associated with HCMV infection (247), this population of cells is further inflated in HIV-1 infection (248, 299) (Fig. 7F-7J), and these elevated frequencies persist despite years of suppressive ART (Fig. 14A). The mechanism by which HIV-1 contributes to this phenomenon, though, is poorly understood. However, reports suggest that expansions of FcRγ^- NK cells are not a direct consequence of HIV-1 infection but rather due to the potentially greater impact of HCMV exposure in HIV-1 infection (224, 248), as evidenced by higher HCMV antibody titers (224, 248, 306), the extent of HCMV reactivation (Table 8), and the magnitude of HCMV-specific T cell responses in HIV-1-infected individuals (473). Although ART successfully suppresses HIV-1 viremia and reduces AIDS-associated mortality, the significant inflammation and elevated levels of markers of innate immune activation induced by HIV-1 infection persist in HIV-1$^+$ individuals despite sustained ART-mediated viral suppression (488, 494-497) (Fig. 16A-16D). This chronic immune activation drives immunosenescence, which subsequently triggers additional inflammation and immune dysfunction (231). HIV-1 infection, in fact, is characterized by an accumulation of late-differentiated T cells with a senescent phenotype and heightened secretion of pro-inflammatory cytokines (308, 498, 499). In particular, HCMV-specific memory CD8$^+$ T cells lacking CD28 are highly expanded in HIV-1$^+$ individuals (233, 500), and this is accompanied by elevated HCMV antibody titers (224, 248, 306), with serum HCMV IgG levels correlating with inflammatory markers (501-503). Furthermore, an inverse relationship exists in HIV-1 infection between the

extent of NKG2C^{+}CD57^{+} NK cell expansion and the fraction of HCMV-specific CD8^{+} T cells expressing CD28. The parallel expansions of highly differentiated HCMV-associated 'memory' NK cell populations with limited functional plasticity and CD28^{-} HCMV-specific T cells imply increased HCMV reactivation to immunogenic levels, reflecting a level of underlying immune dysfunction in HIV-1 infection (233). It is also possible that 'memory' NK cell differentiation and persistence occurs as a compensatory mechanism for poor protective T cell responses in HIV-1^{+} individuals (224), but their expansion to high levels indicates stress on the overall ability of the immune system to control HCMV reactivations (233).

Whereas T cell and monocyte activation induced by HIV-1 viremia is resolved within 24 months of ART (224), activated NK cells continue to persist in those maintaining undetectable levels of plasma viremia (224, 225), suggesting NK cells are more sensitive to immunologic perturbations (224). Based on these data, along with our findings, we surmise that HIV-1 infection accelerates the expansion of FcRγ^{-} NK cells through immune dysfunction and poor HCMV control (Fig. 16A-16D; Table 8). We also posit that the selective expansion of FcRγ^{-} NK cells may, in part, be due to the impact of simultaneous subclinical reactivations of both of these latent viruses, which could occur frequently if a portion of the HCMV-responding, antigen-specific CD4^{+} T cells themselves harbor latent HIV-1, as our previous study suggests (504). As immune cells do not act independently of one another, but rather in a coordinated fashion, dysfunction in one cellular compartment likely skews the delicate balance of the entire immune system, resulting in additional but interrelated layers of dysfunction.

Numerous questions linger related to FcRγ^{-} NK cells, including the precise mechanisms involved in triggering their differentiation, as well as the implications of their expansion in ART-treated HIV-1^{+} individuals. The specialization of FcRγ^{-} NK cells plausibly corresponds with

reduced plasticity, as they appear to have lost a degree of flexibility, hindering their ability to rapidly respond to innate cytokine stimulation as immune helper cells through the production of IFNγ. This, in turn, could affect immune homeostasis and have important implications for future HIV-1 cure interventions aiming to elicit potent CTL responses and for the establishment of primary adaptive immunity to new pathogens such as the novel SARS-CoV-2, as the ability of NK cells to respond to innate signaling with agility and flexibility is critical for the induction of effective primary adaptive T cell responses to viral infections (505). In fact, recent studies suggest that innate immune dysregulation and a similar NK cell immunotype are related to COVID-19 disease severity (506, 507). It remains to be determined whether the reduced responsiveness of FcRγ$^-$ NK cells to innate stimuli, as observed *in vitro* in aviremic HIV-1$^+$ individuals, plays a role in generating functionally impaired CTLs *in vivo*. Moreover, FcRγ$^-$ NK cells could perpetuate inflammation through their hyperactivity and enhanced ability to produce inflammatory cytokines following antibody-mediated stimulation (246-248), thereby contributing to the long-term health consequences linked to persistent innate immune activation (224).

As an added complication to achieving immune homeostasis in HIV-1$^+$ individuals, damage to the gut epithelium during HIV-1 infection presumably results in increased microbial translocation from the gut into the blood stream, contributing to both immune activation and chronic inflammation (494, 508). Interestingly, IL-18 appears to support barrier function in the intestine by acting directly on IL18R1-expressing CD4$^+$ T cells to limit colonic Th17 differentiation, in addition to promoting Foxp3$^+$ Treg cell–mediated control of intestinal inflammation (509). Although NK cells are potent producers of IFNγ (153, 510) (Fig. 5), they also function in parallel with Foxp3$^+$ Treg cells to dampen inflammation and protect against immunopathologies by producing IL-10 in response to innate stimuli (27, 157, 511, 512).

However, FcRγ$^-$ NK cells, which home to mucosal sites (304), demonstrate reduced responsiveness to innate cytokine stimulation (290, 291) (Fig. 10, 15), presumably limiting their regulatory role and capacity to promote immune homeostasis. Moreover, members of the *Prevotella* genus have previously been linked to inflammation and intestinal dysbiosis (513-515), and a recent study demonstrates that intestinal *Prevotella* colonization results in a reduction of short-chain fatty acids, thereby decreasing production of IL-18 and exacerbating intestinal inflammation (516). Of note, a higher prevalence and relative abundance of *Prevotella*, as well as lower levels of the bacterial genus *Bacteroides*, are present in HIV-1$^+$ individuals and prior to HIV-1 acquisition in MSM who later seroconvert compared with HIV-1$^-$ MSM (517-519). This *Prevotella*-rich microbiome is associated with increased numbers of activated colonic T cells (518), along with significantly increased levels of sCD14, sCD163, and IL-6 (517), underscoring the profound impact of disrupted intestinal homeostasis on immune activation and resultant inflammation in the setting of HIV-1 infection.

In summary, we theorize that chronic immune activation contributes to innate immune exhaustion and dysfunction, particularly NK cell dysfunction, resulting in poor HCMV control, as NK cell deficiencies are associated with a higher susceptibility to herpesvirus infections. HCMV reactivation induces the production of pro-inflammatory cytokines, which further enhance the inflammation caused by subclinical HIV-1 replication, bacterial translocation, and immunosenescence. HCMV reactivation also causes the production of HCMV-specific antibodies, driving the expansion of FcRγ$^-$ NK cells. One can also reasonably hypothesize that FcRγ$^-$ NK cells actually perpetuate inflammation through their hyper-reactivity and enhanced ability to produce inflammatory cytokines following antibody-mediated stimulation but their reduced ability to respond to innate stimuli, which is essential for their regulatory role. Ultimately, this chronic

inflammatory state promotes diseases of aging, including malignancies, cardiovascular disease, and neurocognitive disorders, despite highly effective suppressive ART.

On the contrary, the potential exists for FcRγ$^-$ NK cells to be exploited for antibody-based vaccines or immunotherapies due to their superior *in vitro* ADCC activity. The lack of FcRγ potentially favors more efficient killing of infected cells via ADCC in comparison with conventional NK cells, as in the absence of FcRγ that only bears one ITAM sequence, CD16 engagement exclusively involves the adaptor protein CD3ζ, which contains three ITAMs, delivering a stronger signal inside the cell (246, 289). Indeed, FcRγ$^-$ NK cells are more dynamic mediators of antibody-dependent effector responses relative to their FcRγ$^+$ counterparts (Fig. 19B-19D), and total NK cells from HIV-1$^+$ participants demonstrate a stronger capacity to eliminate antibody-opsonized target cells (Fig. 20A). Notably, NK cells also utilize antibodies to enhance the ability of DCs to induce potent HIV-1-specific CTLs (Fig. 20C, 20D). Combining our findings, including that the rare FcRγ$^-$ NK cell population is highly and frequently expanded in HIV-1-infected individuals, with the knowledge that bNAbs lead to durable suppression of viremia via CTL-mediated mechanisms (433), we propose employing bNAbs to direct FcRγ$^-$ NK cells not only to target HIV-1-infected cells but also to facilitate the emergence of potent CTL responses due to their superior capacity to modulate DC function via antibody-mediated signaling (Fig. 21). Although we were unable to explore the *in vivo* implications of the expansion of FcRγ$^-$ NK cells in the present study, our findings highlight the need for nuanced longitudinal studies to discern how their presence in treated chronic HIV-1 infection relates to disease progression, inflammation-associated comorbidities, and long-term control of HIV-1 viremia.

NK cells are uniquely positioned to influence the antiviral response and limit viral spread due to the pleiotropic nature of their effector functions, including the potential to respond directly

to infected cells and to modulate the adaptive immune response through the establishment of a vast communication network encompassing both the innate and adaptive arms of immunity (79, 173, 180, 475). This elaborate series of interactions, with NK cells at the helm, is critical for an optimal immune response. Therefore, improving our understanding of the magnitude of dysfunction suffered by NK cells during HIV-1 infection, including their interactions with DCs, will be imperative for rescuing their function and improving long-term health outcomes through successful immune reconstitution. Strategies for harnessing the natural powers of NK cells to control HIV-1 infection, modulate adaptive immune responses, and promote overall health and immune homeostasis will be integral in maximizing the effectiveness of HIV-1 therapies.

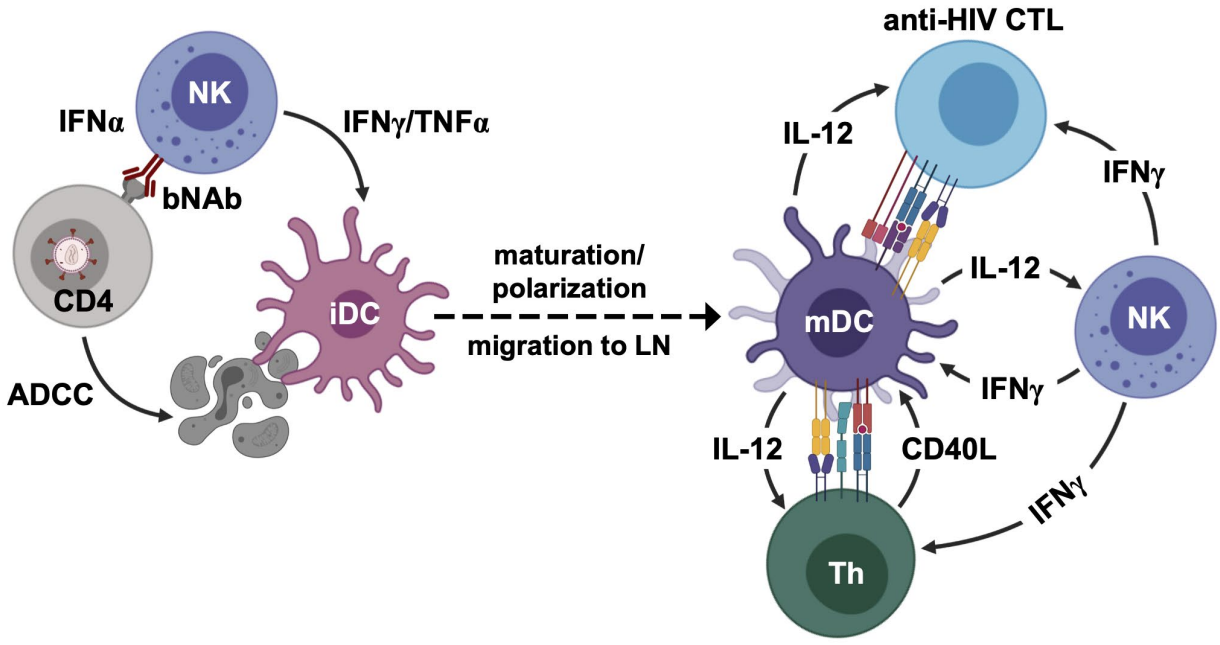


Figure 21. Schematic depicting the mechanism of NK cell help in DC-mediated adaptive immunity to HIV-1

bNAbs support NK cell–mediated killing of an HIV-1-infected $CD4^+$ T cell via ADCC. IFNα synergizes with antibody-dependent signaling to activate the helper function of NK cells, promoting DC maturation and polarization. The DC, carrying environmental cues from the periphery, produces IL-12 in response to CD40L stimulation by a $CD4^+$ T helper cell, and IL-12 acts with NK cell–derived IFNγ to drive cellular-mediated immunity to HIV-1. *Figure created with BioRender.com.*

6.0 PUBLIC HEALTH IMPLICATIONS

Incremental, but remarkable, scientific advances have transformed HIV from a near-death sentence to a manageable chronic condition, beginning in 1983 with the identification of HIV-1 as the causative agent of AIDS (520, 521). This led to the development of diagnostic assays that provided insight, for the first time, into the true magnitude of the global HIV/AIDS pandemic, and an understanding of the HIV-1 replication cycle illuminated mechanisms by which to control the virus. The first antiretroviral drug azidothymidine (AZT), introduced in 1987, enabled a temporary reduction in viral load by inhibiting HIV-1 reverse transcriptase. Two-drug therapy, again, failed to completely suppress the virus (522). However, two major milestones occurred in 1996 that revolutionized the fight against the HIV/AIDS pandemic. First, highly effective combination ART, three-drug treatment regimens including protease inhibitors or nonnucleoside reverse transcriptase inhibitors, durably suppressed plasma HIV-1 viremia below detectable levels (523). Second, the Joint United Nations Program on HIV/AIDS (UNAIDS), an innovative joint program of entities across the United Nations (UN), became operational, urging concerted action by countries in every region in a new global response to the HIV/AIDS pandemic. Global leadership through UNAIDS, together with activists living with HIV, propelled efforts to reverse the course of the pandemic. Notably, access to affordable HIV treatment in developing countries has substantially decreased the number of new HIV infections and averted an estimated 16.2 million AIDS-related deaths since 2001 (524). In 2014, UNAIDS launched their ambitious 90–90–90 initiative, with the aim to diagnose 90% of all people living with HIV, to provide treatment for 90% of those diagnosed, and to achieve viral suppression in 90% of those treated by 2020 (525). In a further commitment to

ending AIDS by 2030, as called for in the 2030 Agenda for Sustainable Development, the 2016 UN Political Declaration outlined the following three goals to be achieved by 2020:

1. Reduce new HIV infections to fewer than 500,000 globally per annum.
2. Reduce annual AIDS-related deaths to fewer than 500,000 globally.
3. Eliminate HIV-related stigma and discrimination (526).

Unfortunately, despite dozens of countries achieving, even exceeding, the UNAIDS 90–90–90 targets, the aggregated data indicate that all of the global targets for 2020 have been missed. For instance, in 2020, 1.5 million people acquired HIV, triple the 2020 target of fewer than 500,000 new infections, and 690,000 AIDS-related deaths were recorded. Moreover, of the 37.6 million people living with HIV globally in 2020, an estimated 27.4 million were receiving treatment. Although this number has more than tripled since 2010, it is still short of the 2020 target of 30 million. In some instances, previous gains are even being reversed (524). Among the factors undermining progress toward the 90–90–90 targets are the marginalization and criminalization of key populations, including MSM, people who inject drugs, transgender people, female sex workers, and people in prisons, who remain underserved by HIV programs in spite of their extremely high risk of acquiring HIV (527, 528). Additionally, COVID-19 has exposed the inadequacy of investments in public health and has presented new challenges, adding enormous pressure on health systems and disrupting the HIV care continuum (529-531), with many countries reporting dips in new HIV diagnoses and treatment initiations in 2020 (524, 528). Thus, the logistical and economic hurdles in delivering optimal, lifelong treatment to the 37.6 million people living with HIV underscore the need to explore therapeutic alternatives that achieve sustained virologic remission in the absence of ART. Accordingly, the goals set forth in the International

AIDS Society Global Scientific Strategy of 2016 have prioritized the development of a safe, affordable, and scalable HIV cure strategy (532).

In addition to providing a basic immunologic understanding of the impact of chronic HIV-1 infection and long-term ART on the phenotypic and functional character of NK cells, our study provides promising avenues for optimizing NK cell responses to improve HIV-1 control and effect a functional cure. The importance of NK cells in anti-HIV-1 immunity has already been widely established; specifically, ADCC activity has been implicated in both vaccine-induced protection from acquisition of infection and phenotypes of viral control (116-119). However, HIV-1 infection disrupts the delicate balance of the innate and adaptive arms of the immune system, and NK cells are particularly sensitive to these immunologic perturbations, resulting in pathologic subset redistribution, a skewed repertoire, and maintenance of an activated phenotype despite undetectable levels of plasma viremia (181, 185, 192, 196, 224, 225). Furthermore, our study demonstrates that the presence of expanded populations of FcRγ^- memory-like NK cells in treated chronic HIV-1 infection negatively impacts the quality of immune help delivered to DCs by NK cells in response to innate cytokine stimulation. This functional shortcoming of FcRγ^- NK cells is counteracted, though, by their superior responsiveness to antibody-mediated signaling, a quality that can potentially be harnessed toward the goal of HIV cure given the growing interest and success of bNAb therapies in animal models (Table 3). Notably, our study also highlights that NK cells utilize antibodies not only to kill target cells but also to activate their helper function, resulting in the polarization of DCs with a heightened ability to induce highly functional CTLs. Combining our findings with the knowledge that bNAbs lead to durable suppression of viremia via CTL-mediated mechanisms (433), we propose a novel HIV functional cure strategy that marries proviral reactivation and bNAb administration, with the latter engaging NK cells, namely

the FcRγ⁻ memory-like population, to enhance their antibody-mediated effector and helper functions. That is, NK cells will boost the kill component of the kick and kill approach directly by eliminating cells harboring reactivated latent HIV-1 via ADCC and indirectly by promoting highly effective DC-mediated cellular immunity to HIV-1.

Although pioneering research has accelerated the global response, HIV remains a pandemic driven by inequalities (524). For this reason, the importance of eliminating societal and economic barriers to the future success of HIV cure efforts cannot be overstated, as disparities in testing and treatment coverage threaten to undermine even the most promising of therapeutic modalities. Therefore, only the equitable distribution of therapies within all subpopulations will allow for the attainment of a once seemingly impossible feat—a global HIV cure.

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