Elucidating the Mechanistic Role of ADAM17 in IL-18 Induced "Memory-Like" Natural Killer Cell Differentiation and Helper Function

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HIV-1 infection causes a skewed phenotypic and functional repertoire of natural killer (NK) cells, which continues to be present in people living with HIV-1 (PLWH) following effective control of HIV-1 viremia with long-term antiretroviral therapy (ART). This is highlighted by the expansion of a rare and highly differentiated population of ADCC hyper-responsive CD16$^+$ FcRγ^- NK cells that exhibit adaptive immune characteristics, such as immunological memory. In addition to the FcRγ^- NK population, NK cells can differentiate into a cytokine-induced "memory-like" population that lacks CD16 expression and acquires characteristics more commonly associated with T helper cells. Our lab has shown that IL-18 stimulation proved to be critical for distinguishing the capacity of an NK cell to differentiate into either a cytolytic FcRγ^- NK cell or a helper -like NK cell as FcRγ^- cells are rendered unresponsive to IL-18 stimulation and maintain CD16 expression. However, the mechanism involved in this IL-18 mediated NK cell differentiation process is still not completely known. The TNFα converting enzyme, ADAM17, has been implicated in the shedding of NK cell receptor CD16, and is therefore an interesting molecule of study for the mechanism of NK cell differentiation. The results of this study highlight the roles of both IL-18 and ADAM17 in the differentiation of NK cells into a functionally distinct "memory-like" NK helper cell subset and implicates a potential role of ADAM17 in the development of FcRγ^- NK cells. Moreover, this study highlights the parallel that exists between NK cells and T cells, with both having the capacity to differentiate into adaptive cytolytic vs helper

cell subsets. Overall, this underscores the need to continue to identify and study the causative mechanisms contributing to NK cell immune irregularities seen in PLWH. A better understanding of how these different NK cell populations arise naturally, how they can be induced, and their role in chronic HIV-1 infection and associated comorbidities can lead to more effective targeting of NK cells in HIV-1 comorbidity and remission studies.

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1.0 Introduction: Natural Killer Cells and the Immune Response

1.1 NK Cells in the Innate Immune Response

The versatile and complex roles natural killer (NK) cells play in the immune system offer a unique perspective into the functionality of immune cell interactions. NK cells are lymphoid progenitor cells that, unlike T and B cells, play a major role in the innate immune response [1]. These cells have the ability to mount an innate killing response to virally infected cells as well as tumor cells [2-4]. Importantly, NK cells are able to mount this response without any prior exposure or priming, making them critical mediators of the innate immune response [3, 5]. Another critical aspect of their innate functionality is their ability to lyse any cell in the immune environment that lacks "self" major histocompatibility complex (MHC) class I due to their mixed repertoire of activating and inhibitory surface receptors. Typically, inhibitory receptors on the NK cells will engage with MHC class I, halting cell lysis. However, if a cell is missing MHC class I and fails to engage with an NK cell inhibitory receptor, activating receptor signaling pathways are triggered, and the NK cell will lyse the target [6-9].

The natural ability of NK cells to lyse target cells is the most common feature of their innate immune function, and this cytolytic capacity is an important first-line mechanism of host defense for most infections.

1.1.1 Classical Phenotypical Characterization of NK Cells

Most often, NK cells are classified by their relative expression of two surface molecules, CD56 and CD16, and their absence of CD3. In the peripheral blood, about 10% of NK cells are characterized as having high expression of CD56 and lack of expression of CD16, also termed CD56^{bright}CD16$^-$ NK cells. These NK cells are unique in their ability to migrate to the lymph node due to their surface expression of CCR7 and CD62L [10, 11]. The other 90% of NK cells in the peripheral blood have a reduced level of CD56, but have high expression of CD16 and are termed CD56^{dim}CD16$^+$ NK cells [12, 13]. The population of CD56^{dim}CD16$^+$ NK cells also displays higher levels of inhibitory NK receptors (iNKRs) that recognize and engage with various MHC class I molecules [14, 15]. The CD56^{bright} population of NK cells is thought to be a younger or immature NK population that will eventually lose CD56 expression and acquire CD16 as they mature [16]. Once these mature or conventional NK cells have the CD56^{dim}CD16$^+$ phenotype, they display a decrease in proliferative capacity and an increase in cytotoxicity [17].

1.1.2 Diverse Effector Functions of NK Cells

Although NK cells are often classified by only a handful of surface molecules, their functionality extends beyond this simple characterization. As previously described, NK cells have an innate ability to lyse cells undergoing stress due to viral infection, tumor transformation, or because of lack of self-MHC presentation. However, NK cells function beyond their cytolytic potential. At the most basic level, CD56^{bright}CD16$^-$ NK cells are recognized for their immunomodulatory function as major cytokine producers with little cytolytic ability [18, 19]. On

the other hand, CD56^{dim}CD16$^+$ NK cells are recognized more for their killing capacity [20, 21] (Fig. 1).
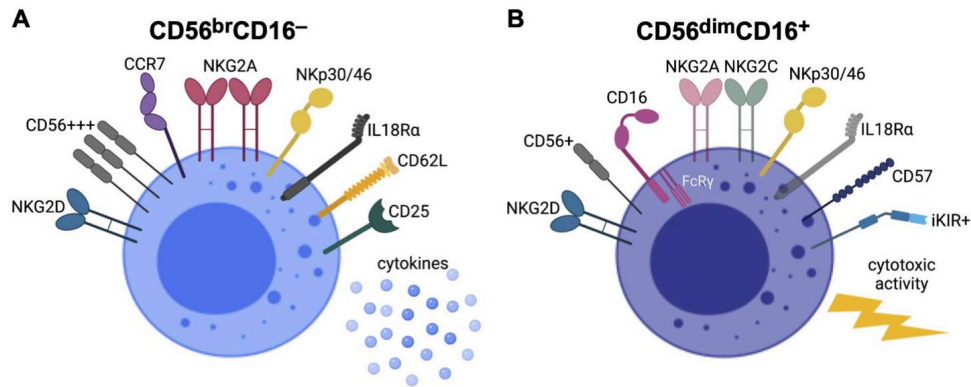
Figure 1. Classical characterization of NK cell subsets

(A) NK cells defined by a CD56brightCD16- phenotype exhibit higher levels of CD56, inhibitory NKG2A, IL-2 receptor CD25, and other cytokine receptors. These cells are defined by their increased ability to produce cytokines upon stimulation, increased expression of inhibitory NK receptors such as NKG2A, as well as traffic to the lymph node due to the expression of CCR7 and CD62L. **(B)** NK cells defined by a CD56dimCD16+ phenotype exhibit higher levels of CD16, activating NK receptors such as NKG2C, and inhibitory immunoglobulin-like receptors (iKIRs), but lower levels of IL-2 receptor CD25. These cells are defined by their increased cytolytic potential (Ref: Anderko et al, 2021) [22].

The increased cytolytic ability of the CD56^{dim}CD16$^+$ NK cells comes from their increased expression of activating receptors, as well as their maintained expression of the low-affinity IgG Fc receptor surface molecule CD16 (or FcγRIIIA) [23-25]. This expression of CD16 allows NK cells to be good mediators of antibody-dependent cellular cytotoxicity (ADCC). When CD16 is engaged with the Fc portion of an antibody on an opsonized cell, an intracellular signaling cascade takes place in the NK cell that leads to target cell lysis and cytokine secretion [26, 27]. CD56^{bright}CD16$^-$ NK cells, however, cannot mediate ADCC due to their lack of CD16 expression. These cells instead are able to quickly produce higher levels of cytokines, such as IFNγ, TNF-α,

and IL-10, in response to innate cytokine stimulation as compared to the CD56^{dim}CD16^{+} population [19, 28]. In addition, these cells express chemokine receptors CCR7 and CXCR3, which make them more abundant in the lymph nodes than the CD56^{dim}CD16^{+} cells [10, 29]. Put together, the presence of the CD56^{bright}CD16^{-} NK cells in peripheral and lymphoid tissues, along with their increased capacity to produce cytokines, allows for them to provide important immune help to other immune cells in the environment to influence the character of the immune response [28].

1.1.3 NK Cell-Mediated "Help" to Dendritic Cells

As mentioned above, besides their killing responses, NK cells an important role by providing immune "help" to other cells by producing an array of effector cytokines [28]. Their impact on dendritic cells (DCs) in particular is important for shaping the character of the adaptive immune response. DCs are antigen-presenting cells that bridge the gap between the innate and adaptive immune response as they carry information from the periphery to the lymph node and subsequently interact with and prime T-cells to mount specific immune responses [30]. NK cells and DCs exhibit a cross-talk that can shape the landscape of the immune environment [31]. DCs produce a variety of NK cell activating cytokines including IL-12, IL-15, and IL18, each of which contributes to NK cell proliferation, cytolytic function, and production of cytokines including IFNγ [31-33].

In addition to the DCs providing immune signaling to the NK cells, the NK cells in turn are able to provide immune help to modulate the phenotype and function of DCs. Activated NK cells produce IFNγ and TNFα, which promote DC maturation and enhance their capacity to produce high levels of IL-12p70 when interacting with CD40L-expressing T helper cells [34]. In

addition, this bidirectional signaling between NK cells and DCs often occurs before T cell priming events. The nature of this communication can contribute to the environmental milieu by providing instruction for the proper development of polarized T helper responses [35, 36]. The NK cytokine production can also promote DC migration to the draining lymph nodes where the DCs can continue to prime effector T cells to mount an appropriate immune response [31]. Importantly, NK cells present in secondary lymphoid organs (SLO) are also able to participate in this NK-DC cross-talk, adding to that immune environment within the lymphoid tissue [37]. Conventional NK cells that are induced to differentiate and migrate from the periphery to the lymph nodes to provide this immune help are phenotypically characterized by their expression of the markers, CD83, CD25, and CCR7 [34].

1.2 NK Cells and HIV-1

NK cells have been shown to be important players in HIV-1 infection, progression, and outcomes. More specifically, their germline-encoded killer receptors interact with HLA molecules presenting HIV peptides, and this interaction has been associated with a delayed progression to AIDS, as well as protection against initial viral infection [38, 39]. Their cytokine and chemokine-producing abilities can also aid in the response to HIV infection. When NK cells produce chemokines, such as CCL3 and CCL4, they can act as competitive ligands to HIV co-receptor CCR5, thereby preventing viral entry into the host cell [40]. Not only do the NK cells produce chemokines to block viral entry, but they also produce cytokines that interact with DCs that have downstream effects on shaping the adaptive response to HIV- infection [41, 42]. In addition, NK

cells also contribute to the effectiveness of HIV-1 vaccines due to their increased ADCC potential when their CD16 receptor is engaged with vaccine-induced antibodies [43, 44] (Fig. 2).
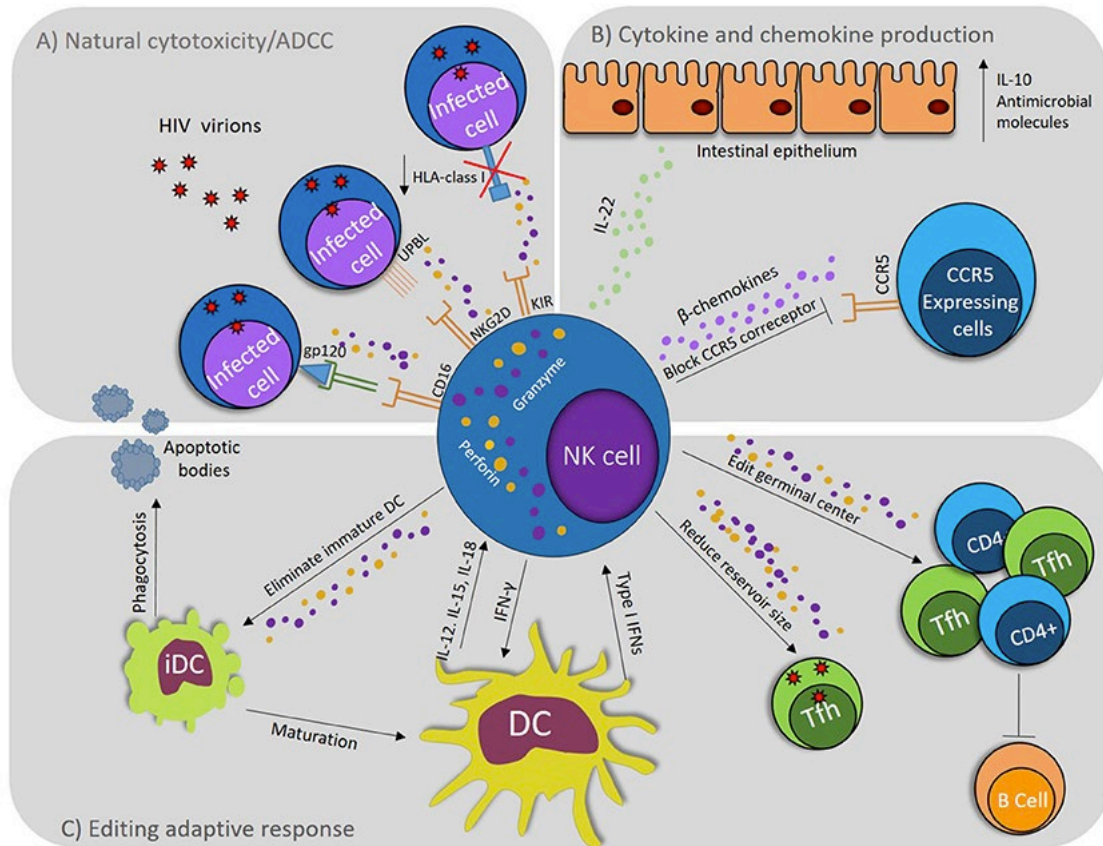
Figure 2. The role of NK cells in HIV-1 infection

(A) NK cells' different surface receptors, such as CD16 and KIRs, can interact with antibodies or HLA presenting HIV peptides in the environment and create a cytolytic environment to control HIV infection. **(B)** NK cells can produce various forms of chemokines that can bind to CCR5, thereby preventing viral entry into the host, as well as other chemokines that increase the production of antimicrobial molecules in the epithelium to protect against the virus. **(C)** NK cells are also able to produce various cytokines in their environment that mature and activate DCs, therefore, having a downstream effect on the adaptive response to HIV infection. NK cells also play a role in editing the germinal center and reducing viral reservoirs in adaptive cells (Ref: Flórez-Álvarez et al. 2018) [45]. Copyright © 2018, Frontiers in Immunology.

1.2.1 NK Cell Dysfunction in HIV-1

Although NK cells can play a protective role in HIV-1 infection, the virus is still able to infect and remain latent in its host and cause immune dysfunction. Part of this immune dysfunction can be seen in the NK cell population itself, causing these cells to become less effective in controlling the virus [40, 46]. Upon HIV infection, the proportion of NK cells shifts and there becomes a depletion in the CD56^{bright}CD16$^-$ NK cells, but an increase in the CD56^{dim}CD16$^+$ cells [46]. Eventually, when untreated, chronic viremia leads to an expansion of a CD56$^-$CD16$^+$ subset of NK cells, which exhibit dysfunctional cytotoxic activity, including ADCC [47, 48]. This dysfunctional subset also shows an increase in NKG2C expression, but a decrease in NKG2A, lending to a more activated state but still functionally impaired [49]. In addition, these NK cells also display weakened cytokine production, which can impair effective immune cross-talk and subsequent adaptive immune responses [46, 47].

The NK cell dysfunction can be slightly restored through the use of antiretroviral therapy (ART) [50]. The levels of NKG2A can be seen to increase on the NK cells once the viremia has been suppressed through treatment, and improvements in ADCC capacity occur [49, 51]. However, even with this phenotypical restoration, the NK cells of those virally suppressed on ART still exhibit a chronic activation state as seen by increased rates of degranulation and expression of HLA-DR [52]. This chronic immune activation status can lead to a pro-apoptotic state in immune cells, therefore allowing the virus to continue to evade its host's defenses [53]. In addition, although ART can restore levels of cytolytic CD56^{dim}CD16$^+$ NK cells, terminal differentiation of this population can lead to an overall decrease in NK cell functional proliferative activity [54].

The continued immune dysfunction, in addition to the ability of the virus to remain latent during ART, has led researchers to focus attention towards a functional cure for HIV-1 [55]. As

part of implementing and testing functional cure strategies, the removal of ART is needed to determine efficacy. Therefore, a need exists to better understand how these dysfunctional immune characteristics arise in chronic HIV infection, and how their effect can be minimized to ensure that study participants will be provided the best chance for successful immune control of the virus upon cessation of ART.

1.2.2 FcRγ Deficient NK Cell Population

Another atypical subset of NK cells commonly seen in HIV infection is a group of NK cells deficient in the intracellular CD16 adaptor protein, FcRγ. Typically, the FcRγ adaptor protein associates with CD16 as either a homodimer or as a heterodimer with an intracellular CD3ζ chain protein leading to an immunoreceptor tyrosine-based activation motifs (ITAMs) signaling cascade upon CD16 engagement [56, 57]. However, those living with HIV exhibit an inflated percentage of this rare population of NK cells that lack the FcRγ adaptor protein that typically associates with CD16, which instead is replaced with a singular CD3ζ protein [58] (Fig. 3).
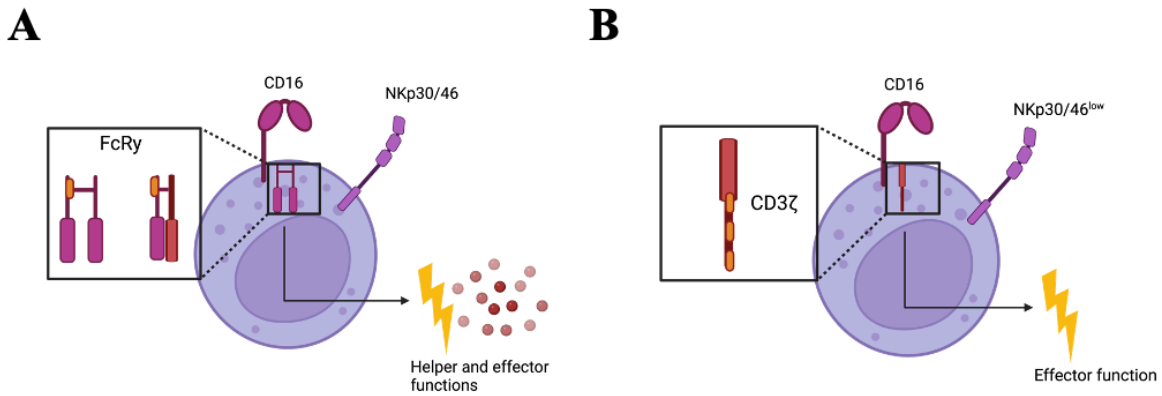
Figure 3. Dysfunctional FcRγ NK cell population

(A) Typical NK cell distributions contain a CD16 adaptor protein, FcRγ, that acts as a heterodimer or homodimer and contains one ITAM molecule. When the NK cell is activated, this signaling cascade leads to a variety of effector functions. **(B)** A subset of NK cells in those living with HIV are deficient in the FcRγ adaptor protein and instead contain a single CD3ζ protein, which has three ITAM molecules and the signaling cascade leads to increased cytotoxic effector functions. *Figure made with BioRender. com.*

As mentioned previously, NK cells respond to innate stimuli in their environment. In particular, NK cells produce helper cytokines IFNγ and TNFα when stimulated by pro-inflammatory cytokines commonly made by DCs. However, there is a two-signal requirement for induction of this NK helper function, and this is most pronounced in the presence of IL-18 in conjunction with other innate cytokines, such as IL-12 or IL-15 [59]. In addition to producing high levels of IFNγ and TNFα, the CD56^{dim}CD16$^+$ NK population also downregulates its CD16 expression upon IL-18 co-stimulation, differentiating these cells into more of a classic NK helper cell phenotype [34].

FcRγ⁻ NK cells do not exhibit this same capacity to differentiate into NK helper cells. Upon innate co-stimulation with IL-18, the NK cells deficient in the FcRγ adaptor protein are unable to produce high levels of IFNγ, nor do they acquire the surface markers, CD25 and CD83 [59].

Moreover, when NK cells with high proportions of FcRγ⁻ cells are put into culture with DCs, the production of IL-12p70 by these DCs is reduced, emphasizing their dysfunctional capacity to effectively cross-talk with DCs [59]. Although FcRγ⁻ NK cells exhibit a reduced ability to provide immune help, these cells do show an increased capacity for ADCC due to their retention of the Fc receptor, CD16 [58]. Also contributing to their increase in CD16 responsiveness is the fact that the FcRγ⁻ NK cells utilize the CD3ζ adaptor protein bearing three ITAMs, instead of one, leading to a more robust signaling cascade [57].

Importantly, this subset of FcRγ⁻ NK cells is also associated with human cytomegalovirus (HCMV) infection and those who are seropositive for HCMV exhibit higher levels of dysfunctional FcRγ⁻ NK cells [60]. In addition, once these NK cells differentiate to an FcRγ⁻ phenotype, ART is not effective at restoring the original phenotype, and these cells are terminally differentiated and specialized for enhanced ADCC function [61].

1.3 NK Cell "Memory-Like" Characteristics

Immunological memory is a characteristic of immune cells typically confined to the adaptive immune response. More recently, there has been evidence supporting the notion that NK cells can exhibit adaptive features, such as immunological memory, despite being innate cells [62]. Although they have germline-encoded receptors, some retain the ability to clonally expand in response to an antigen-specific encounter, as well as maintain populations capable of recall responses, all features of immunological memory [63, 64].

Early studies first demonstrating NK cell memory showed that when NK cells are primed by tumors deficient in MHC class I, these NK cells become more potent effector NK cells, with

increased cytolytic potential and production of IFNγ, when subsequently exposed to tumor antigen [65]. More recently, NK cell memory was shown in an HCMV infection model where NK cells bearing a receptor specific for the viral antigen were able to clonally expand upon infection, and once reactivated, these cells exhibited an increase in cytokine production. In addition, the adoptive transfer of this clonally expanded NK population into HCMV-challenged naïve animals revealed a secondary clonal expansion with heightened responses [66].

Not only has NK cell memory been shown in the context of a viral infection or MHC priming, but there is also growing evidence to support the notion of cytokine-induced memory-like NK populations [67]. Studies have shown that when NK cells are stimulated with innate cytokine combinations (IL-12+15, IL-12+18, or IL-15+IL-18) they produce increased levels of IFNγ upon restimulation with the same innate cytokines. Moreover, both CD56^{bright}CD16$^-$ and CD56^{dim}CD16$^+$ NK cells exhibited this proliferation and increase in cytokine production upon secondary stimulation [68]. Importantly, these NK cells do not exhibit increased cytolytic potential upon re-stimulation, only increased cytokine production [69].

In addition to the cytokine-induced NK cells demonstrating "memory-like" characteristics, the FcRγ$^-$ NK cells also demonstrate similar "memory-like" features [70]. It has been shown that FcRγ$^-$ NK cells clonally expand in response to HCMV-infected cells in an antibody-dependent manner. These expanded cells exhibit increased cytokine production, as well as enhanced CD16 responses. This expansion, however, only takes place in HCMV seropositive individuals, emphasizing the "memory-like" features of these cells and their HCMV association [70].

1.4 Using IL-18 Responsiveness to Characterize NK Cell Subsets

NK cells differentiate into unique roles in the immune response, with each subset expressing a different phenotype. The two "memory-like" NK cell subsets described have been the NK cytokine-induced "memory-like" cells and the FcRγ⁻ cytolytic "memory-like" NK cells. These subsets represent different functionalities of the NK cell population, and the distinction of these populations can be made based on their responsiveness to innate cytokine, IL-18 [59]. IL-18 is a pro-inflammatory cytokine that is made by DCs in the immune environment in response to tissue damage, infection, or transformation [71]. As mentioned previously, IL-18 can be used to prime NK cells to acquire their helper-like phenotype, which includes the downregulation of CD16 and upregulation of CD83, CD25, and CCR7 [34]. However, FcRγ⁻ NK cells do not downregulate their CD16 expression upon IL-18 stimulation and display a lack of IFNγ production, and, therefore, are considered unresponsive to IL-18 [59]. This difference in IL-18 responsiveness between the cytokine-induced NK cells and FcRγ⁻ NK cells is a signature functional trait that can be used to clearly distinguish these two populations *in vitro*.

However, even with the distinguishing differential responsiveness to IL-18, the mechanism of how the IL-18-responsive cells undergo these phenotypical and functional changes is still not greatly understood. One proposed mechanism of responsiveness to IL-18 is the presence of IL-18Rα, where it was shown that FcRγ⁻ NK cells have a diminished expression of IL-18Rα, partially explaining why these cells fail to respond to IL-18 [59].

1.5 ADAM17 and NK Cells

ADAM17 is a TNFα-converting enzyme (TACE) that plays a role in ectodomain shedding and is present on most leukocytes [72, 73]. ADAM17 has been well-studied in neutrophils where it has been shown to cleave TNFα, CD62L, and CD16B [74-77]. More recently, ADAM17 has been looked at in the context of NK cells [78]. Studies have found that ADAM17 is also involved in the cleavage of CD16A on NK cells. Moreover, it was shown that ADAM17 inhibition on NK cells led to increased cytokine production, such as IFNγ, after engagement with CD16A through ADCC-dependent mechanisms [74]. More recently, it was shown that IL-18 stimulation of NK cells plays a role in increasing the surface expression of ADAM17 on NK cells [78]. These characterized mechanisms of ADAM17 have been used to study the effects of ADAM17 blockade in cancer immunotherapy to increase the ADCC potential of the NK cells in response to antibody therapies, but the systemic effects of the blockade are still unknown [79]. In addition, there is still a lack of information related to the functional mechanisms of ADAM17 and its effects on NK cells, specifically regarding how it might contribute to the differentiation of cytokine-induced "memory-like" NK helper cells and FcRγ^- "memory-like" cytolytic NK cells.

2.0 Specific Aims

Despite viral suppression on long-term ART, PLWH still exhibit significant aspects of immune dysfunction. This immune dysfunction is highlighted by the NK cell population where NK cells get terminally differentiated and exhibit various effector functions. These two major differentiated NK populations, the cytokine-induced "memory-like" NK helper cells and FcRγ⁻ "memory-like" cytolytic NK cells, are typically distinguished by their responsiveness to innate cytokine, IL-18. Although IL-18 is capable of phenotypically and functionally distinguishing these populations *in vitro,* the exact functional mechanism of how these populations differentiate *in vivo* remains unknown. *Therefore, I hypothesize that the TACE, ADAM17, plays a functional and mechanistic role in the distinct differentiation of the cytokine-induced "memory-like" NK helper cells and FcRγ⁻ "memory-like" cytolytic NK cells.* To test this hypothesis, I propose the following aims:

Aim 1: Define the role of ADAM17 on IL-18-induced differentiation of "memory-like" NK cells.

    A. Verify the selective loss of CD16 expression in conventional FcRγ⁺ NK cells, and not FcRγ⁻ cells, induced by IL-18 co-stimulation.

    B. Characterize transcriptional changes in NK cells associated with IL-18 co-stimulation using single cell RNAseq.

    C. Determine the impact of ADAM17 inhibition on IL-18 modulation of CD16 and helper marker surface expression on responsive NK cells.

Aim 2: Determine the role of ADAM17 on IL-18-induced "memory-like" NK cell helper activity.

A. Characterize the helper cytokine production profile of IL-18-treated NK cells co-stimulated with various innate cytokines.

B. Determine the impact of ADAM17 inhibition on the helper cytokine profile of IL-18 co-stimulated NK cells.

C. Assess the impact of ADAM17 inhibition on IL-18-mediated NK cell activation of DCs through DC phenotyping and DC cytokine production.

3.0 Materials and Methods

3.1 Cell Culture

Daudi and K562 cells were cultured in RPMI (Gibco) and IMDM (Gibco) containing 10% fetal bovine serum (Gemini), 1% Gentamicin (Gibco), and 0.5% GlutaMAX (Gibco) (cRPMI, cIMDM), maintained at 37°C in 5% CO_2. Mel-285 cells were similarly maintained but cultured in IMDM (Gibco) containing 10% fetal bovine serum (Gemini), 1% Gentamicin (Gibco), and 0.5% GlutaMAX (Gibco). In addition, Mel-285 were passaged with 1X PBS (Cytiva) and 0.25% Trypsin-EDTA (Gibco).

3.1.1 Isolation of Monocytes and Peripheral Blood Lymphocytes (PBLs)

PBMC were isolated from the whole blood of participants from the MACS/WIHS Combined Cohort Study (MWCCS) by a density gradient separation using lymphocyte separation medium (Corning). PBMCs were then separated into monocytes and PBLs using human CD14 MicroBeads (Miltenyi Biotec).

3.1.2 Isolation of Natural Killer (NK) Cells from PBLs

NK cells were purified from PBLs by two different separation techniques. The first technique utilized a magnetic bead negative selection NK cell enrichment kit on previously isolated PBLs (STEMCELL EasySep™). The second technique involved further separating the

PBLs into NK cells by negative selection using human CD3 and CD19 MicroBeads (Miltenyi Biotec).

3.1.3 Generation of Monocyte-Derived Immature DCs (iDCs)

To generate iDCs, isolated monocytes were cultured for six days in 24-well plates (Costar) at a concentration of 7×10^5 cells/well in cRPMI. Cultures were maintained with GM-CSF (1000 IU/ml; Sanofi-Aventis) and IL-4 (1000 IU/ml; R&D Systems).

3.1.4 Inhibition of DC Maturation by NK Cells

Autologous isolated NK cells were added directly to the day 6 iDC cultures at a density of 2 million cells/ml. Following the addition of NK cells, an anti-TACE/ADAM17 antibody (6μg/ml; clone D1(A12), Millipore Sigma) or IgG isotype antibody (6μg/ml; clone MOPC 21, Sigma) was incubated at 37°C in 5% CO_2 with the co-cultures for 30 minutes. Following the 30-minute incubation, co-cultures were cultured for an additional 48 hours in the presence or absence of NK innate stimuli. The NK cell innate stimuli were IL-18 (1μg/ml, MBL International) and IL-12p70 (20 ng/ml, R&D Systems). After innate stimulation, DCs were harvested, washed, and plated for phenotypical and functional analysis using flow cytometry and an IL-12p70 producing ELISA. To analyze the DC's ability to produce IL-12p70, harvested DCs were plated onto a 96-well flat-bottom plate (Costar) at 3.0×10^4 cells/well with or without the addition of recombinant human (rh) soluble CD40L (hereinafter referred to as CD40L; 0.1mg/ml; Enzo Life Sciences). Cultures were incubated for 24 hours after which supernatants were collected to analyze using the IL-12p70 EILSA.

3.1.5 NK Cell Cultures

Purified NK cells were cultured in cRPMI in a 96-well round bottom plate (Costar) at a density of 2 million cells/ml. NK cells were then cultured with or without an anti-TACE/ADAM17 (6μg/ml; clone D1(A12), Millipore Sigma) or IgG isotype antibody (6μg/ml; clone MOPC 21, Sigma) and incubated for 30 minutes at 37°C in 5% CO_2. After incubation, NK cells were cultured for 48 hours in the presence of various innate stimuli. Innate stimulation included combinations of IL-18 (1μg/ml, MBL International), IL-12p70 (20 ng/ml, R&D Systems), IL-2 (6000 IU/ml, Prometheus Laboratories) and IL-15 (20 ng/ml, R&D Systems). After 48 hours, NK supernatants were collected and analyzed using the MESO Scale Diagnostics (MSD) U-Plex Assay Platform to measure various levels of cytokine production. In addition, NK cells were stained for flow cytometry to analyze their phenotypical profiles.

3.2 Antibody Dependent Cellular Cytotoxicity (ADCC) and Kill Assay

Purified effector NK cells were analyzed for their ADCC and killing abilities against a variety of target cells in a co-culture. ADCC and killing were measured by evaluating the depolarization of the target cells' mitochondrial membranes through the use of a fluorescent dye, MitoProbe^{TM} JC-1 (hereinafter referred to as JC-1; Thermo Fisher Scientific).

3.2.1 ADCC Assay Preparation

Activated NK effector cells were prepared by using purified NK cells and plating them on a 24-well plate (Costar) at a density of 2 million cells/ml and stimulating the cells with IL-2 (1000 IU/ml, Prometheus Laboratories) in cRPMI for 24 hours. After the 24 hour incubation, the effector NK cells were plated on a 96-well flat bottom plate (Costar) at a two-fold serial dilution starting at a density of 100,000 cells/well. The Mel-285 cell line was used as the target cell and prepared at a density of 1 million cells/ml. The JC-1 dye ($2\mu M$, Thermo Fisher Scientific) was added to the target cells and incubated for 25 minutes at 37°C in 5% CO_2. In addition, a mitochondrial membrane disrupter, CCCP ($50\mu M$, Thermo Fisher Scientific), was added to target cells as a positive control.

After the 25 minute incubation, target cells were washed with warmed PBS. Following the wash, an anti-ganglioside GD3 antibody (1 mg/ml, clone R24, Abcam) was added to the target cells and incubated for 20 minutes at 37°C in 5% CO_2. Following the 20 minute incubation, target cells were washed with PBS and resuspended in cRPMI. Target cells were plated on the 96-well plate with the effector cells at a density of 10,000 cells/well in an effector-to-target ratio of 10:1, 5:1, 2:1, 1:1. The 96-well plate was then centrifuged at 1000 RPM for 2 minutes and incubated at 37°C in 5% CO_2 for 4 hours before the readout.

3.2.2 ADCC Assay Readout

After the target and effector cells are incubated, the fluorescence of the JC-1 dye in the target cells is measured using the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific). ADCC activity was measured by calculating the ratio of red fluorescence (excitation:

550nm and emission: 600nm) to green fluorescence (excitation: 550nm and emission: 535nm) of the target cells. A smaller red-to-green ratio indicated more cell death of target cells.

3.2.3 Target Only Kill Assay Preparation and Readout

Various target cell lines including, Daudi, K562, and Mel-285 were prepared at a concentration of 1 million cells/ml. The JC-1 dye (2µM, Thermo Fisher Scientific) was added to the target cells and incubated for 25 minutes at 37°C in 5% CO_2. In addition, a mitochondrial membrane disrupter, CCCP (50µM, Thermo Fisher Scientific), was added to target cells as a positive control. After the 25 minute incubation, target cells were washed with warmed PBS and plated on a 96-well plate at a two-fold serial dilution starting at a density of 100,000 cells/well. The 96-well plate was then centrifuged at 1000 RPM for 2 minutes and incubated at 37°C in 5% CO_2 for 4 hours. Following the 4 hour incubation, TritonX-100 (Sigma) was added as an additional positive control. The fluorescence of the target cell assay was read using the Varioskan LUX plate reader (*see ADCC Assay Readout).*

3.3 Single Cell RNA and Abseq Multiomics

Transcriptional and protein expression profiles of differentially activated NK cells were found using the BD Rhapsody[TM] Single-Cell Analysis System. Bioinformatic analysis was done utilizing R programming language and platform.

3.3.1 Single Cell Sample Preparation

Isolated NK cells from PBMC of n = 2 MWCCS donors were stimulated with a variety of innate cytokine stimulation for 6 hours before continuing to cell harvest. Stimulation included combinations of IL-12p70 (20 ng/ml, R&D Systems), IL-18 (1μg/ml, MBL International), and IL-15 (20 ng/ml, R&D Systems). After the 6 hour stimulation, NK cells were harvested and stained for Abseq antibodies CD3 (clone SK7, BD^{TM}), CD16 (clone 3G8, BD^{TM}), and CD56 (clone NCAM16.2, BD^{TM}). In addition to Abseq antibodies, NK cells were stained with a BD^{TM} universal antibody-oligo that contains a unique barcode used to sample tag each NK cell stimulation condition. Following cell labeling, each stimulation condition was equally pooled to create one sample of NK cells to be loaded on a BD^{TM} Rhapsody cartridge. After cell loading, RNA transcripts were captured following the BD^{TM} Rhapsody Single-Cell Analysis System protocol, and whole transcriptome cDNA libraries were made and amplified using reverse transcription and PCR. Whole transcriptome and Abseq sequencing were done at Emory Yerkes National Primate Research Center Genomics Core on the Illumina NovaSeq6000. Sequencing read quality, alignment, and annotation were done using the BD^{TM} Rhapsody WTA Analysis Pipeline on the Seven Bridges Genomics Platform. Sequencing data stored as a Seurat object was then imported into R Studio version 9.1. Further quality control was done to remove multiplets and filter for only cells with <25% mitochondrial reads. Following this filtering, N= 20,022 cells from n=2 samples were analyzed for their differential gene expression. The SCpubr package, version 1.1.2, was used to create a dimensional reduction plot (DimPlot) to visualize the cells in a dimensional reduction embedding [80]. In addition, SCpubr was used to create dot plots and volcano plots to visualize the highly differentiated expressed genes in each stimulation condition of the NK cells.

3.3.2 Single Cell Statistical Analysis

Normalization and differential gene expression on the single cell multiomic data were analyzed using Seurat::SCTransform and Seurat:: FindAllMarkers in the Seurat package version 5. Seurat:: FindAllMarkers differential gene expression was performed with a non-parametric Wilcoxon rank sum test [81].

3.4 Flow Cytometry

PBMC and isolated NK cells and DCs were phenotyped by immunostaining through combinations of various antibody stains. The viability of the cells was checked using the LIVE/DEAD^{TM} Fixable Aqua stain (Thermo Fisher Scientific). After staining for viability, the following antibodies were used for staining in FACS buffer with 1X PBS, 0.5% BSA (Sigma), and 0.1% sodium azide (Sigma): CD3-APC-H7 (clone SK7, BD Biosciences), CD16-PerCP-Cy^{TM}5.5 (clone 3G8, BD Biosciences), CD25-BV711 (clone 2A3, BD Biosciences), CD28-PE (clone CD28.2, Biolegend), CD56-PE-Cy7 (clone N901, Beckman Coulter), CD83-PE (clone HB15a, Beckman Culter), CD86-PE (clone HA5.2B7, Beckman Coulter), CD169-PE (clone 7-239, Biolegend), HLA-DR-PE (clone TU36, BD Biosciences), CCR7-APC (clone 150503, R&D Systems), anti-hTACE/ADAM17 (clone 111608, R&D Systems) and F(ab') anti-mouse IgG-APC (eBioscience). Following surface staining with these antibodies, intracellular staining with an FcRγ-FITC (Milli-Mark®) antibody was done by permeabilizing the cells with the Cytofix/Cytoperm^{TM} Fixation/Permeabilization kit from BD Biosciences. The stained samples

were fixed in 2% PFA (Sigma) and analyzed on the BD LSRFortessa^{TM} cytometer. Flow cytometry data was analyzed using FlowJo version 10.10.

3.5 MSD U-Plex Assay

The supernatants from various cell cultures were collected and analyzed for their cytokine production using the MSD U-Plex Assay Platform. A 96-well plate density was used, and the following cytokine analytes were measured according to the manufacturer's instructions: human IFN-γ, IL-10, IL-12p70, IL-13, IL-23, IL-6, IP-10, MIP-3b, MIP-3a, and TNF-α. Data was acquired on the MESO QuickPlex SQ 120 plate reader and cytokine concentrations were found using the standard curve generated through the MSD Discovery Workbench version 4.0 software.

3.6 IL-12p70 ELISA

The supernatants from DC co-cultures with ADAM17-inhibited NK cells were collected and analyzed for their IL-12p70 production after incubation with CD40L. Supernatants from n=2 DC cultures were analyzed.

3.6.1 ELISA Plate Preparation

A 96-well EIA plate (Costar) was coated in 100µl of 5µg/ml primary IL-12p70 capture antibody (Invitrogen) and incubated at room temperature overnight, covered from light. After the

overnight incubation, the plate was blocked with assay buffer (powdered BSA in 1X PBS) for one hour at room temperature then washed with assay wash buffer (5% TRIS and 0.2% Tween®20 in 1L of Millipore water).

3.6.2 ELISA Assay Procedure

Once the plate was washed, CD40L-stimulated and unstimulated DC supernatants were plated in triplicate at 50µl/well. An 8-point standard curve was generated by plating 50µl of a 1:2 serial dilution of the IL-12p70 standard concentration (R&D Systems). The highest standard concentration was 5000 pg/ml, and the lowest concentration was 39.06 pg/ml. The samples and standards were then incubated at room temperature for 1 hour. After incubation, the secondary, detection antibody (Thermo Fisher Scientific) was diluted in assay buffer, and 50µl was added to each well on the plate and incubated at room temperature for an additional 1 hour. The plate was then washed and 100µl of 1:10 diluted horseradish-peroxidase (HRP)-conjugated streptavidin (Thermo Fisher Scientific) in assay buffer was added to each well and incubated at room temperature for 30 minutes. A final wash was done and 100µl of tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific) was added to each well. After 5-7 minutes, 100uL of ELISA Stop solution (Thermo Fisher Scientific) was added to each well. Following the addition of the stop solution, the plate absorbances were read on the Varioskan LUX Multimode Microplate Reader, and sample IL-12p70 concentrations were determined based on the standard curve.

4.0 Results

4.1 Aim I: Define the Role of ADAM17 on IL-18-Induced Differentiation of "Memory-Like" NK Cells

4.1.1 The Selective Loss of CD16 Expression in Conventional FcRγ⁺ NK Cells, and not FcRγ⁻ Cells, is Induced by IL-18 Co-Stimulation

In typical $CD56^{dim}CD16^+$ NK cell populations, IL-18 stimulation promotes the downregulation of surface molecule CD16 (Fig. 4A). This downregulation is driven by co-stimulation with IL-18. Stimulation with only one innate cytokine, such as IL-12, does not drive downregulation of CD16. Stimulation with only IL-18 drives some of the CD16 downregulation, but when stimulated together, IL-12 and IL-18 push the NK cells to a complete loss of CD16 expression (Fig. 4B) [59]. The NK cell populations that exhibit close to complete loss of CD16 are made up of mostly FcRγ⁺ cells. However, in NK cell populations where there is a high proportion of FcRγ⁻ cells, the same CD16 downregulation does not occur. When NK cells stimulated with innate cytokine combinations do not exhibit significant CD16 downregulation, the population still expressing surface CD16 is characterized by containing a high proportion of FcRγ⁻ cells. On the other hand, the stimulated population that does downregulate CD16 contains a minimal proportion of FcRγ⁻ cells and a majority of FcRγ⁺ cells (Fig. 4C-D).

Selective loss of CD16 on NK cells is a defining feature of these cells, and the FcRγ⁻ cells that do not downregulate CD16 exhibit a unique phenotype not typically seen in NK cell populations. Understanding the mechanism of FcRγ⁻ NK cells' unresponsiveness to innate

cytokines will allow for a better understanding of how these cells function in the immune environment and how they affect the overall immune response.
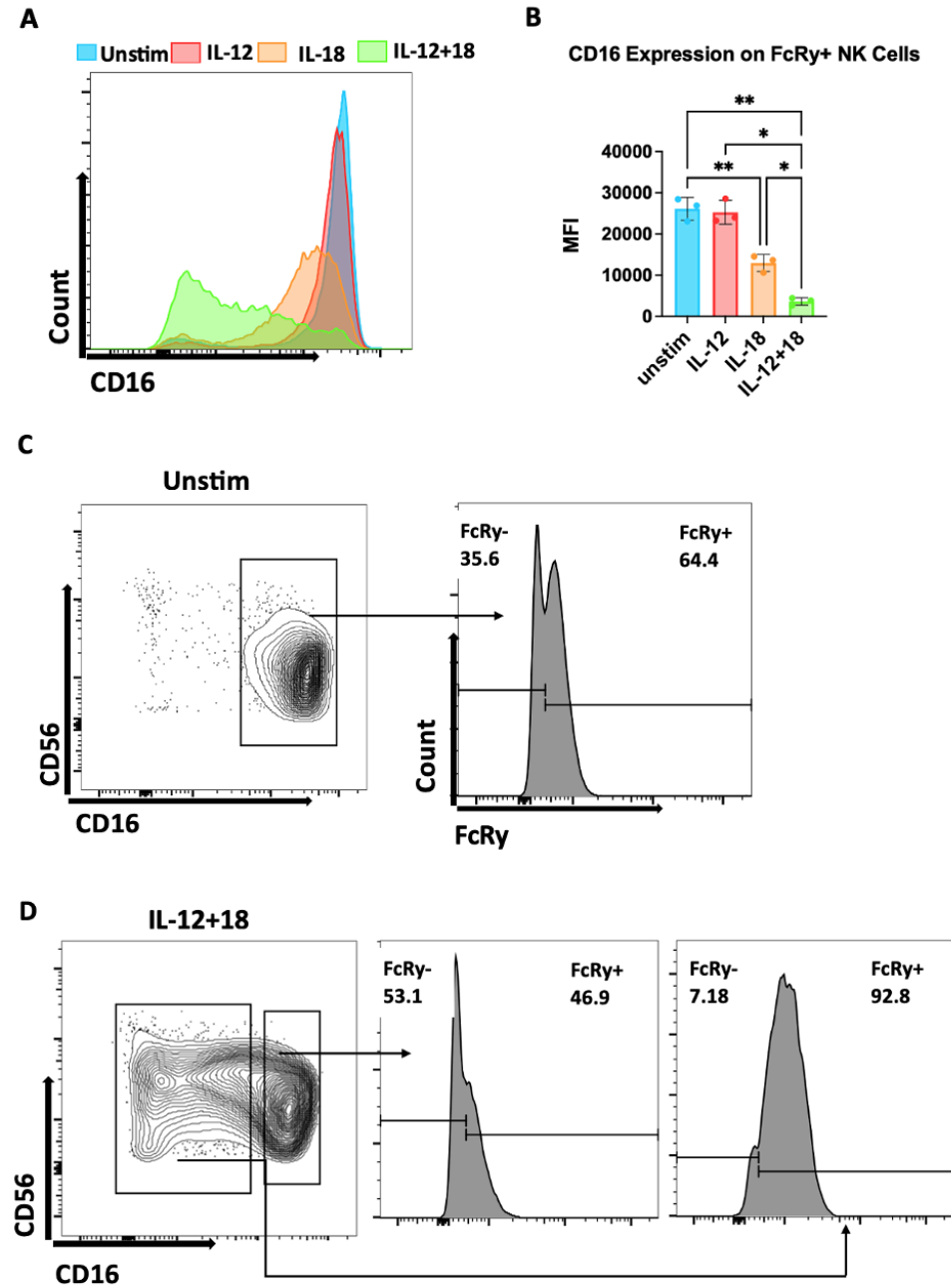

Figure 4. FcRy- NK cells are unresponsive to IL-18 stimulation

NK cells were stimulated with various combinations of IL-12 and IL-18 for 48 hours and then analyzed by flow cytometry for the presence of different phenotypical markers. **(A-B)** NK cells with a high proportion of FcRγ$^+$ show almost complete downregulation of CD16 upon stimulation with IL-12 and IL-18 but do not show the same downregulation with only one cytokine. **(C-D)** NK populations with a high proportion of FcRγ$^-$ cells do not exhibit the same CD16 downregulation when stimulated with IL-12 and IL-18. Statistical significance was determined using a one-way ANOVA (*p=0.05*).

4.1.2 Transcriptional Changes in NK Cells Associated with IL-18 Co-Stimulation are

Confirmed Using Single Cell RNAseq

As previously shown, innate cytokine IL-18 can be used to differentiate the cytokine-induced "memory-like" NK helper cells and the FcRγ$^-$ "memory-like" cytolytic NK cells [59]. The differentiation of these NK subsets has been characterized by surface expression loss of CD16, as well as increased surface expression of various helper receptor molecules, such as CD25 and CD83 [34]. Therefore, to better describe the functional changes that occur in NK cells when differentiated using IL-18, single cell transcriptomics was performed on unstimulated NK cells along with NK cells stimulated with various forms of innate cytokines for a total of six hours.

Stimulation with the various innate cytokines revealed distinct transcriptional clusters defined by their unique differential gene expression (Fig. 5A). Specifically, when looking at the cells that were stimulated with IL-18 in combination with IL-15 there is a significant increase in the percent of cells that express helper markers, CD25, IFNγ, CCL3, and CD83 (Fig. 5B.). This increase in helper markers is only seen in the co-stimulated conditions emphasizing the role that IL-18 plays in differentiating NK cells to a helper-like phenotype. Moreover, the same increase in helper markers is seen in stimulation conditions with IL-18 in combination with IL-12, further emphasizing the effect of IL-18 in NK differentiation (Fig. 5C).
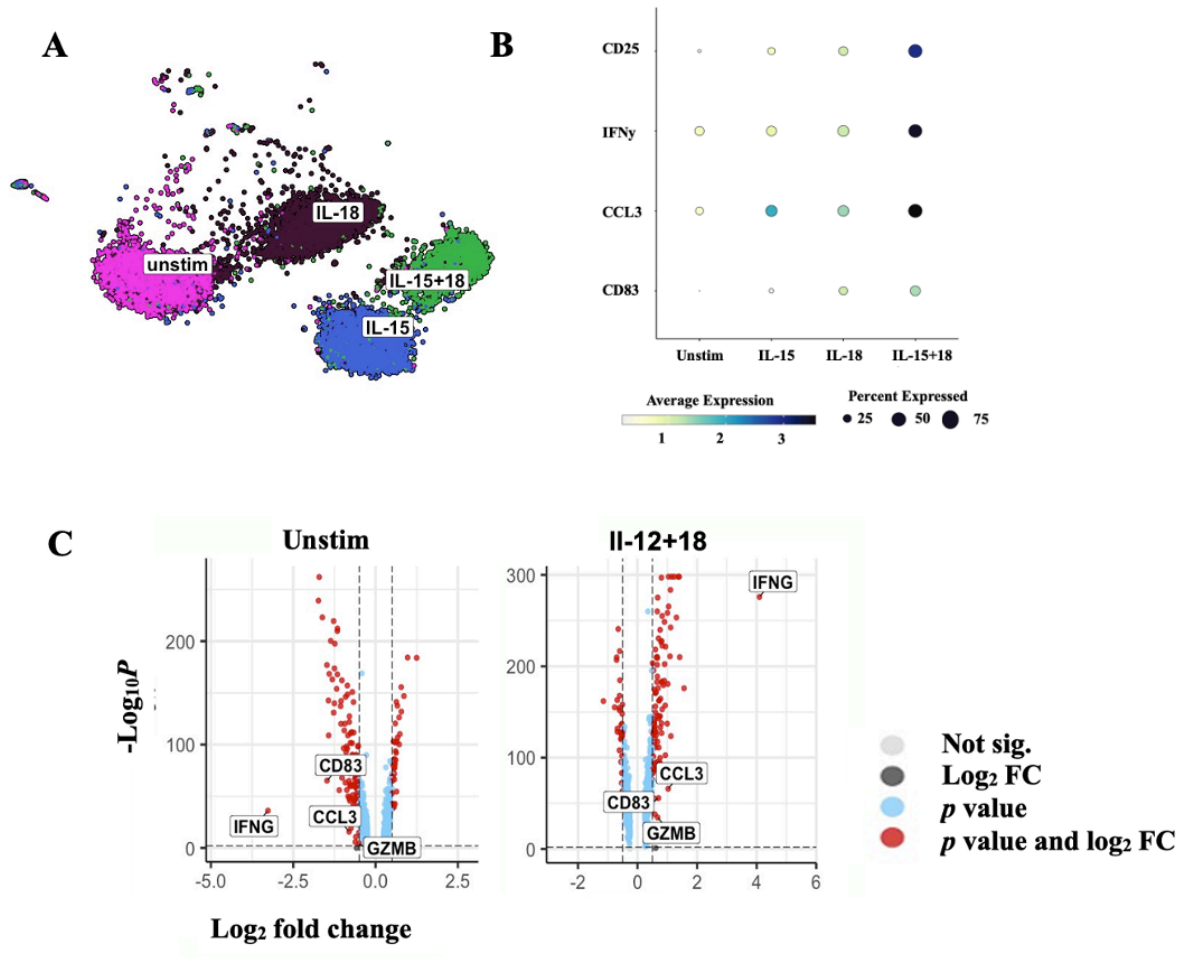
Figure 5. IL-18 drives helper NK cell transcriptional profile

NK cells were stimulated for 6 hours in the presence or absence of various innate cytokines and then captured for single cell analysis using the BD^{TM} Rhapsody Protocol. **(A)** NK cells stimulated with various innate cytokines reveal distinct transcriptional clusters. **(B)** Differential gene expression shows NK cells stimulated with IL-18 in combination with IL-15 exhibit an increase in helper molecules and **(C)** the same increase in helper molecules is seen in stimulation with IL-18 and IL-12. The Dim plot, dot plot, and volcano plot were made using the SCpubr package and statistical analysis was determined using a non-parametric Wilcoxon rank sum test. Fold changes were determined by comparing the gene expression of group A (specified stimulation condition) to the gene expression of group B (the other 3 stimulation conditions combined) using Seurat::FindMarkers.

4.1.3 The Role of ADAM17 Inhibition on IL-18 Modulation of CD16 and Helper Marker Surface Expression on Responsive NK cells

ADAM17 has been implicated in the shedding of CD16B in neutrophils and CD16A in NK cells [74, 77]. To confirm the role of ADAM17 in a population of FcRγ$^+$ NK cells, an ADAM17 inhibitor was used to block its activity in culture. Once the inhibitor was added, the FcRγ$^+$ NK cells were stimulated with both IL-12 and IL-18. Typically, this stimulation condition will lead to a complete loss of CD16 on the NK cells. However, when ADAM17 is inhibited, the stimulated NK cells show only a minor loss of CD16 and phenotypically resemble unstimulated NK cells more than the IL-12 and IL-18 stimulated NK cells (Fig. 6A). This confirmed the role ADAM17 is playing in the CD16 downregulation in cytokine-induced "memory-like" FcRγ$^+$ NK cells, and with this understanding, the next question to be answered is the role of ADAM17 in FcRγ$^-$ NK cells. As stated earlier, FcRγ$^-$ NK cells are unresponsive to IL-18 and therefore do not downregulate their CD16 molecule, despite any innate co-stimulation [59].

To determine if ADAM17 is playing any role in FcRγ$^-$ NK cells not downregulating CD16, the first thing characterized was the presence of ADAM17 on both FcRγ$^+$ and FcRγ$^-$ NK cells. If the FcRγ$^-$ NK cells are unable to downregulate CD16, then perhaps they are missing their ADAM17 molecule altogether and when stimulated with IL-18, there is no ADAM17 available to cleave CD16. However, when NK populations with proportions of both FcRγ$^+$ and FcRγ$^-$ cells are stained for the presence of ADAM17, there is no significant difference in the presence of ADAM17 between the two populations (Fig. 6B). Therefore, if ADAM17 is playing a role in the unresponsiveness of FcRγ$^-$ cells, it is not due to the presence or absence of the molecule, but rather

a mechanistic role that could be occurring intracellularly, and potentially due to interactions with innate cytokine IL-18.

In addition to the role of ADAM17 on CD16 surface expression, it was also determined if ADAM17 inhibition on IL-18-induced phenotypes revealed phenotypical differences on IL-18-responsive FcRγ⁺ NK cells. IL-18 has been found to induce a helper-like role in FcRγ⁺ NK cells, in addition to driving their CD16 downregulation [34]. Therefore, to determine the impact of ADAM17 on the surface molecules that characterize this helper phenotype, ADAM17 was inhibited on the FcRγ⁺ NK cells and then stimulated with IL-12 and IL-18. ADAM17 inhibition revealed no difference in the presence of CD83 on the stimulated cells, but a slight increase in the presence of CD25 (Fig. 6C).

The downregulation of CD16 due to ADAM17 lends to ADAM17 driving a helper-like role in the NK cells. However, inhibition of ADAM17 also leads to an increase in CD25 which alludes to a more cytolytic role of ADAM17 when not inhibited. Therefore, taken together, these results further stimulate the question of ADAM17's role in differentiating "memory-like" NK cells down a helper or cytolytic pathway.
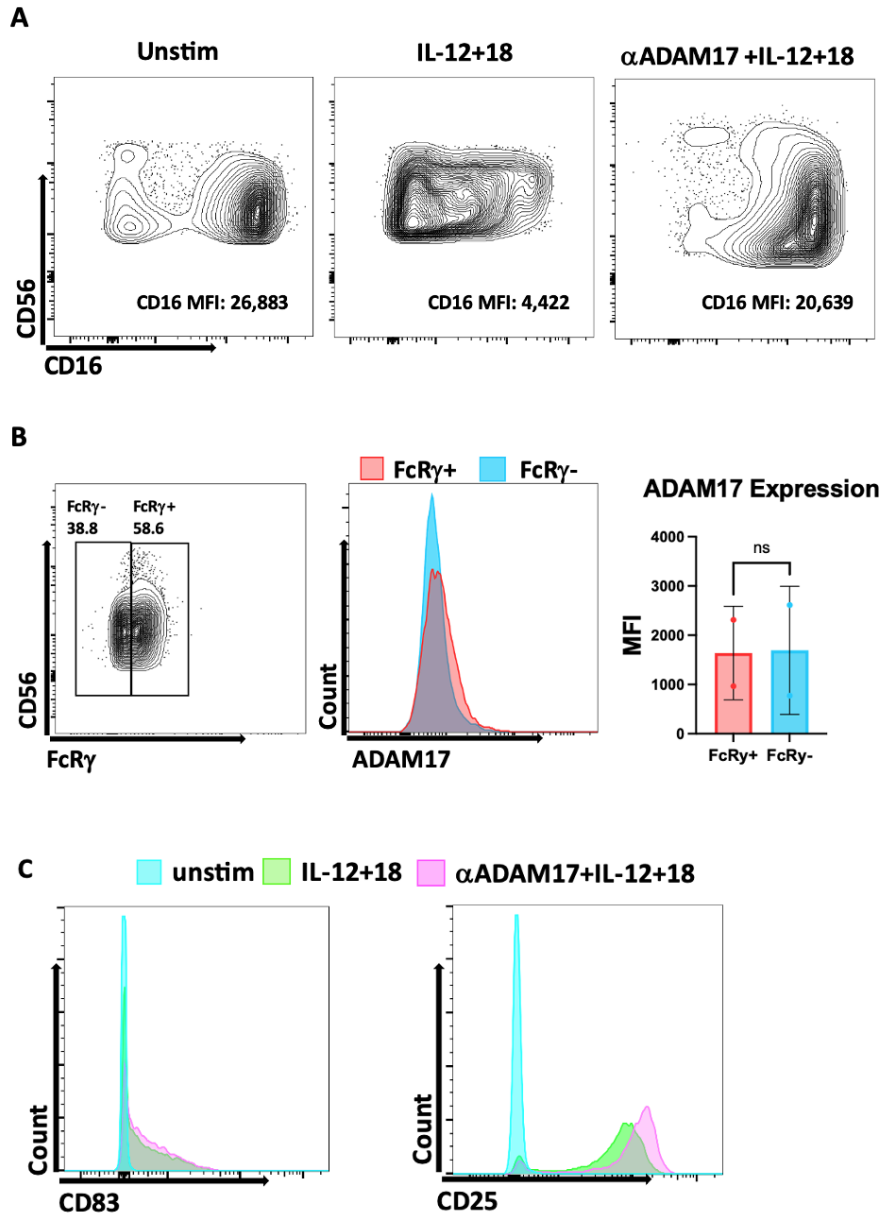
Figure 6. ADAM17 drives phenotypical changes in FcRy+ NK cells

NK cells were inhibited with an anti-TACE molecule and then stimulated for 48 hours with IL-12 and IL-18 for phenotypical analysis using flow cytometry. **(A)** Inhibition of ADAM17 on stimulated NK cells revealed no CD16 downregulation. **(B)** No significant difference in the presence or absence of ADAM17 was observed in the FcRγ$^+$ vs the FcRγ$^-$ NK cell populations. **(C)** ADAM17 inhibition led to an increase in CD25 on stimulated NK cells, but no difference in CD83. Statistical significance was determined using a one-way ANOVA (*p=0.05).*

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4.2 Aim II: Determine the Role of ADAM17 on IL-18-Induced "Memory-Like" NK Cell Helper Activity

4.2.1 Characterize the Helper Cytokine Production Profile of IL-18-Treated NK Cells Co-Stimulated with Various Innate Cytokines

IL-18-responsive NK cells are able to differentiate to a helper-like phenotype when stimulated with various innate cytokines [34, 82]. This helper-like phenotype is characterized by the NK cells' ability to produce different cytokines that interact and educate the immune environment. To better characterize the helper role that these NK cells play in the immune response, NK cells were stimulated with various combinations of innate cytokines, driven by co-stimulation with IL-18. After stimulation, supernatants from the culture were analyzed for NK cell production of numerous cytokines. Analysis of NK cell cytokine production showed that IL-18 co-stimulation with either IL-2, IL-12, or IL-15 results in high levels of type one cytokine, IFNγ (Fig. 7A). The increased IFNγ levels were dependent only on IL-18 co-stimulation and not as much on the other innate cytokine it was stimulated with. NK cell production of TNFα was also increased in all IL-18 co-stimulated conditions but was the highest in conditions stimulated with IL-2 and IL-18 (Fig. 7B). Interestingly, IL-18 co-stimulated NK cells were able to produce increased levels of type two cytokine, IL-13, but this production was only seen in stimulated condition with IL-2 or IL-15 (Fig. 7C).

The increase in NK cell cytokine production upon innate stimulation verifies the role NK cells play in providing a helper-like role in the immune environment. Moreover, these results indicate that not only are NK cells capable of providing immune help, but they can be differentiated to create a type one or type two immune response dependent on their innate stimulation. This

model of differentiation closely resembles the T-helper cell dogma of differentiation where T-helper cells are capable of differentiating down a Th1 or Th2 immune response [83]. Similar to T-helper cell differentiation, NK cell helper differentiation seems to be dependent on the innate cytokines they are co-stimulated with. IL-18 co-stimulation with IL-12 results in high levels of IFNγ and low levels of IL-13 resembling a type one immune response. However, IL-18 co-stimulation with IL-2 or IL-15 results in high levels of IL-13 resembling more of a type two response. Further characterizing the functionality of these differentiated NK cells will allow for a better understanding of the role these various NK cell subsets play in the immune response.
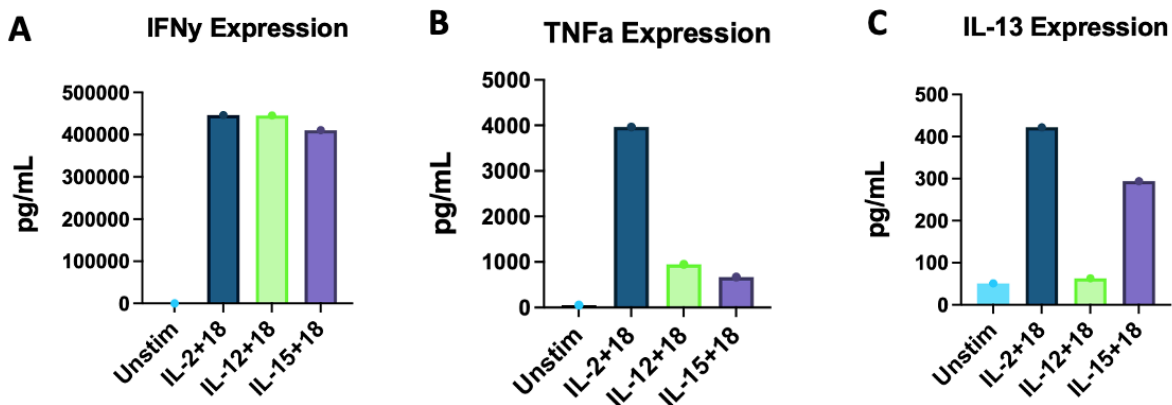
Figure 7. NK cell type 1 and type 2 cytokine production based on innate co-stimulation

NK cells were stimulated with various combinations of innate cytokines for 48 hours and supernatants were harvested for analysis on the MSD platform. **(A)** Co-stimulation with IL-18 and any other innate cytokine results in high levels of type one cytokine, IFNγ. **(B)** Co-stimulation with IL-18 leads to increased levels of type one cytokine TNFα, with the highest levels seen in IL-2 and IL-18 co-stimulation. **(C)** Only IL-18 co-stimulation with either IL-2 or IL-15 leads to the production of type two cytokine IL-13.

4.2.2 Determine the Impact of ADAM17 Inhibition on the Helper Cytokine Profile of IL-18

   Co-Stimulated NK cells

As mentioned above, NK cells can differentiate into different subsets of helper-like cells, producing a variety of innate cytokines. In addition, ADAM17 has been implicated in the downregulation of NK surface molecule CD16, and therefore potentially the mechanism of how these NK cells respond to innate stimulation and differentiate into helper-like cells. Further, ADAM17 is known as a TNFα-converting enzyme (TACE) that cleaves TNFα to its active form on the NK cell surface [76]. Taken together, this points to ADAM17 being involved in the creation of distinct helper-like NK cell subsets. Therefore, to evaluate ADAM17's role in NK cell helper-like differentiation, NK cells were stimulated with various combinations of innate cytokines in the presence or absence of an ADAM17 inhibitor. The supernatants from the culture were analyzed for their differential cytokine production.

Analysis revealed that upon innate stimulation and ADAM17 inhibition, NK cells showed a stable level of expression of IFNγ in comparison to those only co-stimulated (Fig. 8A). However, ADAM17 inhibition did decrease the expression of TNFα by half (Fig. 8B). In addition, inhibition resulted in NK cell expression of innate cytokine IL-10. However, IL-10 was only produced in ADAM17-inhibited NK cells that were co-stimulated with IL-12 and IL-18 (Fig. 8C). Inhibition and co-stimulation with IL-2 or IL-15 and IL-18 did not result in increased levels of IL-10 (Fig. 8D).

The results of these experiments show that ADAM17 may play a role in the functional response of NK cells upon innate stimulation. Although IFNγ levels remain constant, the decrease in NK cell production of TNFα, due to ADAM17 inhibition, can have dramatic effects on NK

cells' abilities to provide immune instruction to cells in the environment, thereby greatly impacting the overall immune response. In addition, IL-10 is a pleiotropic cytokine that can have anti-inflammatory properties or immune-stimulating properties depending on the immune environment [84]. The increased expression of IL-10 when ADAM17 is inhibited points to some kind of role ADAM17 could be playing in modulating the response of NK cells to innate stimulation, and therefore the overall immune response. The induction of IL-10 by IL-12 and IL-18 and no other co-stimulation condition also points to the relationship between IL-18 and ADAM17. Being able to define the mechanism of ADAM17 in modulating NK cell responses will result in a greater understanding of how NK cells differentiate to provide immune help.
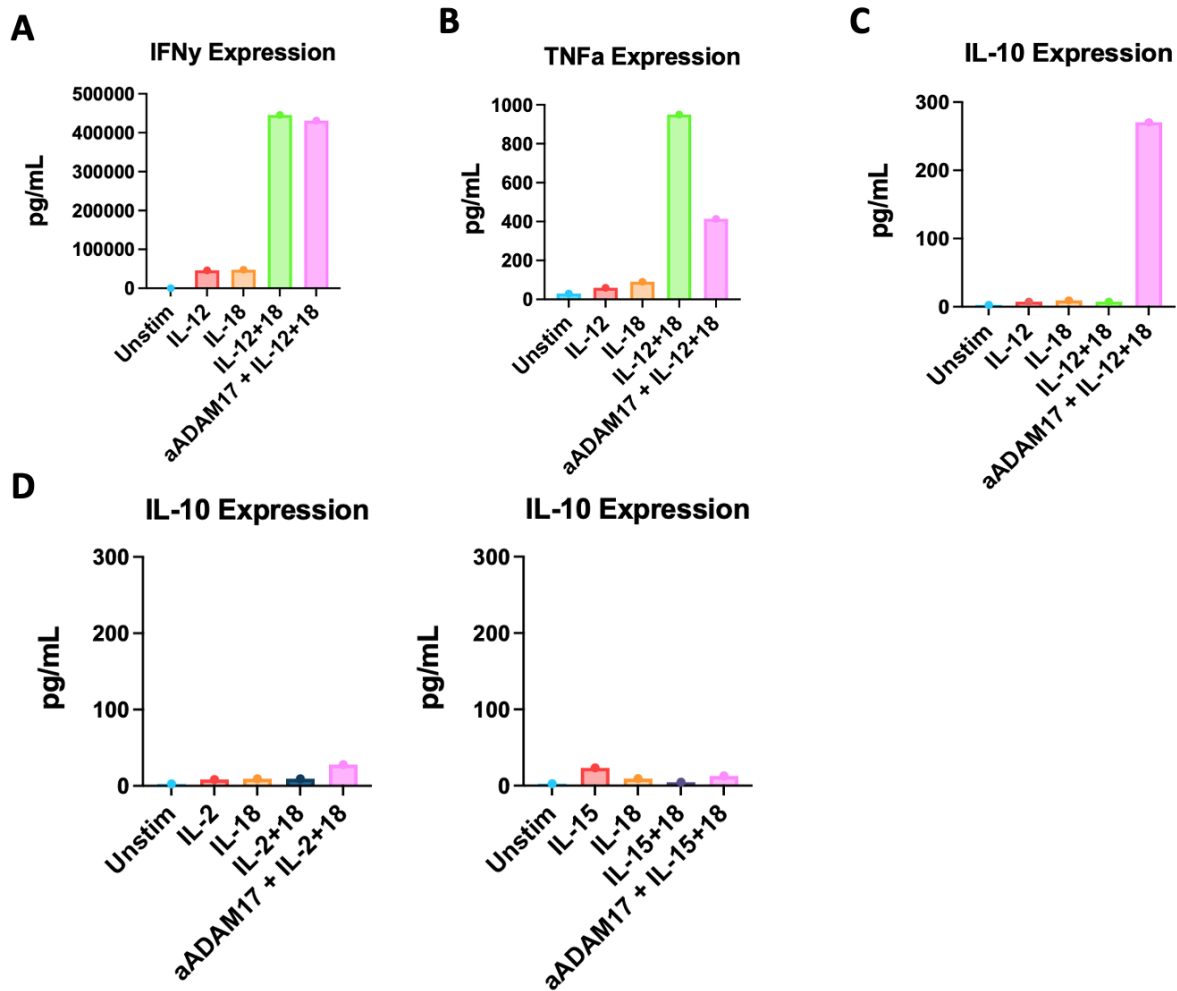
Figure 8. ADAM17 inhibition leads to changes in NK cell cytokine production

NK cells stimulated with various combinations of innate cytokines for 48 hours were inhibited with an anti-ADAM17 molecule and supernatants were collected for analysis on the MSD platform. **(A)** ADAM17 inhibition made no impact on the production of IFNγ from IL-12+18 co-stimulated NK cells. **(B)** TNFα production was decreased by half when NK cells had ADAM17 inhibited during IL-12+18 co-stimulation. **(C)** IL-12+18 co-stimulated NK cells only expressed cytokine IL-10 when ADAM17 was inhibited. **(D)** ADAM17-inhibited NK cells co-stimulated with IL-2 or IL-15+18 did not produce similar levels of IL-10 compared to IL-12+18 co-stimulated NK cells.

4.2.3 The Role of ADAM17 Inhibition on IL-18-Mediated NK Cell Activation of DCs

Characterization of ADAM17-inhibited NK cells revealed the potential mechanistic role of ADAM17 in mediating NK cell differentiation to a helper-like phenotype. When ADAM17 is inhibited, NK cells stimulated with IL-12 and IL-18 produce lower levels of TNFα, and NK cells are known to be able to polarize and mature DCs through the production of IFNγ and TNFα [31]. Therefore, if ADAM17 is inhibited, the question arises if NK cells are still capable of maturing and polarizing DCs, or if their immune crosstalk capabilities have become impaired through an ADAM17-dependent mechanism.

To try and answer this question, DCs were grown and then cultured with autologous NK cells in the presence or absence of ADAM17 inhibition, along with innate cytokine IL-12 and IL-18 co-stimulation. After two days in culture with the NK cells, DCs were harvested and analyzed for phenotypical and functional differences. The DCs' morphological changes were first assessed while still in culture, and results revealed that the DCs co-cultured in the presence of ADAM17-inhibited NK cells more closely resemble an immature phenotype characterized by a rounded shape and short dendrites, similar to the DCs cultured with unstimulated NK cells [85]. On the other hand, the stimulated NK cells that do not have ADAM17 inhibited can mature the DCs, which can be seen by the morphological changes to the cells (Fig. 9A). These morphological changes seen on mature DCs are characterized by an elongated shape and extended dendrites [85].

The immature morphological characterization of DCs in culture with ADAM17-inhibited NK cells points to these DCs not being phenotypically or functionally mature as well. To assess whether the DCs were phenotypically matured, surface marker expression was analyzed. The most notable changes to DCs cultured with ADAM17-inhibited NK cells were a slight decrease in

surface molecule CD86 and a significant increase in Siglec-1 expression (Fig. 9B-D). CD86 and Siglec-1 are both known markers seen on matured DCs [86, 87]. The slight decrease in CD86 but increase in Siglec-1 points to the idea that DCs cultured in the presence of ADAM17-inhibited NK cells could potentially still be phenotypically mature despite their immature morphological appearance.

With this information, the next step was to look to see how functionally mature and polarized the DCs were by characterizing their production of IL-12p70 in response to CD40L stimulation. For DCs to provide immune help to T-cells, they must be polarized and able to produce high levels of IL-12 which in turn activates T-cells into Th1 cells [88]. Therefore, to analyze the ability of the DCs in culture with NK cells to provide immune help to T-cells, DCs from the cultures were harvested and stimulated with CD40L before taking supernatants to analyze with an IL-12p70 ELISA. The results revealed that NK cells alone are unable to produce detectable amounts of IL-12 from the DC culture but when the NK cells are stimulated, the DCs produce high levels of IL-12. Moreover, when the DCs are cultured with ADAM17-inhibited NK cells, the levels of IL-12 decrease slightly but they still produce high amounts of the cytokine (Fig. 9D).

Taken together, the results of this experiment show the ability of ADAM17-inhibited NK cells to act on the maturation of DCs. Although the DCs cultured with ADAM17-inhibited NK cells show an immature morphology, their mature and polarized phenotypical and functional profiles contradict the morphology. Therefore, more work needs to be done to explain the mechanistic role of ADAM17 in DC maturation and its impact on T-cell activation.
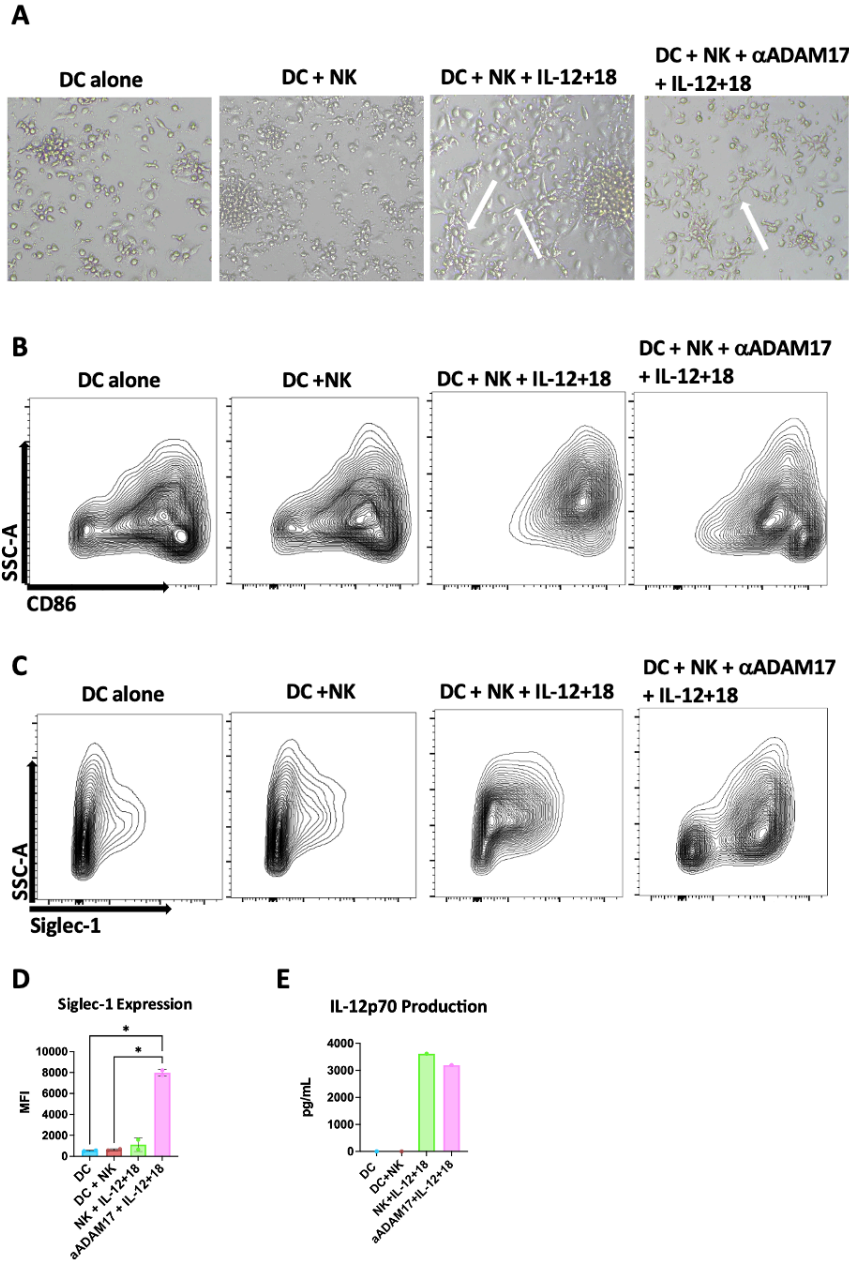
Figure 9. ADAM17 inhibition results in phenotypical changes of DCs in NK cell-mediated help

DCs were cultured for six days and matured with autologous NK cells for two days in the presence or absence of an ADAM17 inhibitor and IL-12 and IL-18. Phenotypical and functional analyses of the DCs were done using flow cytometry and an IL-12p70 ELISA on DC supernatants. **(A)** DCs cultured in the presence of NK cells and IL-12+18 show a matured phenotype with elongated dendrites whereas DCs cultured with ADAM17-inhibited NK cells resemble more of an immature DC cultured with NK cells alone. **(B)** DCs matured in the presence of ADAM17-inhibited NK cells show an increase in the presence of CD86 and **(C-D)** Siglec-1 surface expression. **(E)** DCs matured with NK cells and IL-12+18 show a slight increase in their IL-12p70 production after

stimulation with CD40L compared to those matured with ADAM17-inhibited NK cells. Statistical significance was determined using a one-way ANOVA *(p=0.05)*.

5.0 Conclusions and Discussion

Natural killer cells exhibit various functions in the immune response beyond their innate cytolytic activity. NK cells have been shown to differentiate into distinct subsets characterized by either their helper or cytolytic phenotypes, with both exhibiting "memory-like" features [34, 58, 62]. However, the mechanisms of how these NK cells can differentiate into these distinct subsets are still not fully understood. Therefore, my project focused on elucidating the mechanisms of how these subsets arise, with a focus on the TACE, ADAM17. My findings indicate that ADAM17 plays a contributing role in both NK cell differentiation as well as their function in the immune response.

First, I was able to confirm the use of IL-18 co-stimulation to drive NK cell differentiation into their previously reported NK helper cell phenotype [34, 59] (Fig. 4-5). Furthermore, I showed that when driven by IL-18, the presence of different -co-stimulators (IL-2, IL-12, or IL-15) promoted unique cytokine production profiles of the NK cells (Fig. 7). The NK cells not only acquired the basic phenotype of typical NK helper cells, but they could also be further characterized into different NK helper subsets that produced different patterns of type I or type II cytokines, analogous to that of T helper (Th) cells. Importantly, the overall character of the NK cell cytokine response was dependent on the co-stimulatory factor used with IL-18.

These findings highlight the versatile role NK cells play in the immune response, and their functional ability is more sophisticated than just being limited to their cytolytic function. The flexibility in NK cell helper function closely resembles that of Th cells or the more recently described innate lymphoid cells (ILCs). Th cells can differentiate into distinct subsets that each have a unique role to play in the immune response, such as providing pro-inflammatory, anti-

inflammatory, or even regulatory immune help to cells in the environment [89]. Furthermore, ILCs have been described as non-T cell lymphocytes that secrete various effector cytokines without having antigen specificity [90]. ILCs are characterized by their transcriptional factor profile, as well as the cytokines they produce upon stimulation. For example, ILC1s are most often described as being the most closely related to NK cells because of their Tbet transcriptional profile and production of IFNγ and TNFα. ILC2s, however, are characterized by their ability to produce cytokines such as IL-13 and IL-10 [91]. The work shown here reveals the ability of NK cells to produce a variety of different cytokines, such as IFNγ, TNFα, IL-13, and IL-10 in response to different stimulation conditions further implicating their functional similarity to other lymphocyte populations, such as Th cells and ILCs (Fig. 7 and Fig. 8). Moreover, this also suggests that the tissue-derived ILCs identified *in vivo* may originate from conventional NK cells that undergo helper differentiation through an IL-18 driven pathway, similar to what we describe *in vitro*.

After revealing the flexibility NK cells demonstrate in their ability to carry out different helper functions in the immune response, I next confirmed the presence of the rare and highly differentiated FcRγ⁻ cytolytic NK cell subset in PLWH (Fig. 4). Further, I showed that ADAM17 may be implicated in the differentiation process of this cytolytic "memory-like" subset (Fig. 6). In a typical NK cell population with a low proportion of FcRγ⁻ NK cells, the surface receptor CD16 is downregulated upon innate cytokine stimulation when IL-18 is present [21]. However, in populations with a high proportion of FcRγ⁻ cells, CD16 does not get downregulated when stimulated, and this subset of NK cells becomes highly specialized for their cytolytic and ADCC capabilities [59, 67]. The mechanism of how these FcRγ⁻ NK cells remain unresponsive to innate stimulation and maintain CD16 expression is not currently known. A TNF-converting enzyme, ADAM17, has been shown to be involved in CD16 downregulation on NK cells and neutrophils,

leading me to further assess its role in IL-18-mediated NK cell differentiation. The results here show that inhibition of ADAM17 in an NK cell population with a low proportion of FcRγ^- cells leads to the blocking of CD16 downregulation upon NK cell co-stimulation in IL-18 responsive cells (Fig. 6A). This reveals the role of ADAM17 in NK cell CD16 downregulation, further stimulating the question of whether ADAM17 or ADAM17 dysfunction is also playing a role in the IL-18 unresponsiveness of FcRγ^- cells. However, when comparing the expression level of ADAM17 on FcRγ^+ vs FcRγ^- cells, there does not seem to be a significant difference (Fig. 6B).

Taken together, these results show that ADAM17 is involved in CD16 downregulation in IL-18 responsive NK cells, but its role in FcRγ^- cells remains unknown. It is possible that although the surface expression of ADAM17 on FcRγ^+ and FcRγ^- cells is not different, ADAM17 is still playing an intracellular role in the function of FcRγ^- cells. Recent work has revealed the relationship between IL-18 stimulation and activation of ADAM17 [78]. Researchers from this study were able to demonstrate the intracellular signaling cascade that results from IL-18-induced ADAM17 activation. However, this work focused on the ADAM17-driven downregulation of CD16 in FcRγ^+ NK cells and not the implication of ADAM17 in FcRγ^- NK cells. Therefore, it remains possible that even with comparable surface expression of ADAM17, the FcRγ^- NK cells do not have a properly functioning or stimulated ADAM17 to create the same intracellular cascade that is seen in FcRγ^+ NK cells leading to CD16 downregulation.

To further elucidate the role of ADAM17 in NK cell differentiation, I showed the impact on NK cell phenotype and function when ADAM17 was inhibited (Fig. 6C and Fig. 8). If ADAM17 is involved in the downregulation of CD16, I next explored its involvement in further driving the NK cells down a helper-like path. If ADAM17 is inhibited on the NK cells, the question becomes if NK cells then show a more helper or cytolytic phenotype. The results of my experiment

show that when ADAM17 is inhibited on NK cells with a low proportion of FcRγ⁻ cells, the NK

cells will increase their expression of CD25 but maintain a similar expression of CD83 compared

to NK cells with no ADAM17 inhibition (Fig. 6C). This finding implicated ADAM17 inhibition

as driving more of a helper-like role in the NK cells given it led to an increase in the high affinity

IL-2 receptor, CD25. However, given the unchanged CD83 expression on ADAM17-inhibited NK

cells, these implications still need to be supported by further experimentation. Therefore, I next

went on to characterize the cytokine production in ADAM17-inhibited NK cells (Fig. 8).

ADAM17-inhibited NK cells produced equivalent levels of IFNγ compared to unstimulated NK

cells, however, they produced half the amount of TNFα and showed a dramatic increase in

production of IL-10. The decrease in TNFα was expected given that ADAM17 is a TNFα-

converting enzyme, so when ADAM17 is inhibited, TNFα is unable to be cleaved to its active

form as effectively as when it is present and active. The stable production of IFNγ points to

ADAM17 inhibition as still being able to drive a helper-like role of the NK cells. Moreover, the

increase in IL-10 production implicates ADAM17 inhibition as influencing and regulating the

overall helper cytokine profile of the NK cells. IL-10 has been described as a pleiotropic cytokine

capable of exerting various functions on many different cell types [84]. IL-10 can have an

inhibitory effect on T-helper cell type I cytokine production, such as IFNγ, but also is capable of

causing an enhancement in IFNγ production in the presence of IL-18, as well as upregulation in

MHC expression on cytolytic effector T-cells [92, 93]. The multifaceted functionality of IL-10

points to ADAM17 inhibition still allowing for a helper-like response of the NK cells, but in

addition, drives a regulatory role of the NK cells to promote a more specific immune response

dependent on the immune environment. This regulatory role of NK cells supports the notion

mentioned above of ADAM17 playing a role in NK cells differentiating to distinct subsets with unique roles in the immune response.

Further, the increase in IL-10 expression by ADAM17-inhibited NK cells was only seen in cells co-stimulated with IL-12 and IL-18. IL-10 production was not observed when NK cells were stimulated with IL-2 or IL-15 and IL-18 (Fig. 8). This points to a relationship between IL-12 and IL-18, as well as an interaction with ADAM17. It has previously been shown that NK cell stimulation with IL-12 leads to an increase in the NK cell IL18Rα, and NK cell stimulation with IL-18 leads to an increase in the NK cell IL12Rβ2 [59]. Moreover, studies have shown a relationship between IL-18 and ADAM17, revealing a signaling cascade between IL-18 and ADAM17 that ultimately leads to the downregulation of CD16 [78]. Put together, this implicates the potential of ADAM17 inhibition to act upon this IL-18 signaling cascade, therefore leading to a reciprocal decrease in IL12Rβ2 and IL18Rα. This decrease in functional IL-12 and IL-18 receptors could potentially explain why the cytokine profile of ADAM17-inhibited NK cells differs in IL-12 and IL-18-stimulated NK cells compared to those co-stimulated with IL-12 or IL-15 and IL-18.

Given that ADAM17-inhibited NK cells produce half the amount of TNFα as those without inhibition and the fact that TNFα is needed to mature DCs, I then went on to understand the functional consequences of ADAM17-driven NK cell help. I looked at the effects of ADAM17 inhibition on the ability of NK cells to provide help to and mature DCs. DCs were maintained for six days, and then autologous NK cells were added to the culture and stimulated for an additional 48 hours with or without an ADAM17 inhibitor. The results of this experiment showed morphological differences in DCs cultured in the presence of ADAM17-inhibited NK cells compared to DCs cultured with just stimulated NK cells (Fig. 9A). DCs cultured with ADAM17-

inhibited NK cells looked morphologically immature compared to the DCs cultured only stimulated NK cells. The ADAM17-inhibited cultures had DCs that were more rounded and did not have elongated dendrites, whereas the cultures with only stimulated NK cells had DCs with a more extended shape and elongated dendrites which are characteristics of more mature DCs. The morphological similarities of the ADAM17-inhibited culture compared to the cultures with only unstimulated NK cells reveal the possibility that not only is stimulation required to mature DCs, but also the presence of a functioning ADAM17 molecule.

However, to confirm this possibility, the DCs were phenotypically and functionally characterized to assess maturity and polarization status. The results of the phenotyping revealed that DCs in culture with ADAM17-inhibited NK cells show a significant increase in Siglec-1 expression and a slight decrease in CD86 expression (Fig. 9B-D). This finding indicates that ADAM17-inhibited NK cells are still capable of phenotypically maturing the DCs, despite their decreased TNFα production; although, the decreased TNFα production could have led to the slight decrease in CD86. To confirm the functional character of the DCs, the DCs were exposed to CD40L, and the supernatant was collected and analyzed for IL-12p70 production. Results of this experiment revealed that DCs cultured with ADAM17-inhibited NK cells produce slightly lower, but not significant, levels of IL-12 (Fig. 9D). The IL-12 producing capacity of the DCs further validates the finding that although ADAM17-inhibited NK cells produce lower levels of TNFα, they still can phenotypically and functionally mature DCs, despite the morphological differences.

Interestingly to these results is the striking increase in Siglec-1 expression seen on the DCs cultured with the ADAM17-inhibited NK cells. Siglec-1 is expressed on type-I polarized mature DCs and functions as a sialic-acid-binding immunoglobulin-like lectin that can allow the mature DC to internalize antigens and sustain the adaptive immune response through their antigen-

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presenting capabilities [94-96]. Siglec-1 becomes upregulated upon DC exposure to type one interferons, as well as DC exposure to IFNγ [97]. Given that ADAM17-inhibited and non-inhibited NK cells produce equivalent amounts of IFNγ, this leads to the question as to what the ADAM17-inhibited NK cells are producing that is causing the Siglec-1 upregulation on the DCs. Therefore, I have hypothesized two different situations that could explain the NK cell-mediated upregulation of Siglec-1 on DCs.

The first explanation for the DC Siglec-1 upregulation is the synergy between IL-10 and IL-18. As previously mentioned, ADAM17-inhibited NK cells stimulated with IL-12 and IL-18 produce increased levels of IL-10 (Fig. 8). Furthermore, studies have shown that IL-10 can increase the cytotoxic potential and proliferation of NK cells when combined with IL-18 [98, 99]. Therefore, upon IL-12 and IL-18 stimulation of the ADAM17-inhibited NK cells, the IL-10 produced from these NK cells could potentially be synergizing with IL-18 to produce an increased type I cytokine response that acts upon the DC to upregulate their Siglec-1 expression.

The next explanation for the increased expression of Siglec-1 on the DCs is the relationship between the IL-10 receptor (IL-10R) and type I interferon receptors. Previous work has shown that the IL-10R is structurally related to the interferon receptor with both being classified as a type II cytokine receptor [100]. It is also known that DCs express the IL-10R at high levels [101]. Therefore, the IL-10 production from the NK cells treated with the ADAM17 inhibitor could be acting upon the IL-10R on the DCs, and the signaling cascade from activation of the IL-10R could mimic that of type I interferon receptors resulting in upregulation of Siglec-1.

Overall, the findings presented highlight a clear involvement of ADAM17 in the responsiveness of NK cells to IL-18 and their associated loss of CD16 and differentiation into NK helper cells. This also supports the case that the continued study of ADAM17 and its functional

impact on NK cell differentiation and helper function is of importance. With further investigation, this work could implicate the involvement of ADAM17-driven mechanisms in the establishment of and persistence of the FcRγ⁻ NK cells commonly found in PLWH. In addition, further work with ADAM17 could lead to the exact mechanisms of how the molecule is implicated in NK helper cell differentiation, and more specifically, the type of help (i.e. type 1, type 2, or regulatory) the NK cell may provide. Finally, the findings presented here further stimulate the question as to how ADAM17 aids NK cells in providing help to different immune cells, such as DCs, and therefore leads to an impact on the overall immune response.

6.0 Future Directions

The work presented here offers many different avenues for future investigation. To fully understand the mechanisms of ADAM17-induced differentiation in NK cells, various experiments can be conducted. The first avenue for future exploration would be to determine if ADAM17 or its modulation is involved in the persistence of FcRγ^- NK cells. To confirm the role ADAM17 plays, or fails to play, in the unresponsiveness of these cells, more in-depth intracellular signaling experiments would need to be conducted. The goal of these experiments would be to determine if the ADAM17 molecule in FcRγ^- cells functions in the same way as FcRγ^+ NK cells. Different methods, such as the phospho-flow assay, could be used to elucidate the signaling cascade that results in FcRγ^- versus FcRγ^+ NK cells when ADAM17 is stimulated by IL-18 co-stimulation. Determining the mechanisms of how FcRγ^- NK cells remain unresponsive to cytokine stimulation will allow for a better interpretation of their role in the immune response and how to utilize their therapeutic potential due to their increased ADCC capacity. Furthermore, a better understanding of the immune response to ADAM17 inhibition could lead to more effective utilization of ADAM17 inhibitors in ADCC therapies. Therefore, further studies can be done to test the ADCC capacity of different NK cell subsets in the presence or absence or ADAM17 inhibition.

In addition to investigating ADAM17 in the FcRγ^- NK cell population, further experimentation into the "memory-like" responses of the differentiated NK helper cells can be conducted. The NK cells that differentiate into a helper type I or type II can be re-stimulated to see if they elicit a stronger secondary response that is skewed toward the production of type I or II

cytokines. In addition, further experimentation can be done to see if any other combination of cytokine stimulation can reveal other distinct subsets of NK cells, such as regulatory NK cells.

Next, to best understand the mechanisms that are contributing to ADAM17-driven NK helper cell differentiation, additional intracellular signaling experiments could take place. These experiments could involve comparing the signaling cascade of ADAM17-inhibited NK cells to control NK cells to identify distinct pathways being utilized by ADAM17. Further, a closer investigation into the impact of IL-18 co-stimulation on ADAM17 function is necessary to understand its mechanism of action. Stimulating NK cells with various combinations of innate cytokines and profiling the intracellular effects of ADAM17 inhibition could lead to a more definitive understanding of the mechanism(s) involved in the activation of ADAM17 functioning.

Finally, to elucidate the impact of ADAM17 inhibition on NK cell-DC crosstalk, a more thorough phenotype of the ADAM17-inhibited NK cells should be done. The supernatant analysis of ADAM17-inhibited NK cells did not include testing for type I interferons, which in the future should be explored. This would confirm if ADAM17-inhibited NK cells can produce these cytokines and therefore contribute to the upregulated Siglec-1 on the DCs. In addition, a further look into the function of IL-10 is critical to understanding the impact of ADAM17 on NK cell-mediated immune help. Experiments testing the outcome of DC stimulation with IL-10 alone or in combination with other cytokines that were present in our experiments could provide insight into its effect on DC phenotype, as well as confirm if it can cause upregulation of Siglec-1.

Altogether, these results help to answer questions on the mechanisms of NK cell differentiation but also lead to more questions as to the exact role ADAM17 is playing in this process. Future work involving more in-depth analyses on ADAM17 will hopefully illuminate the answers to these questions.

7.0 Public Health Significance

Even with the use of ART, HIV-1 remains a public health concern over the globe. Access to ART is not always an equitable resource for those in need, and the strict adherence to the drug regime leaves those living with HIV at risk for viral increase and progression to a worsened disease state [102]. For these reasons, and others not mentioned, the search for a cure has been at the forefront of HIV research. However, to implement a cure in the population, PLWH would have to stop their ART regime to assess the efficacy of the cure. Therefore, to ethically interrupt ART, and essentially take PLWH out of remission, a complete understanding of their immune state and its function would need to be understood.

As mentioned earlier, HIV can cause many aspects of immune dysfunction, some of which have yet to be fully characterized. Natural killer cells represent a population of cells that exhibit significant immune dysfunction during HIV-1 acute and chronic infection despite long-term treatment on ART [47, 52, 61]. Therefore, to implement an HIV cure, there is a need for a better understanding of how optimal NK cell function can be fully recovered. The results of the research I presented offer the beginnings of understanding the skewed NK cell populations and the mechanisms of how these populations arise. In addition, my work highlights the therapeutic potential of these dysfunctional cells to be utilized for therapy given their increased capacity for ADCC. Furthermore, I have characterized potential mechanisms for how NK cells can become helper-like cells. These mechanisms can also be utilized in research for HIV cure studies. Current work on an HIV cure has looked into DCs as a way to expose HIV antigens from a latent reservoir and interact with T-cells to essentially "kill" the virus [103]. Characterizing the mechanisms of

how NK cells provide immune help to DCs will allow for a better understanding and use of DCs in any HIV cure strategy.

In addition to characterizing NK cells to address this HIV public health crisis, the research presented here extends beyond just HIV. NK cells are critical for the immune response to various cancers, and a better understanding of their differentiation mechanisms can allow for improved strategies for their utilization in different cellular and immunotherapies. A major type of immunotherapy utilized in numerous solid-tumor cancer trials is monoclonal antibody (mAb) therapy, which induces an ADCC response from NK cells. The increased ADCC activity of NK cells can also lead to enhanced cytolytic responses from T-cells and therefore a better overall immune response to the tumor [104]. The research I have presented shows the mechanism of how NK cells maintain or lose their Fc receptor, the presence of which is critical in their capacity to facilitate ADCC. Understanding ADAM17 and utilizing inhibitors against it could further increase the efficacy of NK cells and mAb therapy for various cancers. Furthermore, elucidating how ADAM17 influences the differentiation of NK helper cells can lead to a better understanding of the role these cells play in the immune response to cancer, other than their capacity to kill. This may lead to their effective utilization in cellular therapies, such as CAR-NK cells where they are not only able to bind and kill a target, but also provide help to other immune cells in the environment leading to better clearance of the tumor.

Overall, the characterization of NK cells and their mechanisms of differentiation is crucial in understanding their role in the immune response. NK cells are no longer thought of as cells with only cytolytic abilities, and my research has shown how they can be better understood and utilized in the treatment of various diseases.

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