Characterization of Galectin-9+ and TIGIT+ Natural Killer Cells in Human Immunodeficiency Virus-1 Infected Individuals

by

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Abstract

NK cells play an integral role in mediating immune responses to viral infections; however, their function is compromised during chronic diseases such as HIV-1, partly due to an increase in expression of inhibitory receptors. Thus, one of the reasons for the reduced immune responses elicited by NK cells in HIV infection, can be the result of engagement of co-inhibitory molecules with their respective ligands. Galectin-9 (Gal-9) is a tandem-repeat type galectin that possesses a wide range of immunomodulatory properties such as interaction with Tim-3, which is upregulated on T cells during chronic conditions. Previously, our group has shown that interaction of Gal-9 on regulatory T cells with Tim-3 on $CD8^+$ T cells renders them more permissible to immunosuppression and subsequently leads to their exhaustion. Thus, we aimed to further study the role of Gal-9 on NK cell function in HIV-infected patients. As a comparison, we also analyzed another inhibitory receptor known as TIGIT. Our data indicates upregulation of surface Gal-9 and TIGIT on NK cells in HIV-infected individuals on antiretroviral therapy, long-term non-progressors and progressors compared to healthy controls. We observed two effector NK cell populations with dichotomous functional potential. TIGIT+ NK cells expressed more cytotoxic molecules (GzmB, perforin and granulysin) but less IFN-γ. Conversely, Gal-9+ NK cells expressed less cytotoxic molecules, but more IFN-γ. Additionally, Gal-9+ NK cells, in contrast to Gal-9- NK cells, co-expressed negligible amounts of GzmB and perforin, which is detrimental to their cytotoxic abilities. However, the functional potential of these Gal-9+ NK cells can be augmented, as the addition of a cytokine cocktail (IL-12, IL-15 and IL-18) enhanced their degranulation and expression of effector molecules. We believe that understanding the underlying pathway associated with Gal-9+ NK cells differential functional potential, can pave the way for potential therapeutic strategies.

Preface

Research Ethics Approval

This thesis is original work done by Melika Motamedi. This research project received the two ethics approvals indicated below by the institutional research review boards at the University of Alberta:

1. Investigating Immune Correlates of Protection in HIV-1 Infection, Protocol #Pro000046064, valid until 2020.
2. Human Galectin-9 as a Novel Weapon to Reactivate Latent HIV, Protocol #Pro000070528, valid until 2020.

In addition, some samples were obtained from the Centre for AIDS Research (CFAR)-University of Washington through an already established Material transfer agreement (MTA).

Contribution of others in this study

I would like to acknowledge the contribution of others in this project. Shima Shahbza who performed the initial studies confirming Gal-9 overexpression on NK cells in HIV-1 patients. Li Fu, a former post-doc of the Elahi lab, conducted the studies determining the functionality of Gal-9+ and TIGIT+ NK cells. Garett Dunsmore who helped conduct Image Stream. Lai Xu who performed some of the experiments and assisted in data analysis. Dr. Robert Harrington at the University of Washington and Dr. Stan Houston, Director of Northern S.H., contributed in HIV-patient recruitment. Dr. Robert Harrington also provided HIV-specimens. Most importantly, a special thank you to all of the HIV-1 infected individuals and healthy controls that generously donated their blood for research, as without their blood donation this project would have been impossible. I also would like to acknowledge the contribution of Shima Shahbaz and Petya Koleva for the blood collection from the HIV-infected and healthy individuals.

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Table of Contents

List of Figures

Chapter 1: Introduction

1.1 What are natural killer cells?

Natural killer (NK) cells are granular lymphocytes that are classically identified under the innate immune system due to their "natural" propensity to kill targets without requiring prior sensitization (1). Over the last couple of decades, the classification of NK cells has become more complex with the recognition that they can form immunological memory and respond to specific antigens, both characteristics of adaptive immunity (2-6). To make matters even more complicated, NK cells are comprised of a highly diverse population with up to 30,000 distinct phenotypes determined to date (7). Furthermore, unlike T-cells that have definitive surface markers such as CD3 that can define their population, NK cells lack a conclusive surface marker for their classification. Thus to identify NK cells, we have to selectively exclude phenotypically defined populations of lymphocytes such as T cells (ex. CD3 and CD5), B-cells (ex. CD19 and CD20) and antigen presenting cells (CD14, CD11b and CD11c) while selectively including markers present on NK cells such as the neural cell adhesion molecule-1 (CD56) and the low affinity Fc gamma receptor 3A (CD16) (8). The CD16 is able to bind to the Fc portion of an antibody coating a pathogen or tumor/viral antigen and activate NK cells to kill the target via antibody dependent cell mediated cytotoxicity (ADCC). It is important to note that neither CD56 nor CD16 are exclusively on NK cells as both of these markers can be found on the surface of other cells including T cells, dendritic cells, and monocytes (9, 10).

1.2 Peripheral blood NK cells

The differential expression of CD56 and CD16 identifies two functionally diverse peripheral blood (PB) NK cell subsets, namely the $CD56^{bright}$ and the $CD56^{dim}$ subsets (11). The $CD56^{bright}$ subsets have a high density of expression for CD56 and can either lack or express low amount of

CD16. In contrast, the $CD56^{dim}$ population of NK cells have low to undetectable expression of CD56 and express CD16. The majority of NK cells in the PB of healthy individuals are $CD56^{dim}$, with $CD56^{bright}$ cells representing the minority (8). The $CD56^{bright}$ NK cells are the immature precursors that give rise to the mature $CD56^{dim}$ NK cells (12-14). Thus, as NK cells develop, they start to lose their expression of CD56 and gain expression of CD16. Not only do these markers represent different stages of NK cell development but they also aid in understanding the distinct functional properties of these two subpopulations. The $CD56^{bright}$ NK cells are known for their immunomodulatory role in producing cytokines such as interferon-gamma (IFN-γ) and tumour-necrosis factor-alpha (TNF-α). However, these subsets of NK cells have low expression of cytolytic mediators such as perforin and granzymes and thus are poor killers (15). In addition, due to their undetectable/low levels of CD16, $CD56^{bright}$ cells do not readily participate in the ADCC mechanism of killing. In contrast, the $CD56^{dim}$ are less involved in cytokine secretion but more cytotoxic due to their increased expression of cytolytic mediators and possessing more of the activating receptor, CD16 (11, 14). Therefore, despite the immense diversity of NK cells, they can generally be divided into two distinct subsets that are specialized in producing cytokines ($CD56^{bright}$) or killing targets via their cytotoxic mediators ($CD56^{dim}$). Due to the heterogeneous population of NK cells we have categorized them into three subpopulations; from CD3 negative lymphocytes we examined CD56 single positive ($CD56^{+}$), double positive ($CD56^{+}CD16^{+}$) and CD16 single positive ($CD16^{+}$) subpopulations. For our research in particular, it was impractical to further subdivide our population into bright and dim subsets due to the rarity of the population of cells examined. However, other research groups have utilized identical (158) or very similar methods of gating for NK cells (112).

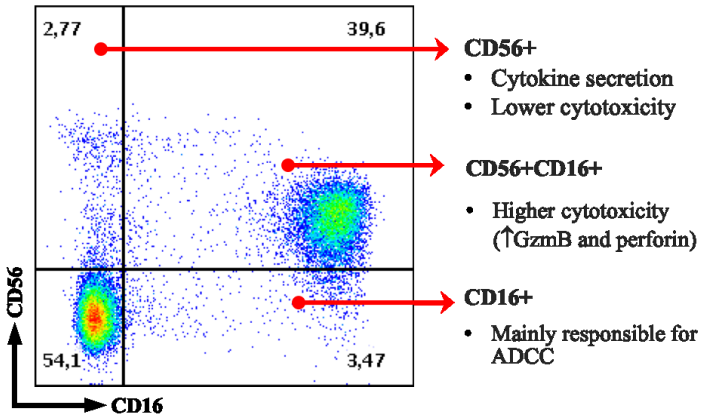

Figure 1-1: Three subpopulation of NK cells. Summary of the subpopulations of NK cells utilized in this study, along with their predominant function.

1.3 NK cell development

NK cells require stromal cells and cytokines present in the bone marrow to develop from common lymphoid progenitors (CLP) (248). NK cell development can be broken down into two stages: commitment and maturation (14). What drives CLP away from the B and T cell lineage is the key acquisition of receptor-β (IL-2Rβ). The cells that first gain the IL-2Rβ are in the committed stage and are classified as NK-cell precursor (NKP) (249). Next NK cells go through stages of maturation to become immature NK cells, pseudomature lytic NK cells, and mature NK cells (14). The acquisition of NK-cell receptor protein 1 (NKR-P1) and CD2 marks the transition into immature NK cells that lack ability to kill. The pseudomature lytic NK cells possess lytic potential and are identified by their surface expression of CD56 and NKG2 receptors. In order for the full maturation process to be complete NK cells need to acquire at least one functional inhibitory receptor that recognizes classical MHCs which are known as Killer Immunoglobulin-like Receptors (KIRs). Additionally, there are key transcriptional factors that play an important role in development of NK cells. More specifically, the transcriptional factors Nfil3 and PU.1 are

shown to be important in the transition of CLP to NKP (250, 251). Whereas the transcriptional factors Eomesodermin and T-bet are required for the final step of NK cell maturation (252).

1.4 Lytic granule proteins

NK cells are capable of killing their target cells by releasing a cargo of deadly proteins located in their lytic granules. The three prominent proteins within the lytic granules of NK cells are granzymes, perforin, and granulysin (GNLY) (16).

1.4.1 Granzymes

Granzymes are a family of serine proteases that are constitutively synthesized and stored in cytotoxic T lymphocytes (CTLs) and NK cells (16). Five granzymes have been identified in humans (A, B, H, K, and M) with Granzyme B (GzmB) being the most extensively studied due to its presence in 3 species widely used in research (mice, rats, and humans) as well as being expressed in both CTLs and NK cells (17, 18). These proteases are capable of inducing apoptosis in the target cell by various mechanisms such as activation of pro-apoptotic proteins, fragmentation of DNA, and release of reactive oxygen species (16, 19). In particular, apoptosis can be carried out by a group of proteases known as caspases that exist as zymogens and become activated upon cleavage of their specific aspartic residues. GzmB has been shown to help activate caspases 3, 7, 8 and 10, which allows the fully functioning caspases to cleave their various substrates (20, 21). However, caspase-3 has been shown to have the most important role *in vivo* (21-23). The direct cleavage of pro-caspase-3 (zymogen) by GzmB activates caspase-3, which itself has the potential to cleave various substrates such as poly(ADP-ribose) polymerase (PARP), Inhibitor of Caspase Activated DNase (ICAD) and gelsolin, which results in

inhibition of DNA repair, DNA fragmentation, and cytoskeletal changes, respectively (24-26). GzmB can also initiate the caspase-independent mechanism of killing by activating the pro-apoptotic protein Bid. Bid associates with other pro-apoptotic proteins leading to mitochondrial outer membrane permeabilization (MOMP), which can result in the death of target cells via necrosis (21, 27). Additionally, the permeabilization of mitochondria can lead to the release of apoptogenic factors such as cytochrome c and second mitochondrial activator of caspases (SMAC). Cytochrome c can combine apoptotic protease-activating factor 1 (APAF-1), pro-caspase-9, and ATP to form the apoptosome complex; this complex can activate pro-caspase-9, which amplifies the caspase cascade by activating pro-caspase-3(21, 28, 29). SMAC is capable of making pro-caspase-3 accessible to GzmB cleavage by inactivating the caspase inhibitor IAP (inhibitor of apoptosis protein) (29, 30). Overall, the breadth of GzmB's impact on the death of target cells is very broad and complex; a simplified summary of its function is depicted below.

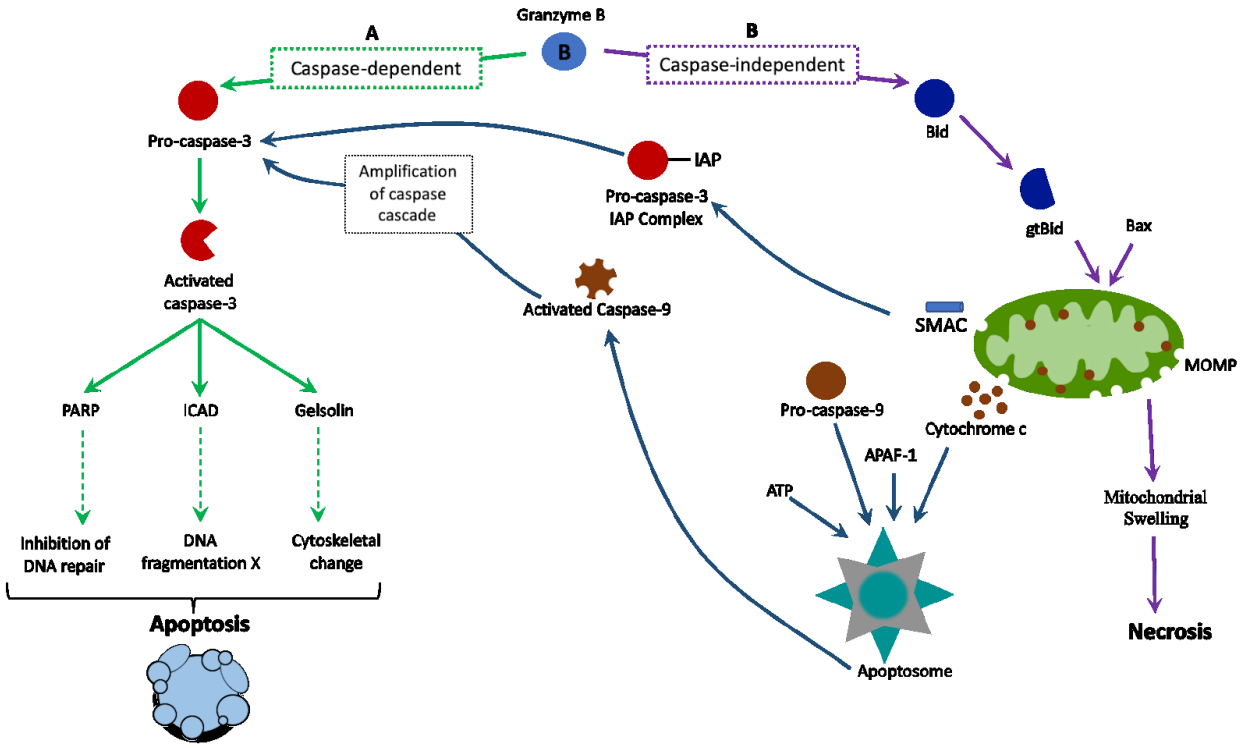

Figure 1-2: Caspase-dependent and independent mechanism of target cell killing mediated by GzmB. (A) The cleavage of pro-caspase-3 by GzmB gives rise to the active form of Caspase-3, which has the potential to cleave various substrates such as PARP, ICAD, and gelsolin and ultimately lead to apoptosis of target cells. (B) GzmB can directly cleave Bid to form the granzyme B-truncated Bid (gtBid), which recruits the pro-apoptotic protein, Bax. Bax oligomerizes to initiate mitochondrial outer membrane permeabilization (MOMP). MOMP can lead to swelling of mitochondria and eventual cell death via necrosis as well as the release of cytochrome c and SMAC. Cytochrome c, along with pro-caspase-9, APAF-1 and ATP form the apoptosome complex which can amplify the caspase cascade by activating caspase-9, leading to caspase-3 activation. The inhibitory activity of IAP on pro-caspase-3 can be removed by the competitive binding of SMAC to IAP/caspase-3 complex which enables the release of uninhibited pro-caspase-3. Adapted from: Lord *et al.* Granzyme B: a natural born killer. Immunol. Rev. 2003 (21).

1.4.2 Perforin

Perforin is a glycoprotein that is capable of oligomerizing and forming membrane-spanning pores in the presence of calcium and neutral pH condition (31). The fact that perforin is inactive under the acidic conditions of granules (~ pH 5) is important in

preventing the degradation of the storage compartment that holds the cytotoxic mediators (31, 32). Previously, it was believed that perforin enables the creation of large pores in the plasma membrane that subsequently allow granzymes to enter the cytosol to interact with their intracellular substrates and mediate apoptosis (16, 32, 33). However, this model has since been criticized for two main reasons. First, the concentration of perforin utilized in various studies to form pores that are sufficiently large to allow the passage of granzymes is not physiologically relevant and leads to necrosis by the loss of membrane integrity rather than apoptosis (34, 35). Second, physiologically relevant concentration of perforin creates very small pores in the membrane that physically cannot allow granzymes to pass through (36, 37). Thus, a revised method for the action of perforin has been proposed based on a different site of action. It has now been accepted that perforin forms small pores in the target cell membrane that allows the passage of small molecules such as calcium, but not granzymes (in particular GzmB). The influx of calcium triggers the membrane repair process, which causes endocytosis of the damaged membrane compartment and simultaneous uptake of perforin and granzymes into a large endosome. It is from this large endosome that perforin mediates its function of forming pores to allow GzmB to escape into the cytosol to mediate its pro-apoptotic function (34). Thus, in order for GzmB to mediate its function it needs to be co-endocytosed with perforin (38).

1.4.3 Granulysin

Granulysin (GNLY) exists in a 15 and 9 kDa isoforms and is an interesting protein with both cytotoxic and immunomodulatory properties (16). The 15 kDa form exists in distinct granules that are devoid of perforin and granzymes and has a role in differentiation of

monocyte to dendritic cells (39). The 9 kDa GNLY is confined to the cytotoxic granules and has cytolytic properties with a broad spectrum of activity against tumors, bacteria, fungi, and parasites (16, 39-41). The smaller form of GNLY has been shown to serve as a chemoattractant for T cells, monocytes and NK cells, as well as activation of immune cells to produce proinflammatory cytokines (16). Whether the 9 kDa GNLY serves as a lytic or immunomodulatory molecule has been shown to be dependent on its local concentration. At high concentrations, which are observed at the immunological synapse, GNLY is able to mediate lysis of target cells. At low concentrations, further from the site of granule release, GNLY serves as a chemoattractant gradient that recruits and activates immune cells at the site of infection (42). To date, no known receptor has been identified for GNLY (16), however crystal structure of the molecule has indicated that the surface of the molecule is positively charged (43). It is believed that GNLY interacts with the negatively charged lipids via electrostatic interaction and subsequently leads to membrane disruption. The fracturing of the membrane results in an influx of calcium, which activates the calcium-dependent potassium pumps and leads to the efflux of potassium ions. The change in the ion concentration is crucial as the blockage of the calcium or potassium channels prevents cell lysis (44). It is believed that the mitochondria can take up the additional calcium and facilitate the opening of pores for cytochrome c release, which can lead to the formation of the apoptosome and eventual apoptosis via the caspase system (45, 46). Furthermore, the decrease in potassium has been shown to lead to apoptosis, due to the critical role that potassium ions play in maintaining caspases in their inactive forms (47). Lastly, it has been shown that GNLY can mediate its function independent from perforin (48, 49).

1.4.4 Lytic granule exocytosis

When NK cells receive activating signals, they are probed to release their lytic granule contents in a directed manner to their target cells. In order to ensure a controlled release of the cytotoxic mediators, an intimate contact is formed between the target cell and the NK cell known as the immunological synapse (IS). There are both lytic and inhibitory synapses depending on whether the activating or inhibitory signals are predominating, respectively (50). Nonetheless, the formation of this interface allows for the exchange of information between the two cells to allow the NK cell to determine whether it should release its lytic contents into the environment. The formation of the lytic synapse occurs in 3 stages known as initiation, effector and termination (51). The initiation stage involves the adhesion of the two cells via various adhesion receptor/ligand interactions (ex. Lymphocyte function-associated antigen 1 (LFA-1) interacting with intercellular adhesion molecule-1 (ICAM-1)), as well as the initial signalling required for cell activation. This is followed by the effector stage where the lytic granules are delivered to the target cell. This stage begins with actin polymerization and accumulation at the synapse, followed by the discrete organization of receptors at the site. NK cells have regional locations such as the central supramolecular activation cluster (cSMAC), which contains the activating receptors, as well as the peripheral SMAC (pSMAC), where the adhesion receptors are located. This arrangement of receptors enables activating signal amplification, allowing for polarization of the lytic granules to the IS. The granules dock onto the microtubules and move into the microtubule-organizing centre (MTOC), which creates a path for the granules to get access to the microfilaments of the F-actin. Next, the granules utilize the scaffold of F-actin to gain access to the central platform of the

synapse where they fuse with the plasma membrane (PM), eventually entering the target cell. The granules that contain the cytotoxic mediators are enclosed by a lipid bilayer that contains Lysosomal-associated membrane protein 1 (LAMP1, CD107a). Thus, granule exocytosis is typically detected by the surface expression of CD107a (16, 52, 53). Lastly, the termination stage occurs whereby the NK cell detaches from the target and renews its cytolytic capacity (51).

1.5 Interferon-gamma

As previously mentioned, the two major functions of NK cells are to kill via their lytic granules or to produce cytokines. NK cells are capable of releasing many anti-inflammatory (ex. Interleukin 5 (IL-5) and IL-13) and pro-inflammatory cytokines (IFN-γ and TNF-α) depending upon the context (54, 55). Amongst the pro-inflammatory cytokines, interferon-gamma (IFN-γ) is the most prominent one produced upon engagements with target cells or in response to stimulation from other cytokines (56, 57). As suggested by its name, interferon-γ has numerous ways to "interfere" with infected cells by promoting antiviral, antitumor and antiproliferative responses. The biological function of IFN-γ is mediated via the activation JAK/STAT 1 pathway (Janus activated kinase/signal transducer and activator of transcription 1), which can ultimately lead to the transcription of various genes that determine the biological response (58, 59). The initially discovered impact of IFN-γ on immune cells was the upregulation of major histocompatibility complex (MHC) class I and II molecules, and the associated machineries involved in antigen presentation such as the transporter associated with antigen processing (TAP) (60-62). It has been shown that IFN-γ can lead to tumor cell apoptosis, however the mechanism is yet to be elucidated. One study found that activation of caspase-1 dependent pathway by IFN-γ

leads to apoptosis of pancreatic cell carcinoma (63). Meanwhile, IFN-γ mediated apoptosis of glioblastoma cell lines occurs via the downregulation of a transcription factor important for survival known as nuclear factor kappa B (NF-κB) (64). Thus, the lack of clarity on the mechanism of IFN-γ tumor apoptosis seems to be dependent on the type of tumor being examined (59). In addition to its direct antitumor effects, IFN-γ can indirectly promote tumor or virally infected cell killing through its involvement in the activation of $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, NK cells, macrophages and dendritic cells (DCs) (65, 66). At the same time, IFN-γ can negatively regulate the function of regulatory T cells (Tregs), thus promoting an overall T-helper (Th) 1 mediated immune response that is needed for the clearance of damaged and virally infected cells (67, 68). In NK cells, cytokines are stored in distinct carriers devoid of lytic molecules and delivered in a polarized manner via the immunological synapse, as well as in a non-polarized manner by being delivered to multiple points around the cell (69). The carriers of cytokines in NK cells are believed to be recycling endosomes (RE) rather than granules, which have been shown to play an important role in cytokine exocytosis in various cells (69-71). The fact the cytokines can be delivered via two separate pathways is important in ensuring that NK cells that are killing their targets can simultaneously secrete their cytokines in multiple directions to orchestrate a better immune response.

1.6 Regulation of NK cell activity

Whether NK cells release the constituents of their secretory vesicles and mediate killing of target cells is dependent on the balance between activating and inhibitory signals transmitted by various receptors. Activating receptors (AR) provide an “on” signal to NK cells, while inhibitory receptors (IR) provide an “off” signal. Thus, the net effect of whether NK cells are activated or

not is determined by the dominant signalling mode (16). There are two models for NK cell activation, the "missing self" and the "induced self" theories (72). The "missing self" theory considers the interaction of MHC class I (a self-molecule present on healthy cells) and KIRs. NK cells possess both activating and inhibiting KIRs that can distinguish between MHC class I allelic variants (73). The activating KIRs bear immunoreceptor tyrosine-based activation motifs (ITAM) which provides an on signal upon interaction with their corresponding MHC class I. The inhibitory KIRs contain the inhibitory motifs (ITIM), which provide an off signal upon engagement with their designated MHC class I. The majority of KIRs are inhibitory (74), thus when there is a downregulation in MHC class I expression, typically observed in cancerous and virally infected cells (75), the NK cell recognizes this missing self and mediates lysis of the cell. The "induced self" complements the "missing self" model because it provides an explanation of why aberrant cells can be killed by NK cells despite displaying MHC class I. In the "induced self" model, aberrant cells can upregulate the expression of activating ligands that can activate the AR on NK cells due to the prevailing activating signal (72). While there are an extensive number of AR on NK cells, our research focused on NKG2D (natural-killer group 2, member D) and the three Natural Cytotoxicity Receptors (NCRs: NKp30, NKp44 and NKp46). These receptors were analyzed due to their vitalness in clearance of HIV-infected cells and their decline in expression on NK cells observed in HIV-infected individuals (76-79). NKG2D is an AR expressed as a homodimer that recruits the adaptor protein DNAX-activating protein 10 (DAP10) upon engagement with its ligand. This adaptor protein recruits and activates PI3K (p85 of phosphatidylinositol-3 kinase) and Grb2 (growth factor receptor-bound protein 2), which allows for the downstream signalling cascade that leads to both cytotoxicity and cytokine-mediated responses (80, 81). The ligands for human NKG2D are two MHC class I chain-related

proteins (MIC-A and MIC-B) as well as six members of the UL16 binding proteins (ULBP) family (82). These ligands are present at relatively low quantities under homeostatic conditions, but their expression is upregulated during infections or oncogenic transformation (83). Due to the potent immune-stimulatory effects of NKG2D on NK cells, it plays an important role in the clearance of stressed cells such as tumors (81). In fact, certain tumors have employed a decoy mechanism of secreting the ligand MIC-A to mask detection and prevent activation of NK cells against the tumor (84). The NCRs are part of the immunoglobulin superfamily that associate with ITAM-bearing adaptor proteins that elicit an activating signal (85). There is a vast array of ligands for NCRs ranging from bacterial, viral, parasitic and cellular origins, which exemplifies their immense polyfunctionality (86). Despite the broad spectrum of ligands already identified, there are still ongoing studies being conducted to identify other potential ligands. Apart from some of the AR discussed, there are other markers to assess NK cell activity such as the ectoenzyme CD38. This enzyme is responsible for producing calcium mobilization agents such as cyclic adenosine diphosphate-ribose (ADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) from nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide 2'-phosphate (NADP+), respectively (87-89). The calcium influx mediated by CD38 allows for the cascade of phosphorylation that activates NF-κB, leading to subsequent NK cell effector function (90). Thus, CD38 is often used as a marker of NK cell activation, as the addition of agonistic monoclonal antibody (mAb) against CD38 results in increased IFN-γ secretion and lysis of target cells (91, 92).

1.7 Important cytokines for NK cells

There are various cytokines that are important for the development and function of NK cells, but for the purposes of our study we will focus on IL-12, IL-15 and IL-18. IL-12 is predominantly produced by macrophages and DCs and associates with a heterodimeric receptor composed of IL-12 Receptor β 1 (IL-12Rβ1) and IL-12Rβ2 (93). The predominant function of IL-12 in NK cells is to enhance the production of cytokines (IFN-γ and TNF-α) and cytotoxic mediators such as perforin and granzymes (94-96). It has been shown that peripheral blood mononuclear cells (PBMCs) from HIV-infected patients produce less IL-12 in response to stimulation, and the addition of IL-12 to NK cells from HIV-infected patients significantly increases their ability to secrete IFN-γ and kill target cells (97). Additionally, there is a vast array of opportunistic infections that can occur when HIV patients progress to acquired immunodeficiency syndrome (AIDS). One of the most commonly seen opportunistic infections in HIV-infected individuals is infection with *Cryptococcus neoformans* that can cause meningitis (98). A study found that addition of IL-12 restored the defective NK cell killing of a *C. neoformans* in AIDS patients via the upregulation the activating receptor NKp30 (99). The cytokine IL-15 binds to a heterotrimeric receptor containing IL-15Rα as well as the two subunits shared with IL-2, known as IL2/15Rβ and the common gamma chain (100). Like IL-12, IL-15 enhances the functionality of NK cells but is also important in their development (101). This is further supported by a study that shows that IL-15Rα knockout mice are deficient in NK cells (102). As with the previous cytokine mentioned, IL-15 has been used to stimulate NK cells from HIV patients on anti-retroviral therapy (ART) and has been shown to clear latently infected $CD4^+$ T cells (103). Lastly, the predominant effect of IL-18 on NK cells is indicated by its original name as IFN-γ-inducing factor (104). In addition, IL-18 and IL-15 together have been shown to induce NK cell

proliferation (105). Unlike IL-12 and IL-15, IL-18 has not been used to enhance NK cell function in HIV patients. This may be due to the fact that HIV-infected individuals have an increased circulation of IL-18 in their plasma compared to seronegative individuals (106, 107). In fact, it has been shown that IL-18 can actually induce the death of NK cells in a dose-dependent manner (108). Nonetheless, the combination of these three cytokines is frequently used in the literature to yield a potent NK cell response (109-112).

1.8 Human Immunodeficiency Virus (HIV)

Despite the discovery of HIV in the 1980s and the ongoing progress to date, there is still no cure for this debilitating disease that has infected over 75 million people worldwide (113). HIV is classified as a retrovirus because it can transcribe its RNA into DNA upon entering its target cell (114). More specifically, the viral RNA is composed of nine genes that encode the necessary material needed to make a new virus (gag, pol, env, tat, rev, nef, vif, vpr and vpu) (253). The gag gene gives rise to several structural proteins such as the nucleoprotein, capsid protein and matrix protein. The gene env is involved in the creation of glycoproteins (gp120 and gp41) that make up the outer shell of the virus. The pol gene provides the code for various enzymes (protease, reverse transcriptase, RNase H, and integrase) that are critical in the creation of a new virus. The remaining six genes are involved in the creation of several proteins that aid in the replication, infectivity and release of the virus from cells. The main cells that are infected with HIV are $CD4^+$ T cells, due to the fact that the virus requires both CD4 and the chemokine receptors CCR5 or CXCR4 to enter the cell (113, 114). However other cells bearing CD4 are susceptible to HIV infection such as monocytes, macrophages, and DCs (115-117); however, these cells are generally more important for the dissemination of the virus throughout the body, rather than

supporting replication, as viral production in these cells is quite low compared to $CD4^+$ T cells (118-120). The fact that the ideal targets for HIV infection is $CD4^+$ T cells and infection can result in their death (121) comes with detrimental consequences for the host, as these cells are crucial for antibody production (122), and the recruitment (123) and activation (124) of a plethora of immune cells. If left untreated HIV infection can severely weaken the immune system to a point where simple infections can lead to life-threatening conditions. However, with the advancements in our understanding of HIV, infected individuals have a significantly higher life expectancies and virtually undetectable viral loads due to the emergence of ART (125). This therapy is a combination of two or more drugs that specifically inhibits essential enzymes needed for HIV replication (126). The Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and Non-nucleoside RTIs (NNRTIs) block the action of the viral enzyme reverse transcriptase that is needed to convert the viral RNA to DNA (254). Another class of ART drug are integrase inhibitors that prevent the integration of viral DNA within the host genome (255). Other classes of ART drugs work by inhibiting viral protease (essential for cleavage of proteins) or acting as a CCR5 antagonist (254). Interestingly, there is a cohort of HIV-infected patients that are capable of maintaining a low viral load and high CD4 counts in the absence of ART known as long-term-non-progressors (LTNPs) (127). The mechanism by which LTNPs are able to control their infection is thought to be related to both genetic and immunological components (128). For example, there have been many reports of genetic polymorphism for the CCR5 gene locus in many LTNPs that results in a truncated protein that cannot escape the endoplasmic reticulum (ER), and thus is not expressed on the cell surface (129-131). In terms of the immune components, LTNPs have been shown to have an increased proportion of human leukocyte antigen (HLA) -B57 and -B27(132-134). These two HLA molecules are known to be protective

due to their presentation of the highly conserved immunodominant region of HIV capsid protein known as Gag- p24 (amino acids 240 to 272) (135, 136); HLA-57 specifically presents amino acids 240 to 249 (137), while HLA-B27 presents amino acids 263 to 272 (138). Additionally, it has been shown that CTL restricted to HLA-B57/27 are less susceptible to Treg-mediated suppression (139). Interestingly, $CD4^+$ T cells from LTNPs are found to be intrinsically resistant to HIV infection due to their reduced ability to support HIV replication (140); this deficit is associated with an overexpression of the cyclin-dependent kinase (CDK) inhibitor known as p21 in $CD4^+$ T cells (141). It has been reported that CDKs are involved in the transcriptional elongation of HIV mRNA, thereby supporting HIV replication (140). The discovery that LTNPs have upregulation of the inhibitor for CDK, p21, has led to proposals to examine inhibitors of CDK for treatment of HIV (142, 143). In addition, the functional quality of $CD8^+$ T cells seems to be different in LTNPs compared to individuals who are unable to control their viral load in the absence of ART (progressors). Specifically, it has been shown that HIV specific $CD8^+$ T cells from LTNPs maintain high proliferative capacity (144) and are highly polyfunctional, as they tend to simultaneously exhibit a more robust immune response by having higher expression for CD107a, IFN-γ, TNF-α, IL-2 and more proliferative capacities (145). Additionally, HIV-specific CD8+ T cells from LTNPs have enhanced cytotoxic ability compared to progressors due to higher expression of granzymes, perforin and granulysin within their cytotoxic granules (146-148). On the other side of the HIV clinical spectrum lies a cohort of HIV-infected individuals termed progressors who are less fortunate and do not possess many of the genetic and immunological components to naturally protect them from the infection. As examined in a systematic review (149), there is considerable heterogeneity in the definition of progressors in the literature. However, this review established that most studies refer to progressors as a group

of HIV individuals with a low CD4 count and high viral load that may eventually progress to AIDS. Unfortunately, some LTNPs eventually progress to AIDS after several years and sometimes after decades in the absence of ART (128). There are several mechanisms by which disease progression is thought to be mediated. As previously mentioned, the epitope recognized by HLA-B27 is very highly conserved, but an infrequent point mutation at residue 264 from arginine to lysine/glycine has been shown to contribute to disease progression by escaping the CTL response (138, 150). Additionally, Elahi *et al*. demonstrated that HLA-B57 restricted CTLs from LTNPs that eventually progressed to AIDS become more susceptible to Treg-mediated suppression (139). The mechanism of suppression was proposed to be mediated by the upregulation of the inhibitor receptor TIM-3 (T cell immunoglobulin and mucin domain 3) on CTLs engaging with their ligand on Tregs. Furthermore, it was shown that after progression these CTLs lose the ability to kill Tregs, which was observed before disease progression. This is further reinforced by a study that showed a significantly higher percentage of Tregs in progressive disease (151).

1.9 Impact of HIV on NK cells

There is a myriad of immunological defects observed in NK cells from HIV-infected patients. Although, acute HIV infection has been associated with a marked increase in the total NK cell population (specifically $CD56^{dim}CD16^{+}$ population), the distribution of NK cells is nearly normalized in chronic and acute infections when patients undergo ART (152). Despite the overall stability of total NK cells in infected patients, HIV infection can modulate the function of NK cells. It has been established that HIV infection of monocytes/macrophages can induce the dysregulated release of several matrix metalloproteinases (MMPs) such as MMP-9 (153).

Moreover, those MMPs are capable of cleaving off CD16 molecules on NK cells, impeding their ability to kill via ADCC (154). Furthermore, NK cells from HIV-infected individuals have been shown to secrete lower amounts of various chemokines such as CCL3 and CCL5 (155). More importantly, these chemokines are capable of competitively binding to the co-receptor CCR5 (156, 157). However, due to the decline in the secretion of these chemokines, the CCR5 co-receptor has a higher chance of being unoccupied due to a reduction of its corresponding ligands in the environment; therefore, the virus has an increased probability of infecting target cells expressing CCR5. Aside from chemokines, production of cytokines such as IFN-γ is lower in NK cells from HIV-infected patients compared to uninfected individuals (158). In additional to the reduction of chemokines/cytokines, chronic conditions such as HIV infection have been shown to skew NK cells to a more unresponsive/exhausted phenotype due to modulation of various receptors. More specifically, the levels of activating receptors such as NKp30, NKp44, NKp46 and NKG2D are downregulated on NK cells (159). Concomitantly, the levels of inhibitory receptors such as NGK2A increases on NK cells in chronic conditions thereby hindering their activation (160, 161). Furthermore, the prototypical KIRs, play an important role in NK cell functions in HIV infection. For instance, individuals with higher expression of activating KIRs such as KIR3DS1 and its interaction with HLA-Bw4 is associated with slower progression towards AIDS (162). On the contrary, the increase in the inhibitory KIR2DL3, has been shown to contribute to increased transmission of HIV from mother to child during delivery (163). In addition, the absence of ligands for inhibitory KIRs in HIV infected patients is associated with a better response (164). Thus, the regulation of NK cell activity is intimately related to the levels of activating and inhibitory receptor expression and their engagement with their ligand. In addition to the inhibitory KIRs, there are various other inhibitory receptors that

are postulated to play a role in NK cell exhaustion such as PD-1 (Programmed cell death protein 1), TIGIT (T cell immunoreceptor with Ig and ITIM domains) and TIM-3 (T cell immunoglobulin and mucin domain 3) (159, 165). These co-inhibitory receptors are extensively investigated in the context of T cell exhaustion (166) and their overexpression has also been reported on NK cells in HIV infection and cancer (159, 167-169). Although transient upregulation of co-inhibitory receptors is required for immune haemostasis, their persistent expression is associated with deteriorated T cell and NK cell effector functions (166). For instance, the overexpression of PD-1 on NK cells results in decreased degranulation capacity and IFN-γ expression (170). In addition, the inhibitory function of TIGIT on NK cells has been well established, as TIGIT blockade resulted in increased cytolytic activity of NK cells through an ITIM-dependent mechanism (171-173). Moreover, upregulation of TIGIT on NK cells is reported to be associated with lower IFN-γ expression, which can be restored upon TIGIT blockade (112). In line with these observations, a recent study has noted the importance of TIGIT, but not PD-1 in NK cell exhaustion (174). This study demonstrated that TIGIT is the most predominant inhibitory receptor expressed on NK cells and that the sole deficiency of TIGIT on NK cells was capable of inhibiting tumor growth *in vivo* (174). Furthermore, blocking TIGIT restored NK cell functions as assessed by the levels of degranulation and cytokine production. Unlike TIGIT and PD-1, the role of TIM-3 in NK cell function is not well-defined and appears to be different. For example, TIM-3 has been shown to be a marker of NK cell maturation, with cells expressing TIM-3 having higher degranulation and cytokine production compared to the negative population (175). However, in the presence of antibody against TIM-3, a significant decline in lysis of target cells by NK cells was noted, indicating that signaling through TIM-3 negatively regulates NK cell cytotoxic functions (175). Similarly, another study

revealed that NK cells from melanoma patients incubated with anti-TIM-3 resulted in decreased degranulation and IFN-γ expression (176). Furthermore, the upregulation of TIM-3 on PB NK cells has been reported in various cancers such as advanced melanoma (176), and lung cancer (177) and has been associated with poor prognostic factors. However, the stimulation of NK cells with recombinant Galectin-9 (rGal-9), the ligand for TIM-3, was shown to enhance the function of NK cells as assessed higher levels of IFN-γ expression (178). Given the promiscuity of TIM-3 binding to numerous ligands, the controversial effects observed are postulated to be dependent on the density and type of ligand associating with TIM-3 (165). Nonetheless, the role of TIM-3 as an inhibitory receptor on NK cells requires further investigation.

1.10 Galectin-9

Gal-9 is a β-galactoside-binding lectin that is ubiquitously expressed at baseline in many tissues as well as various immune cells (T cells, B cells and mast cells) (179). More specifically, Gal-9 is a 34-39 kDa protein with two distinct carbohydrate recognition domains (CRDs) that are linked by a linker peptide (185). The range in the molecular weight of this protein is due to the three different isomers that exist (Gal-9 short, Gal-9 medium and Gal-9 long) which only differ in their relative length of the linker peptide (256). There are multiple reported receptors for Gal-9 including protein disulfide isomerase (PDI), CD44, CD137, TIM-3 and IgE, highlighting its versatile role (180-184). Initially discovered as an eosinophil chemoattractant, Gal-9 has a wide range of immunomodulatory roles such as cell adhesion, migration and apoptosis (184, 185). Other important roles of Gal-9 include regulating the immune response through induction of Tregs and suppression of the proinflammatory Th17 and Th1 cells (186). Gal-9 has been shown to decrease Th17 and Th1 cells by binding to its prototypical receptor, TIM-3, and inducing an

influx of intracellular Ca^{2+} that eventually leads to caspase activation and death of the cells (184). Moreover, Gal-9 is made on free ribosomes and it is secreted non-classically, as it does not contain a signaling sequence to direct it to the ER (185). Its ER/Golgi- independent mechanism of secretion is further highlighted by the fact that Gal-9 secretion is not blocked by the addition of brefeldin A and monensin (187). Interestingly, during the early stages of HIV infection, there is an elevation of plasma Gal-9 (188, 189), which is associated with higher viral load (190). Our group has also observed a similar association, as plasma Gal-9 from individuals with an elevated viral load (> 10, 000 copies/ml) had significantly higher plasma Gal-9 than individuals with lower viral load (< 10, 000 copies/ml) (191). However, upon initiation of ART the levels of this lectin were dramatically reduced (189). Given the association of Gal-9 with viremia, various studies sought to determine the role of Gal-9 in HIV pathogenesis. A study revealed that the addition of recombinant Gal-9 (rGal-9) induced the expression of host cell cytidine deaminase, APOBEC3G (192). The APOBEC3G is an enzyme that induces hypermutation of the viral genome by converting guanine to adenine (193). It was demonstrated that the upregulation of APOBEC3G through the addition of rGal-9 reduced the infectivity of HIV (as assessed by integrated HIV DNA in $CD4^+$ T cells) by 7-fold compared to a negative control (192). Although Gal-9 may contribute to a lower infectivity, it has also been shown to promote HIV entry into cells by modulating the T cell surface redox state (180). Specifically, Gal-9 is capable of increasing the retention of surface PDI, an enzyme that promotes the cleavage of disulfide bonds on the T cell glycoprotein, CD4. It has been shown that the reduction of this glycoprotein is crucial for enabling Gal-9's interaction with the HIV glycoprotein 120 (gp120) to mediate viral entry (194). Furthermore, rGal-9 can reactivate latent HIV reservoirs by inducing HIV transcripts in both the J-Lat HIV latency model and $CD4^+$ T cells (192). Recently

it has been shown that rGal-9 is able to induce HIV transcription via T cell receptor-mediating activation of extracellular signal-regulated kinase 1/2 (ERK1/2) (195, 196). Finding mechanisms of depleting HIV reservoirs by forcing the provirus out of hiding so that it can be seen by the immune system, is known as the "shock and kill" strategy (197). This strategy has gained significant interest, as one of the biggest impediments to the cure of HIV is the presence of latent reservoirs even in the presence of ART (198). Thus, Gal-9 is currently being studied as a potential HIV latency reversal agent. In the context of NK cells, a study by Golden-Mason *et al*. examined the impact of rGal-9 on NK cells that were previously stimulated with IL-12/IL-15 (199). Incubation of these NK cells with Gal-9 resulted in significantly lower IFN-γ expression and downregulation of genes involved in NK cell-mediated cytotoxicity such as CD16, NKp46, NKp30, and perforin (199). These functional impairments mediated by rGal-9 were shown to be independent of the TIM-3, indicating that other receptors may be engaging with Gal-9.

1.11 Hypothesis and aims

One of the hallmarks of immune exhaustion is the upregulation of co-inhibitory receptors on T cell, which leads to overall decreased effector functions such as cytokine production, proliferative capacity and cytotoxicity (166, 200). Upregulation of co-inhibitory receptors such as PD-1, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), LAG-3 (Lymphocyte Activation Gene-3), TIGIT and TIM-3 with T cell exhaustion in HIV-infection is well-defined (201-203). For instance, upregulation of TIM-3 on both $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals is widely studied (204, 205) and its upregulation has been associated with exhausted T cells (206, 207). Moreover, TIM-3 expression has been shown to be negatively correlated with $CD4^+$ counts and positively with viral load (208). As previously mentioned, the level of soluble

Gal-9 (the ligand for TIM-3) is substantially higher in HIV-infected individuals compared to healthy controls (188, 191, 205). Previously, our group has shown that the elevated serum Gal-9 in HIV-infected individuals interacts with TIM-3 on $CD4^+$ T cells and inhibits T cell proliferation, which leads to reduced viral replication in activated $CD4^+$ T cells (191). In another study, our group discovered that Tregs constitutively express Gal-9 on their surface and the interaction of Gal-9 with TIM-3 on $CD8^+$ T cells impairs their function (139). This report found that $CD8^+$ T cells restricted by HLA-B27 and B57 upregulate less TIM-3 compared to non-HLA-B27/57 upon recognition of their cognate epitopes and thus are less susceptible to Treg-mediated suppression; this explains why individuals restricted by HLA-B27/B57 have more polyfunctional CTLs and subsequently, a natural immunity against HIV (139). Based on these evidences, it is clear that Gal-9 plays an important role in HIV pathogenesis. Therefore, we aimed to further study the role of surface Gal-9 expression on NK cells in HIV-infected individuals. To our knowledge, there are no studies that have examined whether Gal-9 is upregulated in HIV-infected individuals and subsequently how Gal-9 overexpression on NK cells modulates their function in the context of HIV. We hypothesized that HIV-1 infection may enhance Gal-9 expression on NK cells, which impairs their functionality. To test our hypothesis, we decided to determine the expression pattern of surface Gal-9 on NK cells in different HIV-infected patients, including those on ART, LTNPs and progressors in comparison to HIV-uninfected controls. In addition, the functional potential of Gal-9+ vs. Gal-9- NK cells in HIV+ individuals were investigated by examining the expression of perforin, GzmB, GNLY, and IFN-γ. Moreover, the impact of cytokine cocktail stimulation (IL-12, IL-15, and IL-18) on the function of Gal-9+ vs. Gal-9- NK cells was determined by assessing degranulation, cytotoxic mediators, and IFN-γ

expression. Lastly, we evaluated the expression levels of other important co-inhibitory receptors such as TIGIT and TIM-3 on NK cells and compared their functionality with Gal-9+ NK cells.

Chapter 2: Materials and Methods

2.1 Study participants

The study cohort was comprised of the following four groups:

1. Healthy controls (HCs): HIV seronegative-individuals (34 subjects).
2. HIV patients currently undergoing antiretroviral therapy (ART) (72 subjects).
3. Long-term non-progressors (LTNP): infected with HIV for more than 11 years, have a CD4 count > 500 cells/ml of blood and plasma viral lead undetectable or < 10,000 copies/ml in the absence of ART (15 subjects).
4. Progressors (Ps): infected with HIV for more than 5 years, have a CD4 count < 400 cells/ml blood and plasma viral load >10,000 copies/ml in the absence of ART (20 subjects).

The study was approved by the institutional research review boards at the University of Alberta (Protocol #Pro000046064 and Pro000070528*)* and written informed consent was obtained from all study participants. In addition, some PBMCs were obtained from the Centre for AIDS Research (CFAR)-University of Washington through an already established Material transfer agreement (MTA).

2.2 Human sample collection and processing

2.2.1 Fresh samples

Fresh blood was obtained from all healthy controls and the majority of HIV-infected individuals on ART. Blood was collected in tubes spray-coated with the anticoagulant, K_2EDTA (Fisher Scientific; 02-657-32) and subsequently centrifuged (1200 rpm, 10 min) to remove the plasma. Next, room temperature Phosphate buffered saline (PBS) solution (Sigma-Aldrich; D8537) was used to dilute the whole blood (1:1). The diluted blood

sample was carefully layered onto 15 ml of Ficoll-Paque media solution (Sigma-Aldrich; IgGE17-5442-03) and the sample was centrifuged (2000 rpm, 10 min) with the brakes off. The buffy coat, containing PBMCs, was collected using a sterile pipette and placed in a sterile 50 ml tube. Enough PBS was added to fill the tube up to 50 ml and the sample was subsequently centrifuged (1400 rpm, 10 min). Lastly, the supernatant was removed, and cells were resuspended in RPMI-1640 Medium (Sigma-Aldrich; R0883) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific; 12483020) and 1% penicillin/streptomycin (Sigma-Aldrich; P4333).

2.2.2 Frozen samples

Previously, cryopreserved PBMCs (10% Dimethyl sulfoxide (DMSO)), 90% FBS) from LTNPs, Progressors, and a few HIV-infected individuals on ART were thawed and utilized for experiments.

2.3 Flow cytometry

2.3.1 Antibodies and analysis

Fluorophore conjugated antibodies with specificity against human cell antigens and cytokines were purchased from Biolegend, Invitrogen, eBioscience or BD bioscience. The antibodies utilized were: anti-Galectin-9 (9M1-3), anti-TIGIT (MBSA43), anti-TIM-3 (7D3), anti-CD3 (HIT3a), anti-CD16 (eBioCB16, 3G8, B73.1), anti-CD56 (CMSSB, B159), anti-granzymeB (GB11), anti-perforin (dG9), anti-GNLY (RB1), anti-IFN-γ (B27), anti-CD107a (H4A3), anti-NKG2D (1D11), anti-NKp30 (p30-15), anti-NKp44(P44-8) and anti-NKp46 (9-E2). LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher

Scientific; L34966) was used to exclude dead cells. After fixation with Paraformaldehyde (PFA 4%), cells were transferred into fluorescence-activated cell sorting (FACS) tubes and acquired using Fortessa-X20 or BD LSR Fortessa-SORP (BD Bioscience). All data was analyzed data using the FlowJo software (version 10).

2.3.2 Surface staining

To stain surface markers, cells (1 x 10^6) were placed in a 96-well round bottom plate and washed in PBS containing 2% FBS by centrifugation (1600 rpm, 3 min). The supernatant was removed and then a master mix was prepared with the corresponding surface antibodies in a manner such that each well would contain 1µl of each antibody in 25 µl of 2% FBS in PBS. The plate was covered with tin foil to protect it from light and was incubated at 4°C for 30 min. After the incubation, cells were washed by adding 175 µl of 2% FBS in PBS into each well and centrifuged (1600 rpm, 3 min). The supernatant was discarded and the cells were fixed in 200 µl of 4% PFA (Sigma-Aldrich; P6148).

2.3.3 Intracellular staining

In order to perform intracellular cytokine staining (ICS), previously surface stained cells were resuspended in 200 µl of Cytofix/cytoperm solution (BD Bioscience; 554714) and incubated in the dark for 20 min at 4°C. Cells were spun down (1600 rpm, 3 min) and washed twice with 1X diluted Perm/Wash buffer (BD Bioscience; 554723). Next, a master mix was prepared with the corresponding intracellular antibodies as described above. The plate was protected from light and incubated at 4°C for 30 min. After the incubation, cells were washed by adding 175 µl of 2% FBS in PBS into each well and centrifuged (1600

rpm, 3 min). The supernatant was discarded and the cells were fixed in 200 µl of 4% PFA (Sigma-Aldrich; P6148).

2.4 Image cytometry

ImageStream analysis was performed on surface-stained PBMCs that were fixed with 4% PFA. Over 15000 images were collected using Amnis ImageStream Mark II (EMD Millipore). Analysis was performed by choosing an aspect ratio > 0.8, using cells that were in focus, and gating for NK cells ($CD3^-$, $CD56^{+/-}$ and $CD16^{+/-}$). Afterwards, the Gal-9+ population was gated on the total NK cells.

2.5 Cytokine stimulation

One million PBMCs were placed in a 96-well round bottom plate and stimulated with a cytokine cocktail containing IL-12 (10 ng/ml), IL-15 (20 ng/ml) and 1L-18 (100 ng/ml). Wells without cytokine cocktail were used as negative controls. Stimulated and unstimulated PBMCs were placed in a total volume of 200 µl with RPMI-1640 Medium (Sigma-Aldrich; R0883) containing 10% FSB (Thermo Fisher Scientific; 12483020) and 1% penicillin/streptomycin (Sigma-Aldrich; P4333). Cultures were incubated for 24 hours with 5% CO_2 in the presence of anti-CD107a (BD Bioscience; 563869). At the 20-hour mark, 1 µl of GolgiStop (BD Biosciences; 554724) was added. Cells were spun down (1600 rpm, 3 min) and the protocol for surface and intracellular staining was conducted immediately.

2.6 Gal-9 blocking studies

One million PBMCs were placed in a 96-well round bottom plate in a total volume of 200 µl with RPMI-1640 Medium (Sigma-Aldrich; R0883) containing 10% FSB (Thermo Fisher Scientific; 12483020) and 1% penicillin/streptomycin (Sigma-Aldrich; P4333). Cells were incubated for 24-hours at 37°C with 5% CO_2 in the presence of anti-CD107a (BD Bioscience; 563869). Some wells received 30 mM of lactose to block Gal-9 (Thomas Scientific; C987F82). At the 20-hour mark, 1 µl of GolgiStop (BD Biosciences; 554724) was added. Cells were spun down (1600 rpm, 3 min) and the protocol for surface and intracellular staining was conducted immediately.

Wells without lactose served as negative controls. Both lactose and lactose-free cultures were placed in a total volume of 200 µl with RPMI-1640 Medium (Sigma-Aldrich; R0883) containing 10% FSB (Thermo Fisher Scientific; 12483020) and 1% penicillin/streptomycin (Sigma-Aldrich; P4333). Cells were incubated for 24 hours with 5% CO_2 in the presence of anti-CD107a (BD Bioscience; 563869). At the 20-hour mark, 1 µl of GolgiStop (BD Biosciences; 554724) was added. Cells were spun down (1600 rpm, 3 min) and stained for surface and intracellular cytokine staining according to our described protocols.

2.7 NK cell enrichment

EasySep Human NK Cell Isolation Kit (Stemcell; 17955) was used to negatively isolate NK cells from fresh PBMCs. Isolated NK cells with 85% to 95% purity were obtained by following the manufacturer's protocol for isolation as shown below.

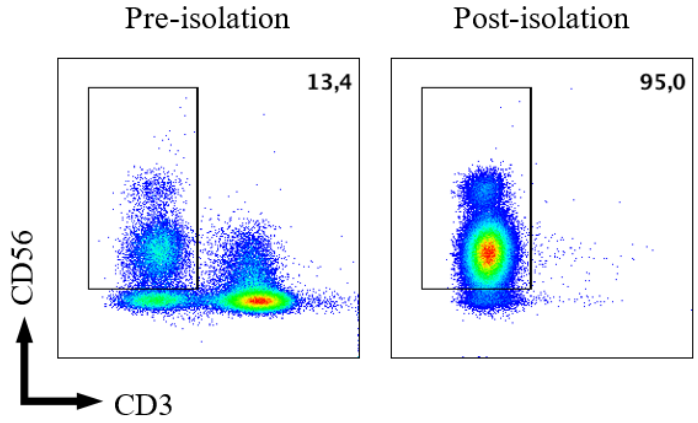

Figure 2-1: Purity plot. Representative plot for the percentage of NK cells ($CD3^{-}CD56^{+}$) before and after isolation by EasySep Human NK Cell Isolation Kit (Stemcell Technologies; 17955).

2.8 Gene expression analysis

Enriched NK cells from HCs and HIV-infected individuals on ART were stored in TRI Reagent (Sigma-Aldrich;T9424). Subsequently, the total RNA was extracted using Direct-zol RNA MicroPrep Kit (Zymo Research;R2060) according to the manufacturer's protocol. The RNA concentration and purity were measured using a NanoDrop 1000 Spectrophotometer. Prior to the quantitative polymerase chain reaction (qPCR) analysis, 200 ng of total RNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription (Qiagen) to contain comparable RNA concentrations. Genomic DNA was removed by incubating the samples with DNA wipeout for 3 min at 42°C, followed by cDNA synthesis for one hour at 42°C. Each Reverse Transcription-PCR (RT-PCR) reaction mixture consisted of 4 µl Quantiscript RT Buffer, 1 µl RT primer mix, and 1 µl Quantiscript Reverse Transcriptase. The gene expression assay was performed on the CFX96 TouchTM Real-Time PCR Detection System (BioRad). Samples were analyzed in duplicates and the qPCR reaction mixtures consisted of 12.5 µl QuantiFast SYBR Green Master Mix (Applied Biosystems), 2.5 µl of each primer assay, 10 ng of template cDNA and the addition of PCR water to reach a final volume of

25 µl. The cycling program contained: a preparation step of 50°C for 2 min, followed by initial denaturation at 95°C for 15 min and forty cycles of 94°C for 15 sec. Primer annealing occurred at 55°C for 30 seconds and extension occurred at 72°C for 30 seconds. After the amplification cycles were completed, melting curves were obtained to check for single distinct peaks of PCR amplicons across all the samples. The melt curves were generated by a stepwise increase of the temperature from 60°C to 95°C (increase of 0.5°C every 10 seconds). The expression levels of Lgals9 genes (Qiagen; QT00014273) were examined and β2- macroglobulin (B2M, Qiagen; QT00088935) was used as a house-keeping gene. The mRNA from healthy controls was used as a reference group for the fold change calculations, where the gene expression of the targeted genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.9 Statistical analysis

The Mann-Whitney test was used to compare differences between groups. The PRISM software was utilized for statistical analysis, with p value <0.05 being considered statistically significant. Results are presented as mean ± standard error of mean (SEM).

Chapter 3: Results

3.1 Changes in NK cell subpopulations in HIV-1 infected individuals

Considering that NK cells are comprised of a heterogeneous population with varying function and capability, we sought to assess the possible changes in NK cell subpopulations in HIV-patients on ART relative to HCs. We observed a significant increase in the $CD56^+$, no change in the $CD56^+CD16^+$, but a significant decrease in the $CD16^+$ population in HIV-infected individuals on ART (Fig.3-1A and 3-1B). Our study divided NK cells into 3 subpopulations; $CD56^+$, $CD56^+CD16^+$ and $CD16^+$. However, these 3 subpopulations can be further subdivided into bright, mid and dim subpopulations depending on the relative expression for each marker (ex. $CD56^{bright}$, $CD56^{dim}CD16^{bright/+}$etc.). Thus, due to the variation in the way that NK cells are categorized, the effect of HIV infection on the subpopulations of NK cells remain unclear in the literature. However, a study conducted with the same gating strategy that we have utilized indicated a decrease in $CD16^+$ population in HIV-infected individuals undergoing ART compared to ART-naive individuals (158). Similar to ART-treated individuals, LTNPs and progressors had a significantly higher $CD56^+$ subpopulation compared to HCs (Fig. 3-1C and 3-1D). However, unlike ART-treated individuals, the double positive population was significantly lower in progressors and LTNPs compared to HCs (Fig. 3-1C and 3-1D). Meanwhile, there was no change in the proportion of $CD16^+$ subpopulation in both progressors and LTNPs (Fig. 3-1C and 3-1D).

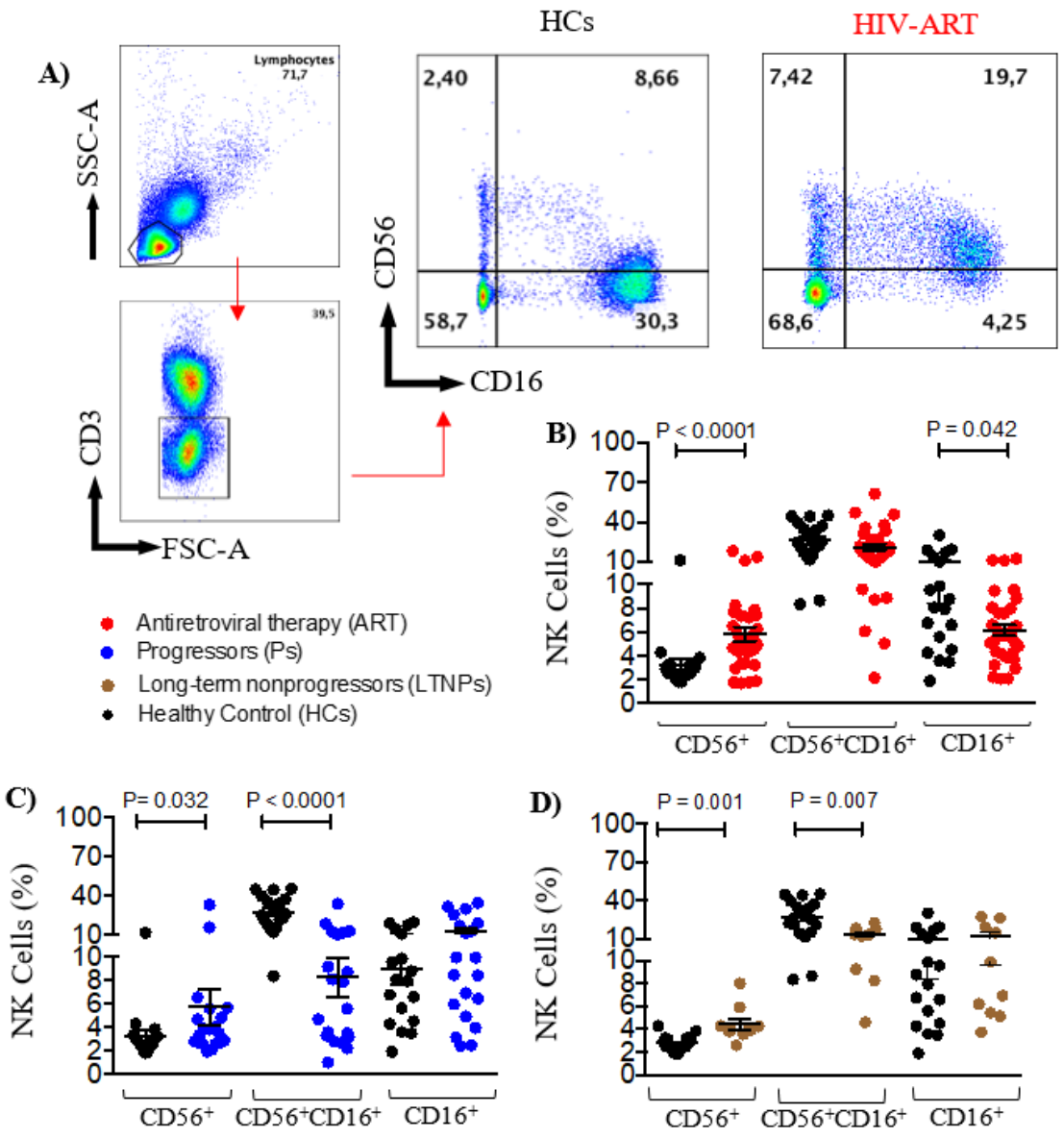

Figure 3-1: Changes in the frequency of NK cell subpopulations in various HIV-infected groups in comparison to HCs. (A) Gating strategy and representative flow cytometry dot plots showing NK cell subpopulations in HCs versus HIV-patients on ART. (B) Cumulative data indicating percentages of the three subpopulations of NK cells in HCs vs. HIV-patients on ART. (C) Cumulative data indicating percentages of the three subpopulations of NK cells in HCs vs. progressors. (D) Cumulative data indicating percentages of the three subpopulations of NK cells in HCs vs. LTNPs. Each point represents data from an individual patient (HC n=19, HIV n=37). Bar, mean ± one standard error. SSC-A, side scatter area; FSC-A, forward scatter area; Healthy Controls (HCs); Antiretroviral therapy (ART).

3.2 Increase in Gal-9 expression in HIV-infected individuals relative to HCs

Next, we evaluated whether NK cells in HIV-infected patients differ in their surface expression of Gal-9, TIGIT and TIM-3. We observed a significant increase in Gal-9 expression on $CD56^+$ (Fig. 3-2A, 3-2B and Fig. 3-3A) and $CD56^+CD16^+$ (Fig. 3-2A, 3-2C and Fig. 3-3B) subpopulations of NK cells in HIV-infected individuals on ART compared to HCs. We also examined the surface expression of the indicated molecules on the two other cohorts of HIV-infected individuals, progressors and LTNPs, both of which were ART-naive. Interestingly, we observed the same pattern of increased surface Gal-9 expression on $CD56^+$ and $CD56^+CD16^+$ subpopulations as observed in ART-treated individuals when compared to HCs (Fig. 3-3A and 3-3B). Furthermore, we observed that progressors express significantly higher levels of Gal-9 on the $CD56^+CD16^+$ subpopulation compared to the LTNPs (Fig. 3-3B). However, there was no significant difference in the expression levels of Gal-9 on the $CD16^+$ subpopulation of HIV-patients on ART, progressors or LTNPs when compared HCs (Fig. 3-3C). To our knowledge, this is the first report of its kind showing the overexpression of Gal-9 on NK cells in HIV-infected individuals.

To further characterize NK cells, we measured TIGIT expression and observed an increase in TIGIT on only the $CD56^+$ NK cell population in HIV-patients on ART compared to HCs (Fig. 3-2A, 3-2B and Fig. 3-3A). A similar observation was made when TIGIT expression was examined in progressors and LTNPs (Fig. 3-3A). Examining the $CD56^+CD16^+$ subpopulations, we observed that LTNPs have significantly higher TIGIT expression compared to HCs, progressors and HIV-patients on ART (Fig. 3-3B). A similar expression pattern was observed in the $CD16^+$ subpopulation; although the difference between TIGIT expression on LTNPs versus

progressors was not statistically significant, it followed the same trend as the double positive populations (Fig. 3-3C). In agreement with our observations, NK cells have been shown to express TIGIT (171, 174) and this co-inhibitory receptor has been shown to be upregulated on NK cells in HIV-infected individuals (112).

Finally, we investigated the expression of TIM-3, the receptor for Gal-9, on NK cell subpopulations in various cohorts of HIV-infected patients. Compared to HCs, there was a decrease in TIM-3 expression on $CD56^{+}$ population in only the HIV-patients on ART (Fig. 3-2A, 3-2B and Fig. 3-3A). Interestingly, it has been reported that NK cells have the highest TIM-3 expression amongst lymphocytes (175, 178) and TIM-3 has been shown to be a marker of maturation and activation, as it can be upregulated on immature NK cells upon cytokine stimulation (175). However, it has been reported that TIM-3 is downregulated on NK cells in HIV-infected individuals compared to HCs (190). Similarly, we found ART-treated individuals had lower TIM-3 expression than both progressors and LTNPs in the $CD56^{+}$ subpopulation of NK cells (Fig. 3-3A). A statistically lower mean fluorescent intensity (MFI) for TIM-3 on $CD56^{bright}$ populations of NK cells in HIV-patients on ART has been noted when compared to progressors but that statistical significance has not been observed when compared to LTNPs (190). Lastly, we observed an upregulation of TIM-3 in both the $CD56^{+}CD16^{+}$ and $CD16^{+}$ subpopulations of NK cells in LTNPs compared to progressors, patients on ART and HCs (Fig. 3-3B, 3-3C).

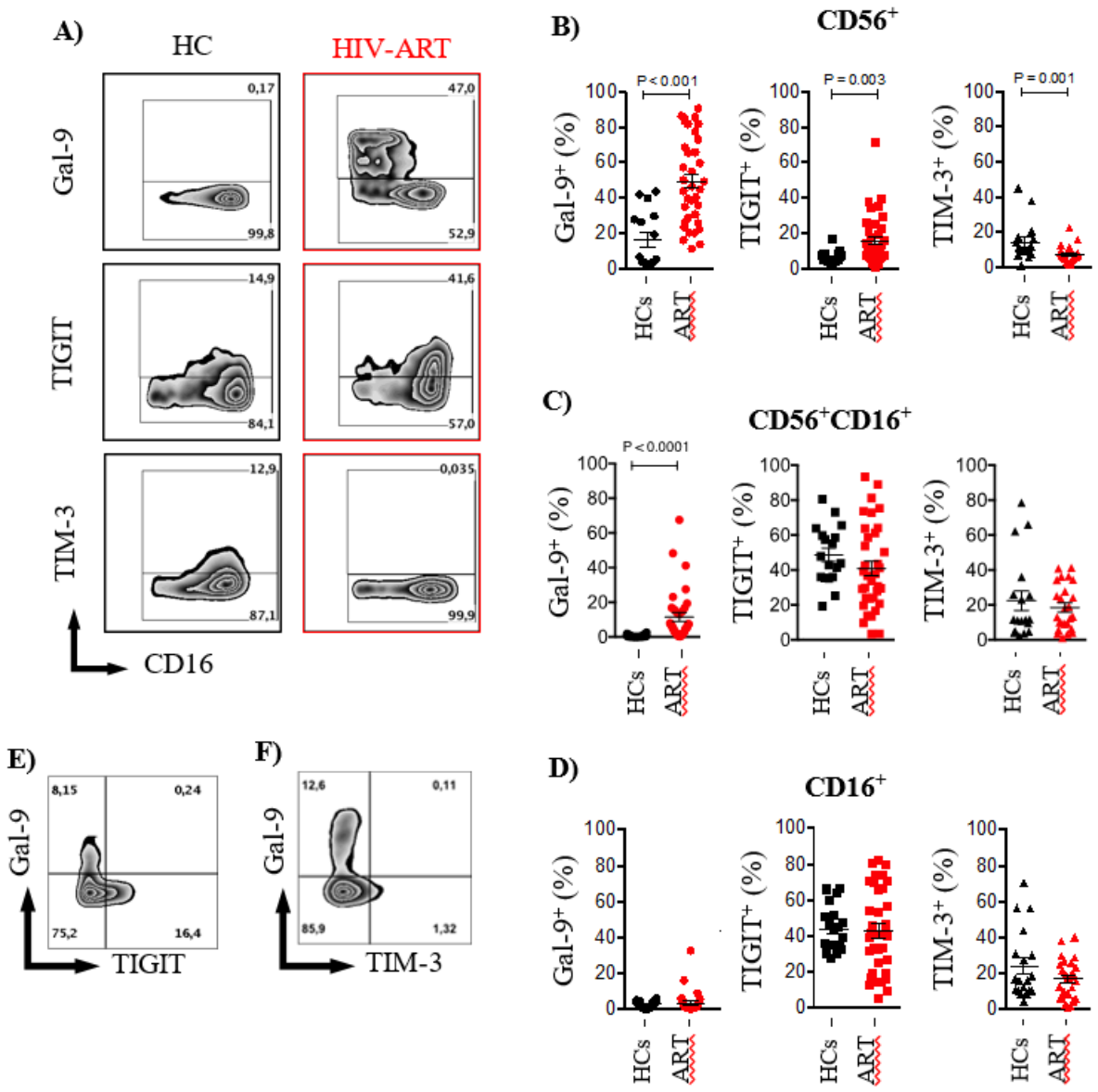

Figure 3-2: Significant increase in the surface expression of Gal-9 and TIGIT but decrease in TIM-3 on NK cells in HIV patients on ART versus HCs. (A) Representative flow cytometry dot plots of $CD56^{+}CD16^{+}$ NK cell in HCs vs. HIV-patients on ART for Gal-9, TIGIT and TIM-3. (B) Cumulative data indicating percentages of surface Gal-9, TIGIT and TIM-3 on $CD56^{+}$ NK cells in HCs versus HIV patients on ART. (C) Cumulative data indicating percentages of surface Gal-9, TIGIT and TIM-3 on $CD56^{+}CD16^{+}$ NK cells in HCs versus HIV patients on ART. (D) Cumulative data indicating percentages of surface Gal-9, TIGIT and TIM-3 on $CD16^{+}$ NK cells in HCs versus HIV patients on ART. (E) Representative flow cytometry dot plot showing co-expression of Gal-9 and TIGIT on the $CD56^{+}CD16^{+}$ population of NK cells. (F) Representative flow cytometry dot plot showing co-expression of Gal-9 and TIM-3 on the $CD56^{+}CD16^{+}$ population of NK cells. Each point represents data from an individual patient (HCs n=19, HIV n=37). Bar, mean ± one standard error. Galectin-9 (Gal-9), T-cell immunoreceptor with Ig and ITIM domains (TIGIT); T-cell immunoglobulin and mucin domain 3 (TIM-3).

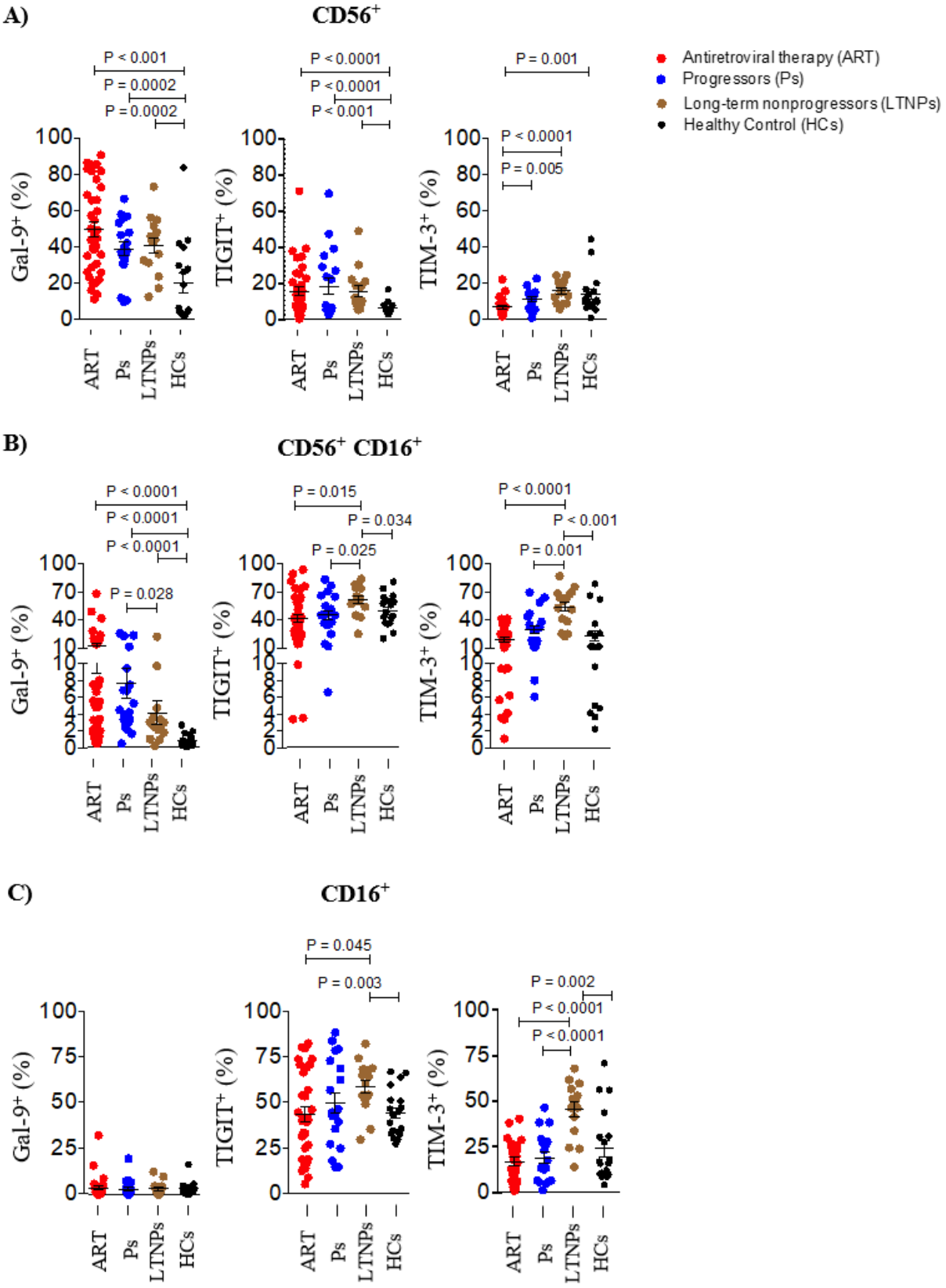
A)
CD56+
P < 0.001
P = 0.0002
P = 0.0002
P < 0.0001
P < 0.0001
P < 0.001
P = 0.001
P < 0.0001
P = 0.005
Gal-9+ (%)
TIGIT+ (%)
TIM-3+ (%)
ART
Ps
LTNPs
HCs
Antiretroviral therapy (ART)
Progressors (Ps)
Long-term nonprogressors (LTNPs)
Healthy Control (HCs)
B)
CD56+ CD16+
P < 0.0001
P < 0.0001
P < 0.0001
P = 0.028
P = 0.015
P = 0.034
P = 0.025
P < 0.0001
P < 0.001
P = 0.001
C)
CD16+
P = 0.045
P = 0.003
P = 0.002
P < 0.0001
P < 0.0001

Figure 3-3: Expression of surface Gal-9, TIGIT and TIM-3 in various HIV-infected individuals (on-ART, Ps, LTNPs) compared to HCs. (A) Cumulative data indicating percentages of surface Gal-9, TIGIT and TIM-3 on $CD56^+$ NK cells in HCs versus various HIV-infected individuals (on-ART, Ps, LTNPs). (B) Cumulative data indicating percentages of surface Gal-9, TIGIT and TIM-3 on $CD56^+CD16^+$ NK cells in HCs versus various HIV-infected individuals (on-ART, Ps, LTNPs). (C) Cumulative data indicating percentages of surface Gal-9, TIGIT and TIM-3 on $CD16^+$ NK cells in HCs versus various HIV-infected individuals (on-ART, Ps, LTNPs). Each point represents data from an individual patient (HCs n=19, on-ART n=37, Ps n=20, LTNPs n=15). Bar, mean ± one standard error. Progressors (Ps); Long-term non-progressors (LTNPs).

3.3 NK cells contain intracellular Gal-9

Given that HIV-infected individuals have significantly higher amounts of circulating Gal-9 in their plasma (133, 134) and that Gal-9 has the capacity to bind to carbohydrates on the surface of NK cells, we wanted to determine whether there was a difference in intracellular Gal-9 levels in NK cells from HIV-patients on ART compared to HCs. Using imaging cytometry, we observed surface expression of Gal-9 on NK cells that were $CD56^+CD16^+$ and $CD16^+$ (Fig. 3-4A). Next, utilizing the same patient sample, we permed the cells and observed that $CD56^+$, $CD56^+CD16^+$, and $CD16^+$ NK cells all contained intracellular Gal-9 (Fig. 3-4B). Additionally, in HCs we observed that all three subpopulations of NK cells had intracellular Gal-9 (Fig. 3-4C). Having observed that NK cells from HIV patients have higher levels of Gal-9, specifically the $CD56^+$ and the $CD56^+CD16^+$ subpopulations compared to HCs, we wondered whether the level of Gal-9 transcripts would be higher in HIV patients. NK cells were isolated from 7 HCs and 7 HIV patients on ART and the level of Gal-9 transcripts was examined. However, we did not observe a significant difference in the level of Gal-9 mRNA between HIV patients on ART and HCs (Fig. 3-4D). As such, our observations indicated that all NK cells constitutively express Gal-9 intracellularly and there was no difference in mRNA levels between HIV-infected individuals and HCs.

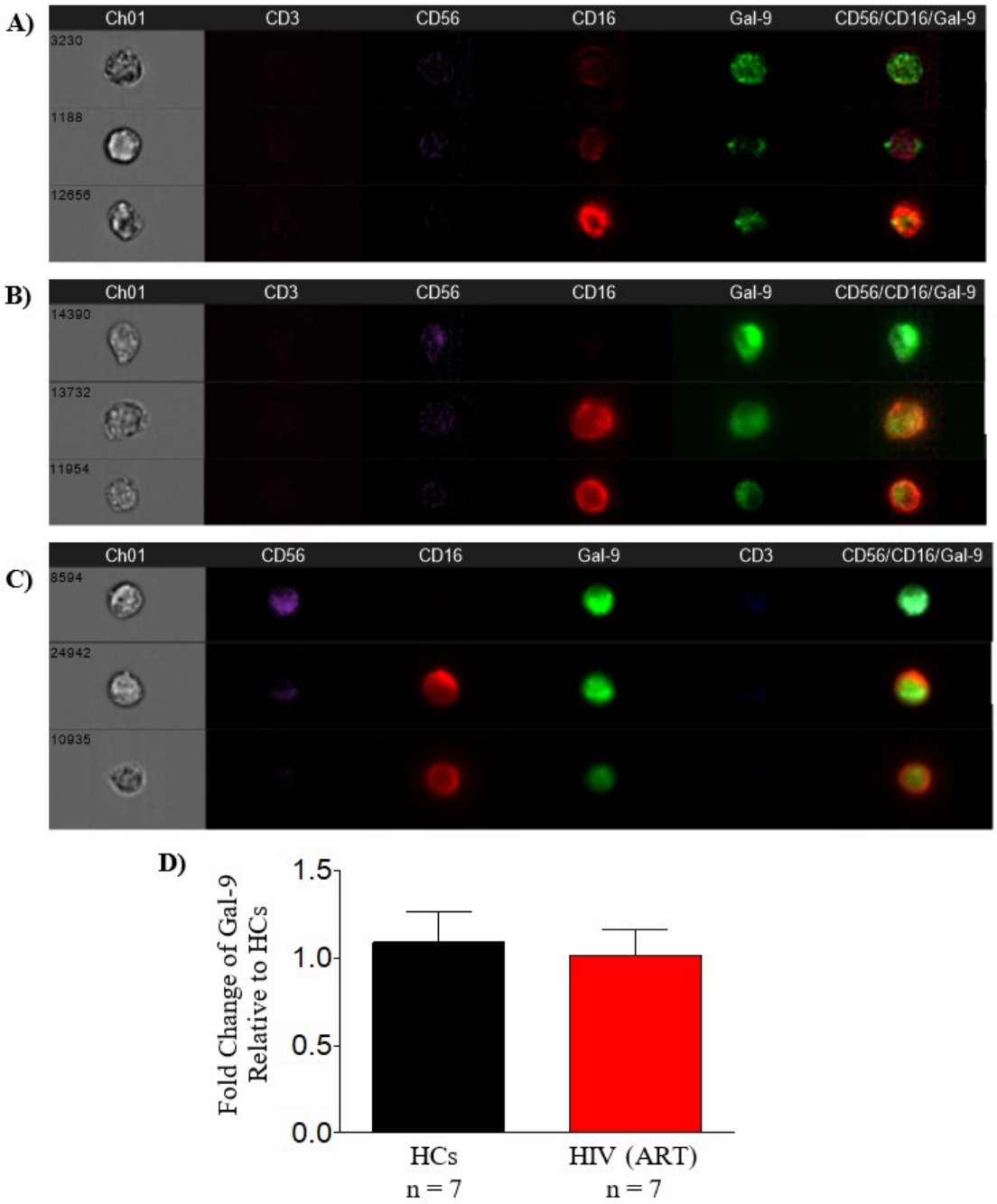

Figure 3-4: Expression of surface and intracellular Gal-9 on NK cells. (A) Representative of >15,000 images of NK cells taken using Amnis ImageStream Mark II, showing surface Gal-9 expression in HIV-infected individual on ART. (B) Representative of ~3000 images of NK cells taken using Amnis ImageStream Mark II, showing intracellular Gal-9 expression in NK cells from the same HIV-infected individual on ART. (C) Representative of ~3000 images of NK cells taken using Amnis ImageStream Mark II, showing intracellular Gal-9 expression in NK cells from HCs. (D) Expression of Gal-9 mRNA in isolated NK cells from HCs versus HIV-infected individuals on ART.

3.4 Gal-9+ NK cells express lower cytotoxic mediators (GzmB, perforin and GNLY) compared to Gal-9- NK cells, which is in contrast with TIGIT+ NK cells

Given that we observed significantly higher expression of Gal-9 and TIGIT on NK cells from HIV patients, we next examined the functional potential of NK cells from patients on ART based on the expression of different markers for cytotoxicity. We observed that Gal-9+ NK cells expressed significantly lower GzmB compared to the Gal-9- NK cells in all three subpopulations (Fig. 3-5A, 3-5C-5E). Interestingly, the opposite expression pattern was observed with TIGIT; TIGIT+ NK cells expressed greater GzmB compared to the TIGIT- counterparts on all three subpopulations of NK cells (Fig. 3-5B-5E). Comparing the two positive populations, we observed that Gal-9+ NK cells express significantly lower GzmB than TIGIT+ NK cells in all three subpopulations (Fig. 3-5C-5E). In addition, there was a significantly higher expression of GzmB on TIM-3+ compared to TIM-3- in only the $CD56^{+}CD16^{+}$ subpopulation of NK cells.

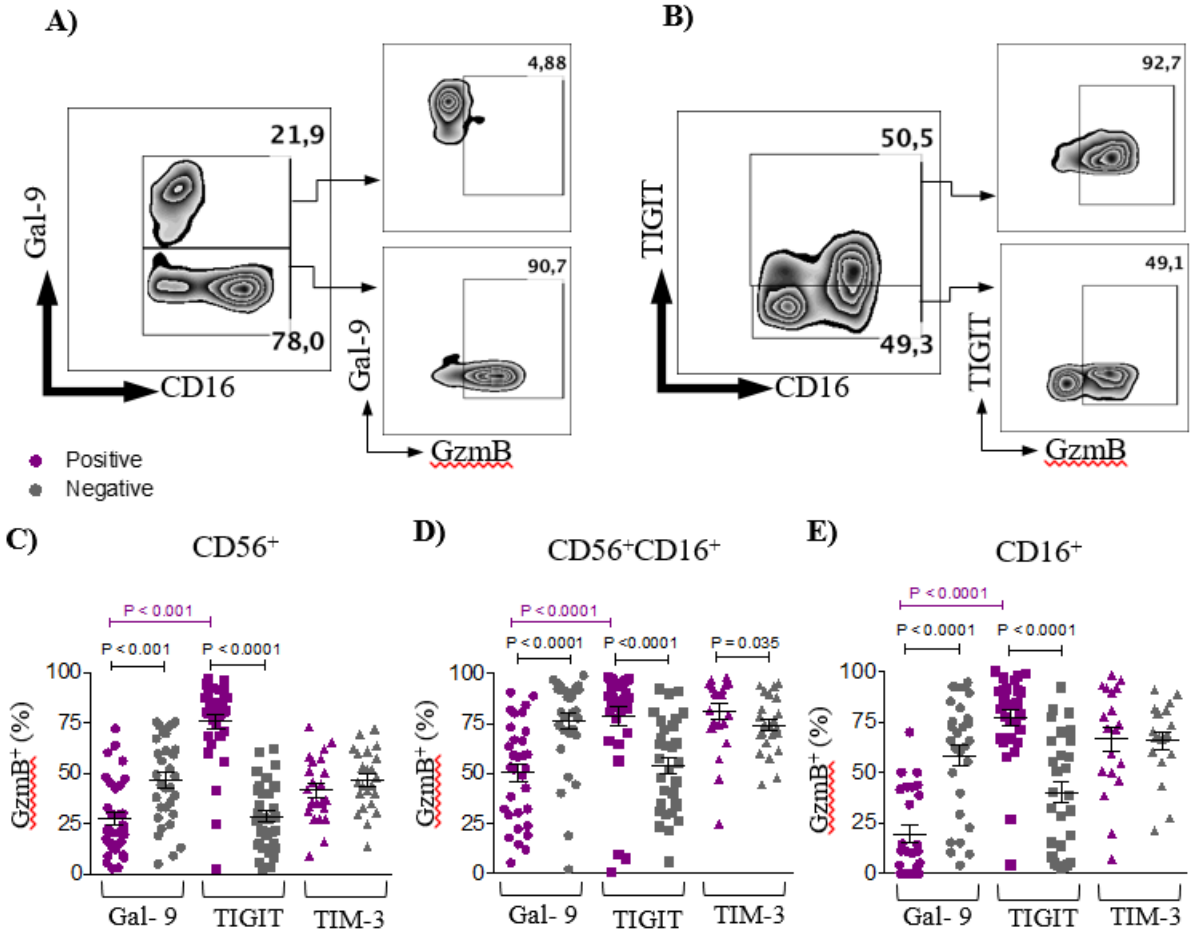

Figure 3-5: Gal-9+, compared to Gal-9- NK cells, express significantly lower levels of GzmB, which is in contrast with TIGIT+ NK cells. (A) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$ population showing GzmB expression in Gal-9+ versus Gal-9- NK cells in HIV-infected individuals on ART. (B) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$ population showing GzmB expression in TIGIT+ versus TIGIT- NK cells in HIV-infected individuals on ART. (C) Cumulative data indicating percentages of GzmB expression in $CD56^{+}$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-.(D) Cumulative data indicating percentages of GzmB expression in $CD56^{+}CD16^{+}$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-.(E) Cumulative data indicating percentages of GzmB expression in $CD16^{+}$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. Each point represents data from an individual patient (n = 32). Bar, mean ± one standard error. Granzyme B (GzmB).

In addition, we examined perforin expression on Gal-9+, TIGIT+, and TIM-3+ NK cells and their negative counterparts. Similar to GzmB, we observed that Gal-9+ NK cells expressed

significantly lower perforin compared to the Gal-9- NK cells in all three subpopulations (Fig. 3-6A, 3-6C-6E). In contrast, TIGIT+ NK cells expressed greater levels of perforin compared to the TIGIT- population in all three subpopulations of NK cells (Fig. 3-6B, 3-6C-6E). Comparing the two positive populations, we observed that Gal-9+ NK cells expressed lower perforin than TIGIT+ NK cells in all three subpopulations (Fig. 3-6C-6E). As observed with GzmB expression, there was a significantly higher expression of perforin on TIM-3+ compared to TIM-3- NK cells in the $CD56^{+}CD16^{+}$ subpopulation.

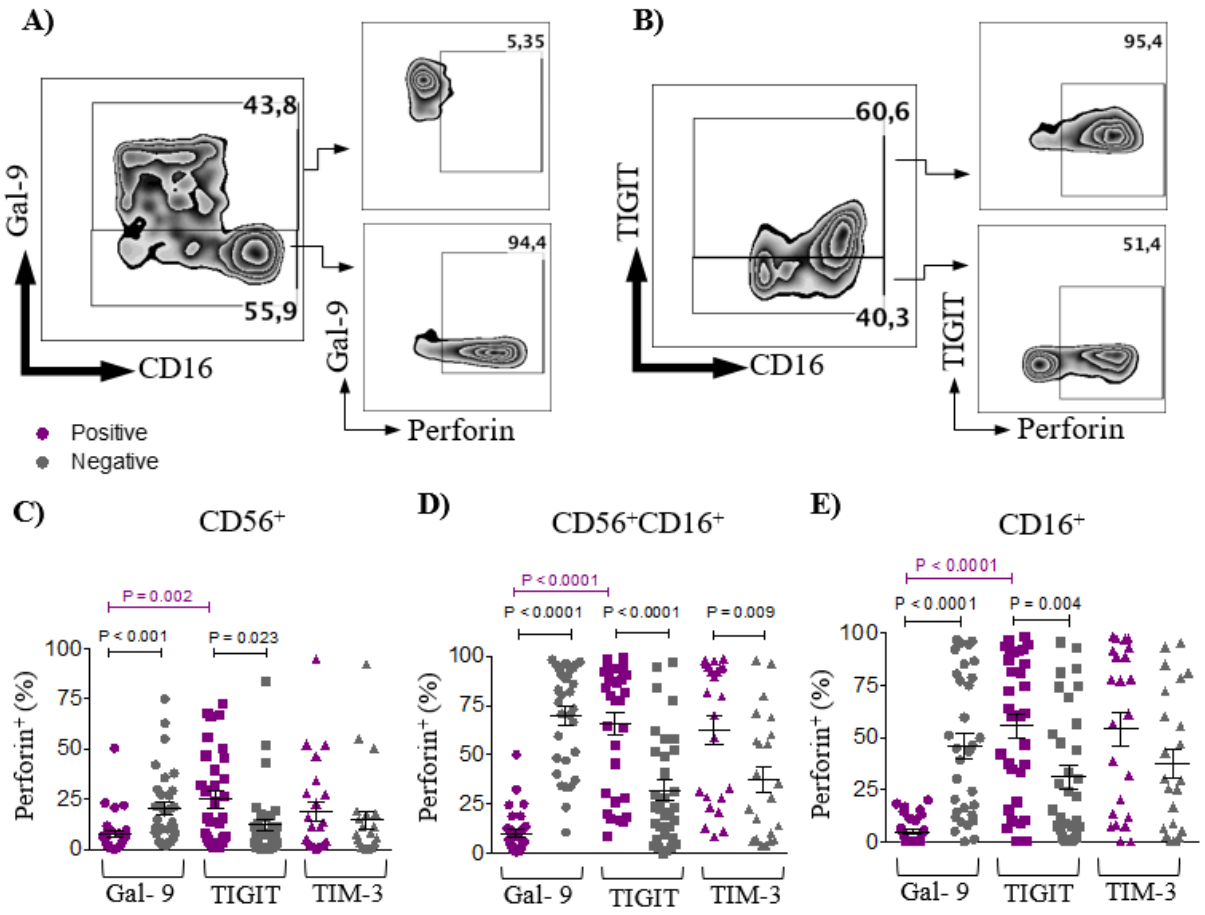

Figure 3-6: Gal-9+, compared to Gal-9- NK cells, express significantly lower levels of perforin which is in contrast with TIGIT+ NK cells. (A) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$ population showing perforin expression in Gal-9+ versus Gal-9- NK cells in HIV-infected individuals on ART. (B) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$

population showing perforin expression in TIGIT+ versus TIGIT- NK cells in HIV-infected individuals on ART. (C) Cumulative data indicating percentages of perforin expression on $CD56^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+, and TIM-3-. (D) Cumulative data indicating percentages of perforin expression in $CD56^+CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+, and TIM-3-. (E) Cumulative data indicating percentages of perforin expression in $CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. Each point represents data from an individual patient (n = 32). Bar, mean ± one standard error.

Our current understanding of the mechanism of function of GzmB and perforin is that perforin induces a transient calcium influx that initiates the membrane repair mechanism that allows the cell to endocytose both perforin and GzmB in a large endosome. From this large endosome, perforin can punch holes in the membrane and allow GzmB to escape into the cytosol to initiate apoptosis of the cells (34, 35). Knowing that the presence of GzmB and perforin together is essential for their function, we next examined the co-expression of these two cytotoxic effectors in Gal-9+/- and TIGIT+/- NK cells. We observed that Gal-9+ NK cells co-expressed significantly lower amounts of GzmB and perforin than Gal-9- NK cells in all three subpopulations (Fig. 3-7A-7D). In sharp contrast, we found that while TIGIT+ and TIGIT- NK cells both co-expressed GzmB and perforin, co-expression was significantly higher in TIGIT+ NK cells, which stresses the dichotomous functionality of Gal-9+ and TIGIT+ NK cells in HIV infection (Fig. 3-7A-7D).

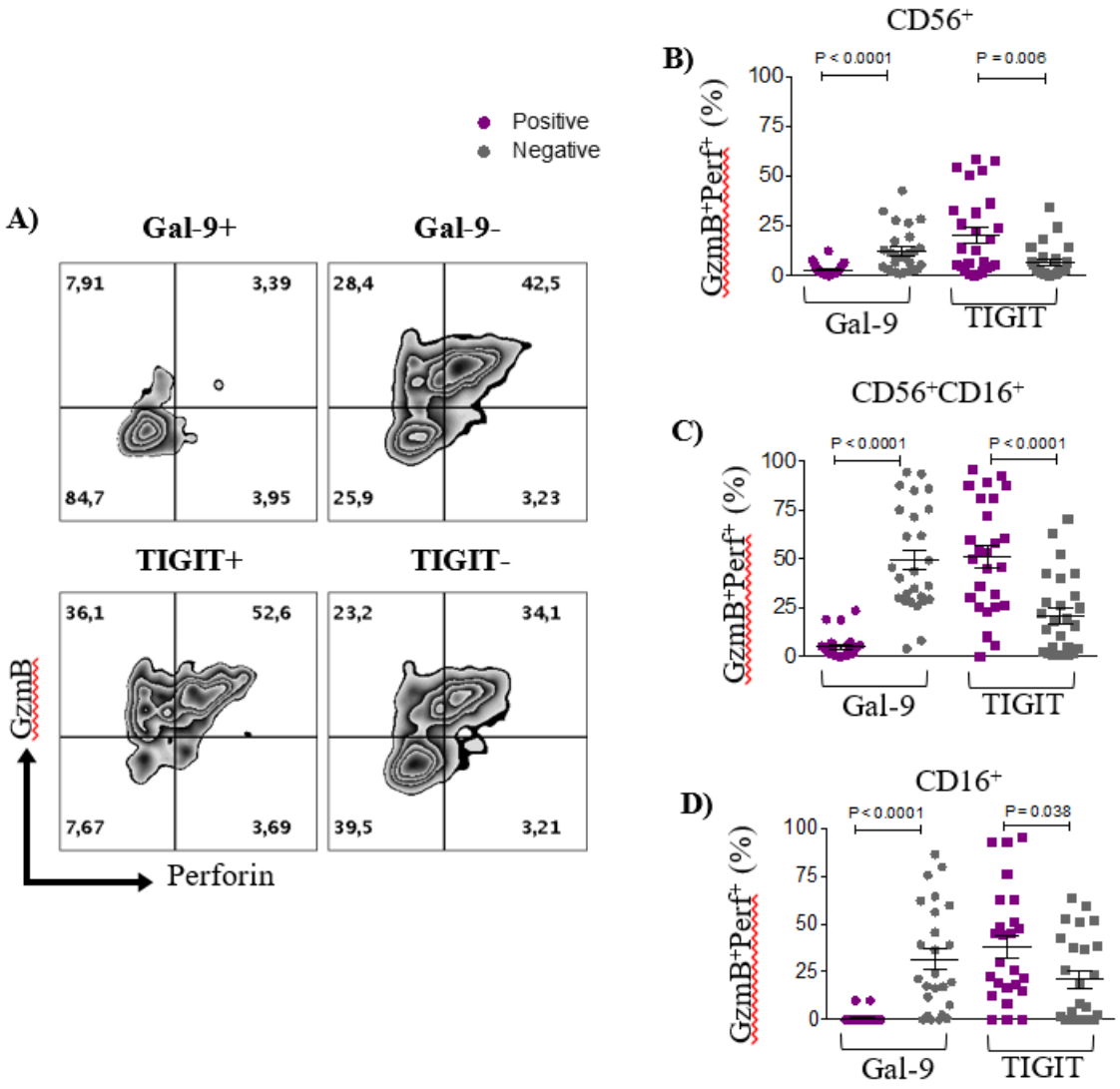

Figure 3-7: Significantly lower co-expression of GzmB and perforin by Gal-9+ NK cells. (A) Representative flow cytometry dot plots showing GzmB and perforin co-expression in Gal-9+, Gal-9-, TIGIT+ and TIGIT- NK cells in HIV-infected individuals on ART. (B) Cumulative data indicating percentages of GzmB and perforin co-expression on $CD56^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+ and TIGIT-. (C) Cumulative data indicating percentages of GzmB and perforin co-expression on $CD56^+CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+ and TIGIT-. (D) Cumulative data indicating percentages of GzmB and perforin co-expression on $CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+ and TIGIT-. Each point represents data from an individual patient (n = 25). Bar, mean ± one standard error.

To be more certain of the cytotoxic differences between these populations of NK cells, we examined the expression of another cytotoxic mediator known as GNLY. We observed the same

expression pattern as the previous cytolytic effectors. Gal-9+ compared to Gal-9- NK cells expressed significantly lower levels of GNLY in all three subpopulations of NK cells (Fig. 3-8A, 3-8C-8E). In contrast, TIGIT+ NK cells expressed significantly higher GNLY compared to TIGIT- NK cells in all three subpopulations (Fig. 3-8B, 3-8C-8E). Comparing Gal-9+ versus TIGIT+ NK cells, we observed that Gal-9+ NK cells expressed significantly lower GNLY in all three subpopulations (Fig. 3-8C-8E).

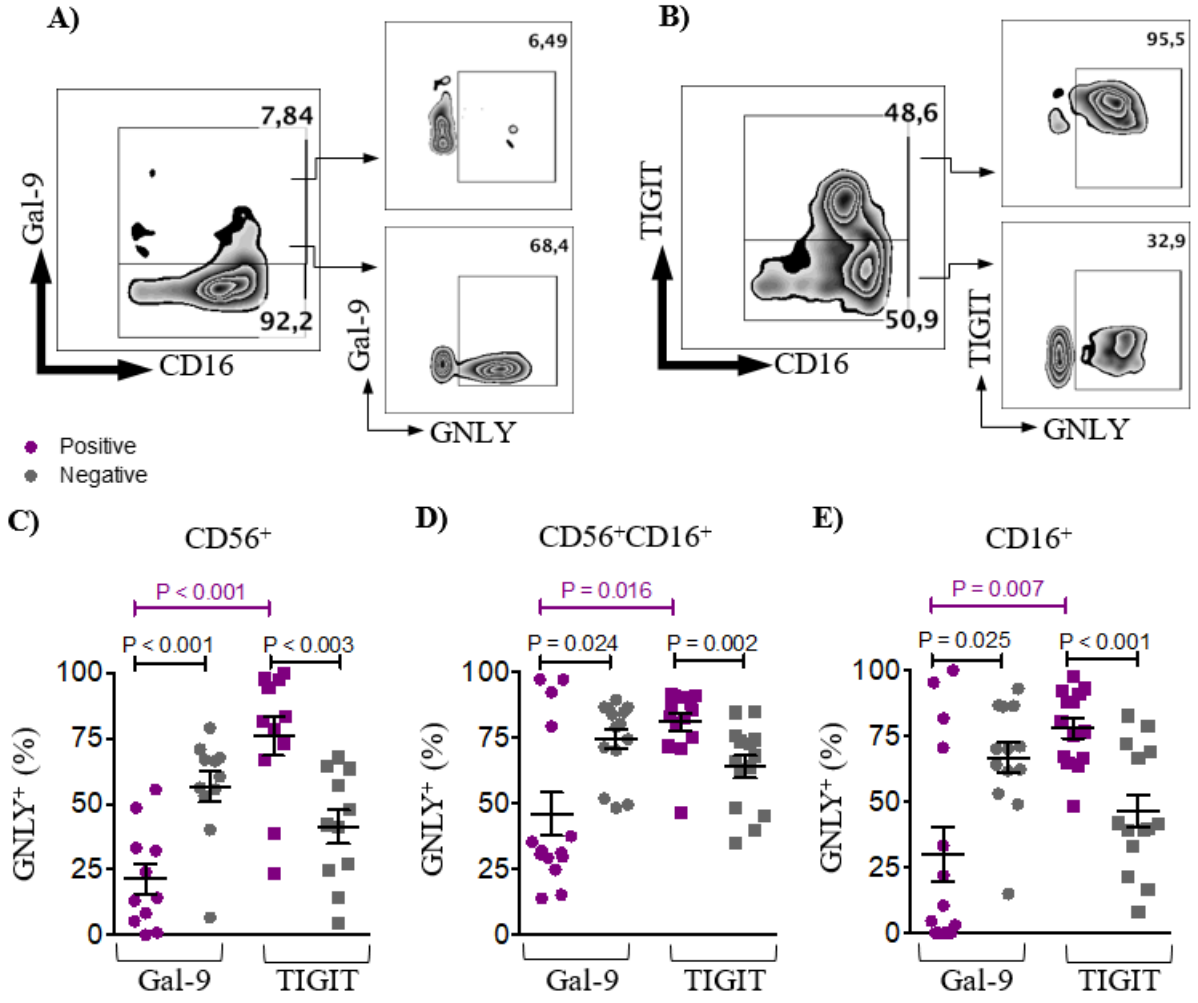

Figure 3-8: Gal-9+, compared to Gal-9- NK cells, express significantly lower levels of GNLY, which is in contrast with TIGIT+ NK cells. (A) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$ population showing GNLY expression in Gal-9+ versus Gal-9- NK cells in HIV-infected individuals on ART. (B) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$

population showing GNLY expression in TIGIT+ versus TIGIT- NK cells in HIV-infected individuals on ART. (C) Cumulative data indicating percentages of GNLY expression in $CD56^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-.(D) Cumulative data indicating percentages of GNLY expression in $CD56^+CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. (E) Cumulative data indicating percentages of GNLY expression in $CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. Each point represents data from an individual patient (n = 15). Bar, mean ± one standard error. Granulysin (GNLY).

Based on our observations, Gal-9+ NK cells had impaired expression of cytolytic molecules (GzmB, perforin and GNLY) compared to their Gal-9- siblings. Therefore, we decided to determine whether this was due to their inability to secrete such molecules or whether they were constitutively degranulating. To answer this question, we assessed the expression of CD107a, a marker for degranulation, in Gal-9+ and Gal-9- NK cells. We observed that Gal-9+ NK cells at baseline (i.e. without stimulation) had significantly higher CD107a expression in comparison to Gal-9- NK cells from HIV patients on ART (Fig. 3-9A and 9B). Thus, our data suggest that Gal-9+ expression results in constitutive degranulation in NK cells.

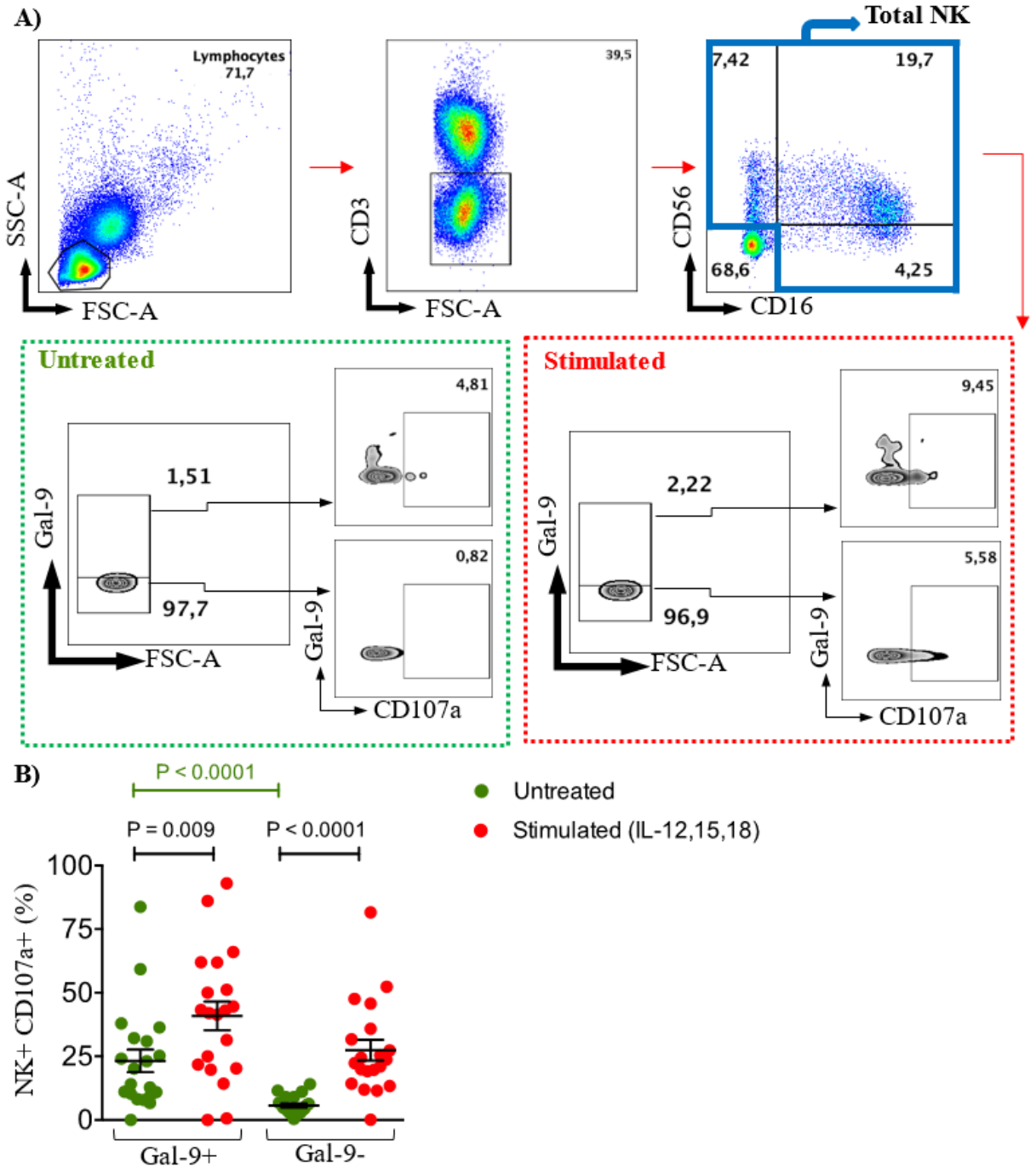

Figure 3-9: Addition of exogenous cytokine cocktail enhances CD107a expression in Gal-9+ and Gal-9- NK cells from HIV patients on ART. (A) Gating strategy and representative flow cytometry dot plots for CD107a expression. (B) Cumulative data indicating percentages of CD107a expression in Gal-9+ versus Gal-9- in total NK cells. Stimulated cells were treated with IL-12 (10 ng/ml) IL-15 (20 ng/ml) and IL-18 (100 ng/ml). Each point represents data from an individual patient. Bar, mean ± one standard error.

3.5 Gal-9+ NK cells express significantly higher IFN-γ compared to Gal-9- NK cells, which is in contrast with TIGIT+ NK cells

Knowing that NK cells are notorious for expressing IFN-γ, we were curious to determine whether Gal-9+/- NK cells differ in their expression for this cytokine. Interestingly, we observed the opposite expression pattern compared to the cytotoxic mediators. We found that Gal-9+ NK cells expressed significantly higher amounts of IFN-γ compared to the negative population in all three subpopulations (Fig. 3-10A, 3-10C-10E). In contrast, TIGIT+ NK cells expressed significantly lower levels of IFN-γ compared to TIGIT- NK cells in only the $CD56^+$ subpopulation, but there was a general trend towards lower IFN-γ in the double positive and $CD16^+$ subpopulations (Fig. 3-10B-10E). We also observed that TIM-3+ NK cells in the $CD56^+$ subpopulation expressed significantly more IFN-γ compared to TIM-3- NK cells (Fig. 3-10C-10E).

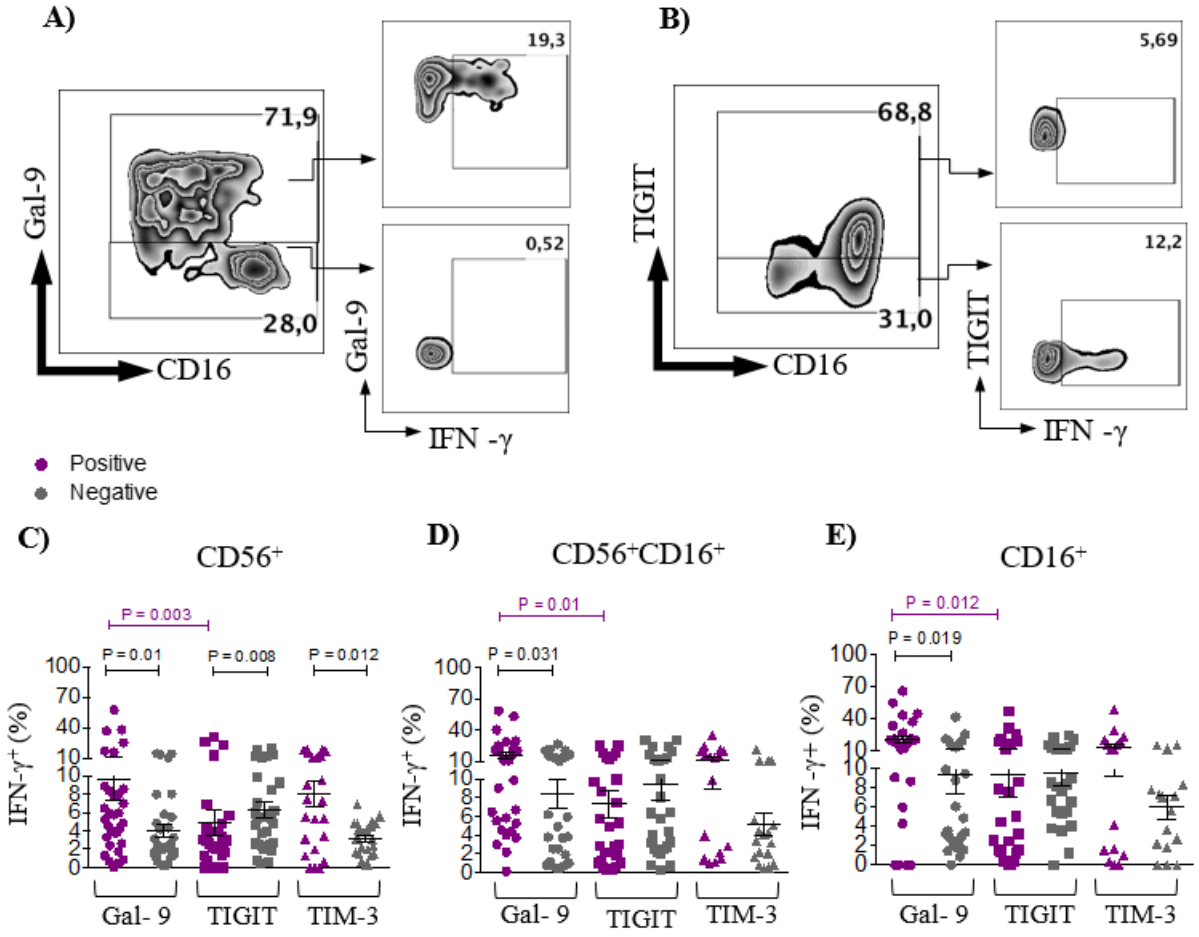

Figure 3-10: Gal-9+, compared to Gal-9- NK cells, express significantly higher levels of IFN-γ, which is in contrast with TIGIT+ NK cells. (A) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$ population showing IFN-γ expression in Gal-9+ versus Gal-9- NK cells in HIV-infected individuals on ART. (B) Representative flow cytometry dot plots from $CD56^{+}CD16^{+}$ population showing IFN-γ expression in TIGIT+ versus TIGIT- NK cells in HIV-infected individuals on ART. (C) Cumulative data indicating percentages of IFN-γ expression in $CD56^{+}$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. (D) Cumulative data indicating percentages of IFN-γ expression in $CD56^{+}CD16^{+}$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. (E) Cumulative data indicating percentages of IFN-γ expression in $CD16^{+}$ NK cells that were Gal-9+, Gal-9-, TIGIT+, TIGIT-, TIM-3+ and TIM-3-. Each point represents data from an individual patient (n = 35). Bar, mean ± one standard error. Interferon γ (IFN-γ).

3.6 Expression for CD38 is not different in Gal-9+ vs. Gal-9- and TIGIT+ vs. TIGIT- NK cells

Thus far we have observed that Gal-9+ NK cells express fewer cytotoxic mediators (GzmB, perforin, GNLY) but more IFN-γ compared to Gal-9- NK cells. Additionally, there was no substantial co-expression of Gal-9+TIM-3+ or TIGIT+Gal-9+ (Fig. 3-2E). Thus, there seem to be two distinct populations of NK cells, Gal-9+ and TIGIT+, with differential expression for markers of cytotoxicity. Thus, our next step was to examine whether these NK cells differ in their level of activation as assessed by CD38, which is a marker of cytolytic function (91). However, we discovered that there was no difference in the expression levels of CD38 when comparing Gal-9+ versus Gal-9- or TIGIT+ versus TIGIT- NK cells (Fig.3-11A-11C). Furthermore, there was no significant difference in levels of CD38 expression when comparing Gal-9+ versus TIGIT+ NK cells in all three subpopulations (Fig.3-11A-11C).

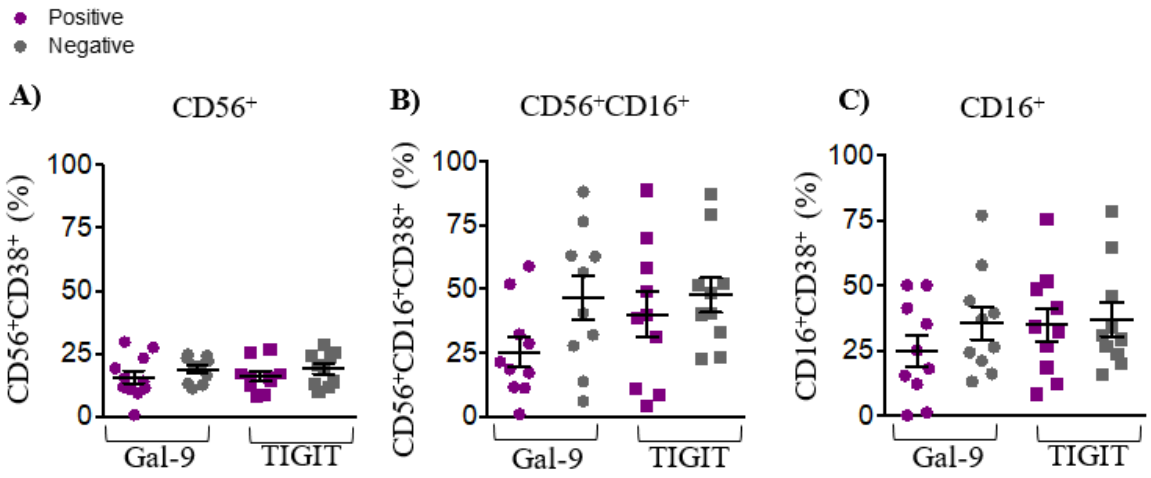

Figure 3-11: No significant difference in CD38 expression on Gal-9+ versus Gal-9- NK cells or TIGIT+ versus TIGIT- NK cells in HIV-infected individuals on ART. (A) Percentages of CD38 expression on $CD56^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+ and TIGIT-. (B) Percentages of CD38 expression on $CD56^+CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+ and TIGIT-. (C) Percentages of CD38 expression on $CD16^+$ NK cells that were Gal-9+, Gal-9-, TIGIT+ and TIGIT-. Each point represents data from an individual patient.

3.7 Expression of activating receptors on Gal-9+ and Gal-9- NK cells

As state above, there was no difference in the level of activation as assessed by CD38 expression. However, we know that NK cells rely on a balance between activating and inhibitory receptors, with the net effect being determined by which signal prevails. Therefore, we wanted to further investigate whether there was differential expression of several activating receptors on the Gal-9+ versus Gal-9- NK cells. We first examined the potent activating receptor NKG2D which plays a critical role in NK cell mediated clearance of HIV-infected $CD4^+$ T cells (209). However, we did not observe any significant difference in the expression of NKG2D when comparing Gal-9+ versus Gal-9- in all three subpopulations of NK cells (Fig. 3-12A-12C). We also investigated the expression of the NCRs (NKp30, NKp44 and NKp46), which associate with ITAM-bearing adaptor proteins that elicit an activating signal (85). Examination of the three NCRs revealed that Gal-9+ NK cells in the $CD56^+$ and $CD16^+$ subpopulations had lower expression of NKp46 compared to Gal-9- NK cells (Fig. 3-12A and 12C). Low expression of NKp46 on NK cells is linked to a poor prognosis, as decreased levels of NKp46 have been associated with viremia (158) and lower expression of NKp46 has been reported in AIDS patients compared to non-AIDS individuals (210).

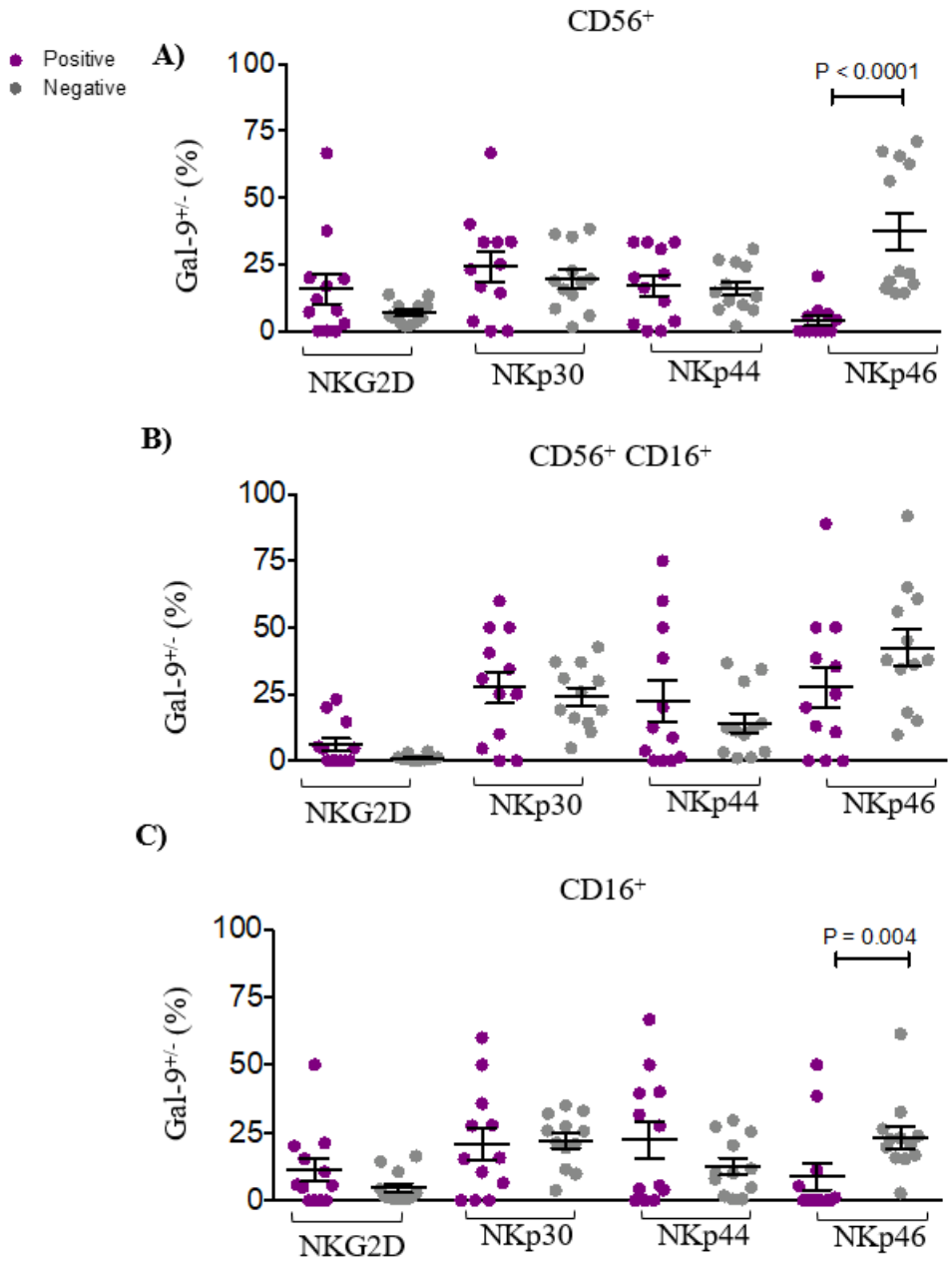

Figure 3-12: No significant difference in the expression of activating receptors on Gal-9+ versus Gal-9- NK cells except for lower expression of NKp46 on Gal-9+ NK cells in HIV-infected individuals on ART. (A) Percentages of NKG2D, NKp30, NKp44, NKp46 expression on $CD56^+$ NK cells for Gal-9+ or Gal-9-. (B) Percentages of NKG2D, NKp30, NKp44, NKp46 expression on $CD56^+CD16^+$ NK cells for Gal-9+ or Gal-9-. (C) Percentages of NKG2D, NKp30, NKp44, NKp46 expression on $CD16^+$ NK cells for Gal-9+ or Gal-9-. Each point represents data from an individual patient. Bar, mean ± one standard error. Natural Killer Group 2D (NKG2D); Natural cytotoxicity receptors (NCR) which include NKp30 (CD337), NKp44 (CD336) and NKp46 (CD335).

3.8 Exogenous cytokine cocktail enhances functional potential of Gal-9+ and Gal-9- NK cells *in vitro*

We next evaluated the impact of exogenous cytokines (IL-12, IL-15 and IL-18) on the function of Gal-9+ and Gal-9- NK cells of HIV-patients on ART. The combination of these three cytokines is frequently used in the literature to yield a potent NK cell response (109-112). We found that Gal-9+ and Gal-9- NK cells were both able to significantly increase their expression of the degranulation marker CD107a in the presence of these cytokines (Fig.3-9A and 9B). We also observed that the cytokine cocktail (IL-12, IL-15 and IL-18) significantly enhanced the expression of GzmB, perforin and IFN-γ in both Gal-9+ and Gal-9- NK cells (Fig. 3-13A-13C). As we discussed above, Gal-9+ NK cells expressed lower levels of GzmB and perforin but higher levels of IFN-γ. However, this cytokine cocktail can modify their effector functions, although the effect was not specific to Gal-9+ NK cells. Despite Gal-9+ NK cells having lower expression of effector molecules both in the untreated and stimulated conditions with cytokines (Fig. 3-13A and 13B), the cytokine cocktail enhanced the activity of both Gal-9+ and Gal-9- NK cells (Fig. 3-13C).

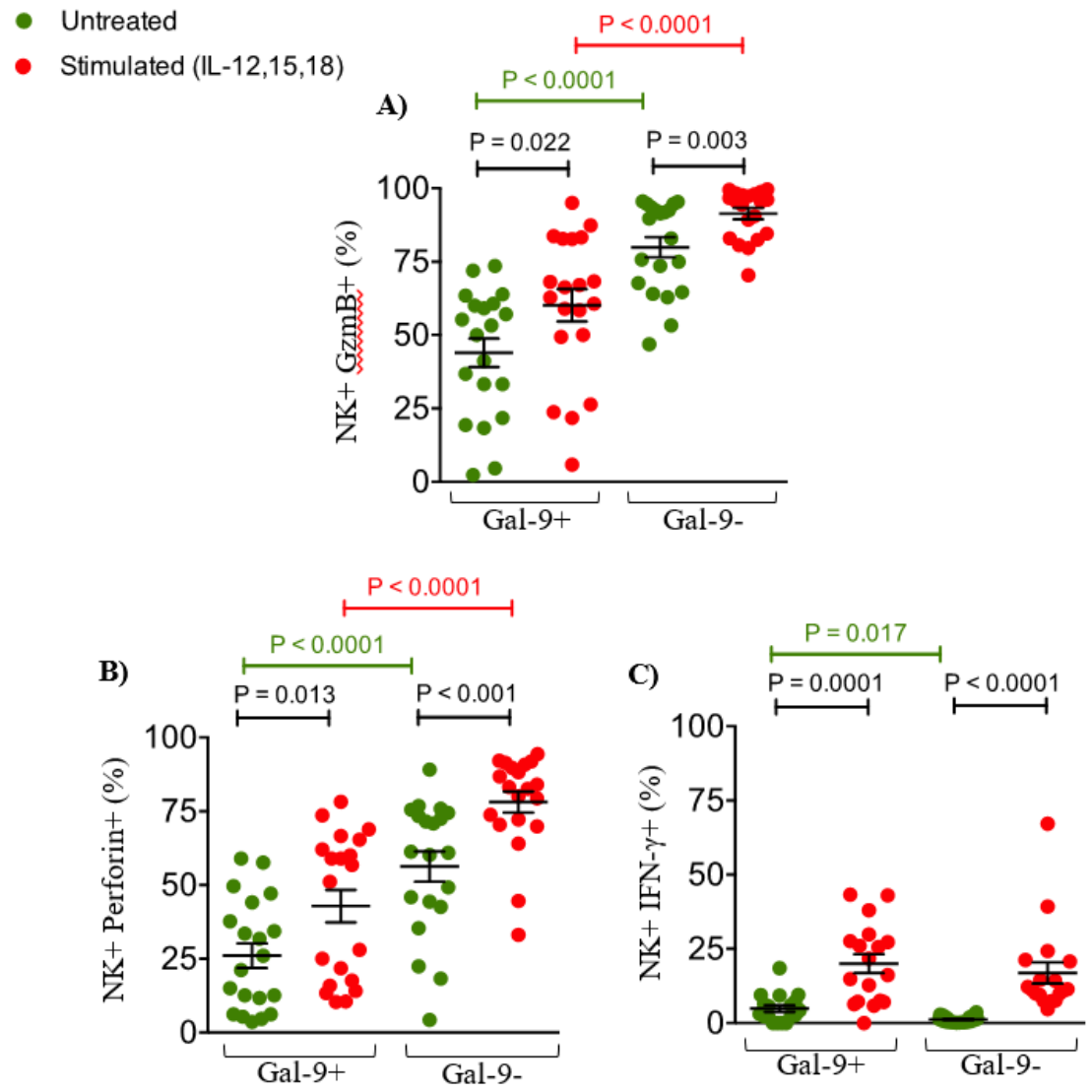

Figure 3-13: Addition of exogenous cytokine cocktail enhances GzmB, perforin and IFN-γ expression in Gal-9+ and Gal-9- NK cells of HIV-patients on ART. (A) Cumulative data indicating percentages of GzmB expression in Gal-9+ versus Gal-9- in total NK cells. (B) Cumulative data indicating percentages of perforin expression in Gal-9+ versus Gal-9- in total NK cells. (C) Cumulative data indicating percentages of IFN-γ expression in Gal-9+ versus Gal-9- in total NK cells. Stimulated cells were treated with IL-12 (10 ng/ml), IL-15 (20 ng/ml) and IL-18 (100 ng/ml). Each point represents data from an individual patient. Bar, mean ± one standard error.

3.9 Lactose does not impact the markers of functionality for Gal-9+ NK cells

Lactose can bind to Gal-9 and competitively inhibits the interaction of Gal-9 with its receptors (e.g. TIM-3) (180, 211, 212). Therefore, we decided to block Gal-9 using lactose (30mM) to determine whether Gal-9+ NK cell impairment is the consequence of cell-cell interactions (Gal-9 interaction with TIM-3). However, we observed that lactose did not have any impact on CD107a, GzmB, perforin or IFN-γ expression in Gal-9+ NK cells (Fig. 14A-14D). These data suggest that Gal-9 expression may intrinsically affect NK cell function.

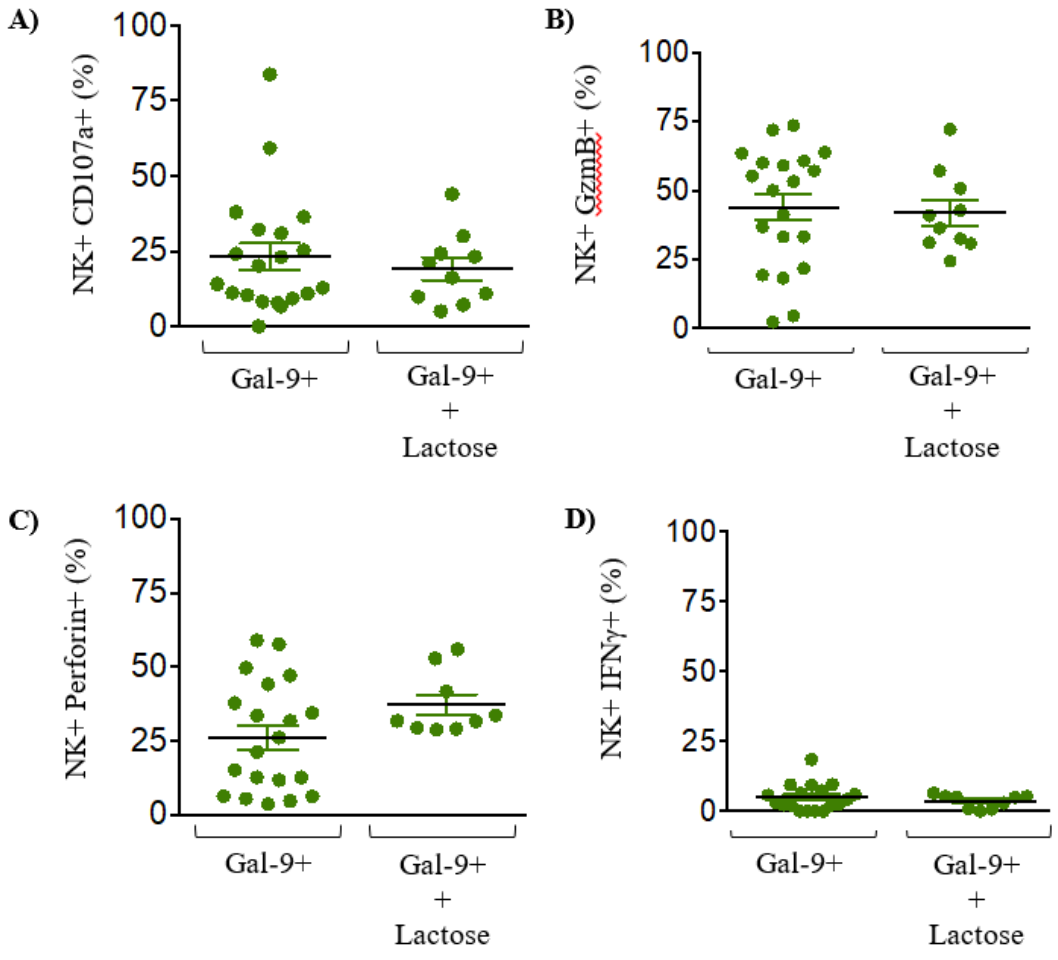

Figure 3-14: Addition of lactose does not impact the expression of CD107a, GzmB, perforin or IFN-γ in Gal-9+ NK cells from HIV-infected individuals on ART. (A) Percentages of CD107a expression in Gal-9+ NK cells with and without lactose (30 mM). (B) Percentages of GzmB

expression in Gal-9+ NK cells with and without lactose. (C) (A) Percentages of perforin expression in Gal-9+ NK cells with and without lactose. (D) Percentages of IFN-γ expression in Gal-9+ NK cells with and without lactose. Each point represents data from an individual patient. PBMCs were cultured with lactose for 24 hours.

3.10 Addition of exogenous cytokines enhances functional potential of TIGIT+ and TIGIT- NK cells *in vitro*

Since the cytokine cocktail enhanced effector functions of Gal-9+ and Gal-9- NK cells *in vitro*, we decided to determine how these cytokines would impact TIGIT+ and TIGIT- NK cells. We discovered that TIGIT+ and TIGIT- NK cells can also benefit from this cocktail and significantly increased the expression of CD107a, GzmB, perforin and IFN-γ (Fig. 15A-15D). Additionally, compared to TIGIT- NK cells, TIGIT+ NK cells expressed more GzmB under both stimulated and untreated conditions (Fig. 15B). In contrast, compared to TIGIT- NK cells, TIGIT+ NK cells expressed less IFN-γ when stimulated with these cytokines (Fig. 15D).

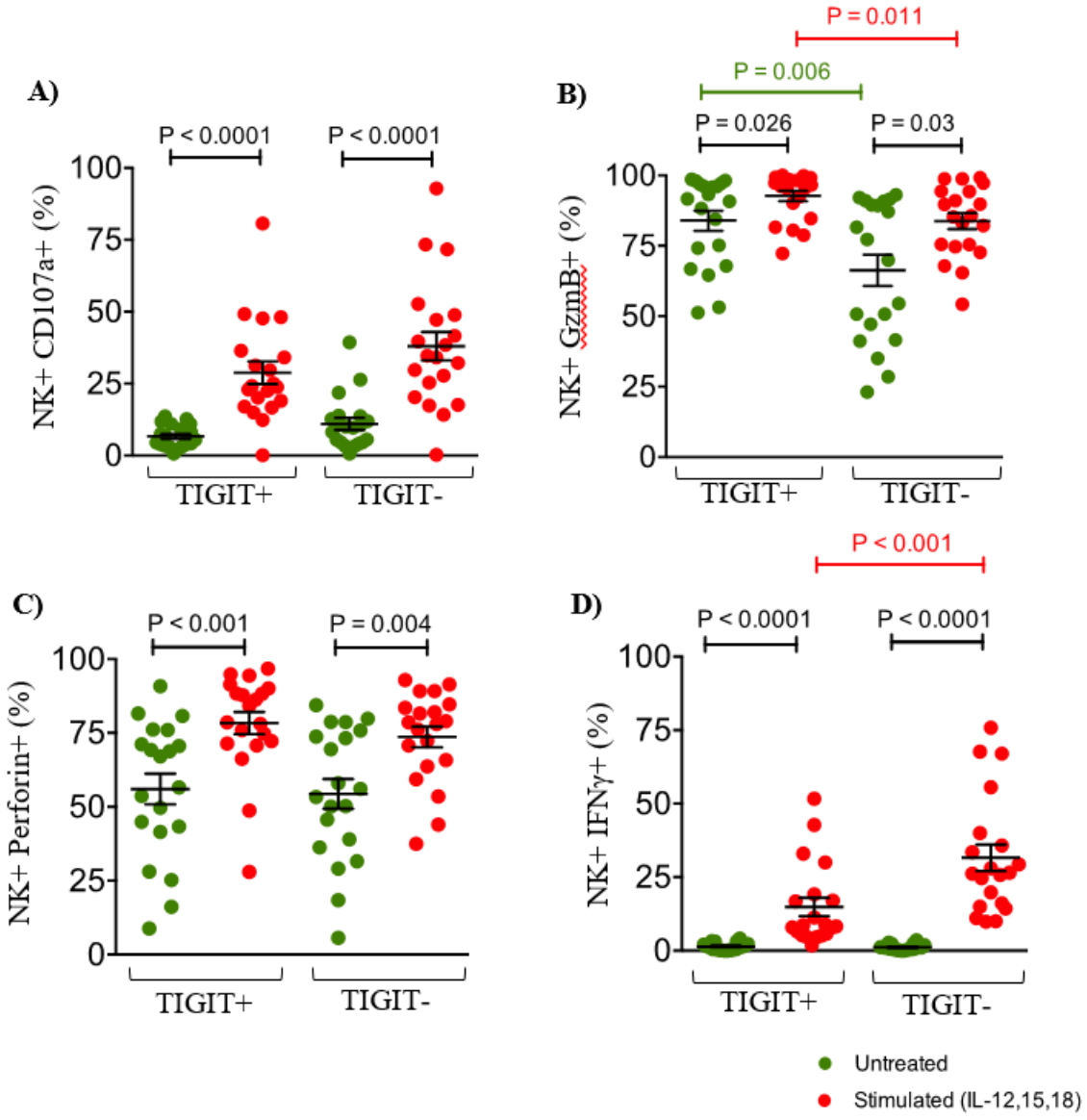

Figure 3-15: Addition of exogenous cytokine cocktail enhances CD107a, GzmB, perforin and IFN-γ expression in TIGIT+ and TIGIT- NK cells in HIV-infected individuals on ART. (A) Cumulative data indicating percentages of CD107a expression in TIGIT+ versus TIGIT- in total NK cells. (B) Cumulative data indicating percentages of GzmB expression in TIGIT+ versus TIGIT- in total NK cells. (C) Cumulative data indicating percentages of perforin expression in TIGIT+ versus TIGIT- in total NK cells. (D) Cumulative data indicating percentages of IFN-γ expression in TIGIT+ versus TIGIT- in total NK cells. Stimulated cells were treated with IL-12 (10 ng/ml), IL-15 (20 ng/ml) and IL-18 (100 ng/ml). Each point represents data from an individual patient. Bar, mean ± one standard error.

Chapter 4: Discussion

4.1 Discussion

NK cells play a pivotal role in the eradication of HIV-infected cells, as an increase in their activity is associated with resistance to infection and progression to AIDS (213). For example, a cohort of intravenous drug users (IDU) who were HIV-exposed but remained uninfected were found to have increased NK cell cytolytic and cytokine production capabilities in comparison to IDU who underwent seroconversion (214). Thus, understanding how HIV infection impacts the reconstitution and function of the heterogenous NK cell pool is clinically important for therapeutic approaches. Although a subset of NK cells that express CD4 as well as the coreceptors CXCR4 and CCR5, have been identified that can be infected with HIV *in vitro* (257), the *in vivo* infection of NK cells has not been demonstrated (158). This indicates that the direct infection of NK cells with HIV, does not play a major role in the alteration of NK cells observed in HIV infected individuals. We observed that HIV-infected individuals on ART differ in their repertoire of PB NK cells, with an increase in the $CD56^+$ subset and a decrease in the $CD16^+$ subpopulation. Various studies have indicated that the $CD56^+$ populations are the more immature precursors to the $CD16^+$ subsets for various reasons such as longer telomeres of $CD56^+$ compared to $CD16^+$ subsets and the ability of $CD56^+$ to differentiate into $CD56^{dim}/CD16^+$ NK cells upon activation (15, 215, 216). Importantly, these two subsets have distinct functional properties; the $CD56^+$ subpopulation is more involved in cytokine secretion, while the $CD16^+$ subpopulation is more cytolytic due to its ADCC mechanism of killing and higher content of perforin and granzymes in its granules (11, 217). Hence, we have observed a shift in the NK cell population from a more mature and cytotoxic subset ($CD16^+$) to a more immature ($CD56^+$) population in HIV patients on ART. It is important to note that in healthy individuals, over 90% of PB NK cells consist of $CD56^{dim}/CD16^+$ and less than 10% comprise the $CD56^{bright}/CD56^+$

subpopulation (11, 218). Thus, there appears to be a complete reversal in the composition of NK cells from HIV patients on ART compared to what is seen under normal conditions. Given the distinct functions performed by these two subsets of NK cells ($CD56^+$ and $CD16^+$), the identification of this abnormal shift in the NK cell population seen predominantly in ART-treated patients can pave way for strategies to reconstitute NK cells towards a more balanced distribution. For example, the utilization of cytokines such as IL-2 or IL-12 has been shown to favour differentiation of $CD56^{bright}/CD56^+$ NK cells towards the more terminally mature $CD16^+$ NK cells, indicating the potential for modulation of NK cell subsets via external factors (218). Apart from the immaturity of the $CD56^+$ subpopulation, the increase of this subset in HIV patients is potentially unfavourable due to their lack of responsiveness to target cell stimulation; it has been shown that in contrast to the $CD16^+$ subset, $CD56^{+/bright}$ subpopulations fail to upregulate CD107a and IFN-γ upon stimulation with MHC-devoid K562 target cells (152). However, it is important to note that our research only examined PB NK cells which does not necessarily provide a cohesive picture of how HIV affects the population of NK cells that resides in various tissues. For example, the $CD16^+$ population express high amounts of CXCR1 and CX3CR1 which aid in migration towards sites of infection and inflammation (258). Therefore, the decrease in the $CD16^+$ population observed in HIV patients on ART may be just due to their increase migration into sites of inflammation rather than a decrease in the pool of this subset. In addition, we also observed an increase in the $CD56^+$ NK cell subpopulation in progressors and LTNPs. However, the $CD56^+CD16^+$ NK cell subset was significantly diminished in these patients. Thus, the reduction in the double positive subset of NK cells in LTNPs and progressors that are ART-native, suggests that ART may maintain the frequency of the $CD56^+CD16^+$ subpopulation in HIV-infected individuals. In contrast, we did not observe any changes in the

frequency of $CD16^+$ NK cell subpopulations in LTNPs and progressors. However, we are unable to define the mechanism underlying this phenotype in these HIV-infected individuals, which merits further investigation.

Aside from the quantitative changes in NK cell populations, we observed that both the $CD56^+$ and $CD56^+CD16^+$ NK cell subpopulations had an elevated surface expression of Gal-9 in HIV-infected individuals compared to HCs. Furthermore, in all HIV-infected individuals the expression of Gal-9 on the $CD16^+$ population was very low and insignificant compared to HCs. It is intriguing that Gal-9 is predominantly expressed by the more immature subsets and not the mature $CD16^+$ subsets. Further investigation would be required to determine whether the enhanced Gal-9 expression seen predominantly on immature NK cells is playing a role in the inhibition of NK cell maturation. Within the HIV cohort, the only difference in Gal-9 expression was in the $CD56^+CD16^+$ population in which the progressors expressed significantly more Gal-9 compared to the LTNPs. The implication of this observation is unclear; however, we do know that viral load in progressors are significantly higher than in LTNPs (219). Given the fact that a positive correlation between plasma viral load and plasma Gal-9 has been reported (190), the higher viremia in progressors is reported to be associated with higher plasma Gal-9 (191); this may explain the increase in surface Gal-9 expression on the double positive population due to the fact that Gal-9 is a protein that can associate with carbohydrates on NK cell surfaces. Thus, the higher plasma Gal-9 may potentially favour the indiscriminate association of Gal-9 to carbohydrate structures on NK cells. However, the association between the viral load and plasma Gal-9 cannot fully explain these observations, as the correlation coefficient was only 0.37, indicating a weak association (190). This is supported by another study showing an insignificant

difference in the plasma level of Gal-9 between chronically infected non-controllers/progressors, LTNPs and HIV patients on ART (188). Furthermore, we know that the majority of HIV patients on ART have an undetectable viral load and despite that, they have high surface Gal-9 expression on their NK cells which is not different from progressors. Thus, progressors may have other mechanisms in place that promote elevated surface Gal-9 on the double positive NK cells. One possibility is that the chronic immune activation observed in progressors may contribute to the elevated Gal-9 expression. It has been shown that surface Gal-9 can be upregulated on Jurkat cells upon stimulation (220). Since progressors, who are mostly viremic, have more activated NK cells (221) this constant activation state may be contributing to the elevated surface Gal-9 expression, similar to the activated Jurkat cells. Therefore, increased levels of Gal-9 on $CD56^+$ and $CD56^+CD16^+$ populations in ART-treated and ART-naive patients (progressors and LTNPs) might be an indication that HIV infection alone, but not ART treatment is the contributing factor in Gal-9 overexpression. The mechanism behind overexpression of Gal-9 on NK cells in HIV infected individuals needs to be explored further. For example, Gal-9 has been shown to bind to the adhesion molecules CD44 (183) and the inducible stimulatory molecules, CD137 (182), which are both expressed on NK cells. Given the chronic immune activation present in HIV infected individuals, the expression levels of the activating receptor CD137 could be explored further. It is possible that the elevated plasma Gal-9 in HIV infected patients is contributing to upregulation of Gal-9 on NK cells by attaching to receptors such as CD137.

Assessment of TIGIT expression revealed higher levels of this inhibitory receptor on the $CD56^+$ population in all HIV-infected patients compared to HCs. A recent study indicated that NK cells from HIV-infected patients express higher TIGIT levels compared to uninfected individuals

(112). However, we delved deeper and found that the increased TIGIT on NK cells from HIV patients is predominantly from the $CD56^+$ subpopulation. It has been widely established that chronic conditions such as HIV and cancer can promote immune exhaustion (159, 167, 169). T cells are typically the focus of immune exhaustion studies; however, we now recognize that many other immune cells such as NK cells can become dysfunctional under severe or chronic infection. NK cell exhaustion may be mediated by various mechanisms such as the increased expression of suppressive factors in chronic conditions. For example, HIV infection has been shown to increase expression of the NKG2A ligand, HLA-E, on $CD4^+$ T cells and concomitant evasion of NK cell lysis by infected cells (222). Additionally, HIV-infected patients have increased plasma levels of transforming growth factor beta 1 (TGF-β1), an immunosuppressive cytokine, compared to HCs (223). TGF-β1 has been shown to promote NK cell exhaustion by downregulating the activating receptors NKp30 and NKG2D (224). TGF-β1 has also been shown to decrease degranulation, cytokine expression (IFN-γ and TNF-α), and lysis of K562 target cells (225). Nonetheless, the cardinal feature of immune exhaustion is the upregulation of inhibitory receptors, with the recent discovery that TIGIT is the most important marker for NK cell exhaustion in comparison to a panel of other inhibitory receptors such as PD-1 (174). Thus, it is not surprising that TIGIT is upregulated on NK cells in chronic conditions such as HIV infection. However, the reasoning for why it is predominantly expressed by the $CD56^+$ population requires further investigation. Furthermore, the ligand for TIGIT, CD155, is highly expressed by $CD4^+$ T cells from HIV-infected patients compared to HCs, which can further inhibit the function of NK cells (112). Interestingly, the expression of TIGIT by double-positive NK cells in LTNPs is significantly higher than in HIV-ART, progressors and HCs. Although the same trend exists in the $CD16^+$ population, the difference between progressors and LTNPs is not statistically

significant. The fact that LTNPs have higher levels of exhaustion markers, such as TIGIT, than progressors is counterintuitive, since LTNPs are spared from disease progression by having a higher CD4 count and lower viral load than progressors. Additionally, immune exhaustion of T-cells can occur in LTNPs even in the presence of negligible viral loads, indicating that high viremia, which is observed in progressors, is not a distinguishing factor for the occurrence of immune dysfunction (226).

Lastly, we observed that TIM-3 expression by the $CD56^{+}$ subpopulation is significantly lower in HIV patients on ART than the ART-naive (progressors and LTNPs) and uninfected patients. This observation is consistent with other studies, as a decrease in MFI of TIM-3 on $CD56^{bright/+}$ subpopulations has been observed in ART-treated patients compared to ART-untreated individuals (such as progressors and LTNPs in this study) (190). Along the same line of work, another study found that $CD56^{high}$ NK cells from HIV-infected patients expressed elevated levels of TIM-3 prior to ART, but that difference was diminished six months post-ART (147). Although the role of TIM-3 in NK cell biology is controversial, there have been several reports indicating that TIM-3 is an NK cell activation marker and thus contributes to the activity of NK cells (175, 178, 227). In agreement, it has been proposed that uncontrolled viral load in the absence of ART leads to NK cell activation and the subsequently higher TIM-3 expression (228). However, this does not explain why LTNPs and progressors, with opposite spectrums of viral loads, express higher levels of TIM-3 compared to HIV-patients on ART. The discrepancy between these observations may be due to the fact that ART impacts TIM-3 expression on NK cells regardless of the viral load. TIM-3 expression on NK cells has been associated with contradicting functions (e.g. activation versus exhaustion) (176, 177). For example, increased

TIM-3 expression by NK cells has been observed in macaques chronically infected with Simian Immunodeficiency Virus (SIV) (229); thus, disease chronicity might explain upregulation of TIM-3 expression on $CD56^{+}CD16^{+}$ subpopulations of NK cells in the LTNPs. Overall, considering the variable effects we observed in different NK cell populations and different HIV-groups, our observations do not support a definite role for TIM-3 on NK cell function.

As previously mentioned, HIV-infected patients have elevated levels of plasma Gal-9, which is capable of interacting with many β-galactoside-containing oligosaccharides via the N and C terminal carbohydrate recognition domains (CRD) (230). In fact, it has been shown that these two CRDs have different affinities and are capable of mediating dual functions depending on which side binds to the carbohydrate (231). Nonetheless, we wanted to ensure that the increased Gal-9 observed in HIV patients was intrinsic to NK cells. Imaging cytometry revealed that NK cells from all three subpopulations expressed intracellular Gal-9 in both HIV patients on ART and HCs. Furthermore, we showed quantitatively that surface Gal-9 expression was the highest in the $CD56^{+}$ subpopulation ($\mu = 49.4\%$), followed by the $CD56^{+}CD16^{+}$ ($\mu = 11.5\%$) and then the $CD16^{+}$ subpopulation ($\mu = 3.3\%$). The levels of intracellular Gal-9 followed the same trend, with the highest intensity of intracellular Gal-9 being expressed by the $CD56^{+}$ subpopulation, followed by the $CD56^{+}CD16^{+}$ subpopulation and the weakest by the $CD16^{+}$ subpopulation. Comparison of Gal-9 mRNA levels within NK cells from HIV patients on ART and HCs revealed no significant difference. Although the quantity of mRNA is often used as a proxy for protein abundance, there have been reports that this association is weak, with a correlation coefficient of 0.4 (232). This is because the volume of transcript present in the cell does not provide insight into the translation/post-translational modifications that may impact how much

Gal-9 is ultimately expressed. Furthermore, since Gal-9 lacks a signal peptide for its secretion, the association between its expression and secretion is not necessarily correlated (179).

Next, we investigated the functional potential of NK cells by examining the expression of GzmB, perforin, GNLY and IFN-γ. We found that Gal-9+ NK cells expressed significantly lower amount of cytolytic molecules (GzmB, perforin and GNLY) than Gal-9- NK cells in all three subpopulations. In contrast, they expressed significantly higher levels of IFN-γ than Gal-9- NK cells in all three subpopulations. More importantly, in all three subpopulations, we observed that Gal-9+ NK cells co-expressed significantly less GzmB and perforin when compared to Gal-9- NK cells. Since co-expression of GzmB and perforin is essential for NK cell mediated cytotoxicity (38), lower co-expression of these cytolytic molecules suggests that the killing ability of Gal-9+ NK cells is impaired. In contrast, we observed that TIGIT+ versus TIGIT- NK cells expressed significantly more GzmB, perforin and GNLY in all three subpopulations compared to TIGIT- NK cells. Unlike their Gal-9+ counterparts, TIGIT+ NK cells co-expressed GzmB and perforin, although this expression was lower than that exhibited by TIGIT- NK cells. In further contrast to Gal-9+ NK cells, TIGIT+ NK cells expressed significantly lower levels of IFN-γ compared to TIGIT- NK cells. Although lower IFN-γ expression by TIGIT+ NK cells of HIV-infected patients have been reported (112), it is surprising to see TIGIT+ NK cells express higher levels of cytotoxic mediators than TIGIT- NK cells. However, the regulation of NK cells is complex and dependent on an array of activating and inhibitory receptors, thus there may exist other unexplored activating receptors on TIGIT+ NK cells that contribute to the increased functional phenotype observed. For example, competition from CD226, which binds to the same ligands as TIGIT (233), could dampen TIGIT signaling and in turn cause the observed high

cytotoxic potential of TIGIT+ NK cells. We found that Gal-9+ and TIGIT+ NK cells are two distinct populations with dichotomous functional potential: Gal-9+ NK cells co-express significantly lower quantities of cytolytic molecules but more IFN-γ. Conversely, TIGIT+ NK cells express higher levels of cytolytic molecules but less IFN-γ in HIV-infected individuals on ART. Unlike Gal-9 and TIGIT, TIM-3 did not appear to have a clear role besides increasing expression of GzmB and perforin in the $CD56^{+}CD16^{+}$ subpopulation. Although the interaction of TIM-3 with its ligand has been shown to suppress NK cell cytotoxicity, high expression of TIM-3 on NK cells marks a functionally mature population with high cytotoxicity (175). Thus, it is not unusual for TIM-3+ NK cells to have higher expression of GzmB and perforin in the $CD56^{+}CD16^{+}$ subpopulation, which is known for its cytotoxicity (14).

Due to the differential expression of various effector molecules by Gal-9+/- and TIGIT+/- NK cells, we decided to investigate the activation status of these cells based on the expression of CD38 and NK cell activating receptors. Our results showed no difference in CD38 expression between Gal-9+ and Gal-9- or TIGIT+ and TIGIT- NK cells in any of the three subpopulations. Moreover, when the expression levels of NKG2D, NKp30, NKp44 and NKp46 were examined, only downregulation of NKp46 was observed in Gal-9+ NK cells (specifically the $CD56^{+}$ and $CD16^{+}$ subpopulations) compared to Gal-9- NK cells. The downregulation of NCRs (NKp30, NKp44 and NKp46) in HIV-infected patients has been reported in the literature (78, 168, 234, 235). For instance, HIV-infected viremic patients (158) and individuals who have progressed to AIDS (210) have been found to exhibit significantly lower NKp46 expression. Moreover, by blocking NKp46, NK cell lysis of HIV-infected $CD4^{+}$ T cells was compromised, indicating that NKp46 interaction is crucial for the clearance of HIV-infected cells (236). Another report has

also indicated that NKp46 blockade abrogated NK cell-mediated cytolysis of target cells (237). These reports support the notion that NKp46 plays an important role in NK cell activation and function, which suggests that decreased expression of this receptor on Gal-9+ NK cells reduces their ability to kill virally-infected cells.

Due to the dysfunctional cytolytic capacity of Gal-9+ NK cells, we decided to examine the effects of a cytokine cocktail (IL-12, IL-15 and IL-18) on NK cell functions *in vitro*. This cytokine cocktail has been reported to enhance IFN-γ production and concomitant killing of leukemia target cells (109). In addition, the synergistic effect of this cytokine cocktail enhances NK cell function to such an extent that it is utilized to stimulate NK cells for adoptive cell therapy (110, 111). Therefore, we used the same combination and concentrations of IL-12, IL-15 and IL-18 in our cytokine cocktail to stimulate NK cells from HIV-infected patients (112). Upon cytokine stimulation, we observed enhanced NK cell functional potential in Gal-9+ NK cells as measured by the levels of CD107a, GzmB, perforin and IFN-γ. This indicates that the impaired cytolytic abilities of Gal-9+ NK cells are partially reversible, although this effect was not specific to Gal-9+ NK cells. Of note, the same enhanced effector functions were observed for both TIGIT+ and TIGIT- NK cells. These observations suggest that the cytokine cocktail enhances NK cell functions regardless of the inhibitory molecules they express.

Interestingly, we observed that at baseline, Gal-9+ NK cells, have a greater degranulation capacity than their Gal-9- siblings. This suggests that lower perforin, GzmB and GNLY expression in Gal-9+ NK cells may be due to constant and indiscriminate degranulation rather than a lower granules content. However, if Gal-9+ NK cells degranulate more in the steady state

than Gal-9- NK cells, it intriguing as to why this does not impact IFN-γ expression. However, a study revealed that NK cells may degranulate in the absence of cytokine secretion, as CD107a was shown to be expressed on cells not secreting cytokines (52). Thus, the level of degranulation may not always correlate with cytokine secretion, which may explain why we observe low expression of cytotoxic mediators but high IFN-γ expression by Gal-9+ NK cells. To determine whether the interaction of Gal-9 with its ligands contributed to the observed phenotype, we blocked Gal-9 using lactose. The addition of lactose, however, did not alter the expression of CD107a, perforin, GzmB or IFN-γ expression. This suggests that the impaired function observed in Gal-9+ NK cells may not be due to cell-cell interactions (e.g. Gal-9 interaction with TIM-3), but an intrinsic pathway mediated by surface expression of Gal-9 on NK cells. Understanding how surface Gal-9 impacts NK cell function merits further investigation.

Our observations indicate that expression of Gal-9 is associated with a population of NK cells with enhanced IFN-γ production but reduced cytotoxic capabilities. Higher IFN-γ expression by Gal-9+ NK cells can play an important role in HIV pathogenesis. IFN-γ can upregulate MHC class I, which increases the ability of cytotoxic T cells to recognize infected cells (60-62). Additionally, IFN-γ can activate various phagocytic cells, induce oxidative burst, and recruit immune cells to the site of infection, thereby controlling HIV replication (239). Although this cytokine plays a crucial role in the acute phase of the disease (240), prolonged IFN-γ production can trigger systemic inflammation and promote HIV pathogenesis (241, 242). For example, a skewing towards IFN-γ-producing cells has been observed in progressive disease (243). The detrimental impact of IFN-γ is likely not due to its action alone, but rather its combination with various other pro-inflammatory cytokines that can, over time, lead to chronic immune activation.

Immune cells such as our $CD4^+$ T cells that are under prolonged exposure in a proinflammatory milieu can become exhausted, which is marked by progressive loss of effector functions and proliferative capacity (244). Thus, a pro-inflammatory environment is beneficial for viral suppression in the initial stages of disease but becomes detrimental under prolonged conditions (242).

Taken together, these findings reveal that at earlier stages of infection, a proinflammatory environment is critical for orchestrating a robust immune response to mediate viral control. However, hyperimmune activation observed in chronic infections can lead to the exhaustion of immune cells and progression of disease. As HIV is an intracellular pathogen, the increased presence of Gal-9+ NK cells that possess lower markers of cytotoxicity suggests that they are inadequate at killing virus-infected cells. Furthermore, these Gal-9+ NK cells express high levels of IFN-γ, which may be contributing to immune activation often seen in HIV-infected patients. However due to the antiviral responses mediated by IFN-γ, whether Gal-9+ NK cells are actually less functional than Gal-9- NK cells should be addressed. Although, IFN-γ production is required for killing HIV-infected cells, this is an indirect mechanism (leads to the activation of a plethora of immune cells such as T cells, macrophages and DCs) in comparison to perforin and GzmB-mediated cytotoxicity (57). Importantly, we now recognize that although ART is capable of decreasing viral load in patients, it does not eradicate the latent HIV reservoir that can be reactivated in response to immune activation to produce new viruses (245). Recently, it has been shown that IFN-γ responses by CTL are not required for reducing the HIV reservoir, but rather the clearance of $CD4^+$ infected cells (which is a measurement of the reservoir) is mediated by the GzmB response (246). Although this was a CD8 study, a parallel can be drawn when compared

to NK cells and may indicate that the IFN-γ responses are potentially less important than that of GzmB in mediating clearance of HIV-infected cells. Furthermore, it has been shown that chronic IFN-γ signaling can promote functional impairment of $CD8^+$ T cells as assessed by their decreased ability to kill target cells (247). Therefore, we believe that Gal-9+ NK cells may play a detrimental role in HIV pathogenesis. On one hand, their ability to kill target cells is impaired because of lower expression of cytolytic molecules (perforin, GzmB and GNLY). On the other hand, although IFN-γ facilitates indirect killing via activation of other cells, higher expression of IFN-γ in the course of chronic disease can contribute to hyperimmune activation and the subsequent immune exhaustion in HIV-infected individuals.

Furthermore, we have shown that impaired NK cell function associated with Gal-9+ expression is partially reversible, which indicates their plasticity and potential for therapeutic interventions. More specifically, finding out what combination of cytokine predominately increase the expression of cytotoxic mediators but are less involved in IFN-γ expression would allow us to reverse the phenotype observed in Gal-9+ NK cells. Thus, better understanding the mechanism underlying Gal-9 overexpression in NK cells or how their functions can be modulated may serve as a therapeutic strategy in HIV-infection.

4.2 Future directions

More studies are required to better understand the role of Gal-9 expression in NK cells. First of all, we need to confirm and identify the source of Gal-9; whether it is intrinsic to NK cells or whether soluble Gal-9 from the plasma is adhering to carbohydrates on NK cells. In order to achieve this, ELISA will be used to determine the concentration of Gal-9 present in the plasma

of HIV patients. Next, isolated NK cells will be treated with different concentrations of recombinant Gal-9. Following this, the surface expression of Gal-9 will be enumerated to determine whether there is any correlation between the surface Gal-9 expression and the recombinant Gal-9 added into the NK cells. Such studies will determine if higher plasma Gal-9 may be contributing to its surface expression on NK cells. In addition, understanding how IL-12, IL-15 or IL-18 influence Gal-9 expression by NK cells is critical. Evaluating the expression levels of the cytokine receptors (CD212, CD215, CD218a) may facilitate this understanding. Importantly, it would be interesting to look at recently infected patients compared to chronically infected individuals to see whether acutely infected patients have a higher proportion of Gal-9 expressing NK cells. Additionally, examining the activating receptor CD226 on NK cells would be important since both CD226 and TIGIT interact with the same ligand CD155. Thus, the density of these two opposing receptors on TIGIT+ NK cells would be important for understanding which signal would dominate. Lastly, single cell RNA sequencing can be performed on Gal-9+ and Gal-9- NK cells to obtain a more comprehensive understanding of the differences between these two populations.

4.3 Limitations

We extrapolated that Gal-9+ NK cells were less cytotoxic due the fact that they contained lower levels of GzmB, perforin, and GNLY vs Gal-9- NK cells. Unfortunately, we were unable to perform a cytotoxicity assay to compare the ability of Gal-9+ vs. Gal-9- NK cells to kill target cells, due to feasibility issues. NK cells are rare in frequency and we have been able to obtain ~400,000 NK cells from 100 million PBMC. Re-isolating Gal-9+ from Gal-9- has been impossible using magnet isolation and we did not have access to a BSL-2 sorting facility for

HIV-infected specimens. Thus, we were only able to examine the functional potential of these NK cells through the expression of various markers, rather than the true functionality of these cells. Another factor that should be considered when interpreting our results is the sample preparation. All samples from HIV patients on ART were freshly collected blood samples from which PBMCs were obtained. However, some of the LTNPs and all of the progressors were cryopreserved samples. There is a general consensus that cryopreservation can impact the functional and phenotypic characteristic of cells (179). To be more certain that frozen and fresh samples do not differ in their phenotype or function, we could have tested fresh versus frozen samples. Additionally, we were unable to measure the levels of IL-12, IL-15 and IL-18 in the plasma of HIV-infected individuals and thus we were unable to investigate the *in vivo* relevance of the concentration of these cytokines utilized. Finally, we treated total PBMCs with a cytokine cocktail and observed that all NK cells (Gal-9+, Gal-9-, TIGIT+ and TIGIT- NK cells) were able to enhance their functionality as measured by CD107a, GzmB, perforin and IFN-γ expression. We cannot conclude from this observation that the dysfunctionality of NK cells is intrinsic or extrinsic due to the presence of other immune cells. Therefore, conducting similar studies on isolated NK cells will enable us to answer this question.

4.4 Conclusion

Taken together, our findings revealed that HIV patients on ART have a higher proportion of the immature $CD56^+$ subpopulation and lower proportion of the mature $CD16^+$ subpopulation compared to uninfected individuals. In addition to a change in the repertoire of NK cells, HIV-infected individuals have an increased percentage of Gal-9+ NK cells compared to seronegative individuals. These Gal-9+ NK cells exhibit lower expression of cytotoxic mediators (GzmB,

perforin and GNLY) but higher expression of IFN-γ compared to their Gal-9- counterparts. The expression pattern of cytotoxic mediators and IFN-γ by TIGIT+ NK cells was the opposite of what was observed for Gal-9+ NK cells. Furthermore, Gal-9+ NK cells degranulate significantly more at baseline compared to Gal-9- NK cells, suggesting an indiscriminate degranulation. Moreover, the addition of exogenous cytokines enhanced the level of degranulation, cytotoxic mediators, and IFN-γ expression for both Gal-9+ and Gal-9- NK cells. To our knowledge, this is the first study to report the existence of Gal-9 on the surface of NK cells and phenotype this population in HIV patients. We hope that the characterization of this expanded population of NK cells will assist us in better understanding how the functionality of NK cell subtypes can be enhanced in HIV-infected individuals.

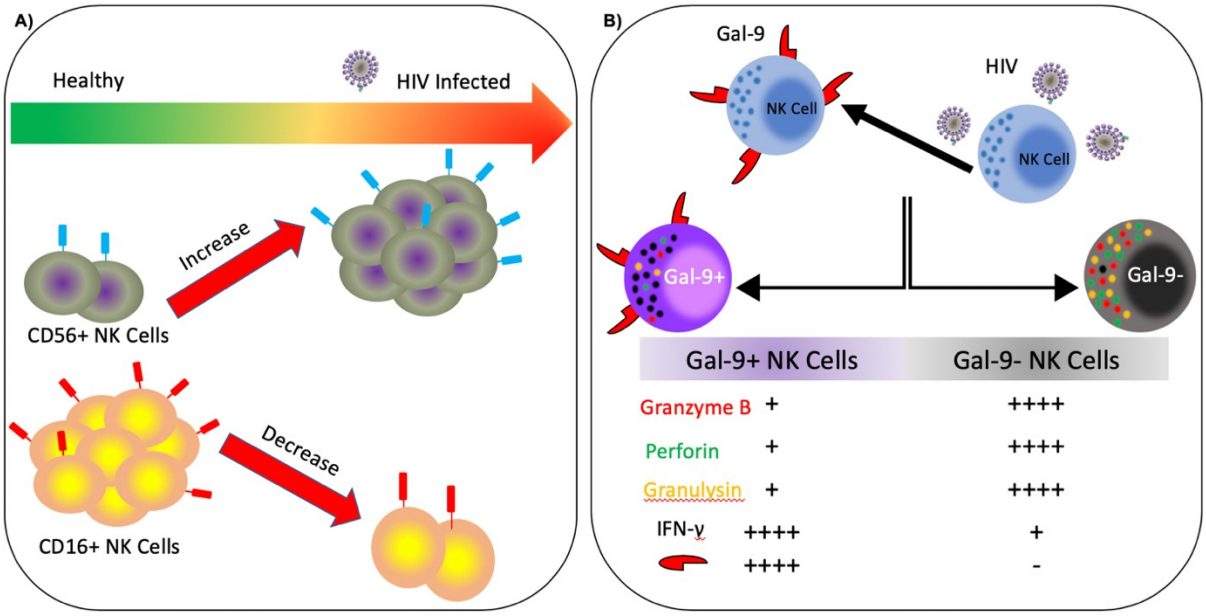

Figure 4-1: Summary of major findings for HIV-infected individuals on ART. (A) Increase in $CD56^+$ and decrease in $CD16^+$ subpopulations of NK cells in HIV-infected individuals on ART. (B) We observed an increase in Gal-9+ NK cells in HIV-infected individuals which was associated with lower GzmB, perforin, and granulysin expression compared to Gal-9- NK cells. In contrast, these Gal-9+ NK cells had higher IFN-γ expression.

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