

**The Impact of HIV-1 Infection on NK Cell Phenotypes and Non-classical Helper Functions**

by

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Rhianna Bronson

University of Pittsburgh, 2022

Although treatment with antiretroviral therapy (ART) results in HIV viral suppression to undetectable viral loads, systemic inflammation continues to persist over long-term administration of ART. Exactly how this state of chronic inflammation affects NK cell phenotypes and their non-classical helper functionality remains to be fully determined. Here we report that a subpopulation of CD56<sup>dim</sup> NK cells lacking FcR $\gamma$  (FcR $\gamma$ -), which are deficient in providing immune help in response to innate stimuli, are highly expanded in the peripheral blood of HIV+ individuals as far out as four years on ART. Surface and intracellular staining, followed by quantification through flow cytometry, identified phenotypic and functional differences of FcR $\gamma$ - NK cells and revealed that these differences do not return to conventional baselines over time. Since this helper deficient FcR $\gamma$ - NK cell phenotype accumulates in the periphery of people living with HIV (PLWH), we hypothesize that this may translate into a decreased capacity among PLWH to generate effective primary adaptive immune responses elicited by the novel COVID-19 vaccines. Therefore, we performed a detailed characterization of peripheral blood NK cell and T cell phenotypes in PLWH at time points before and after COVID-19 vaccination as part of a larger multi-research group HIV project. These data will be included along with vaccine induced antibody responses, cellular responses, and HIV reservoir data as part of a multiparameter machine learning approach to determine the overall impact(s) of HIV infection and NK cell subset distribution on COVID-19 vaccine outcomes. Our initial results suggest that an increased percentage of FcR $\gamma$ - peripheral blood NK cells is associated with lower vaccine-induced antigen-specific T cell responses to the

SARS-CoV-2 spike protein in overnight and 10-day cultures. Determining the effect of HIV infection on COVID-19 vaccination responses in individuals on ART, provides important insight into the immunocompromised state of PLWH and how this may affect vaccine efficacy, leading to future changes in immunization recommendations in the HIV+ population.

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## Preface

To Lux, for being a constant figure of love and support. Thanks for following me all the way from Tennessee to Pennsylvania.

To my family, for supporting my education and research wholeheartedly (even though it involved me moving up North). I love y'all.

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To those living with HIV/AIDS, thank you for your generous support of research. Your perseverance keeps us all moving forward, and no research would be possible without you.

To those we have lost to HIV/AIDS, your legacy continues and the holes you left in our community will never be filled. I hope to continue to try to give back, even in some small way, to the community that has always loved and embraced me.

## **1.0 Introduction**

### **1.1 Human Immunodeficiency Virus (HIV)**

Human Immunodeficiency virus (HIV) is a retrovirus that primarily infects and kills CD4+ T-cells, colloquially known as helper T-cells. If left untreated, HIV infection will progress to acquired immunodeficiency syndrome (AIDS). An individual is said to have progressed to AIDS if the number of their CD4+ cells falls below two hundred cells per cubic millimeter of blood (200 cells/mm<sup>3</sup>) or they develop one or more opportunistic infections regardless of their CD4+ count (1). HIV was first reported in the United States on June 5, 1981, in a report regarding Pneumocystis pneumonia in previously healthy, gay men in Los Angeles (2). However, evidence suggests that the virus has existed in the continental United States since the mid to late 1970s (3). Since the primary populations affected by this virus were gay men, intravenous drug users, immigrants and racial minorities, the United States government did little to address the epidemic at the time (4).

Today, HIV/AIDS is estimated to impact at least thirty-eight million people worldwide. Of those people, around 75% have accessed antiretroviral therapy (ART) and 66% are successfully virally suppressed (1). While ART is highly effective at reducing viral loads and preventing transmission, it does not fully restore functional immunity, causing continuous systemic inflammation despite adherence to the drug regimen. Chronic inflammation has been linked to several detrimental health effects including but not limited to associations with diseases of aging and immune senescence, which is characterized by alterations in soluble markers, increases in cellular exhaustion and expression of immune checkpoint markers in T-cells, as well as decreases in polyfunctional T-cell responses.

## 1.2 NK Cells

The specific interest of this project is to understand how long-term ART treatment and chronic inflammation affects natural killer (NK) cell phenotypes and functionality. NK cells are traditionally known for their innate capacity to kill transformed, stressed, and/or antibody-tagged targets such as virally infected or cancerous cells. Despite their name, they also play a significant role as cytokine-producing “helper” cells through their ability to mediate the adaptive immune response (5). Specifically, they have the capacity to educate and modulate dendritic cell (DC) function (Supplemental Figure 1). For example, innate stimuli can drive DC production of cytokines such as IL-18 and IL-12 which induce NK cells to produce IFN $\gamma$  and TNF $\alpha$  (5). These NK cell-derived factors in turn can drive the maturation and Type 1 polarization of DCs, which are characterized by an enhanced ability to promote a cellular immune response through the production of IL-12 (5). NK cell deficiencies are commonly associated with defects in anti-viral immunity and, most notably, chronic herpesvirus infections (6).

NK cells are traditionally divided into two subsets: CD56<sup>dim</sup> CD16<sup>+</sup> and CD56<sup>bright</sup> CD16<sup>-</sup> cells. CD56<sup>bright</sup> CD16<sup>-</sup> NK cells are a younger, less differentiated population that comprises about 10% of peripheral NK cells. They are stronger producers of cytokines and chemokines in response to cytokine stimulation, and they have the ability migrate to lymph nodes through expression of CCR7 (7). They also contain longer telomeres than their CD56<sup>dim</sup> counterparts, lending credence to the proposed model of differentiation from CD56<sup>bright</sup> into CD56<sup>dim</sup> (8). CD56<sup>dim</sup> cells, on the other hand, are known for their cytolytic effector function, being described as a functionally more mature and differentiated subset that accounts for around 90% of peripheral NK cells. These cells also induce CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) (7). It is, however,

important to note that the divisions of these subsets have become more blurred since one can adopt the characteristics of the other.

In addition to DC crosstalk and adaptive modulation, NK cells have also recently been shown to present phenotypes that demonstrate “memory-like” adaptive behavior. Cytokine-induced (IL-18/IL-12) helper CD56<sup>dim</sup> CD16<sup>-</sup> NK cells are strong producers of IFN $\gamma$  and have shown enhanced responses to rechallenge as far as three weeks post-stimulation (9). In reference to a non-specific antigen response, some prefer to refer to these cells as “trained” or “primed.” However, specific antigen responses have been observed from NK cells in the context of MCMV models. This cytokine-induced “memory” phenotype shows decreased expression of CD16 and increased expression of CCR7, CD25, and CD83 (9).

### **1.2.1 NK Cells and HIV Infection**

A rare subset of these “memory-like” NK cells within the CD56<sup>dim</sup> subset have been found to be highly expanded in HIV+ individuals (Supplemental Figure 2) (10; 11; 12). This specific phenotype is characterized by a loss of FcR $\gamma$  (referred to hereafter as FcR $\gamma$ <sup>-</sup> NK cells) and was originally documented in relation to cytomegalovirus (CMV) infection. These FcR $\gamma$ <sup>-</sup> NK cells show enhanced antibody-dependent functional responses in a non-pathogen specific manner. While the accumulation of FcR $\gamma$ <sup>-</sup> NK cells is associated with previous CMV infection, the functional responses are not restricted to CMV-infected cells. As stated, these cells display non-specific increased ADCC functionality and can persist long-term, which is an important feature of a memory phenotype (12). Previously gathered data has shown that FcR $\gamma$ <sup>-</sup> NK cells are relatively absent from community controls, and since CMV seroprevalence in the population of men who have sex with men (MSM) has been described as higher than the non-MSM population, this

accounts for detected populations of FcR $\gamma$ - NK cells in MSM HIV- donors. CMV seropositivity and HIV infection also have an additive effect, as CMV+ HIV+ donors display higher frequencies of FcR $\gamma$ - NK cells compared to CMV+ HIV- individuals (13).

While this “memory-like” phenotype displays increased ADCC function, they are also characterized by an impairment in their production of IFN $\gamma$  which may impact NK cell crosstalk with DCs and subsequent modulation of the adaptive response (Supplemental Figure 1). In short, as these cells increase their ADCC function beyond that of FcR $\gamma$ + NK cells, they lose responsiveness to innate stimuli, leading to the compromise of their helper function. In addition to their deficit in IFN $\gamma$  production, FcR $\gamma$ - NK cells fail to downregulate CD16 and to upregulate CCR7, CD25, and CD83 in response to cytokine stimulation (13), resulting in an accumulation of NK cells lacking the ability to respond to innate signals in the periphery of HIV+ individuals.

### **1.3 Specific Aims**

#### **1.3.1 Aim 1**

To determine the impact of HIV and long-term ART on NK cell phenotypes and function in people living with HIV (PLWH).

Hypothesis: Long-term ART and viral suppression will not restore conventional NK cell phenotypes, and high numbers of memory-like FcR $\gamma$ - NK cells will persist. The frequency of these FcR $\gamma$ - NK cells will correlate with increased expression of markers of chronic inflammation and exhaustion.

Rationale: While it has been documented that FcR $\gamma$ - NK cells accumulate in the periphery of PLWH, it is unclear whether long-term treatment with ART will diminish this population or if this “memory-like” phenotype will persist.

### **1.3.2 Aim 2**

To determine the relationship between NK cell phenotypes and COVID-19 vaccine-induced adaptive immunity in PLWH.

Hypothesis: HIV+ donors will have differing immune cell phenotypes from HIV- donors that may affect antigen-specific T-cell responses to COVID-19 vaccination. Additionally, individuals with a high frequency of FcR $\gamma$ - NK cells will have lower antigen-specific T-cell responses to COVID-19 vaccination

Rationale: The importance of understanding the immunocompromised state of PLWH has been highlighted during the recent SARS-Cov-2 pandemic. This understanding is not only important for COVID-19 disease outcomes, but now also gains a new relevance after the public release and FDA authorization of COVID-19 vaccinations. Knowing that an altered, innate stimuli (IL-18 and IL-12) unresponsive NK cell phenotype accumulates in the periphery of people infected with HIV, we hypothesized that this may have effects on the adaptive immune response elicited by the COVID-19 vaccines. This hypothesis is based on the ability of NK cells to promote DC maturation and type-1 polarization through the production of factors including IFN $\gamma$  and TNF $\alpha$ . These matured DCs then secrete cytokine signals, such as IL-12, a critical factor for the induction and persistence of an effective Th1 and CTL response. FcR $\gamma$ - NK cells may hinder the magnitude of T-cell responses to the vaccine given their lack of cytokine expression and terminal differentiation status. Here, our goal is to determine if evidence can be obtained, using the

Pittsburgh MWCCS cohort, which would indicate whether the FcR $\gamma$ - NK cell population has an impact on the effectiveness of the SARS-CoV-2 vaccination. This aim is one small part of a larger COVID-19/HIV project involving the work of multiple laboratories and collaborators (Supplemental Figure 6) (NIH/NIAID R01 AI167711-01).

## 2.0 Approach

### 2.1 Participants

This project utilized samples from two separate groups of donors for each aim. The first aim comprised of a longitudinal, quantitative, *in vitro* assessment of the phenotypic and functional characteristics of NK cells. The analysis was derived from CMV seropositive participants of the AIDS Clinical Trials Group (ACTG) A5321 cohort who initiated ART during chronic infection and have well-documented viral suppression. This is a unique cohort that is both well documented and highly sampled (14). We assessed peripheral blood mononuclear cell (PBMC) specimens obtained during viral control at 1 year and 4 years post-ART initiation. N=60 samples were analyzed in total by the laboratory; however, the scope of this thesis covers an N=10 derived from this group, all of which had FcR $\gamma$ - NK cell frequencies at  $\geq 10\%$  of total peripheral NK cells. The work required one vial of cryopreserved PBMCs at 5e6 cells /vial from each donor for each time point.

**Table 1: Demographics of the ACTG Cohort (N=60)**

<i>Age at initiation of ART, median (Q1, Q3), years</i>	<i>39 (34,46)</i>
<i>Female</i>	<i>20%</i>
<i>Race/Ethnicity:</i>	
<ul style="list-style-type: none"><li><i>White, non-Hispanic</i></li></ul>	<ul style="list-style-type: none"><li><i>33 (55%)</i></li></ul>
<ul style="list-style-type: none"><li><i>Black, non-Hispanic</i></li></ul>	<ul style="list-style-type: none"><li><i>11 (18%)</i></li></ul>
<ul style="list-style-type: none"><li><i>Hispanic (regardless of race)</i></li></ul>	<ul style="list-style-type: none"><li><i>15 (25%)</i></li></ul>
<ul style="list-style-type: none"><li><i>American Indian/Alaskan Native</i></li></ul>	<ul style="list-style-type: none"><li><i>1 (2%)</i></li></ul>

Pre-therapy plasma HIV-1 RNA, median (Q1, Q3), <i>log<sub>10</sub>copies/mL</i>	4.6 (4.2, 4.9)
Pre-therapy CD4+ cell count, median (Q1, Q3), <i>cells/mm<sup>3</sup></i>	284 (175, 365)
Year 4 of ART CD4+ cell count, median (Q1, Q3), <i>cells/mm<sup>3</sup></i>	607 (470, 783)
Pre-therapy CD4:CD8 cell ratio, median (Q1, Q3)	0.3 (0.2, 0.4)
Year 4 of ART CD4:CD8 cell ratio, median (Q1, Q3)	0.8 (0.5, 1.0)
Art regimen (initial, at time of last sample collection) <ul style="list-style-type: none"> <li>• NNRTI-based</li> <li>• PI-based</li> <li>• INSTI-based</li> <li>• Other</li> </ul>	<ul style="list-style-type: none"> <li>• 62%, 65%</li> <li>• 35%, 33%</li> <li>• 0%, 2%</li> <li>• 3%, 0%</li> </ul>

The second aim of this study used PBMCs collected from December 2019 to December 2021 through the MACS/WIHS Combined Cohort Study (MWCCS) clinic. We selected 250 participants that have been immunized with mRNA vaccines and requested archived PBMC samples (collected in 2019 prior to the COVID-19 pandemic) and post-pandemic PBMC samples collected during the annual core visits. These samples were then used in a longitudinal, quantitative, *in vitro* assessment of the phenotypes of immune cell populations and the functional characteristics of NK cells, specifically. In the scope of this thesis, only MACS (a study of gay and bisexual men), not WIHS (a study of women who had other risk factors for HIV) samples have been evaluated (N=25; 17= HIV+, 8=HIV-). The average ages of the HIV+ and HIV- donors were

62 years and 60 years, respectively. In HIV+ donors, their samples were collected at an average of 63 days post-vaccination, and for HIV- donors, the average was 47.5 days post-vaccination. The average ages did not vary between those with high frequencies (>10%) and low frequencies (<10%) of FcR $\gamma$ - NK cells, which were 63 years and 62 years, respectively. Additionally, there was no difference in CD4+ counts between these two populations. The average time after vaccination that samples were collected was 73 days for the high FcR $\gamma$ - group and 53 days for the low FcR $\gamma$ - group (Table 2).

**Table 2: Demographics of tested MACS participants (N=25)**

<i>Donor</i>	<i>CD4+ Count</i>	<i>Vaccine</i>	<i>Days from Vax</i>	<i>Age</i>	<i>Race</i>	<i>FcR<math>\gamma</math>- frequency (Pre- vaccination)</i>
34373	200-500	Pfizer-Biotech	96	58	White, non-Hispanic	1.23%
30061	200-500	Pfizer-Biotech	107	79	White, non-Hispanic	70.6%
30692	200-500	Moderna	73	61	White, non-Hispanic	0.092%
34025	>500	Pfizer-Biotech	105	64	White, non-Hispanic	79.1%
34058	>500	Pfizer- Biotech	38	42	White, non-Hispanic	49.5%
34310	>500	Moderna	47	59	Black, non-Hispanic	3.23%
34211	>500	Pfizer-Biotech	113	52	White, non-Hispanic	23.4%
33153	>500	Pfizer-Biotech	4	71	White, non-Hispanic	16.5%
30378	>500	Pfizer-Biotech	6	62	White, non-Hispanic	2.48%
34127	Unknown	Pfizer-Biotech	70	68	Unknown	52.9%
30004	Unknown	Moderna	63	72	Unknown	4.93%
30484	Unknown	Pfizer-Biotech	34	60	White, non-Hispanic	2.16%
34314	Unknown	Unknown	Unknown	55	White, non-Hispanic	22.1%

30438	Unknown	Unknown	Unknown	61	White, non-Hispanic	2.22%
30770	Unknown	Unknown	Unknown	81	White, non-Hispanic	40.7%
30381	Unknown	Unknown	Unknown	63	White, non-Hispanic	47.9%
34356	Unknown	Unknown	Unknown	40	White, non-Hispanic	17.7%
30067	HIV-	Moderna	34	72	Unknown	2.12%
34068	HIV-	Moderna	17	42	Unknown	2.67%
30712	HIV-	Pfizer-Biotech	34	64	Unknown	3%
35018	HIV-	Moderna	31	38	Unknown	0.84%
34047	HIV-	Pfizer-Biotech	97	53	Unknown	0.64%
30267	HIV-	Moderna	72	69	Unknown	1.08%
31027	HIV-	Unknown	Unknown	71	White, non-Hispanic	1.05%
33034	HIV-	Unknown	Unknown	72	Black, non-Hispanic	1.96%

## 2.2 PBMC Cultures

For Aim 1, cryopreserved PBMCs from 1 year (time point 1) and 4 years (time point 2) post-initiation of ART were thawed and cultured in complete IMDM (10% fetal bovine serum and 0.5% gentamicin) in 6-well plates at a density of 1e6 cells/well. The cells were then harvested, washed, and stained for analysis. Phenotypic and functional profiles were determined by flow cytometry (*see flow cytometry*) on NK cell subsets in culture media alone or following 24-hour stimulation in the presence of IL18 (1 µg/ml; MBL International) + IL-12 (rhIL-12p70; 100 ng/ml; Biolegend).

For Aim 2, cryopreserved PMBCs at  $1.5 \times 10^6/\text{mL}$  were suspended in RPMI supplemented with 1% glutamax and 0.5% Gentamicin from each donor at their pre- and post-COVID-19 vaccination time points. The cells were then harvested, washed, and stained for analysis. Phenotypic and functional profiles were determined by flow cytometry (*see flow cytometry*) on NK cell and T-cell subsets.

### 2.3 Flow Cytometry

The Live/Dead Fixable Aqua Dead Cell Stain was used for viability exclusion, and the following antibodies (Abs) were used for immunostaining in Aim 1: CD3-allophycocyanin-H7, CD56-PE-Cy7, CD57-BV421, CD16-PerCP-Cy5.5, NKG2A-allophycocyanin, NKG2C-PE, NKp46-PE, and KLRG1-APC. Surface staining was done in FACS buffer ( $1 \times$  PBS solution, 0.5% BSA, and 0.1% sodium azide). Harvested PBMCs were first stained with 100ul of Live/Dead cell stain (prepared at a 1  $\mu\text{l}$  stain:500  $\mu\text{l}$  PBS ratio) and incubated at room temperature for 20 minutes. Ab stain mixtures and corresponding fluorescence minus one controls (FMO) were then prepared for surface staining. Abs used for labeling of the FMO controls were CD3, CD56, and CD16. Cells were washed and 50 $\mu\text{l}$  of the prepared stain mixtures added, followed by incubation at  $4^\circ\text{C}$  for 30 minutes. After incubation of the surface stains, the cells were washed before intracellular staining (ICS). For staining of intracellular protein expression, cells were fixed with BD Cytfix/Cytoperm (BD Biosciences), incubated for 20 minutes, and permeabilized using BD Perm/Wash (BD Biosciences). Cells were then labeled with a prepared mixture of anti-FcR $\gamma$ -FITC and anti-IFN- $\gamma$ -Alexa Fluor 700 in BDPerm/Wash. After ICS, cells were incubated for 30 minutes and stored in 200 $\mu\text{l}$  of FACS buffer until data acquisition using a BD LSRFortessa flow cytometer. Data were

analyzed using FlowJo version 10.8.1, with expression levels based on comparison with fluorescence minus one samples. NK cell gating strategies can be referenced in Supplemental Figure 3.

The overall percentage of NK cells within the PBMC population was measured at baseline by flow cytometry. The percentages of the of CD56<sup>bright</sup>/CD16<sup>-</sup> and CD56<sup>dim</sup>/CD16<sup>+</sup> subsets and the expression of activating (NKG2C) and inhibitory (NKG2A) receptors was assessed within the total NK cell population. Particular attention was directed toward measurement of the percentage of FcR $\gamma$ <sup>-</sup> vs. FcR $\gamma$ <sup>+</sup> NK cells and their co-expression of activation (NKG2C), differentiation (CD57), and exhaustion-associated markers (KLRG1). Functional assessment was assessed by observing IL-18 and IL-12 responsiveness of NK cells, which was determined by measuring changes in their capacity to produce IFN $\gamma$ .

For Aim 2, the flow cytometry staining was split into two panels. One panel to focus on NK cell subsets (Panel 1) and the other panel to focus on T-cell phenotyping as well as overall monocyte and B-cell percentages (Panel 2). The following Abs were used for the Panel 1 immunostaining: CD3-allophycocyanin-H7, CD56-PE-Cy7, CD57-BV421, CD16-PerCP-Cy5.5, NKG2A-allophycocyanin, NKG2C-PE, and CD38-BV605. The following Abs were used for the Panel 2 immunostaining: CD3-allophycocyanin-H7, CD4-Pacific Blue<sup>TM</sup>, CD8-PE-Cy7, CD16-PerCP-Cy5.5, CD14-PE, CD19-AlexaFluor <sup>®</sup>700, CD45RA-BV605, and CD27-FITC. Surface and intracellular staining was accomplished using the same sample preparations as used for the Aim 1 analysis mentioned above. ICS with anti-FcR $\gamma$ -FITC was used only in Panel 1. Panel 2 required only surface staining. Samples for Panel 1 were stored in 200 $\mu$ l FACS buffer until data acquisition while samples for Panel 2 were stored in 200 $\mu$ l of 2% PFA. Data was acquired using a BD LSRFortessa flow cytometer and were analyzed using FlowJo version 10.8.1, with expression

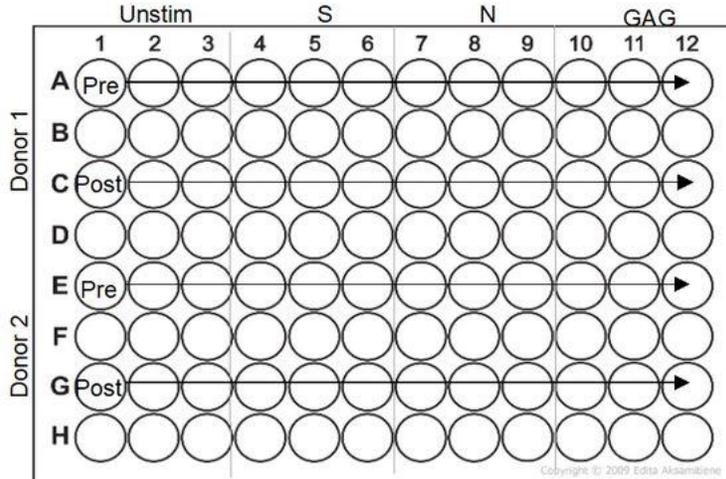
levels based on comparison with fluorescence minus one samples. T-cell gating strategies can be referenced in Supplemental Figure 4.

## **2.4 ELISpot (Enzyme-Linked Immune Absorbent Spot)**

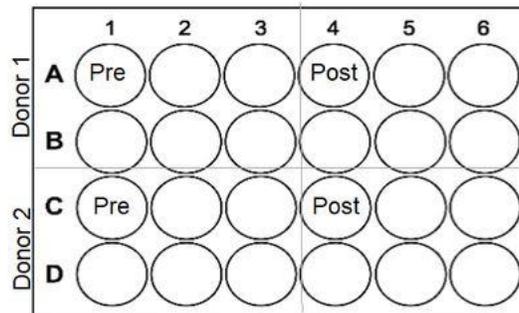
ELISpot assays were conducted using Antihuman interferon- $\gamma$  mAb1-D1K, Mabtech (3420-3-250), Antihuman IFN- $\gamma$  mAb 7-B6-1 biotinylated, Mabtech (3420-6-1000), Vectastain Elite ABC kit, Vector (pk-6100), DAB Sigmafast with metal enhancer tablet, Sigma (D0426-50set), 10xPBS, and PBS 0.5% BSA solution (2.5g BSA + 500mL 1xPBS). Peptide mixes were prepared as follows: for S: 1.2mL RPMI plus 48 $\mu$ l peptide, for N: 1.2mL RPMI plus 48 $\mu$ l peptide, for GAG: 1.2mL RPMI plus 24 $\mu$ l GAG peptide.

On day -1, plates were coated in coating mix (55 $\mu$ l of anti-human IFN $\gamma$  added to 5.5mL 1xPBS). Plates were then stored overnight at 4°C. On day 0, ELISpot plates were blocked by washing with 1xPBS and adding 150 $\mu$ l RPMI to each well. Plates were then incubated at 37°C for 2 hours. PBMCs were then thawed, counted, and resuspended in RPMI to 1.5e6cells/mL.

For the overnight ELISpot, 100 $\mu$ l of each peptide mix was added to the plate in triplicate as laid out below. 100 $\mu$ l of cells was then added to the assigned wells, and then the plate incubated at 37°C for 18 hours or overnight.

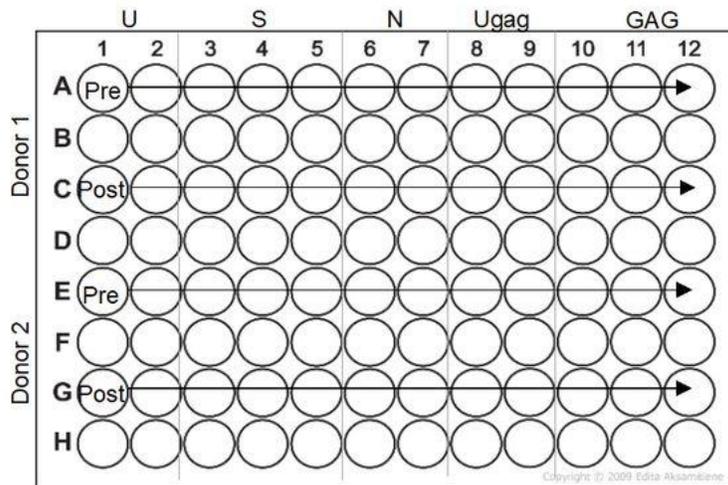


For 10-day cultured ELISpots, cells were cultured in a 24-well flat bottom plate, two wells per sample. 20µl S and 20µl N peptides/mL were added to one well per sample. Then, 10µl Gag peptide/mL was added to one well per sample.



On day 1, the overnight ELISpot was decanted and washed. Biotin antibody was prepared with 5mL PBS/0.5% BSA solution and 10µl Biotin antibody. The wash was decanted from the plate and 50µl of Biotin solution was added to each well, then the plate incubated at 37° for two hours. The plate was then washed with PBS/Tween20 and 100µl of ABC peroxidase added to each well. The assay was then incubated for 45 minutes at room temperature in the dark. The plate was then washed and 100µl of Dab solution was added to each well. Plates were then left to dry and read the following day.

On day 4, the 10-day cultured cells were fed with 10µl IL-2/mL and 10µl IL-7/mL to each culture well. On day 7, 500µl of fresh RPMI was added on top of each well and cells were fed again with 10µl IL-2 and IL-7/mL. On day 9, ELISpot plates were coated as previously described. Cell cultures had 1mL removed and replaced with 1mL fresh RPMI, then returned to the incubator until harvest. On day 10, ELISpot plates were blocked as previously described, and the cells were harvested from culture and resuspended to 500,000 cells/mL. 100µl of the respective peptide mixes and 100µl of the respective cells was added to the ELISpot plate as shown below. The ELISpot plate was then incubated at 37°C overnight. On day 11, plates were prepared to be read in the same manner as previously described for the overnight cultures (15).



## 2.5 Statistical Analysis

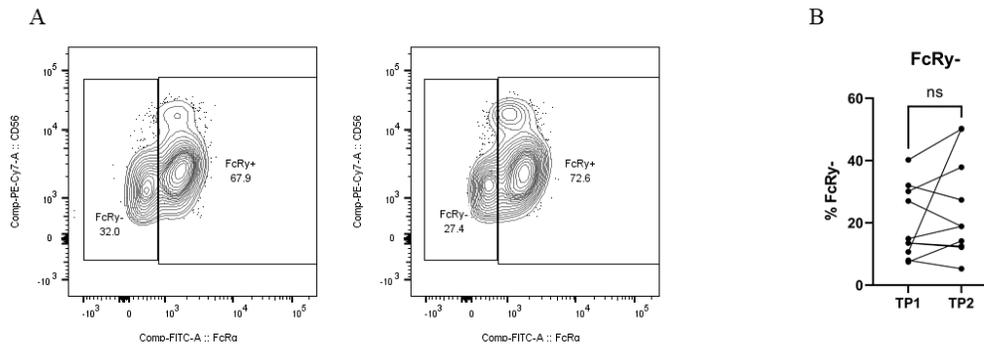
Data were analyzed using GraphPad Version 9. Normality was assessed using the Shapiro-Wilk test, and data that did not follow a normal distribution was analyzed by nonparametric statistics (i.e., Wilcoxon matched-pairs signed rank test, Mann-Whitney test, or Kruskal-Wallis

test). Data following a normal distribution was assessed using Student's paired t-tests for related groups or unpaired t-test for independent data sets. One-way ANOVA with correction for multiple comparisons by Tukey's HSD post-hoc test was used to measure relationships between three or more groups. When two independent variables were present, two-way ANOVA with correction for multiple comparisons by Sidak's multiple comparisons post-hoc test was used. When performing statistical analysis for the ELISpot data, donors noted to have been previously infected with COVID-19 were excluded from the dataset.

### 3.0 Results

#### 3.1.1 FcR $\gamma$ - NK cell populations do not decrease on long-term ART

Previously published cross-sectional data has documented that FcR $\gamma$  mRNA and protein expression in NK cells did not return to control levels in virally suppressed HIV+ patients with median duration of 11.5 years of treatment vs. a treatment naïve group, measured by RT-PCR and immunoblotting of mRNA and protein extractions, respectively (16). However, longitudinal data of potential changes in the FcR $\gamma$ - NK cell population was not obtained. Here, we confirmed that the frequency of FcR $\gamma$ - NK cells was not changed over time in a longitudinal data set (one- and four-years post-initiation of ART) obtained by flow cytometry analysis of intracellular expression of FcR $\gamma$  (Figure 1A-B).



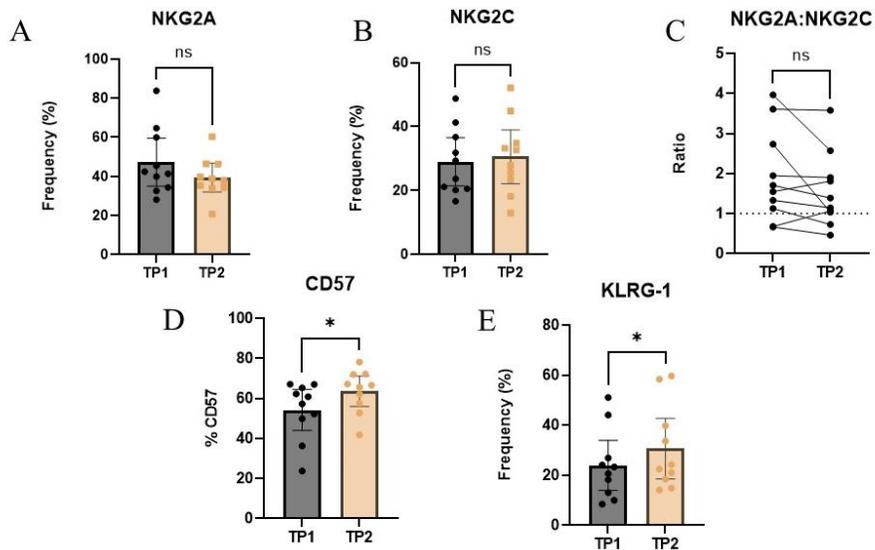
**Figure 1: Percentage of FcR $\gamma$  NK cells at 1 and 4 years post-ART**

**(A) FACS plots from flow cytometry analysis of a representative donor showing the difference in the percentage of FcR $\gamma$ - and FcR $\gamma$ + NK cells at 1-(left) and 4-years (right) post-ART initiation. NK Cells gated by CD3-CD56+.** **(B) Graphical representation of the percentage of FcR $\gamma$ - NK cells from 1-4 years on ART**

**(N=10). Significance calculated by Student's paired T-test  $\alpha=0.05$ .**

### 3.1.2 Markers of exhaustion and differentiation increase over time in overall NK cell populations

Initial phenotyping began with observing the frequency of relevant markers in overall NK cell populations. No changes were noted in the frequency of NKG2A, an inhibitory receptor, or NKG2C, its activating counterpart, over four years of ART. Subsequently, the NKG2A:NKG2C ratio was also unchanged across this period (Figure 2A-C). NKG2A is associated with the more immature subset of CD56<sup>bright</sup> NK cells, implying a skew towards mature and differentiated phenotypes that do not correct over time. However, the frequencies of CD57 and KLRG-1 increased over four years on ART (Figure 2D-E). CD57 is a differentiation marker of replicative senescence, and KLRG-1 is a marker of exhaustion.



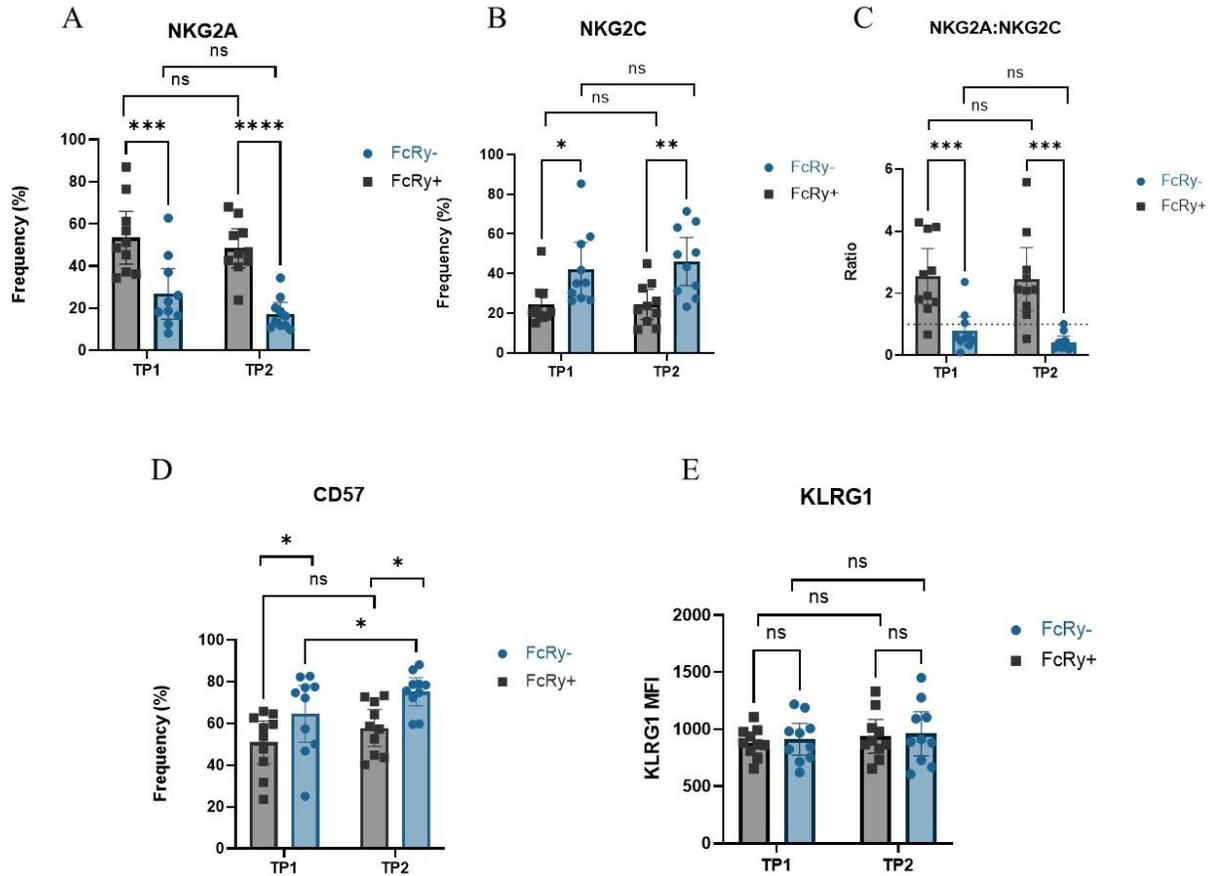
**Figure 2: Expression of markers in overall NK cell populations**

(A) Percentage of NK cell expression of NKG2A from 1 year to 4 years on ART. (B) Percentage of NK cell expression of NKG2C from 1 to 4 years on ART. (C) Ratio of NKG2A:NKG2C in NK cells. Dotted line at

**Y=1 to denote that the conventional ratio is >1. (D) Frequency of CD57 in overall NK cells. (E) Frequency of KLRG-1+ NK cells. Statistical significance measured by paired Student's t-test.  $\alpha=0.05$**

### **3.1.3 Phenotypic differences of FcR $\gamma$ - NK cells do not correct over time**

FcR $\gamma$ - NK cells show decreased frequency of NKG2A and increased frequency of NKG2C (Figure 3A-B). This is consistent with previously published phenotyping data relating to HIV associated FcR $\gamma$ - NK cells and FcR $\gamma$ - NK cells reported in relation to HCMV seropositivity (10). The frequency of these markers does not change over time (Figure 3A-B), and the inversed phenotype, in comparison to conventional NK cells, leads to a significant reduction in the typical NKG2A:NKG2C ratio, which also does not correct over time (Figure 3C). This data also supports the documented inverse correlation of CD57 and NKG2A, as FcR $\gamma$ - cells display a higher expression of CD57 (17). This expression of CD57 increases in the FcR $\gamma$ - population at four-years post-initiation of ART (Figure 3D). However, KLRG-1 does not appear to be substantially different between FcR $\gamma$ - and conventional NK cells (Figure 3E). In the many parameters looked at, there were no remarkable corrections in FcR $\gamma$ - NK cell phenotypes and subsets to conventional levels over four years on ART.



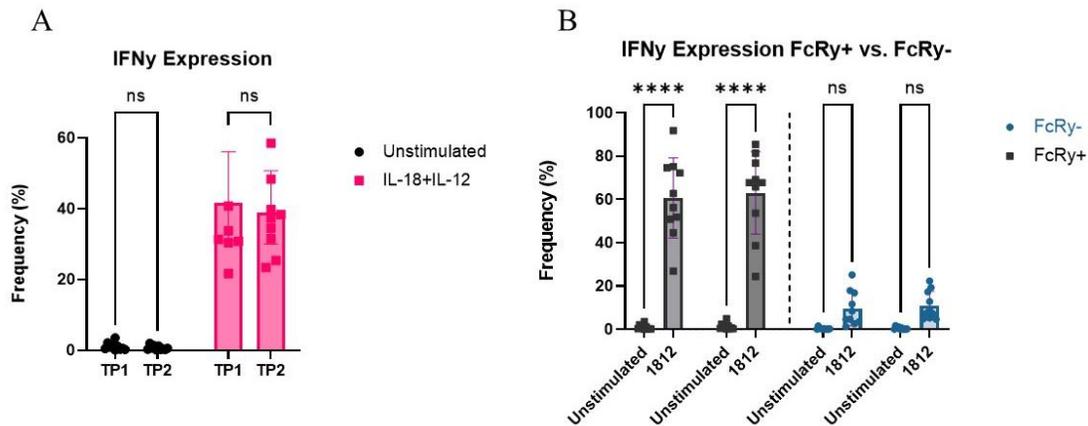
**Figure 3: Phenotypic differences between FcR $\gamma$ - and FcR $\gamma$ + NK cells**

. (A) Percentage of FcR $\gamma$ - vs. FcR $\gamma$ + NK cell expression of NKG2A from 1 to 4 years on ART. (B) Percentage of FcR $\gamma$ - vs. FcR $\gamma$ + NK cell expression of NKG2C from 1 to 4 years on ART. (C) Ratio of NKG2A:NKG2C in FcR $\gamma$ - vs. FcR $\gamma$ + NK cells. Dotted line at Y=1 to denote that the conventional ratio is >1. (D) Percentage of FcR $\gamma$ - vs. FcR $\gamma$ + NK cell expression of CD57. (E) MFI (mean fluorescence intensity) of FcR $\gamma$ - vs. FcR $\gamma$ + NK cell expression of KLRG-1. Statistical significance measured by two-way ANOVA.  $\alpha=0.05$ .

### 3.1.4 Functional differences of FcR $\gamma$ - NK cells do not correct over time

Our previous data above shows that FcR $\gamma$ - NK cell populations are remaining steady, and since these cells have been defined as IL-18-unresponsive and lack the capacity to produce the amount of IFN $\gamma$  comparable to conventional NK cells, it is likely that the levels of IFN $\gamma$  expression

would also remain unchanged. As predicted, the overall production of IFN $\gamma$  after stimulation with IL-18 and IL-12 did not change over time (Figure 4A). Figure 4B confirms that the FcR $\gamma$ - population yields this IL-18+IL-12-unresponsive phenotype that remains over time and does not improve after 4 years on ART.



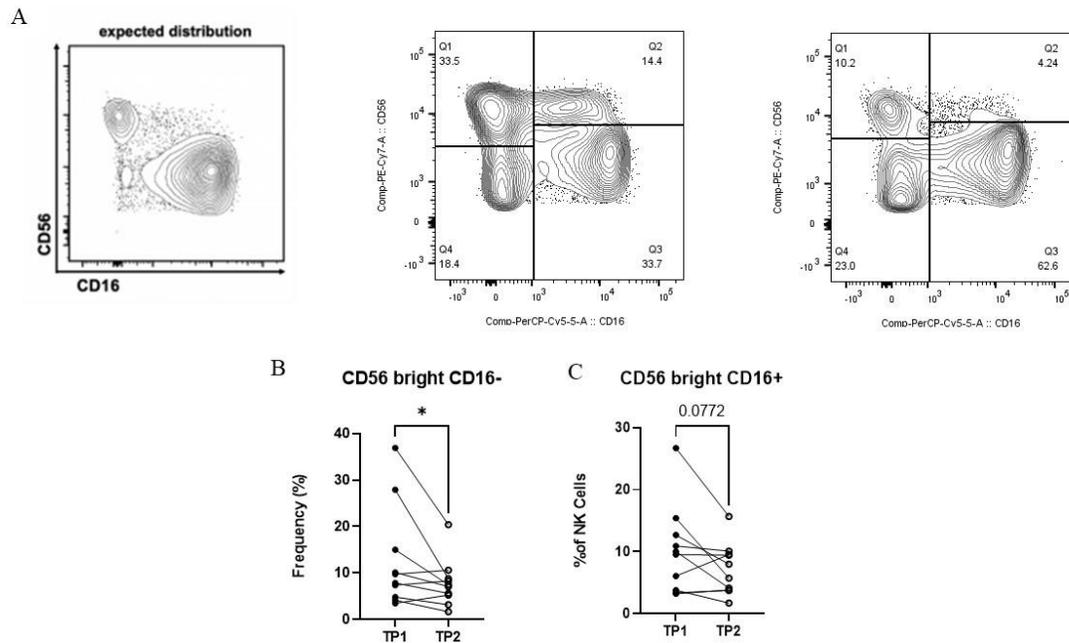
**Figure 4: IFN $\gamma$  expression over time**

(A) Overall IFN $\gamma$  production in unstimulated and stimulated NK cells from 1 to 4 years post-ART. Stimulated NK cells were cultured for 24hr with IL-18 and IL-12 (B) FcR $\gamma$ - vs. FcR $\gamma$ + IFN $\gamma$  production in stimulated and unstimulated NK cells. Statistical significance calculated by two-way ANOVA  $\alpha=0.05$ .

### 3.1.5 Irregular NK cell differentiation patterns noted in HIV+ individuals on long-term ART

As explained previously, NK cells are typically divided into two main subsets: CD56<sup>bright</sup> CD16- and CD56<sup>dim</sup> CD16+, with the CD56<sup>bright</sup> differentiating into the CD56<sup>dim</sup>. Within the observed data set, an irregular distribution of NK cells was noted in seven out of ten donors. These individuals display an unusual grouping of CD56<sup>bright</sup> CD16+ NK cells as well as larger accumulations of CD56<sup>dim</sup> CD16- cells (Figure 5A). The skewed distribution is also reflected in an increase in CD56<sup>bright</sup> CD16- cells, which usually comprise about 10% of peripheral NK cells

and, instead, ranged from typical to as high as 37% (Figure 5B). However, the most drastic of these unexpected distributions occurred at the first time point, around one-year post-ART initiation. At time point two, four years post-ART, these donors still display larger than average accumulations of these irregular subsets, but they tend to decrease over time (Figure 5C).



**Figure 5: Irregular NK cell subset distributions/differentiation patterns noted in some HIV+ donors.**

(A) FACS plots from flow cytometry analysis of CD56/CD16 NK cell subset distributions. Quadrant 2 shows an irregularly high number of CD56 bright, CD16+ NK cells in comparison to an HIV- control. 1 year on ART (left) and 4 years on ART (right). (B). CD56 bright CD16- NK cell populations over time. (C). Irregular CD56 bright CD16+ NK cell populations over time. Statistical significance calculated by Student's paired T-test (B) and Wilcoxon matched paired signed-rank test (C).  $\alpha=0.05$

### **3.1.6 Select immune cell phenotypes do not change pre- and post-COVID-19-vaccination but differ between HIV+ and HIV- individuals**

With the knowledge that FcR $\gamma$ - NK cells are dysfunctional in their production of IFN $\gamma$ , we speculated that there could be a negative impact on the adaptive response elicited by COVID-19 vaccination in PLWH. Additionally, HIV+ donors would have differing phenotypes from HIV- donors that could affect antigen-specific responses to COVID-19 vaccination. Current vaccine studies have included only a few PLWH, who maintain persistent, elevated inflammation and do not fully recover humoral and cellular immune reactivity despite ART treatment and successful viral suppression. Here, we show little difference in immune cell populations before and after vaccination, but the differences between HIV- and HIV+ individuals' immune cell repertoire is highlighted by this data.

In observing CD8+ cell frequency, both overall and at the level of distinct memory populations, there is a significant difference in HIV- and HIV+ donors. Overall CD8+ T-cell frequencies are elevated in HIV+ individuals pre- and post-COVID-19 vaccination compared to HIV- individuals (Figure 6A), and this difference is seen in the effector (CD45RA- CD27-) and central memory (CD45RA- CD27+) CD8+ T cell populations (Figure 6B-C). Digging deeper into sub analysis of the HIV+ donors, those with high (>10%) FcR $\gamma$ - NK cells have higher frequencies of overall CD8+ T cell populations pre-COVID vaccination (Figure 6D). However, it is important to note that only eight HIV- donors have been evaluated compared to seventeen HIV+. Additionally, the CD4:CD8 ratio does not differ pre- and post- vaccination in HIV+ donors, and those with elevated levels of FcR $\gamma$ - NK cells do not have a significantly different ratio when compared to donors with low (<10%) FcR $\gamma$ - cells (Supplemental Figure 5).

Due to the nature of HIV and its tropism for CD4<sup>+</sup> cells, we expected to see lower frequencies of CD4<sup>+</sup> T-cells overall and in the memory populations. As predicted, HIV<sup>+</sup> donors have lower overall frequencies of CD4<sup>+</sup> T-cells both pre- and post-vaccination (Figure 6E). CD4<sup>+</sup> effector memory cells, however, display a higher frequency pre- and post-vaccination in the HIV<sup>+</sup> donors (Figure 6F).

Despite the main focus of the COVID-19 vaccine phenotyping being T-cells, other immune cell populations were measured, albeit less in depth. Classical CD14<sup>++</sup> CD16<sup>-</sup> overall monocytes displayed an increase after vaccination, and HIV<sup>+</sup> individuals may have higher frequencies than observed in the HIV<sup>-</sup> donors post-COVID-19 vaccination (Figure 6G-H). B-cell levels show no notable change pre- and post-vaccination, but the finding of lower B-Cell populations in PLWH at the post-vaccination time points agrees with previously published data (Figure 6I-J) (18).

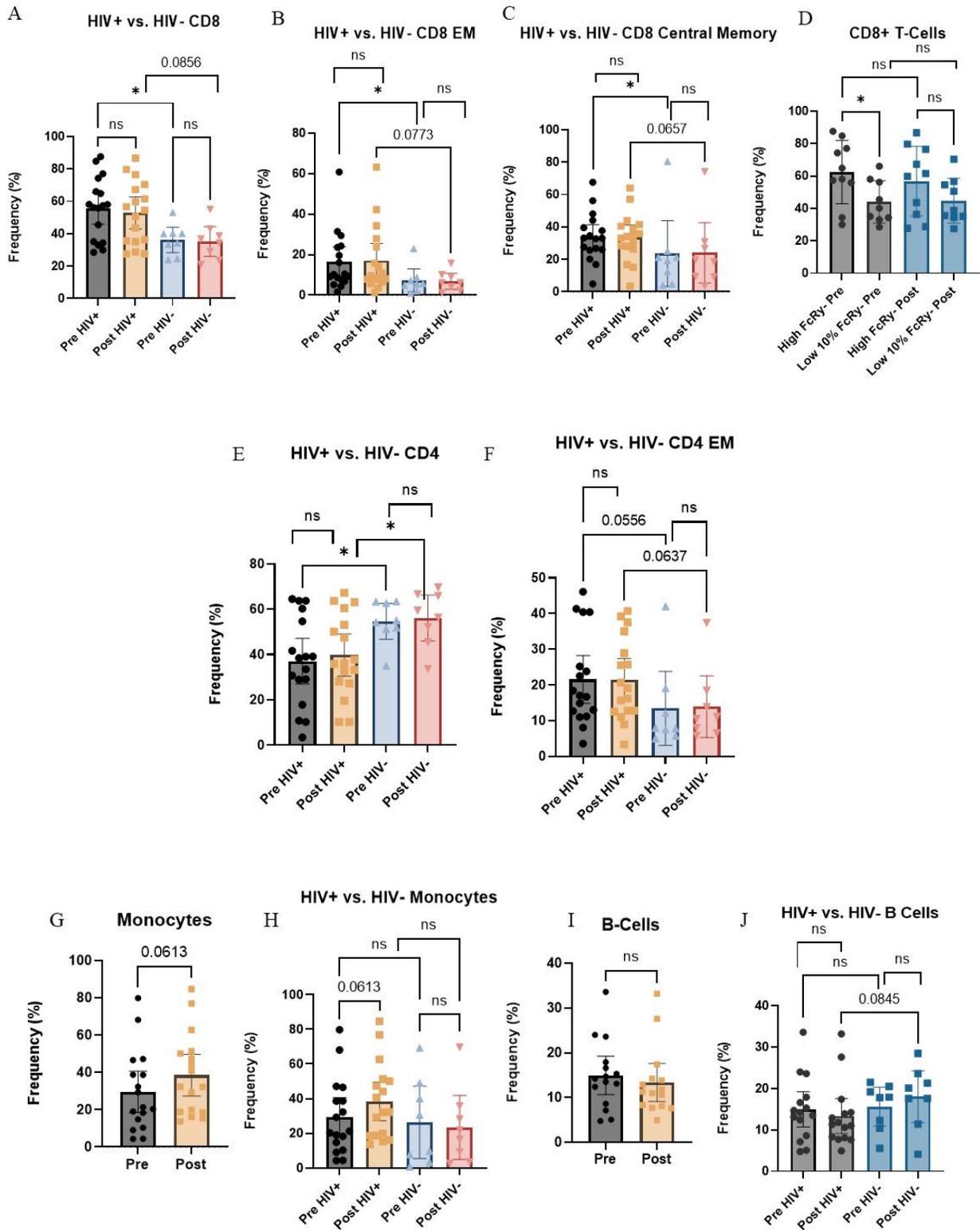


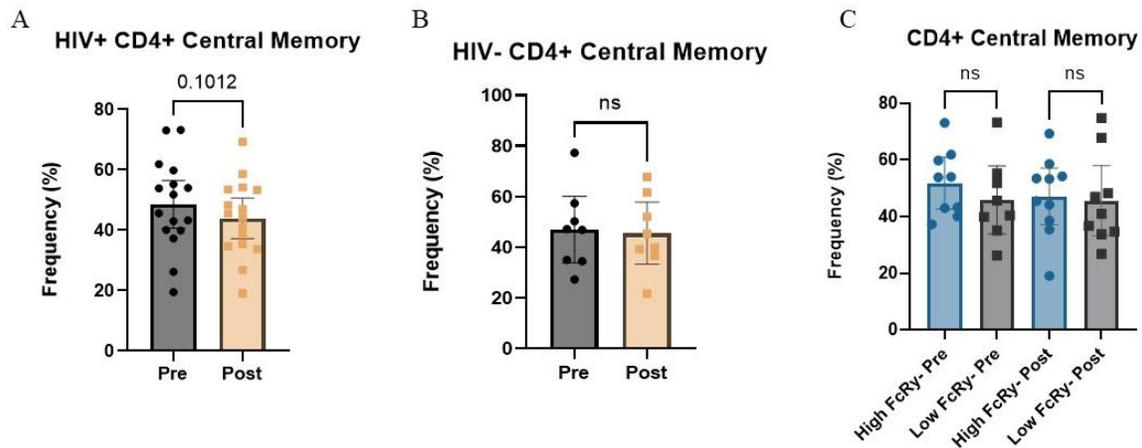
Figure 6: An overview of notably different immune cell phenotypes across donors

**(A-D): CD8+ T-cell population frequencies pre- and post-COVID-19-vaccination measured by flow cytometry analysis. (A). Overall CD8+ T-cell frequencies in HIV+ vs. HIV- donors. (B). CD8+ effector memory phenotype frequencies in HIV+ vs. HIV- donors. (C). CD8+ central memory phenotype frequencies in HIV+ vs. HIV- donors. (D). Overall CD8+ T-cell frequencies in donors with >10% FcR $\gamma$ - NK cells vs. donors with <10% FcR $\gamma$  NK cells. (E-F): CD4+ T-cell population frequencies pre- and post-COVID-19-vaccination measured by flow cytometry. (E). Overall CD4+ T-cell frequencies in HIV+ vs. HIV- donors. (F) CD4+ effector memory phenotype frequencies in HIV+ vs. HIV- donors. (G-J): Monocyte and B-cell population frequencies measured by flow cytometry analysis pre- and post-COVID-19-vaccination. (G). Overall classical (CD14<sup>++</sup> CD16<sup>-</sup>) monocyte frequencies. (H). Classical monocyte frequencies in HIV+ vs. HIV- donors. (I). Overall B-cell (CD3<sup>-</sup> CD19<sup>+</sup>) frequencies. (J). B-cell frequencies in HIV+ vs. HIV- donors. Statistical analysis performed by Mann-Whitney, unpaired Student's t-test, paired Student's t-test, and one-way ANOVA.**

$\alpha=0.05$ .

### **3.1.7 CD4+ Central Memory cells decrease in HIV+ post-COVID-19 vaccination**

A notable finding that was observed during the sub analysis of T-cell memory phenotypes was that CD4+ central memory cells tend to decrease after vaccination in HIV+ individuals (Figure 7A-B). Upon further analysis, CD4+ central memory phenotype frequencies were not affected by the percentage of FcR $\gamma$ - NK cells in the periphery (Figure 7C).



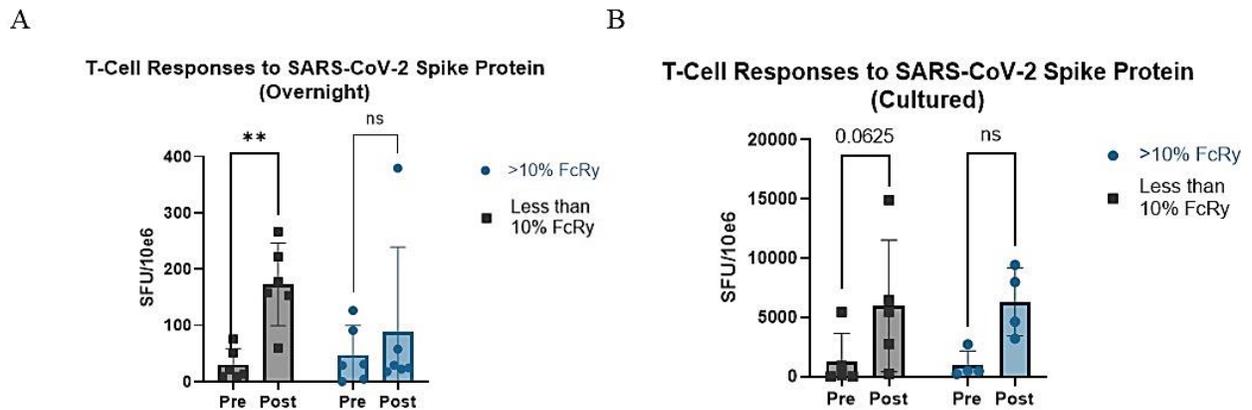
**Figure 7: Percentages of CD4 Central Memory cells pre- and post-vaccination**

(A) Overall CD4+ central memory phenotype frequency in HIV+ donors. (B) Overall CD4+ central memory phenotype frequency in HIV- donors (C) CD4+ central memory phenotype frequency in donors with over 10% FcR $\gamma$ - NK cells vs. less than 10% FcR $\gamma$ - NK cells. Statistical significance calculated by paired t-test and two-way ANOVA at  $\alpha=0.05$ . Central memory phenotype defined by CD45RA- CD27+.

### 3.1.8 A higher percentage of FcR $\gamma$ - NK cells is associated with lower T-cell responses to SARS-CoV-2 Spike protein after overnight culture

One of the most important contents of this in-depth phenotyping project is correlating the phenotypic data with findings from other simultaneous projects under the scope of the broad COVID-19/HIV project utilizing the MWCCS cohort (Supplemental Figure 6). As previously mentioned, we hypothesized that an increase in the frequency of FcR $\gamma$ - cells within the NK cell populations of HIV+ individuals would correlate with lower or deficient adaptive responses elicited by the COVID-19 vaccines. Here, specifically, antigen-specific T-cell responses were measured by IFN $\gamma$  ELISpot, and the results compared to populations of HIV+ donors with greater than or less than 10% FcR $\gamma$ - NK cells. In the overnight cultures, there is a significant difference in

the magnitude of IFN $\gamma$  producing T-cells between donors with elevated levels of FcR $\gamma$ - NK cells vs. lower levels. Donors with higher frequencies of FcR $\gamma$ - NK cells do not look to generate a significantly different IFN $\gamma$  response after overnight culture when comparing pre- and post-vaccination (Figure 8A). When looking at 10-day ELISpot cultures, we see a similar trend as in the overnight ELISpots where individuals with less than 10% FcR $\gamma$ - NK cells produce a greater IFN $\gamma$  response after vaccination (Figure 8B).



**Figure 8: Differences in T-cell IFN $\gamma$  responses to stimulation with SARS-CoV-2 Spike protein between FcR $\gamma$ - and conventional NK cell populations**

(A) CD8+ T-cell production of IFN $\gamma$  after overnight culture, measured in spot forming units per one million cells. (B) CD8+ T-cell production of IFN $\gamma$  after 10-day culture, measured in spot forming units per one million cells. Statistical significance calculated by Wilcoxon matched pairs signed rank test  $\alpha=0.05$

## 4.0 Discussion

This research indicates that the previously documented impact of HIV infection on the NK cell repertoire does not correct over time with ART treatment. Additionally, these effects, especially the expansion of FcR $\gamma$ - NK cells in the periphery, may contribute to lower antigen-specific T-cell responses elicited by COVID-19 vaccines. We observed that the overall population of FcR $\gamma$ - NK cells in the periphery does not change over four years of ART treatment (Figure 1B). The exact mechanism of how HIV infection results in the loss of FcR $\gamma$  remains unexplored, however, we show that this change persists. Furthermore, while the low NKG2A and high NKG2C expression that help to define this unique FcR $\gamma$ - NK cell phenotype continues to remain constant over time, markers of differentiation (CD57) and exhaustion (KLRG-1) increase over the course of four years post-ART (Figures 2,3).

These FcR $\gamma$ - NK cells are also unable to regain any of their functionality related to the expression of IFN $\gamma$  (Figure 4). Overall, these cells remain functionally unresponsive over four years with consistent ART treatment. This is not to conclude that NK cells will never return to their conventional population phenotypes, but at least over the course of four years, there is no notable mitigation of the impact of HIV on the NK cell repertoire. HIV infection seems to result in the long-term disruption and alteration of peripheral NK cell populations.

Another notable effect of HIV on peripheral NK cells was observed during longitudinal phenotyping. In addition to the accumulation of FcR $\gamma$ - NK cells, HIV infection drives the dysregulation of typical NK cell subset distributions. This was defined by a substantial increase in the number of CD56<sup>bright</sup> CD16+ NK cells, which we refer to as an “intermediate population” since the CD56<sup>bright</sup> CD16- NK cells differentiate into the CD56<sup>dim</sup> CD16+ cells (7). Recall that the

typical distribution of NK cell subsets is around 10% CD56<sup>bright</sup> CD16<sup>-</sup> and 90% CD56<sup>dim</sup> CD16<sup>+</sup>. Not only was this irregular subset noted in HIV<sup>+</sup> donors, but also the CD56<sup>bright</sup> CD16<sup>-</sup> percentages were uncommonly high (Figure 5B). Unlike the accumulation of FcR $\gamma$ -NK cells, this irregular subset looks to be decreasing over time, which in turn allows the traditional subsets to return to more typical levels (Figure 5B-C). This implies that continued treatment with ART may be renormalizing NK cell subsets into the conventionally observed groupings. From this data and considering that CD56<sup>bright</sup> CD16<sup>-</sup> cells differentiate into CD56<sup>dim</sup> CD16<sup>+</sup> cells, it can be hypothesized that HIV infection and the state of chronic inflammation that persists despite viral suppression may be inhibiting NK-poiesis or instigating the release of immature NK cells from the bone marrow into the periphery.

Having established the phenotypic and functional characteristics as well as the persistence of the FcR $\gamma$ - NK cell population, we hypothesized that the dysfunction of these cells in producing IFN $\gamma$  would hinder NK cell modulation of the adaptive response and, subsequently, the COVID-19 vaccine-elicited responses of the adaptive immune system. Additionally, HIV<sup>+</sup> donors would have differing phenotypes from HIV<sup>-</sup> donors that may affect antigen-specific responses to COVID-19 vaccination. Testing this in the context of COVID-19 vaccination is both timely and important for the continuation of the accumulation of data on COVID-19 vaccine responses in the immunocompromised population. The investigation began with phenotyping of immune cell populations pre- and post-vaccination. While most cellular phenotypes remained the same before and after vaccination, there were notable differences in populations between HIV<sup>+</sup> and HIV<sup>-</sup> donors. CD8<sup>+</sup> T-cells were measured at consistently higher frequencies in the HIV<sup>+</sup> population vs. the HIV<sup>-</sup> controls. This difference was detected in overall CD8<sup>+</sup> T cell frequencies as well as the specific effector and central memory populations (Figure 6A-D). This increase in circulating

CD8+ T cells could be attributed to the state of chronic inflammation and low-level immune activation that persists in the HIV+ population.

Inversely, CD4+ T-cells were measured at lower frequencies in HIV+ donors than HIV- (Figure 6E). This is unsurprising considering that the main cellular targets of HIV infection are CD4+ T-cells. What is surprising, however, is the increased populations of CD4+ effector memory cells in HIV+ individuals vs. those that are HIV-, regardless of vaccination status (Figure 6F). CD4+ effector memory cells are most often discussed, in the context of HIV infection, in terms of the persisting HIV reservoir (19). However, it is unclear if HIV+ individuals have a skewed persistence or production of CD4+ effector memory cells.

What we find interesting is the potential decrease in CD4+ central memory cells in HIV+ donors post-vaccination (Figure 7A). This decrease does not look to be an artifact of potential FcR $\gamma$ - NK cell effects on the population (Figure 7C). Despite the lack of statistical significance, this trend is notably absent from the HIV- controls, and warrants attention. This potentially coincides with work from the laboratory of Dr. Nicolas Sluis-Cremer, who hypothesize that COVID-19 vaccination in PWLH will result in an increase in HIV transcription. This proposed increase in HIV expression could be driven by one of two mechanisms: (I) vaccination reactivation of latent, replication-competent HIV from CD4+ T cells due to T cell activation; and/or (II) vaccination upregulation of chronically infected, virus-producing CD4+ T cells nonspecifically through the release of activating cytokines. CD4+ central memory cells are frequently implicated in housing a sizable portion of the HIV reservoir and could be decreasing because of a kick-and-kill effect potentially elicited by COVID-19 vaccination (19). This is not out of the realm of possibility as tetanus toxoid has been reported to increase transient HIV viremia after vaccination

(20). This is a major potential finding if correlation with the HIV reservoir data yields significant results.

Lastly, we wanted to draw associations between FcR $\gamma$ - NK cell population abundance and antigen-specific responses to the COVID-19 vaccines. In the overnight ELISpot cultures, only individuals with low FcR $\gamma$ - NK cells showed a significant antigen-specific IFN $\gamma$  response to SARS-CoV-2 Spike protein (Figure 8A), whereas no differences in HIV Gag responses were noted between individuals with high or low prevalence of FcR $\gamma$ - NK cells (Supplemental Figure 7). In the 10-day cultures, donors with low frequencies of FcR $\gamma$ - NK cells also showed increased, but not significant, IFN $\gamma$  production after vaccination in relation to those with high FcR $\gamma$ - NK cells in the periphery (Figure 8B). In response to this data, we propose that individuals with high FcR $\gamma$ - NK cell populations show diminished or delayed Spike responses, these notably being primary responses to a novel antigen. Meanwhile, the FcR $\gamma$ - NK cell populations had insignificant effect on the responses of long-term memory cells (HIV Gag protein) over time. This could be attributed to the overall diminished ability of FcR $\gamma$ - NK cells to perform their helper functions, and from the current ELISpot results we hypothesize that a higher frequency of FcR $\gamma$ - NK cells in the periphery will negatively impact the early adaptive response to vaccination.

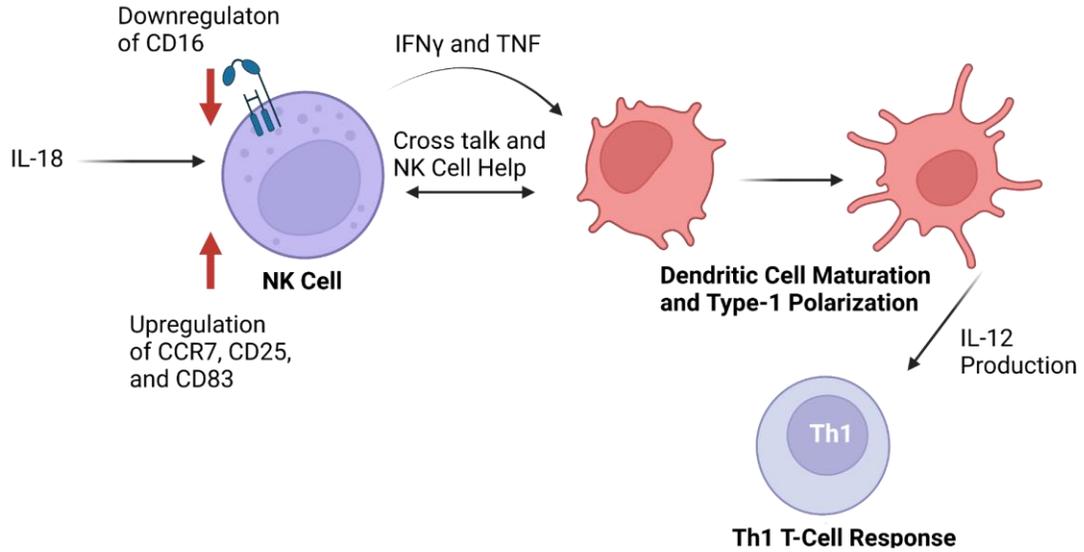
#### **4.1.1 Public Health Relevance**

The importance of NK helper functionality in facilitating an appropriate adaptive response has been further illustrated by this data and understanding the consequences of HIV-mediated dysfunction on the NK cell repertoire is critical for continued research into restoring immune functionality and improving the long-term health outcomes of PLWH. This is due in large part to

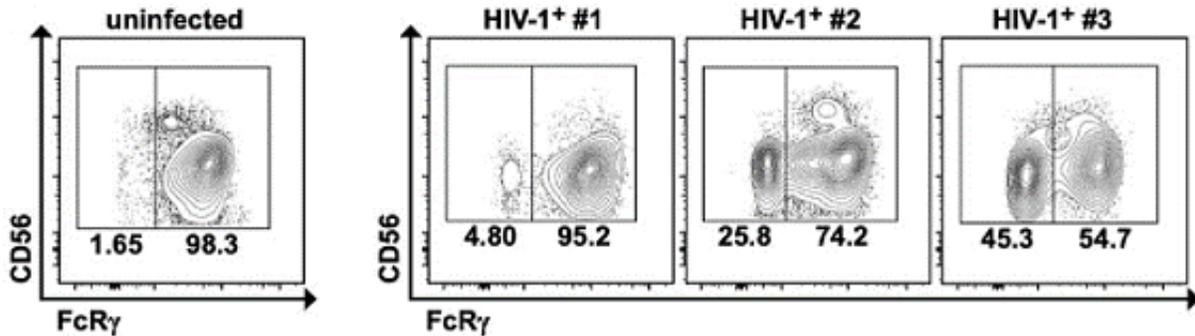
the particularly unique nature of NK cells to mediate both arms of the immune system in addition to their innate antiviral capabilities.

The results on potentially impaired COVID-19 vaccine responses provokes the question of whether these outcomes would be replicated in experiments with other vaccines. In the scope of public health, this also raises questions about boosters or altered vaccination regimens to account for differences in the immune responses of PLWH and the HIV- population. This becomes even more important given the fact that surveillance analysis in the MWCCS indicates that PLWH are twice likely to have a positive test for COVID-19 than HIV seronegative men and women. It has also been established that PLWH are at a higher risk of severe COVID-19 disease and negative outcomes (21). Here, we highlight the need for vaccine and treatment trials focused on PLWH as increased awareness of the dysfunctional and weakened immune state of PLWH not only improves prevention efforts and disease outcomes, but also has broader applications to other groups of immunocompromised individuals.

Supplemental Figures



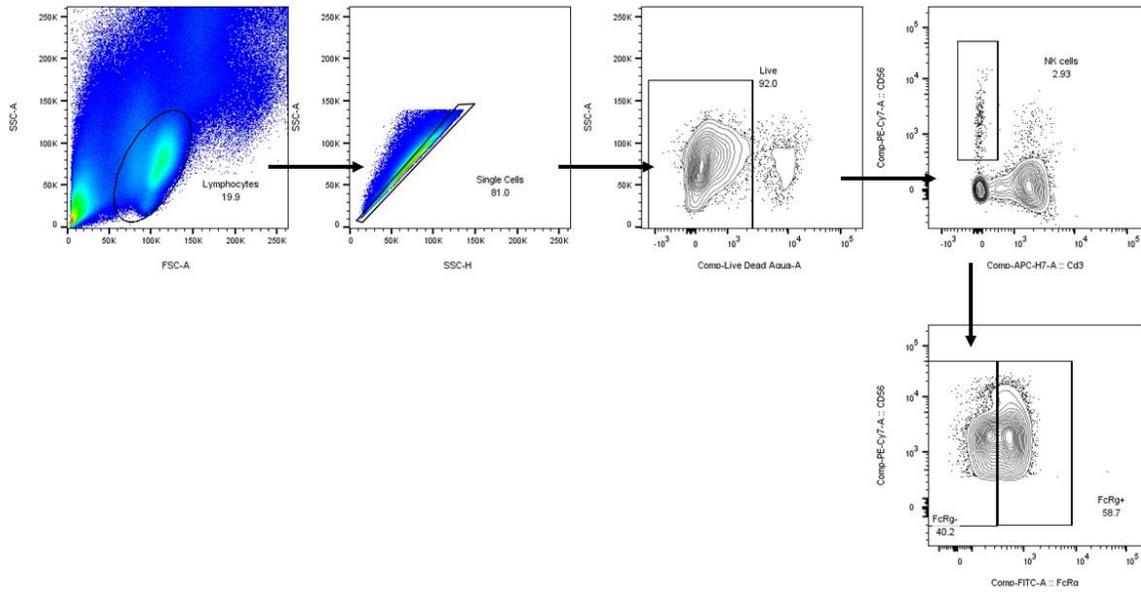
Supplemental Figure 1: NK cells modulate dendritic cell function and educate the adaptive response



Supplemental Figure 2: Identification of the FcR $\gamma$ - NK cell subset in HIV+ MSM

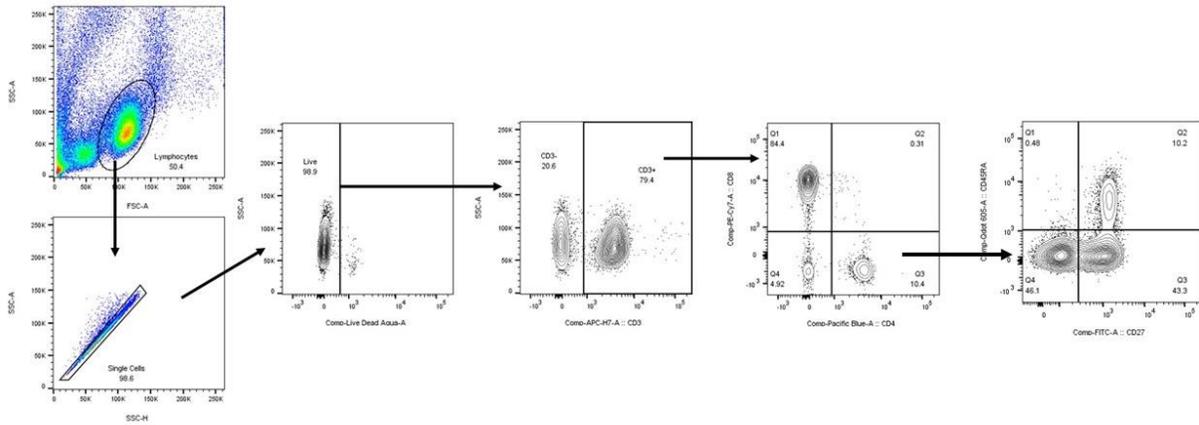
Figure adapted from: *IL-18 Responsiveness Defines Limitations in Immune Help for Specialized FcR $\gamma$ - NK Cells*. Renee R. Anderko, Charles R. Rinaldo and Robbie B. Mailliard. 12, s.l. : The Journal of Immunology,

2020, Vol. 205.



**Supplemental Figure 3: NK cell gating**

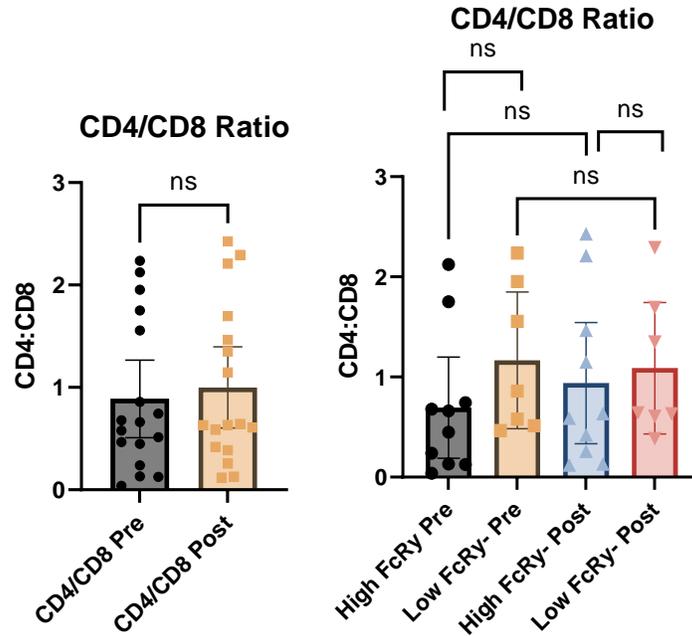
**Initial gates were created based on the FMO. NK cells distinguished by CD3-CD56+.**



**Supplemental Figure 4: CD4+ and CD8+ gating**

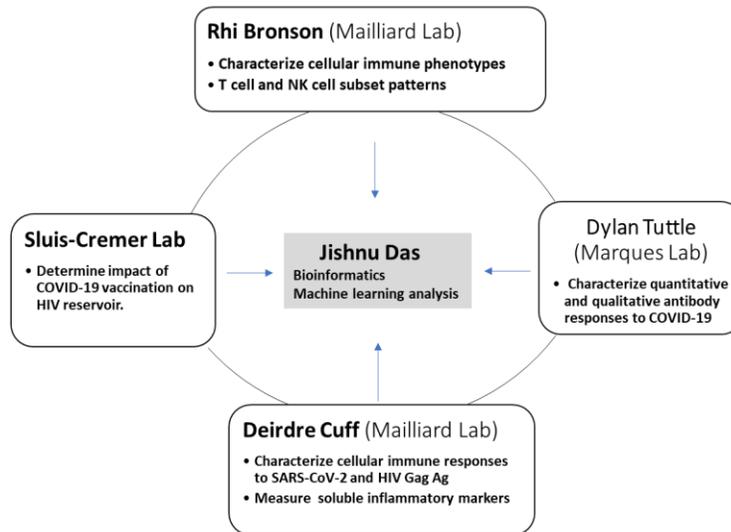
**Initial gates were created based on the FMO. Memory phenotypes distinguished by CD45RA and CD27.**

**Naïve: CD45RA+CD27+. Central: CD45RA-CD27+. Effector: CD45RA-CD27-**

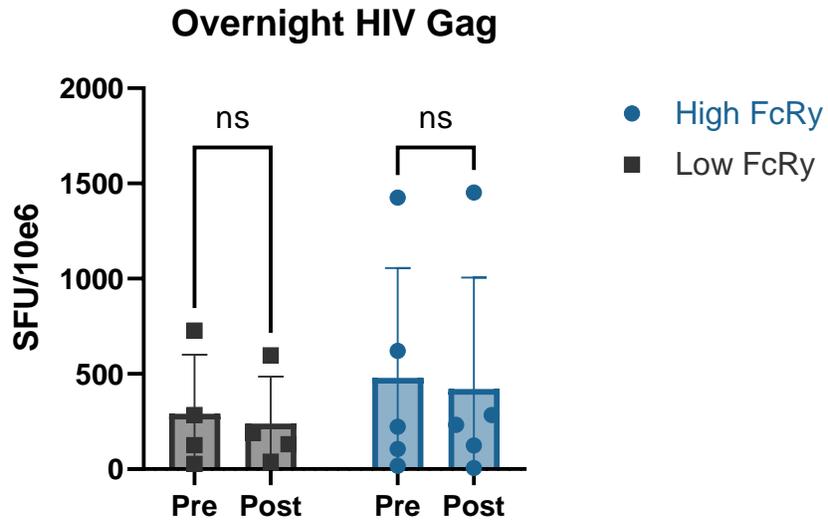


Supplemental Figure 5: CD4:CD8 Ratios

Overall CD4:CD8 ratios pre- and post-vaccination (left). CD4:CD8 ratios pre- and post-vaccination comparing high vs. low frequencies of Fc $\gamma$ R- NK cells (right). Statistical significance measured by paired t-test (left) and Kruskal Wallis test with Dunn's test for multiple comparisons (right).  $\alpha=0.05$



Supplemental Figure 6: MWCCS COVID-19 and HIV project design



Supplemental Figure 7: T-cell responses to HIV Gag

T-cell responses to HIV Gag protein measured by IFN $\gamma$  ELISpot overnight culture. Statistical significance calculated by two-way ANOVA with Sidak's multiple comparisons post hoc test  $\alpha=0.05$ .

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