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Title: TIGIT is upregulated by HIV-1 infection and marks a highly functional adaptive and mature subset of natural killer cells

Running Head: TIGIT function on NK cells in HIV

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Key Words: TIGIT, NK cells, HIV, adaptive, mature

Disclosures/Grant Support: This work was supported by: NIH Ruth L. Kirschstein Institutional National Research Service Award T32AI007502 and TL1TR001084 (EV), NIH/NIAID K08 K08AI138640 (EV), ITI/Bill & Melinda Gates Foundation Pilot Grant (CAB), NIH/NIAID DP2 AI1219301 (CAB), NIH/NIDA Avant Garde Award for HIV Research DP1 DA045089 (CAB), Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Diseases (CAB), Grant # PJT-148529 from the Canadian Institutes of Health Research and by the Réseau SIDA from the Fonds de Recherche du Québec en Santé (MR). CAB is an investigator of the Chan Zuckerberg Biohub. The content of this work is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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ABSTRACT

Objective: Our objective was to investigate the mechanisms that govern natural killer (NK) cell responses to HIV, with a focus on specific receptor-ligand interactions involved in HIV recognition by NK cells.

Design and Methods: We first performed a mass cytometry-based screen of NK cell receptor expression patterns in healthy controls and HIV^+ individuals. We then focused mechanistic studies on the expression and function of T cell immunoreceptor with Ig and ITIM domains (TIGIT).

Results: The mass cytometry screen revealed that TIGIT is upregulated on NK cells of untreated HIV^+ women, but not in antiretroviral-treated women. TIGIT is an inhibitory receptor that is thought to mark exhausted NK cells; however, blocking TIGIT did not improve anti-HIV NK cell responses. In fact, the TIGIT ligands CD112 and CD155 were not upregulated on $CD4^+$ T cells *in vitro* or *in vivo,* providing an explanation for the lack of benefit from TIGIT blockade. TIGIT expression marked a unique subset of NK cells that express significantly higher levels of NK cell activating receptors (DNAM-1, NTB-A, 2B4, CD2) and exhibit a mature/adaptive phenotype ($CD57^{hi}$, $NKG2C^{hi}$, $LILRB1^{hi}$, $FcR\gamma^{lo}$, Syk^{lo}). Furthermore, $TIGIT^+$ NK cells had increased responses to mock-infected and HIV-infected autologous $CD4^+$ T cells, and to PMA/ionomycin, cytokine stimulation and the K562 cancer cell line.

Conclusions: TIGIT expression is increased on NK cells from untreated HIV^+ individuals. Although TIGIT does not participate directly in NK cell recognition of HIV, it marks a population of mature/adaptive NK cells with increased functional responses.

INTRODUCTION

Natural killer (NK) cells are among the first responders to viral infections and can swiftly recognize and kill virus-infected cells [1]. These responses are traditionally thought to be non-specific, as NK cell function is primarily mediated by the expression of germ-line encoded receptors rather than antigen-specific receptors [2]. However, recent evidence has revealed that NK cells are capable of antigen-specific adaptive responses against viruses, such as cytomegalovirus, Epstein-Barr Virus, Varicella Zoster Virus, and influenza virus [1,3–9].

HIV infection profoundly alters the NK cell compartment, with expansion of a $CD56^-CD16^+$ subpopulation [10,11], downregulation of several activating NK cell receptors, including FcRγ (FcεRIγ), NKp30, and NKp46 [12–14], and impairment of NK cell function [15–18]. NK cells also play a critical role in the immune response against HIV [19,20]. NK cells may protect from HIV acquisition, as increased NK cell activity is associated with lower risk of acquiring HIV in highly exposed individuals [21–23]. Consistent with this, genotypes of specific NK cell receptors and human leukocyte antigens are enriched in exposed seronegative individuals [21,24–26]. The expression of specific NK cell receptor/HLA class I ligand pairs (KIR3DL1/KIR3DS1 with HLABw4-80I) is also associated with slower disease progression [27,28] and NK cells from long term non-progressors show increased function compared to HIV^+ typical progressors [12,29]. Altogether, these data raise the possibility that specific NK receptor-ligand interactions may contribute to HIV control and could be used as targets to improve HIV-specific NK cell function.

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T cell immunoreceptor with Ig and ITIM domains (TIGIT) is an inhibitory receptor expressed on T cells and NK cells [30,31]. The TIGIT inhibitory signal is mediated by ligation with its high affinity ligand, the poliovirus receptor (CD155 or PVR), and its low affinity ligand CD112 (Nectin-2 or PVRL2) [30,32,33]. TIGIT has been previously associated with $CD4^+$ T cell, $CD8^+$ T cell and NK cell exhaustion both in the setting of chronic viral infections and malignancy [31,34–40]. Blockade of the TIGIT/CD155/CD112 pathway to improve the function of T cells and NK cells against solid cancers is currently under investigation [41,42].

In HIV-1 infection, TIGIT marks exhausted T cells, correlates with disease progression and is decreased on $CD4^+$ T cells from elite controllers [43,44]. Early initiation of antiretroviral treatment (ART) in HIV infected individuals does not return TIGIT/CD115 to normal levels on $CD8^+$ T cells [37]. Additionally, the co-expression of TIGIT with immune checkpoint inhibitor PD-1 marks $CD4^+$ T cells harboring latent virus [45–47]. These data suggest that the TIGIT/CD155/CD112 pathway in T cells could contribute to HIV pathogenesis.

In NK cells, less is known regarding the role of TIGIT during HIV infection. A recent report showed that TIGIT is increased on NK cells from HIV-1 infected patients and that TIGIT blockade improves NK cell responses to cytokines *ex-vivo* in these patients [48]. Here, we used blood samples from Beninese women to study the effect of HIV infection on the NK cell compartment, with a particular focus on TIGIT expression and function.

METHODS:

Study subjects and sample processing

Cryopreserved peripheral blood mononuclear cells (PBMCs) were obtained from a study of commercial sex workers in Cotonou, Benin. HIV-1-infected women were enrolled from a sex-worker clinic and HIV-1-uninfected women were enrolled from a general health clinic, as described previously [49,50]. PBMCs were obtained from 20 untreated HIV-infected women, 20 HIV-infected women receiving ART and 10 healthy women (Table S1). Written informed consent was obtained from all subjects. The study was approved by the Comité National Provisoire d'Éthique de la Recherche en Santé in Cotonou and the Centre Hospitalier de l'Université de Montréal (CHUM) Research Ethics Committees.

For mechanistic *in vitro* studies using healthy donors, leukoreduction system (LRS) chambers were purchased from the Stanford Blood Bank. PBMCs were purified using Ficoll density gradient centrifugation and cryopreserved in fetal bovine serum (FBS) with 10% DMSO.

Cell isolation

For profiling PBMCs from Beninese women, $1x10^6$ PBMCs were stained for mass cytometry with the ligand antibody panel (Table S2). NK cells were purified from PBMCs by magnetic-activated isolation via negative selection (Miltenyi) and stained with the NK cell antibody panel (Table S3).

For the *in vitro* co-cultures, cryopreserved PBMCs from healthy donors were used to purify $CD4^+$ T cells and NK cells by magnetic-activated isolation via negative selection (Miltenyi).

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Antibody conjugation, mass cytometry staining and data acquisition

Antibodies were conjugated using MaxPar® ×8 labeling kits (Fluidigm). To ensure antibody stability over time, antibody panels were lyophilized into single-use pellets prior to use (Biolyph). Cells were stained for mass cytometry as described previously [51,52] and resuspended in 1× EQ Beads (Fluidigm) before acquisition on a Helios mass cytometer (Fluidigm).

***In vitro* HIV infection**

Replication competent (Q23-FL) HIV-1 was made by transfection and titrated as described [51]. $CD4^+$ T cells were activated for 2 days with plate-bound anti-CD3 (clone OKT3, eBioscience, 10 µg/ml), soluble anti-CD28/CD49d (clones L293/L25, BD Biosciences, 1 µg/ml each) and phytohemagglutinin (eBioscience, 2.5 µg/ml). $CD4^+$ T cells were infected overnight with Q23-FL via ViroMag R/L magnetofection (Oz Biosciences) at a multiplicity of infection of 20. HIV infection was as measured by intracellular p24.

TIGIT blockade assay

Cells were cultured in RPMI media containing 10% FBS (Thermo Fisher Scientific) and 1% penicillin/streptomycin/amphotericin (Thermo Fisher Scientific) (RP10). NK cells were incubated in 24-well plates at a concentration of $2x10^6$ cells/ml in for 3 days at 37°C with 5% CO2, with addition of rhIL-2 (R&D Systems, 300 IU/ml). After 3 days, NK cells were washed with fresh media and plated in 96-well plates (80,000-100,000 cells/well). Blocking mIgG1κ anti-hTIGIT antibody (eBioscience, clone MBSA43, 10 µg/ml) or a mIgG1κ isotype control (eBioscience, clone P3.6.2.8.1, 10 µg/ml) were added. Cells were incubated at 4°C for 20 minutes, washed with FACS wash buffer (PBS + 2% FBS) and resuspended in media.

CD112 and CD155 mRNA expression levels

RNA from resting, mock-infected or HIV-infected $CD4^+$ T cells was isolated using the RNeasy Mini Kit with QIAshredder columns (Qiagen). cDNA was produced using the SuperScript III First Strand Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR (qPCR) was achieved with Taqman Universal Master Mix (Applied Biosystems) with the following commercially bought Taqman Assays, using fluorescein amidite-minor groove binder (FAM-MGB) probes: Nectin-2/CD112 (Thermo Fisher Scientific), PVR/CD155 (Thermo Fisher Scientific), and Human GAPDH endogenous control (Applied Biosystems). qPCR was run on the Applied Biosystems Step One Plus Real Time PCR System. Transcription levels were normalized to GAPDH. mRNA fold change expression was calculated using the double delta Ct analysis method with resting $CD4^+$ T cells as the control group [53].

NK cell:$CD4^+$ T cell co-cultures, cytokine stimulation and determination of NK cell function

All cells were cultured in RP10. NK cells (80,000-100,000/well) were incubated with HIV-infected or mock-infected autologous $CD4^+$ T cells (320,000/well at a 1:4 effector:target ratio), 0.4 µl/200µl of cell stimulation cocktail (phorbol 12-myristate 13-acetate (PMA) and ionomycin, eBioscience), a cocktail of IL-12, IL-15 and IL-18 (rhIL-12, R&D Systems, 5ng/ml; rhIL-15, Pepro-Tech, 20ng/ml; rhIL-18, R&D Systems, 0.5 µg/ml), or with the cancer cell line K562

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(ATCC, used at passage 2-10, 300,000 cells/well at a 1:3 effector:target ratio). Cells were incubated for 4 hours at 37°C with 5% CO2, in the presence of brefeldin, monensin (eBioscience) and anti-CD107a-APC-H7 antibody (BD Biosciences, Clone H4A3). After incubation, cells were stained with LIVE/DEAD™ Fixable Yellow Staining Kit (Thermo Fisher Scientific) for 20 minutes at room temperature, then fixed (BD FACS Lyse), permeabilized (BD FACS Perm II) and stained with anti-IFN-γ-V450 (BD Biosciences, Clone B27), anti-TIGIT-APC (eBioscience, Clone MBSA43), anti-CD3-PerCP-Cy5.5 (Biolegend, Clone UCHT1), anti-HIVp24-FITC (Beckman Coulter, Clone KC57) for 30 minutes at 4°C. Data was acquired on a MACSQuant® Analyzer Flow Cytometer (Miltenyi).

Data analysis

Bead normalization was performed before downstream analyses (https://github.com/nolanlab/bead-normalization) [54] . To ascertain the quality of the stains, data were visualized using FlowJo v10.5.3. FAS-L, Ki-67 and CXCR6 were excluded from subsequent analyses due to poor staining. The original data were uploaded in ImmPort (www.immport.org). Serial negative gating was performed to identify NK cells from any contaminating cells remaining after purification (Figure S1a). Examples of stains for NK markers are shown in Figure S2a. Samples with cell number <1000 were excluded from analyses; the number of subjects used for each analysis is specified in the figure legend. The open source statistical package R (https://www.r-project.org/) was used for all statistical analyses [55]. Normalized signal intensities were transformed using the asinh function with a cofactor of 5 prior to generalized linear model (GLM) analysis, multidimensional scaling (MDS) and Uniform Manifold Approximation and Projection (UMAP) visualization. For the GLM analysis, we used the custom-made package “CytoGLMM” [56,57]. This package implements a generalized linear model with bootstrap resampling to identify markers predictive of clinical treatment groups. The “CytoGLMM” package was also used to create a MDS projection. For comparison between groups, we used the Wilcoxon rank-sum test. When appropriate, p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. UMAP embeddings were calculated using the R package “uwot”, with n_neighbor = 5 and min_dist = 0.2, as described [58]. Gated NK cells from all samples were pooled into treatment groups, to facilitate visualization. Embeddings were visualized in Cytobank (www.cytobank.org).

RESULTS

TIGIT is upregulated on NK cells from untreated HIV-infected women

To investigate the effect of HIV-1 infection on NK cells, we used CyTOF to profile the NK cells of twenty untreated HIV-1-infected women, twenty HIV-1 infected women on ART and ten healthy women from the same geographical area (Figure 1a). To analyze this multidimensional dataset, we first used a custom-made generalized linear model with bootstrap resampling (Figure 1b). This model confirmed that HIV-1 infection is associated with a profound alteration of the NK cell phenotype, with higher levels of CD38 and lower levels of Siglec-7 predicting chronic HIV-1 infection as previously described [59,60]. This analysis also confirmed a recent finding that checkpoint inhibitor TIGIT is upregulated in untreated HIV-1-infected individuals [48].

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Additionally, our data revealed that TIGIT is not elevated on NK cells from antiretroviral-treated HIV-1-infected women (Figure 1b and 1c).

TIGIT expression is increased in CD56^{dim} and CD56^{-} NK cells from untreated HIV-1^{+} women

To further explore the nature of this increased TIGIT expression during HIV-infection, we evaluated TIGIT expression within different NK cell subpopulations. NK cells are classically divided into three distinct subpopulations: CD56^{bright}, which secrete cytokines at high levels, CD56^{dim}, a more cytotoxic subpopulation, and CD56^{-}, a subpopulation of NK cells that are thought to be functionally impaired and are expanded during chronic viral infections, including HIV [10,61–63]. Here we confirmed that CD56^{-} NK cells are increased in HIV-infected individuals, regardless of treatment status (Figure 2a and 2b). In addition, we found that TIGIT expression is higher on CD56^{-} NK cells from untreated HIV-1 infected women compared to healthy controls, and that TIGIT expression is increased in the CD56^{dim} subpopulation in untreated HIV-1^{+} women compared to treated women (Figure 2c).

TIGIT blockade does not alter HIV-specific NK cell responses

TIGIT blockade increases *ex vivo* NK cell responses to cytokines in HIV-1 infected women [48] and elicits improved anti-tumor responses in mouse models [35]. To explore the effect of TIGIT blockade on NK cell responses against HIV *in vitro*, we treated NK cells from healthy donors with an anti-TIGIT monoclonal antibody and measured NK cell responses (CD107a and IFN-γ) by flow cytometry after incubation with mock-infected or HIV-infected autologous CD4^{+} T cells (Figure 3a). Blockade of TIGIT did not improve NK cell responses to mock-infected or HIV-infected CD4^{+} T cells, when compared to untreated NK cells or cells treated with an isotype control (Figures 3b and 3c).

TIGIT ligands are not upregulated by HIV infection

The TIGIT inhibitory signal is mediated by ligation with CD155 (high affinity) or CD112 (low affinity) [30,32]. To investigate the role of the TIGIT-CD155 and TIGIT-CD112 pathways in the setting of HIV infection, we measured the expression of CD155 and CD112 on HIV-infected CD4^{+} T cells *ex vivo* and *in vitro*. CD155 and CD112 were both expressed at low levels on CD4^{+} T cells from HIV^{+} women and there was no difference in expression between healthy women, untreated HIV^{+} women and treated HIV^{+} women (Figures 3d and S2b). CD155 and CD112 were not differentially expressed in HIV-infected or mock-infected CD4^{+} T cells *in vitro* when measured by CyTOF (Figures 3e and S2c). Additionally, CD112 and CD115 transcription levels did not differ between mock-infected and HIV-infected CD4^{+} T cells (Figure 3f) with a median fold change of HIV relative to mock-infected CD4+ T cells 1.15 (interquartile range = 0.94-2.4) for CD155 and 1.07 (interquartile range = 0.93-3.13) for CD112.

TIGIT expression is increased in adaptive/mature NK cells

To characterize NK cells with increased TIGIT expression, we gated on TIGIT and compared TIGIT^{+} and TIGIT^{-} NK cells from our Beninese women (Figure 4a). Using MDS, we found that TIGIT expression marks a distinct population of NK cells that was not explained by HIV infection or treatment status (Figure 4b-d). We then focused our analysis on features distinguishing

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TIGIT$^+$ and TIGIT$^-$ NK cells. We found that TIGIT$^+$ NK cells express higher levels of activating markers (DNAM-1, NTB-A, 2B4, CD2) and exhibit an adaptive/mature phenotype (CD57^{hi}, NKG2C^{hi}, LILRB1 (ILT2, CD85j)^{hi}, FcRγ^{lo}, Syk^{lo}) (Figures 4e and 4f). To follow-up on this finding, we used UMAP to visualize NK cell subsets and found that adaptive/mature NK cells (CD57^{hi}, NKG2C^{hi}, LILRB1^{hi}, NKG2A^{lo}, NKp46^{lo}, NKp30^{lo}, FcRγ^{lo}, Syk^{lo}) were separated on the right side of the UMAP1 axis (Figure 4g). Additionally, when we colored the UMAP plot by TIGIT expression, we found that TIGIT expression co-localized with markers of adaptive/mature NK cells (Figure 4h).

TIGIT expression is associated with increased NK cell responses

As mature NK cells are typically associated with higher functional activity, we sought to better characterize the function of TIGIT$^+$ and TIGIT$^-$ NK cells. We isolated NK cells from healthy donors and cultured them with mock-infected or HIV infected autologous CD4$^+$ T cells (Figure 5a). We then measured the functional responses (CD107a and IFN-γ) of TIGIT$^+$ and TIGIT$^-$ NK cells by flow cytometry (Figures 5b-e). We found that TIGIT$^+$ NK cells had increased responses to mock-infected and HIV-infected autologous CD4$^+$ T cells (Figures 5b and 5c). To better define whether this difference was specific to HIV, we also measured the responses to PMA/ionomycin, a cocktail of IL-12, IL-15 and IL-18, and the leukemia cell line K562 (Figures 5a, 5d and 5e). TIGIT$^+$ NK cells also exhibited increased responses to PMA/ionomycin, cytokine and tumor cells, compared to TIGIT$^-$ NK cells (Figures 5d and 5e).

DISCUSSION

NK cells undergo significant changes during chronic HIV infection [16,64], with expansion of a hypofunctional CD56$^-$CD16$^+$ NK cell subpopulation [10,11], downregulation of several activating NK cell receptors [12–14], and adaptive reconfiguration [65]. Some of these changes are not observed after treatment or in the setting of natural HIV control [66], indicating that diminished NK cell function could contribute to disease pathogenesis. To better understand the role of different NK cell receptors in the pathogenesis of HIV, we used CyTOF, a technique that allows the detection of over 40 receptors simultaneously, to profile the NK cells from infected and uninfected HIV$^+$ women. A particularly striking finding was that TIGIT is upregulated in untreated HIV infection, yet its expression is normal in the setting of ART. TIGIT upregulation on NK cells from HIV-infected male patients has been recently described [48]. Here, we demonstrate that TIGIT is upregulated in HIV-infected women and that this upregulation does not occur in women receiving ART. Further, TIGIT upregulation is more prominent on CD56$^-$/CD16$^+$ NK cells, a subpopulation thought to be functionally exhausted [10,11].

TIGIT is an inhibitory receptor that has been associated with hypofunctional/exhausted NK cells in cancer patients and mouse models of solid tumors [31,35] and with exhausted CD8$^+$ T cells in hematologic malignancy and in HIV infection [36,39,43]. As a result, several studies have explored the use of TIGIT blockade to rescue T and NK cell function. Blocking TIGIT is associated with improved anti-tumoral response in tumor-bearing mice and in patients with colorectal cancer [35], and with improved NK cell responses to cytokine stimulation in HIV$^+$ patients [48]. However, we found that TIGIT blockade does not improve HIV-specific antiviral

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function in healthy donors. This discrepancy could be attributed by the lower expression of TIGIT by healthy individuals, as high expression levels are likely required to see the results of blockade.

The lack of a benefit for TIGIT blockade may also be explained by the fact that TIGIT is simply not engaged during NK cell recognition of HIV-infected cells. We found that TIGIT ligands CD155 and CD112 are not upregulated on $CD4^+$ T cells by HIV infection in *vitro*, consistent with previous reports [67–69]. We also found that TIGIT ligands were not upregulated in HIV^+ women, though this differs from the findings of Yin et al. [48], who reported elevated CD155 expression on $CD4^+$ T cells from HIV-infected individuals. Notably, Yin et al. found that CD155 levels were very low and that blocking CD155 did not improve NK cell responses. Thus, together the data suggest that the TIGIT/CD155/CD112 pathway may not be directly involved in NK cell responses to HIV-infected cells.

While we did not find that TIGIT itself plays a key role in immune response to HIV, we noted that TIGIT marks a distinct NK subpopulation and is coexpressed with several activation markers (DNAM-1, NTB-A, 2B4, CD2). We also show that in our cohort of HIV-infected women, $TIGIT^+$ NK cells exhibit a more adaptive/mature phenotype ($CD57^{hi}$, $NKG2C^{hi}$, $LILRB1^{hi}$, $FcR\gamma^{lo}$, Syk^{lo}) and adaptive NK cells demonstrated higher TIGIT expression in unsupervised clustering via UMAP. This is consistent with the notion that NK cells undergo an adaptive reconfiguration during HIV infection [65]. Notably, Sarhan et al. demonstrated that TIGIT was expressed at lower levels in adaptive NK cells from healthy donors [70]. Our finding that TIGIT is expressed more highly on adaptive NK cells could reflect unique differentiation pathways driven by HIV infection. Alternatively, the differences in TIGIT expression between our studies may be due to differential downregulation of TIGIT in response to cytokine stimulation, given that Sarhan et al. evaluated cytokine-treated NK cells rather than PBMCs directly *ex vivo.*

Adaptive NK cells show increased responses to FcγRIIIa triggering [71–73], are expanded in HIV-infected patients and robustly respond to HIV peptides [14]. Here we demonstrate that $TIGIT^+$ NK cells have higher functional activity against mock-infected and HIV-infected $CD4^+$ T cells, compared to $TIGIT^-$ NK cells. Further, higher functional responses also occur in the presence of non-HIV stimuli (PMA/ionomycin, cytokines and K562). This increased activity could be explained by the co-expression of activating receptors, which may overwhelm the inhibitory signal provided by TIGIT, or by changes in the activation threshold during adaptive reconfiguration. Alternatively, a recent report showed that TIGIT could contribute to NK cell licensing [74]. In this paradigm, the presence of TIGIT may be required during NK cell maturation to ensure the acquisition of optimal effector function.

Some limitations should be noted in our study. First, the limited sample availability from a well curated study of untreated and treated HIV^+ women only allowed us to profile NK cells from a small sample size of only female subjects. To overcome this, functional experiments were done on both female and male subjects. Additionally, the functional experiments were carried on healthy, HIV-negative subjects. This is particularly important for the blocking assays as viremic HIV^+ individuals have higher TIGIT expression and thus may be more responsive to TIGIT

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blockade. Unfortunately, we did not have sufficient samples from viremic HIV^+ patients to perform functional studies. Finally, our CyTOF panel did not exhaustively cover all known markers of adaptive NK cells and expression of PLZF, DAB2 and EAT2 was not assessed. CXCR6 was included in our original panel, but was excluded from analyses due to poor antibody staining.

In summary, our work demonstrates that TIGIT is overexpressed on NK cells of untreated HIV^+ women, but fully corrected by ART. We find that TIGIT does not directly participate in the NK cell response to HIV-infected cells, but rather that TIGIT marks a mature NK cell subpopulation with adaptive features that is enhanced in functional responses to virus-infected cells, tumors, and cytokine stimulation. These results need to be confirmed in independent cohorts that evaluate both the direct functional contributions of TIGIT and the role of ART in regulating TIGIT expression.

ACKNOWLEDGEMENTS

We are grateful to the Beninese study participants. We are indebted to N. Geraldo, A. Gabin, C. Assogba and C. Agossa-Gbenafa for their clinical expertise, to M. Massinga-Loembe, G. Ahotin, L.Djossou, and E. Goma for their technical assistance and to G. Batona and other field workers who helped with recruitment of commercial sex workers.

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FIGURES

Figure 1: TIGIT is upregulated on NK cells from untreated HIV-infected women. (A) Schematic of study design and experiment. **(B)** A generalized linear model with bootstrap resampling was used to identify markers predictive of the study groups. Log-odds are logarithm of ratios of the probability that a cell belongs to each study subject. An increase in the parameter coefficient corresponds to the strength of the classification power, with the 95% confidence interval represented by line surrounding the point estimate. **(C)** Mean Signal Intensity of TIGIT on NK cells from healthy women (n=10), untreated HIV^+ women (n=20), treated HIV^+ women (n=17). * = unadjusted p-value≤0.05, ** = unadjusted p-value≤0.01, ns = non-significant.

Figure 2. TIGIT expression is increased in $CD56^{dim}$ and $CD56^-$ NK cells of untreated $HIV\text{-}1^+$ women. (A) Representative CyTOF plots of gating of NK cells based on CD56 expression. **(B)** Frequency of $CD56^{bright}$, $CD56^{dim}$ and $CD56^-$ NK cells in healthy women (n=10), untreated HIV^+ women (n=20), treated HIV^+ women n=17). **(C)** Mean Signal Intensity of TIGIT on NK cell subsets from healthy women (n=10), untreated HIV^+ women (n=20), treated HIV^+ women (n=17). * = adjusted p-value≤0.05, ** = adjusted p-value≤0.01, ns = non-significant.

Figure 3. TIGIT blockade does not alter HIV-specific NK cell responses to HIV and TIGIT ligands are not upregulated by during HIV infection. (A) Schematic of TIGIT blockade assay and NK cell:$CD4^+$ T cell co-cultures. **(B)** Representative flow cytometry plots of CD107a and IFN-γ production, expressed as frequency of positive cells, by NK cells alone (NK) or after co-culture with mock-infected (mock) or HIV-infected (HIV) autologous $CD4^+$ T cells. Cells were incubated alone (NK), pre-treated with isotype control (Isotype) or a blocking anti-TIGIT

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antibody (αTIGIT). **(C)** Summary plot of frequency of $CD107a^+$ and $IFN\text{-}\gamma^+$ NK cells after 4 hour co-culture with mock-infected or HIV-infected autologous $CD4^+$ T cells. **(D)** Mean Signal Intensity of CD112 and CD155 expression measured by mass cytometry in healthy women (n=9), untreated HIV^+ women (n=20), treated HIV^+ women (n=17). **(E)** Mean Signal Intensity of CD112 and CD155 expression measured by mass cytometry in mock-infected and HIV-infected $CD4^+$ T cells. **(F)** Fold change in the *NECTIN2* (CD112) and *PVR* (CD155) transcript levels, normalized to *GAPDH*, in mock-infected and HIV-infected $CD4^+$ T cells, relative to resting $CD4^+$ T cells. ns = non-sigificant.

Figure 4. TIGIT expression is increased in adaptive/mature NK cells. (A) Representative CyTOF plots of gating for $TIGIT^+$ and $TIGIT^-$ NK cells from healthy women (n=9), untreated HIV^+ women (n=17), treated HIV^+ women (n=16). **(B)** Multidimensional Scaling (MDS) of $TIGIT^+$ and $TIGIT^-$ NK cells colored by TIGIT expression. **(C)** MDS of $TIGIT^+$ and $TIGIT^-$ NK cells colored by group (Healthy, Untreated HIV and Treated HIV). **(D)** Vectors driving the variance of the MDS. **(E)** Mean Signal Intensity of DNAM-1, NTB-A, 2B4, FcRγ, Syk and LILRB1 expression in $TIGIT^+$ and $TIGIT^-$ NK cells from healthy women, untreated HIV^+ women and treated HIV^+ women. **(F)** Frequency of $CD2^+$, $CD57^+$ and $NKG2C^+$ NK cells from $TIGIT^+$ and $TIGIT^-$ NK cells from healthy women, untreated HIV^+ women and treated HIV^+ women. **(G)** UMAP plot of pooled NK cells from healthy women, untreated HIV^+ women and treated HIV^+ women colored by markers of adaptive/mature NK cells (NKG2C, CD57, LILRB1, NKG2A, NKp46, NKp30, Syk, FcRγ) . **(H)** UMAP plot of pooled NK cells from healthy women, untreated HIV^+ women and treated HIV^+ women, colored by TIGIT expression. * = adjusted p-value≤0.05, ** = adjusted p-value≤0.01, *** = adjusted p-value≤0.001.

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Figure 5. TIGIT expression is associated with increased NK cell responses. (A) Schematic of $CD4^+$ T cell:NK cell co-cultures or stimulation and representative flow cytometry plot of gating of $TIGIT^+$ and $TIGIT^-$ NK cells. **(B)** Representative flow cytometry plots of CD107a and IFN-γ production, expressed as frequency of positive cells, by $TIGIT^+$ and $TIGIT^-$ NK cells after 4 hour co-culture with mock-infected (mock) or HIV-infected (HIV) autologous $CD4^+$ T cells. **(C)** Summary plot of the frequency of TIGIT-gated $CD107a^+$ and IFN-γ^+ NK cells after 4 hour co-culture with mock-infected or HIV-infected autologous $CD4^+$ T cells. **(D)** Representative flow cytometry plots of CD107a and IFN-γ production by $TIGIT^+$ and $TIGIT^-$ NK cells after 4 hour stimulation with PMA/ionomycin (PMA/i), a cocktail of IL12, IL-15 and IL-18 (IL12-15-18) or the leukemia cell line K562 (K562). **(E)** Summary plot of the frequency of TIGIT-gated $CD107a^+$ and IFN-γ^+ NK cells after 4 hour stimulation with PMA/ionomycin (PMA/i), a cocktail of IL12, IL-15 and IL-18 (IL12-15-18) or the leukemia cell line K562 (K562). * = adjusted p-value≤0.05, ns = non-sigificant.

Figure S1. Gating Schemes. (A) Gating scheme used for negative gating of NK cells by CyTOF. NK cells were already purified with magnetic bead isolation prior to staining. Negative gating was performed to ensure further NK cell purity for downstream analyses. **(B)** Gating scheme used for analysis of $CD4^+$ T cells by CyTOF. **(C)** Gating scheme used for analysis of NK cells and $CD4^+$ T cells by flow cytometry.

Figure S2. Gating strategy. (A) Representative CyTOF plots of the expression of NK cell markers from one of the treated HIV^+ individuals. **(B)** Representative CyTOF plot of $CD4^+$ T cell expression of CD112 and CD155 from one healthy woman, one untreated HIV^+ woman and one treated HIV^+ woman. **(C)** Representative CyTOF plots of CD112 and CD155 expression on mock-infected or HIV-infected $CD4^+$ T cells. **(D)** Representative flow cytometry plot of p24

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expression in mock-infected and HIV-infected $CD4^+$ T cells. The median infection level for the blocking assay was 31.4% (range: 14.5-55.5%). The median infection level for the $TIGIT^+$ vs $TIGIT^-$ comparison was 38.0% (range: 14.6%-70.5%).

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SUPPLEMENTAL TABLES

Table S1: Study group demographics

	Healthy Women n=10	Treated HIV^+ Women n=20	Untreated HIV^+ Women n=20	p-value
Age, Mean (SD), Years	34 (7)	38 (7)	40 (9)	ns*
Years of sex work at recruitment visit, Mean (SD)	n/a	3.2 (1.5)	3.6 (1.4)	ns*
Use of oral contraceptives, Number of subjects	0	1	0	ns*
Vaginal douching, Number of Subjects	10	20	20	ns*
Number of clients in last 7 days, Mean (SD)	n/a	17 (17)	22 (16)	ns*
Condom always used with client in the last 7 days, Number of subjects	n/a	14	13	ns*

n/a = non applicable; n = number of subjects, SD = standard deviation, ns = non-significant, * = p-values for comparison between groups were calculated with the Kruskal-Wallis test for age, the Wilcoxon signed-rank test for years of sex work and number of clients and with Fisher's exact test for use of oral contraceptives, vaginal douching and condom use.

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Table S2: Ligand Panel

Isotope	Ligand Panel	Source	Clone
89Y	HLA-DR	Biolegend	L243
Qdot	CD19	Life technologies	SJ25-C1
115In	CD3	Biolegend	UCHT1
139La	CD7	Biolegend	6B7
141Pr	CRACC	Biolegend	162.1
142Nd	CD304 (BDCA-4)	Biolegend	I2C2
143Nd	PAN HLA	Biolegend	W6/32
144Nd	HLA-C	Millipore	DT9
145Nd	CD8	Biolegend	SK1
146Nd	CD48	Biolegend	BJ40
147Sm	CD303 (BDCA-2)	Fluidigm	201A
148Nd	ICAM1 (CD54)	Biolegend	HA58
149Sm	HLA-G	Biolegend	87G
150Nd	p24	Abcam	ab9071
151Eu	CD4	Biolegend	OKT4
152Sm	CD123	Biolegend	6H6
153Eu	CXCL13	R&D Systems	53610
154Sm	CCR2	Biolegend	K036C2
155Gd	HLA-E	Biolegend	3D12
156Gd	CD95	Biolegend	DX2
157Gd	Nectin-1 (CD111)	Biolegend	R1.302
158Gd	MICA	R&D	159227
159Tb	DR4 (CD261)	Biolegend	DJR1
159Tb	DR5 (CD262)	Biolegend	DJR2-2
160Gd	CD1c	Biolegend	L161
161Dy	ULBP-1	R&D Systems	170818
162Dy	CD11c	Fluidigm	Bu15
163Dy	ULBP-2-5-6	R&D Systems	165903
164Dy	Nectin-2 (CD112)	Biolegend	TX31
165Ho	PVR (CD155)	Biolegend	SKII.4
166Er	HLA-Bw4	Miltenyi	REA274
167Er	NKp80	Biolegend	5D12
168Er	HLA-Bw6	Miltenyi	REA143
169Tm	CD14	Biolegend	M5E5
170Er	CD11b	Biolegend	ICRF44
171Yb	LFA-3 (CD58)	Biolegend	TS2/9
172Tb	CD33	Biolegend	WM53
173Yb	CD141	Fluidigm	1A4
174Yb	CD56	BD Pharmingen	NCAM16.2
175Lu	CD64	Biolegend	10.1
176Yb	MICB	R&D Systems	236511
209Bi	CD16	Fluidigm	3G8

Table S3: NK Panel

Isotope	NK Panel	Source	Clone
89Y	CD57	Biolegend	HCD57

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Qdot	HLA-DR	Life technologies	Tu36
115In	CD3	Biolegend	UCHT
141Pr	CD38	Biolegend	HIT2
142Nd	CD69	Biolegend	FN50
143Nd	CD33	Biolegend	WM53
143Nd	CD14	Biolegend	M5E5
144Nd	CD2	Biolegend	RPA-2.10
145Nd	LILRB1	Biolegend	GHI/75
146Nd	CD19	Biolegend	HIB19
147Sm	CD8	Biolegend	SK1
148Nd	FcRg	Millipore	Polyclonal
149Sm	CD4	Biolegend	SK3
150Nd	Syk	Biolegend	4D10.2
151Eu	CD62L	Biolegend	DREG-56
152Sm	Ki-67	Biolegend	Ki-67
153Eu	KIR2DS4	R&D Systems	179315
154Sm	KIR2DS2	Abcam	Poly-clonal
155Gd	NKp46	Biolegend	9E2
156Gd	NKG2D	Biolegend	1D11
157Gd	TIGIT	R&D systems	741182
158Gd	2B4	Biolegend	C1.7
159Tb	DNAM-1	BD biosciences	DX11
160Gd	FAS-L	Biolegend	NOK-1
161Dy	NKp30	Biolegend	P30-15
162Dy	Siglec-7	Biolegend	S7.7
163Dy	NKG2C	R&D Systems	134522
164Dy	NKp44	Biolegend	P44-8
165Ho	CD96	Biolegend	NK92.39
166Er	KIR2DL1	R&D Systems	143211
167Er	CD94	Biolegend	DX22
168Er	CXCR6	Biolegend	K041E5
169Tm	PD1	Biolegend	EH12.2H7
170Er	KIR2DL5	Miltenyi	UP-R1
171Yb	NKG2A	R&D Systems	131411
172Tb	NTB-A	Biolegend	NT-7
173Yb	KIR3DL1	BD biosciences	DX-9
174Yb	CD56	BD biosciences	NCAM16.2
175Lu	KIR2DL3	R&D systems	180701
176Yb	Perforin	Abcam	B-D48
209Bi	CD16	Fluidigm	3G8

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FIGURE 1

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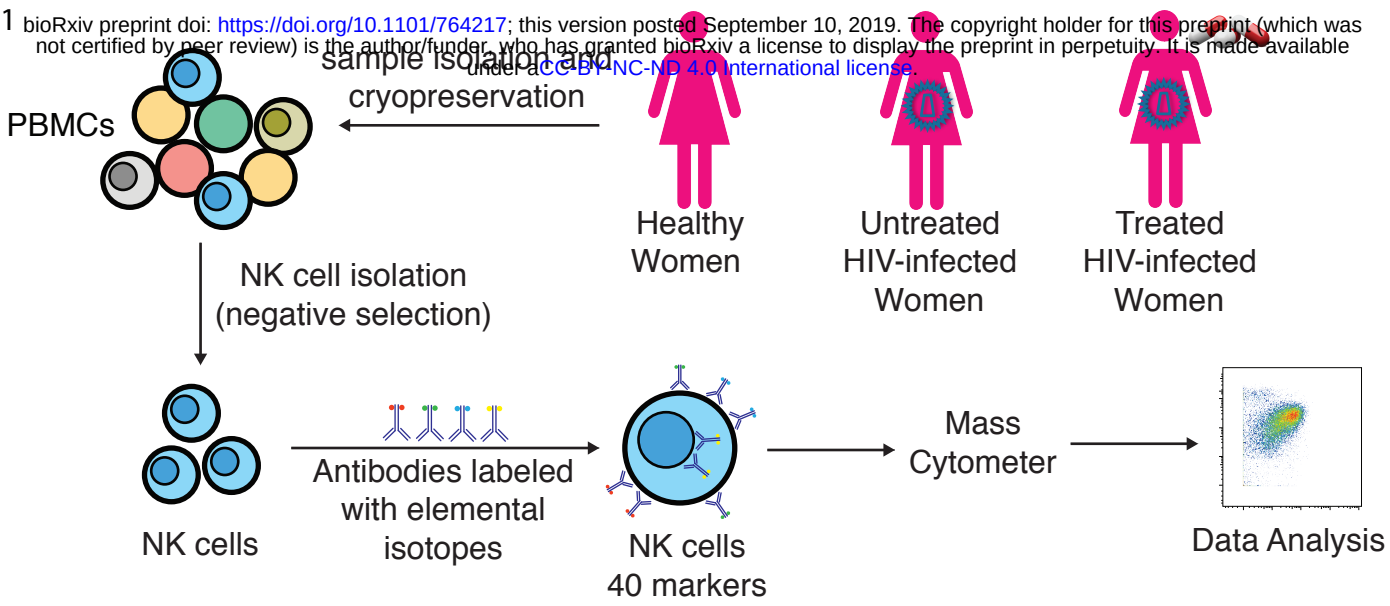

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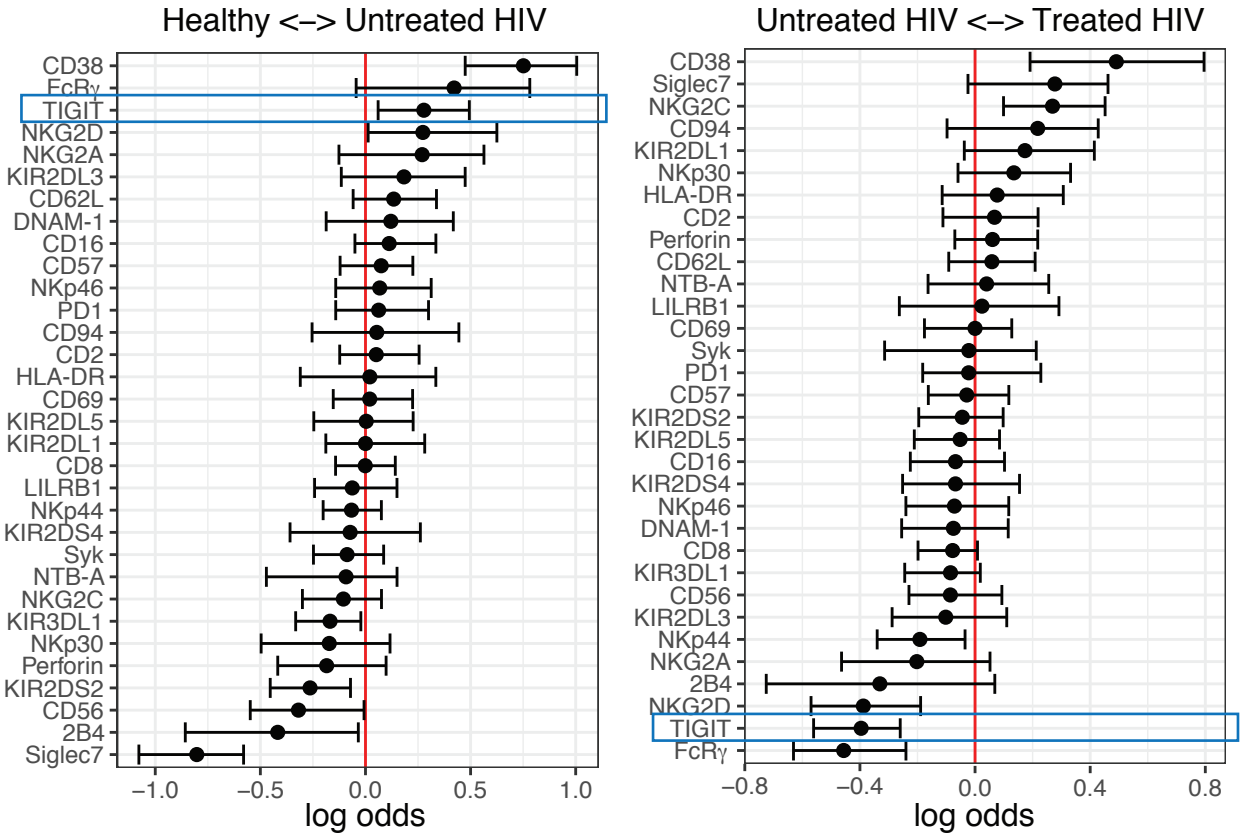

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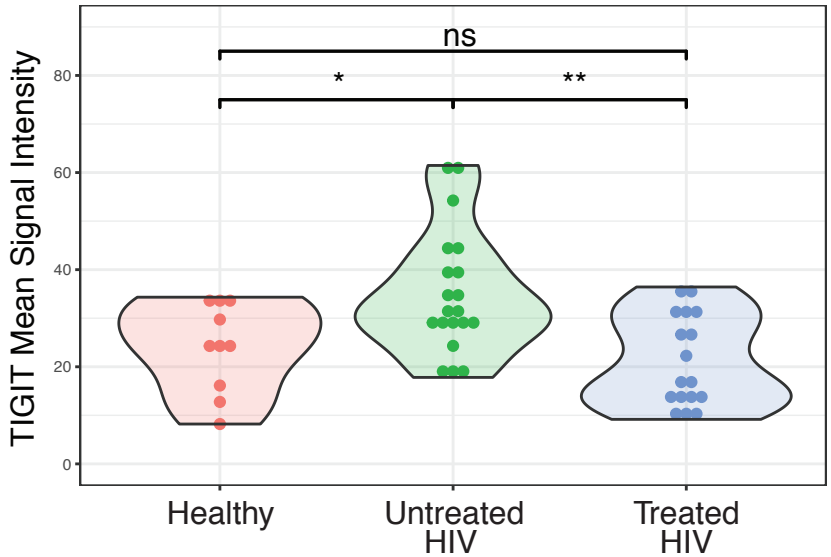

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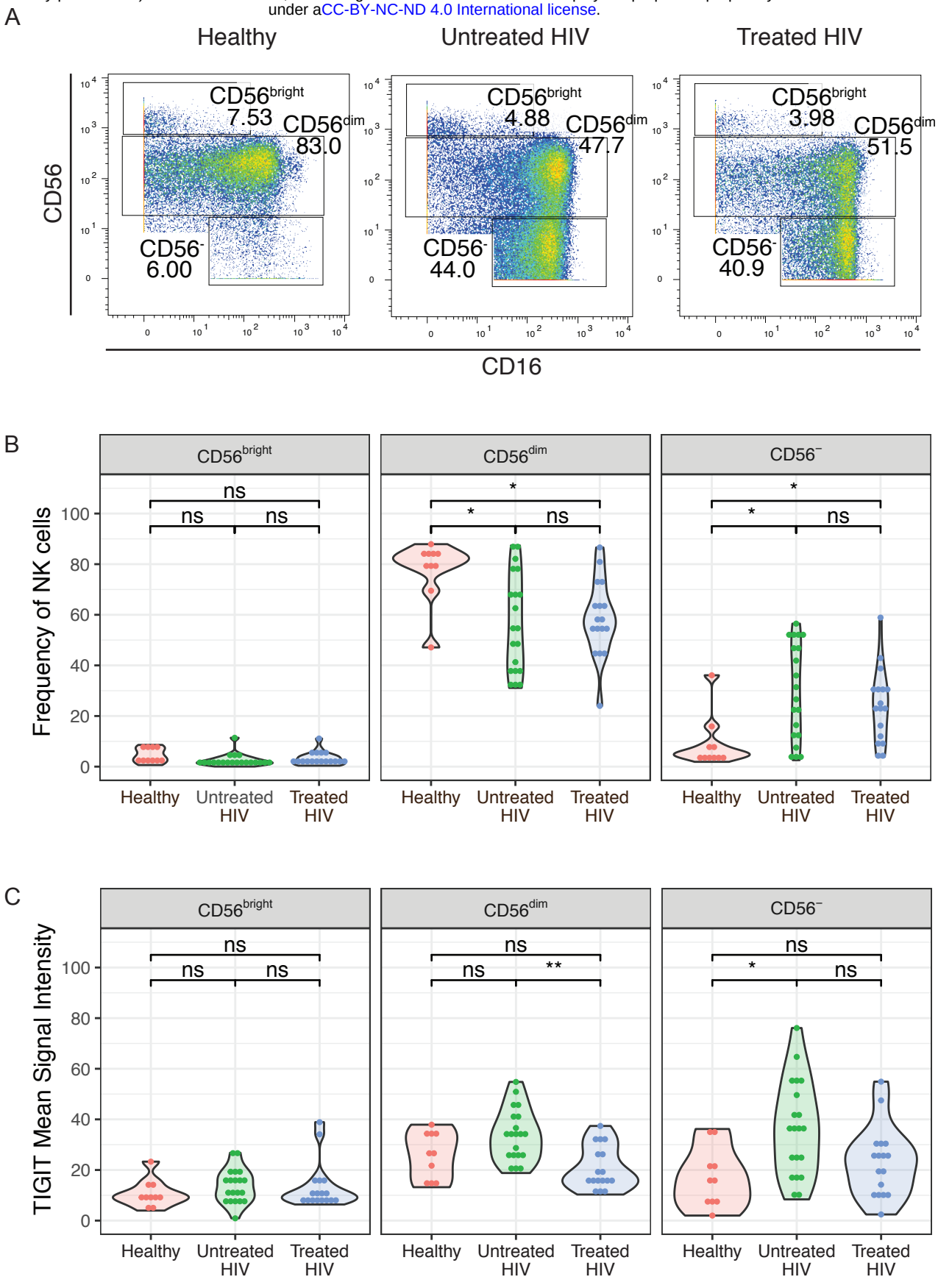
FIGURE 2



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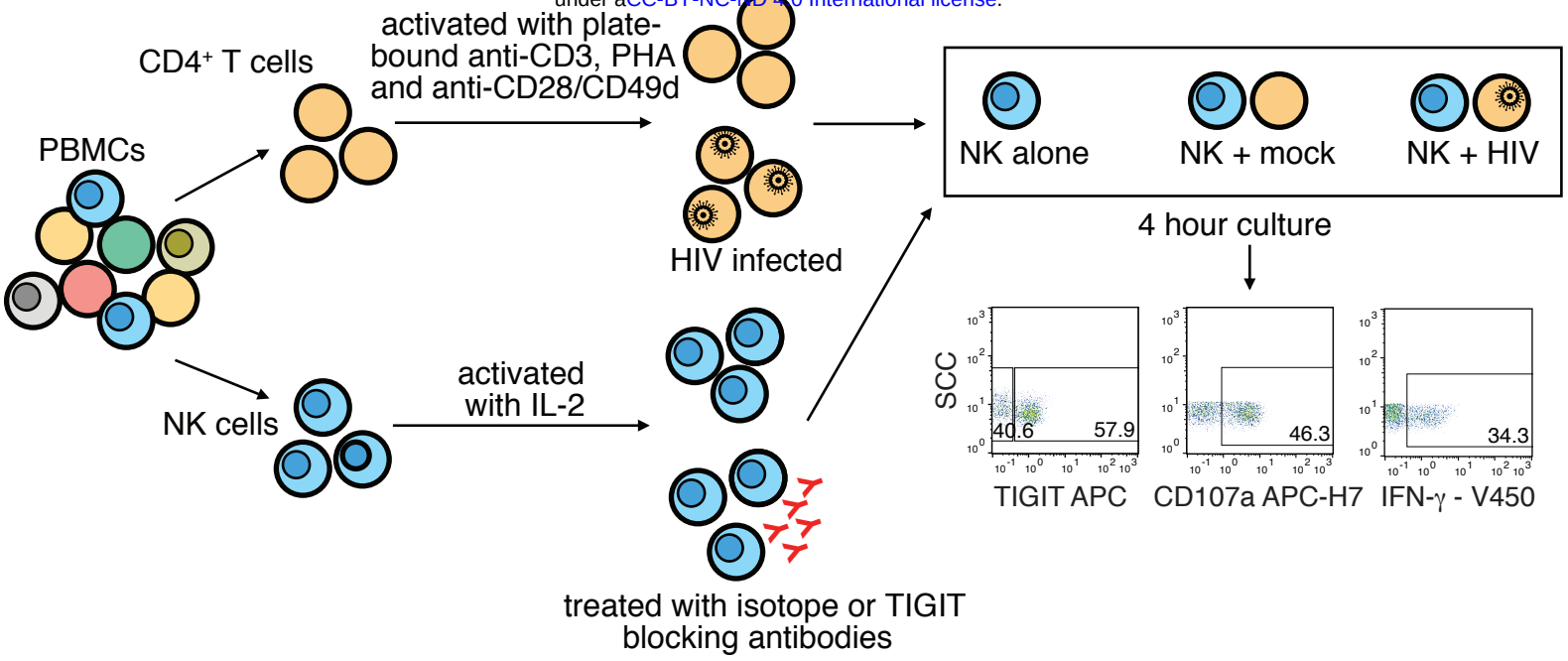

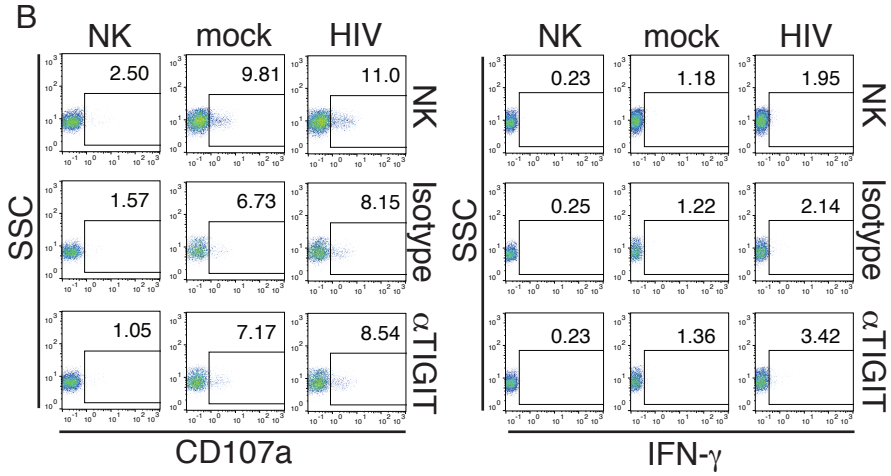

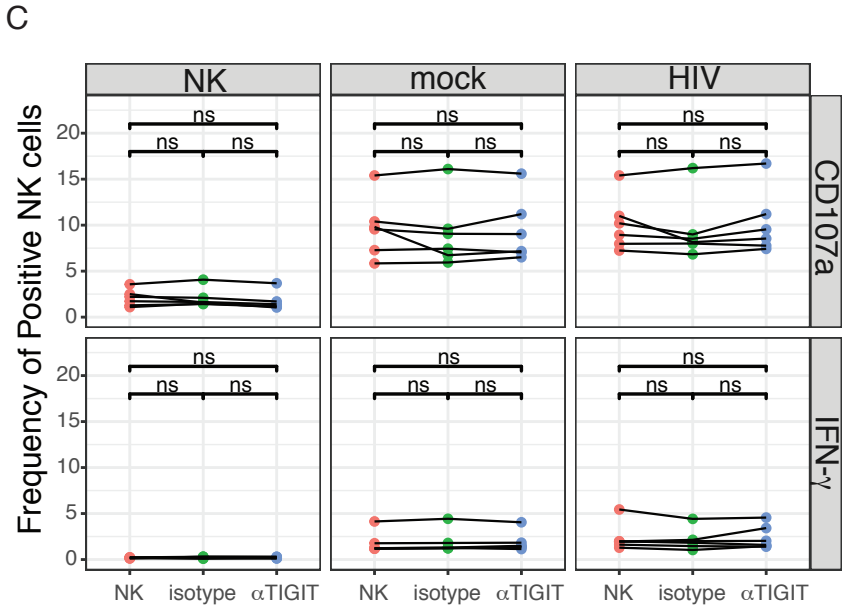

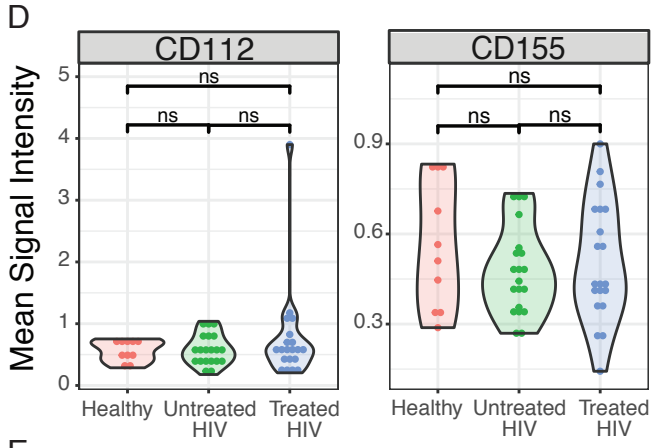

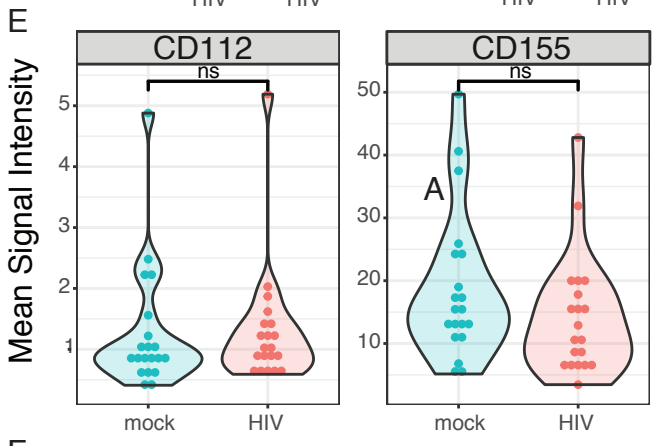

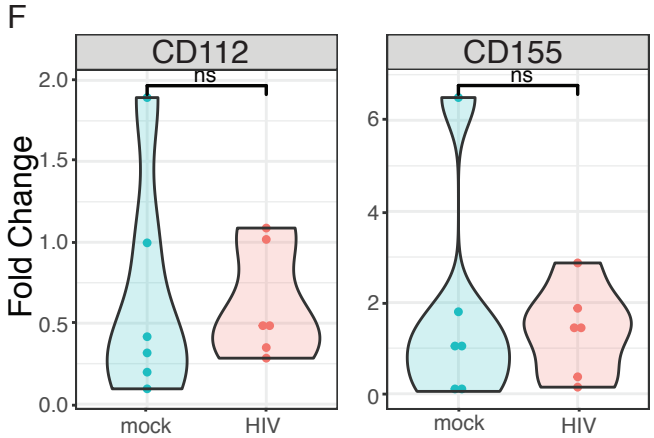

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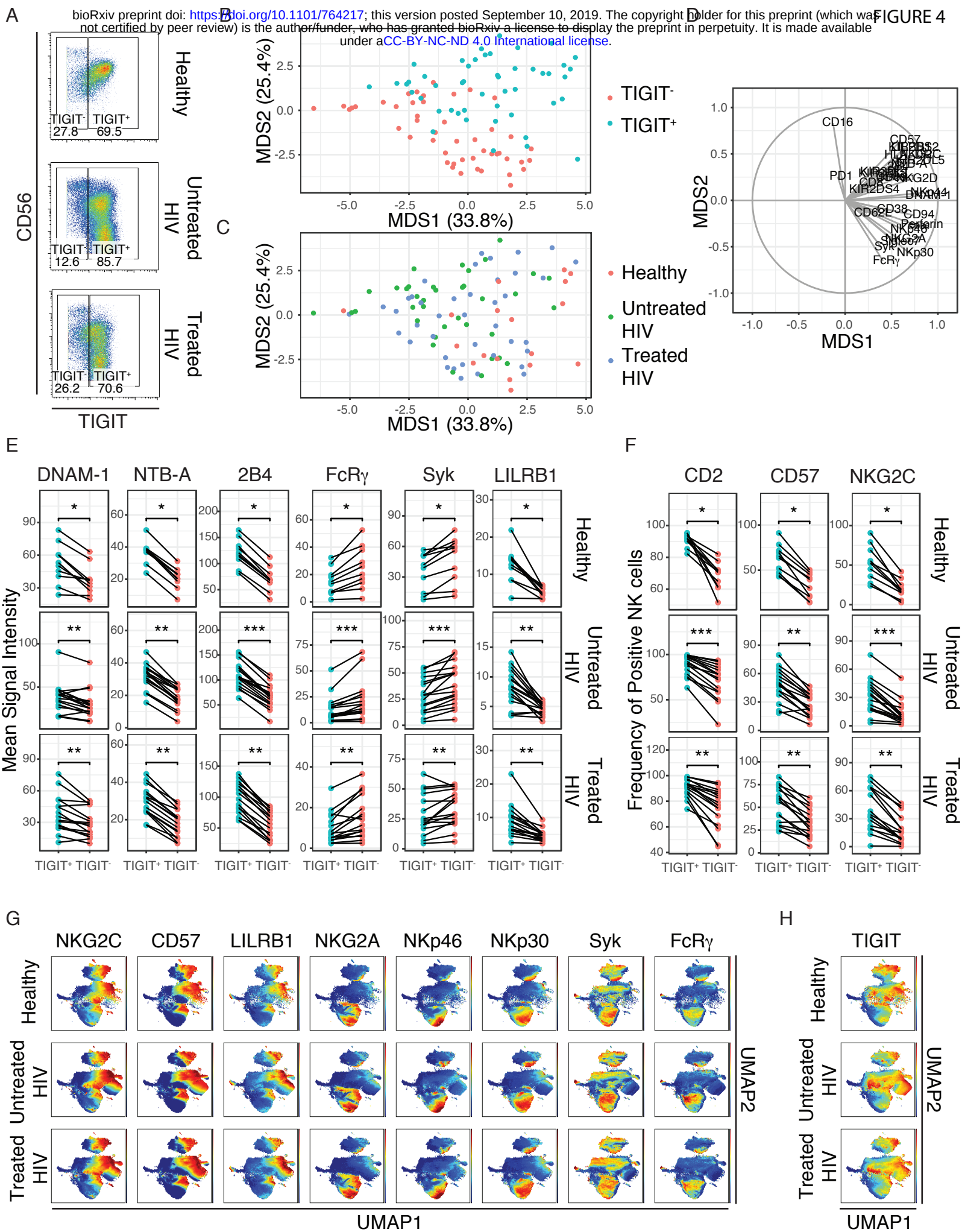

FIGURE 5

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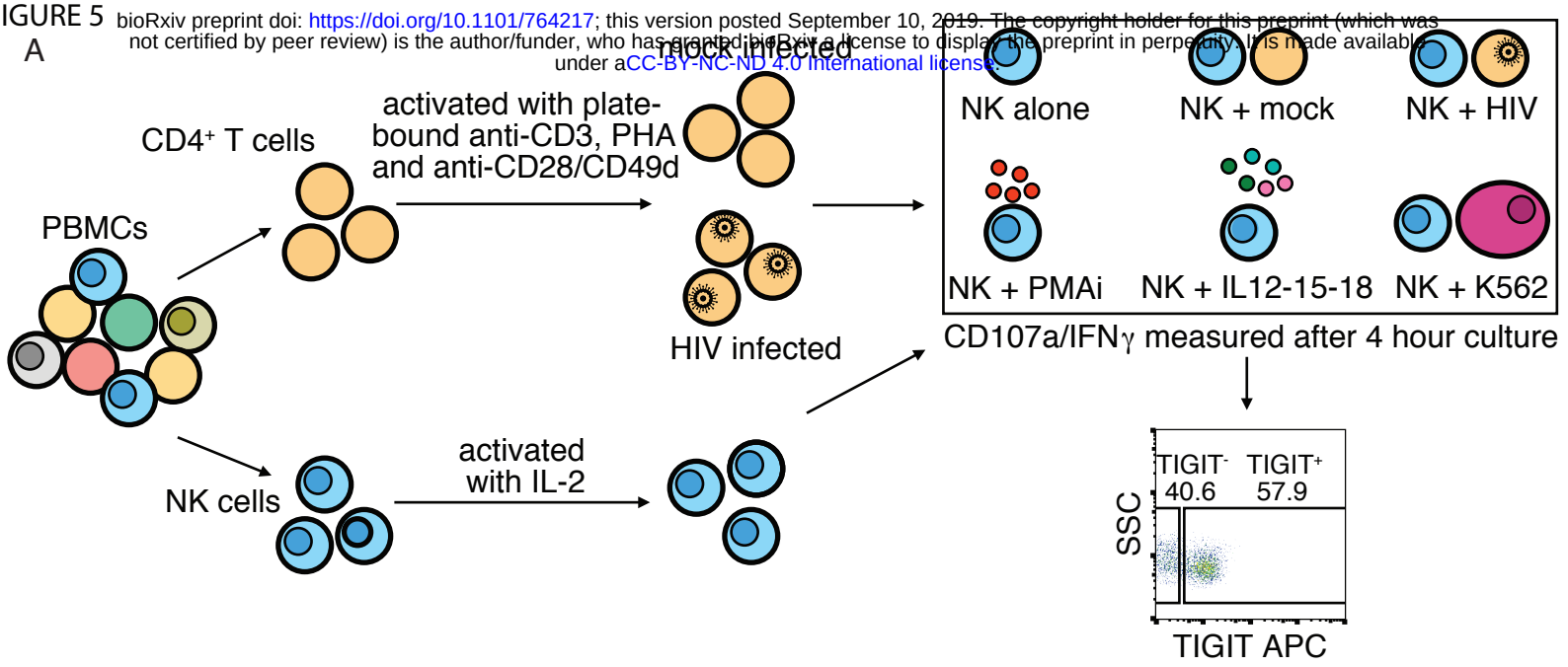

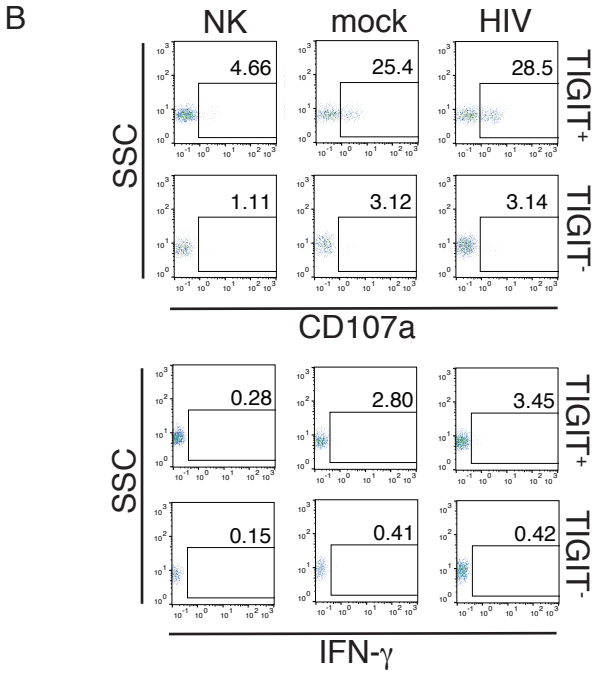

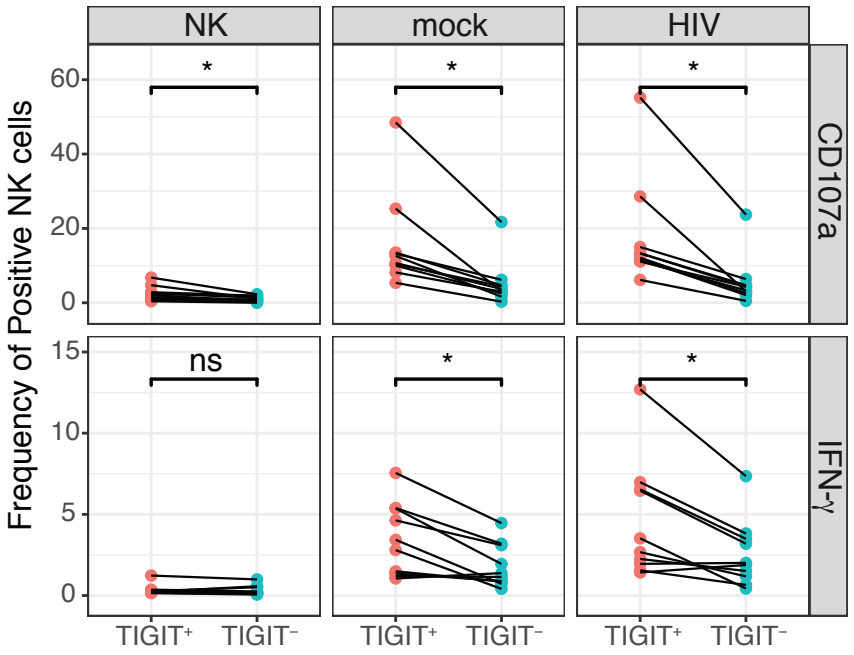

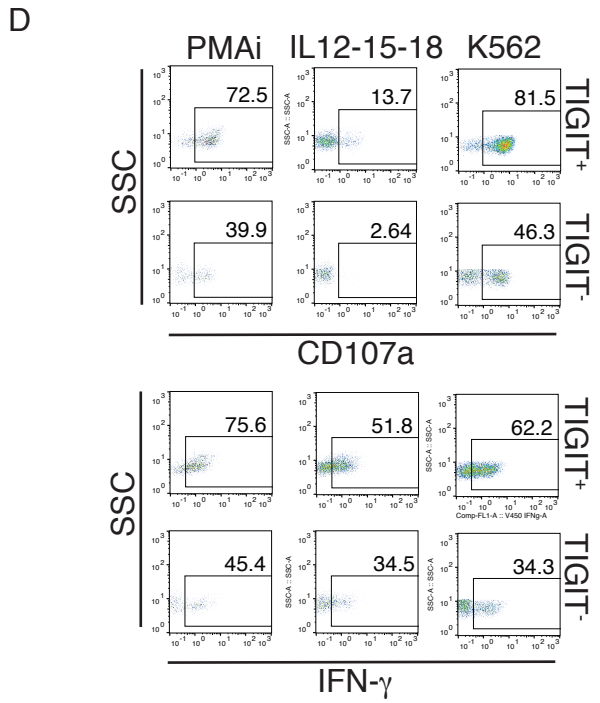

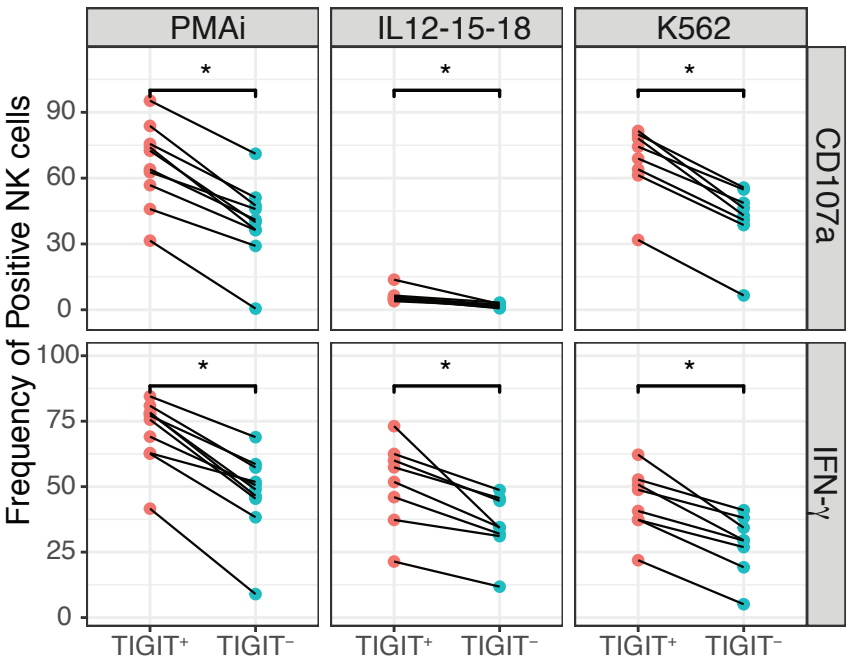

FIGURE S1

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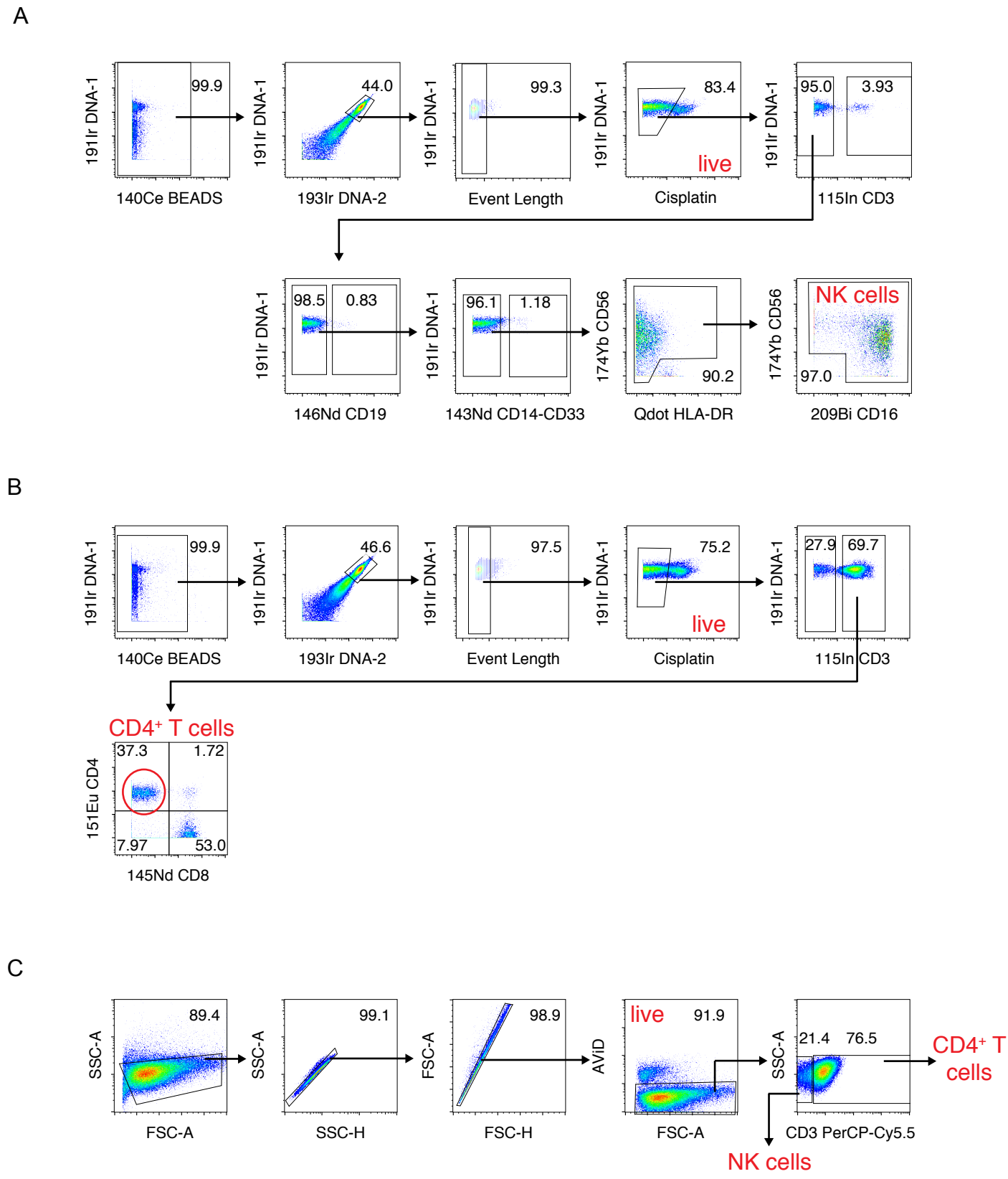

FIGURE S2

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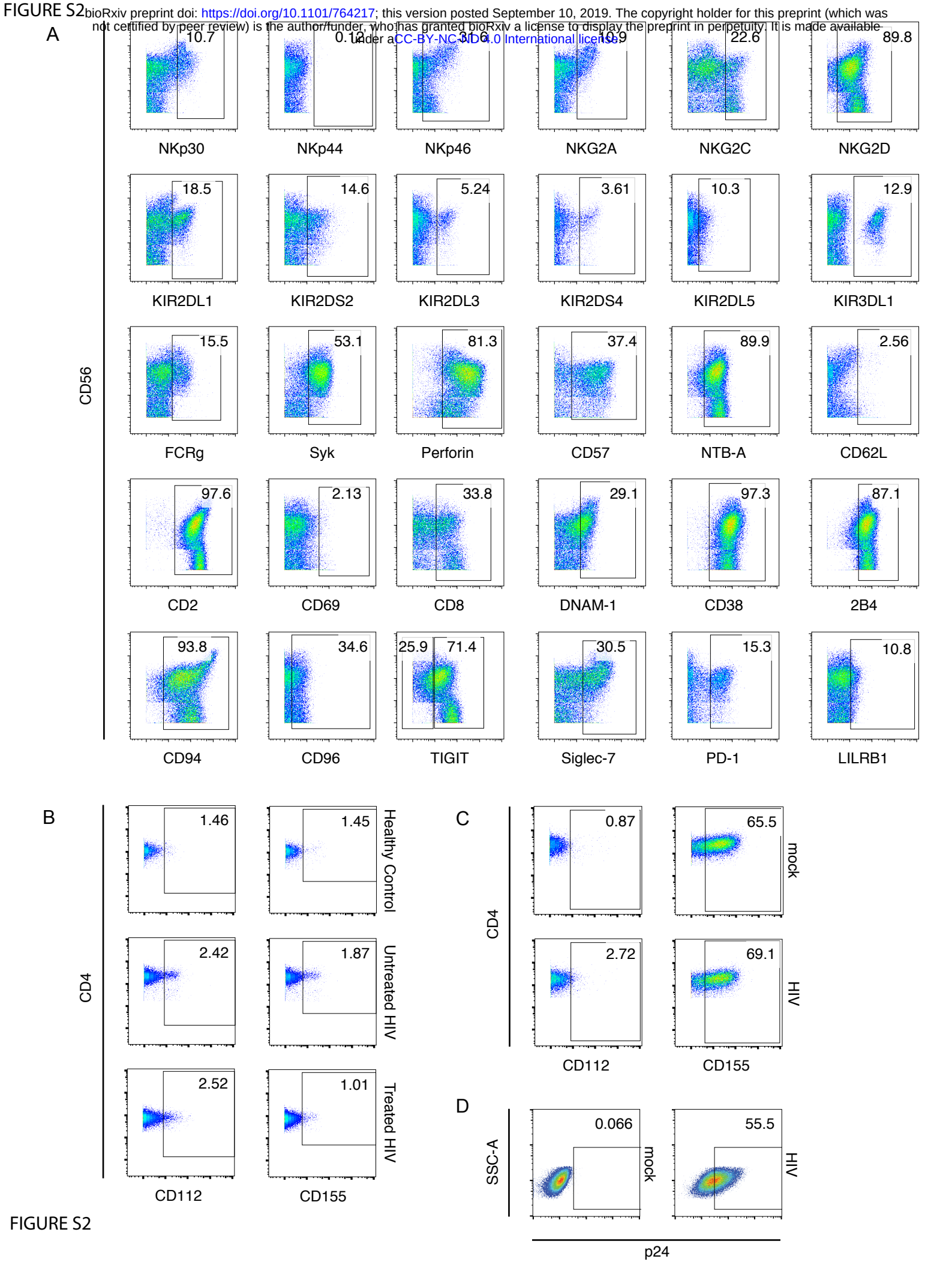

FIGURE S2