THE ROLE OF TISSUE RESIDENT NK CELLS DURING HOMEOSTASIS, PRIMARY AND SECONDARY INFECTION

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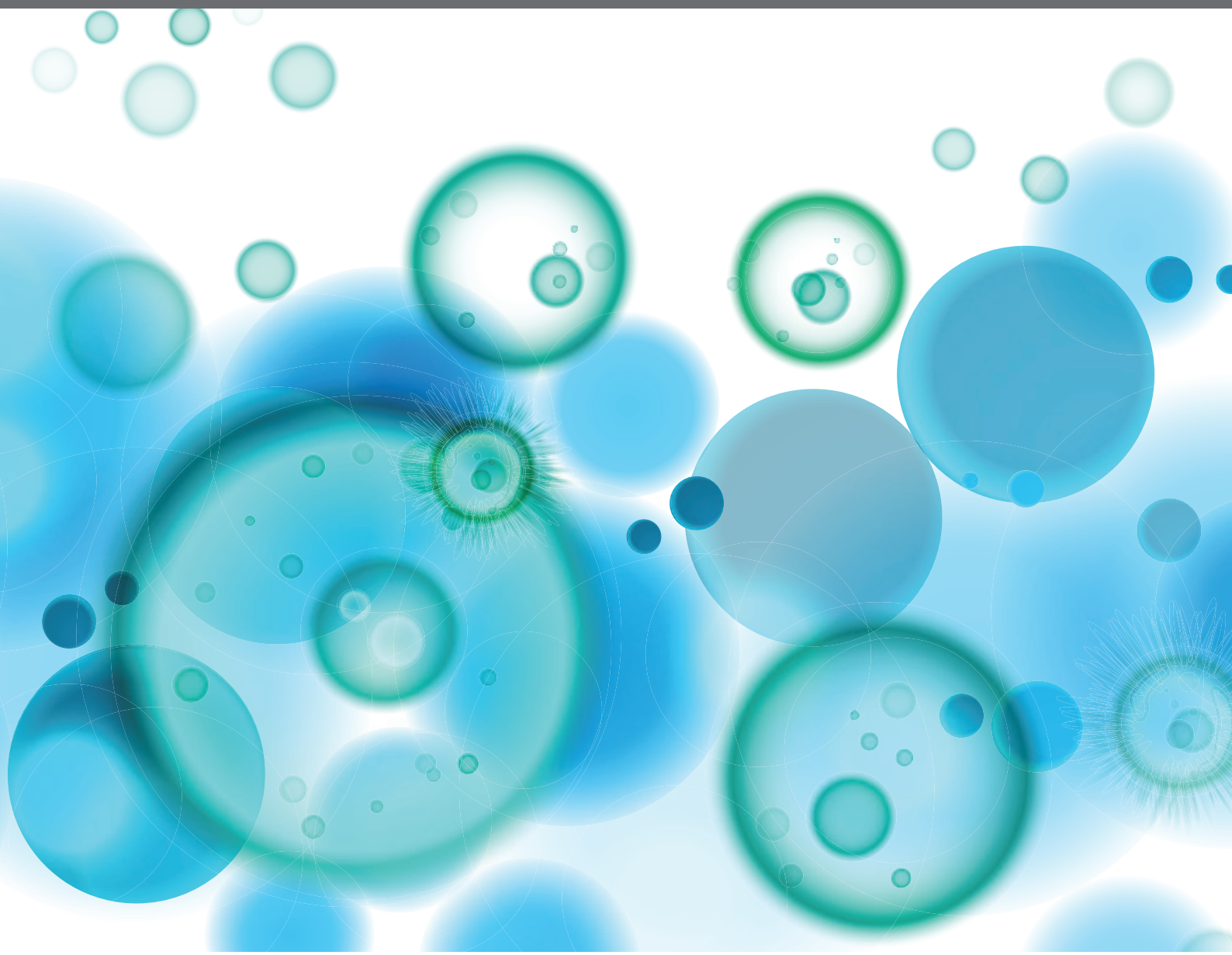

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THE ROLE OF TISSUE RESIDENT NK CELLS DURING HOMEOSTASIS, PRIMARY AND SECONDARY INFECTION

Topic Editors:
Silke Paust, The Scripps Research Institute, United States
Marcus Altfeld, Leibniz-Institut für Experimentelle Virologie, Germany

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Table of Contents

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Treated HIV Infection Alters Phenotype but Not HIV-Specific Function of Peripheral Blood Natural Killer Cells

*Nancy Q. Zhao[1,2], Anne-Maud Ferreira[3], Philip M. Grant[2], Susan Holmes[3] and Catherine A. Blish[1,2,4]**

[1] Immunology Program, Stanford University School of Medicine, Stanford, CA, United States, [2] Department of Medicine, Stanford University School of Medicine, Stanford, CA, United States, [3] Department of Statistics, Stanford University School of Medicine, Stanford, CA, United States, [4] Chan Zuckerberg Biohub, San Francisco, CA, United States

Natural killer (NK) cells are the predominant antiviral cells of the innate immune system, and may play an important role in acquisition and disease progression of HIV. While untreated HIV infection is associated with distinct alterations in the peripheral blood NK cell repertoire, less is known about how NK phenotype is altered in the setting of long-term viral suppression with antiretroviral therapy (ART), as well as how NK memory can impact functional responses. As such, we sought to identify changes in NK cell phenotype and function using high-dimensional mass cytometry to simultaneously analyze both surface and functional marker expression of peripheral blood NK cells in a cohort of ART-suppressed, HIV+ patients and HIV- healthy controls. We found that the NK cell repertoire following IL-2 treatment was altered in individuals with treated HIV infection compared to healthy controls, with increased expression of markers including NKG2C and CD2, and decreased expression of CD244 and NKp30. Using co-culture assays with autologous, *in vitro* HIV-infected CD4 T cells, we identified a subset of NK cells with enhanced responsiveness to HIV-1-infected cells, but no differences in the magnitude of anti-HIV NK cell responses between the HIV+ and HIV− groups. In addition, by profiling of NK cell receptors on responding cells, we found similar phenotypes of HIV-responsive NK cell subsets in both groups. Lastly, we identified clusters of NK cells that are altered in individuals with treated HIV infection compared to healthy controls, but found that these clusters are distinct from those that respond to HIV *in vitro*. As such, we conclude that while chronic, treated HIV infection induces a reshaping of the IL-2-stimulated peripheral blood NK cell repertoire, it does so in a way that does not make the repertoire more HIV-specific.

Keywords: NK cells, HIV-1, memory, innate immunity, CyTOF

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***Correspondence:**
Catherine A. Blish
cblish@stanford.edu

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INTRODUCTION

Natural killer (NK) cells are critical effector cells of the innate immune system that can rapidly recognize and kill virally infected and tumor cells. NK cells express an array of activating and inhibitory receptors; the integration of signaling between these receptors determines NK cell activation and functional activity. This includes the release of cytolytic granules to induce target cell

apoptosis, as well as the secretion of cytokines and chemokines including IFN-γ, MIP-1β (CCL4), and TNF-α .

Epidemiological and experimental evidence have highlighted the role of NK cells in the acquisition and disease progression of HIV-1. Increased constitutive NK cell activity is associated with protection from HIV acquisition in highly exposed seronegative individuals (1, 2). Similarly, the expression of the NK cell receptor KIR3DL1 and its cognate ligand HLA-Bw4-80I is associated with slower disease progression and improved suppression of autologous HIV-infected CD4 T cells (3–5). Specific NK cell receptors have also been implicated in HIV recognition and targeting. NKG2A-expressing NK cells have improved activity against HIV (6–8), and NKG2D ligands are upregulated on HIV-infected cells (9). As such, changes in expression of NK cell receptors can impact their ability to target HIV.

Chronic, untreated HIV infection is associated with significant changes in the NK cell repertoire, the most well-defined of which is the loss of $CD56^{+}$ NK cells, and the concomitant expansion of a $CD56^{neg}$ NK cell subset (10). $CD56^{neg}$ NK cells are functionally impaired and thought to be exhausted, demonstrating reduced cytotoxicity and IFN-γ production (11–13). In addition, the expression of the inhibitory receptor Siglec-7 (14), as well as the expression of the activating receptors NKp30, NKp44 and NKp46 (15), are decreased in chronic, viremic HIV infection, whereas the expression of the inhibitory receptor TIGIT is increased (16, 17). After treatment with antiretroviral therapy (ART), the patterns of $CD56^{+}$ and $CD56^{neg}$ NK cell subsets are restored to levels similar to seronegative, healthy individuals (12). However, less is known regarding how other NK cell subsets, as well as how the NK cell repertoire as a whole, may be altered in the setting of virological control by ART. In addition, the functional outcomes of these alterations, in particular with regards to how they may impact HIV-specific responses, are not well understood.

Contrary to their classic designation as an innate immune cell type, recent work has demonstrated the ability of human NK cells to form memory against viruses including cytomegalovirus, Epstein-Barr virus and varicella-zoster virus (18–24). In non-human primates, infection with simian immunodeficiency virus (SIV) or SHIV generates antigen-specific NK cells that react with presented Gag and Env. In addition, vaccination with Ad26 vectors containing Gag and Env antigens from HIV and SIV generates long-lived, antigen-specific NK cells, even in the absence of continuous antigen stimulation (25), raising the possibility that human NK cells in infected individuals could be similarly capable of generating and retaining memory responses against HIV antigens even without ongoing viral exposure. As such, we sought to understand whether previous HIV infection altered the functional capacity of peripheral blood NK cells to respond against a second, *in vitro* stimulation with autologous HIV-infected cells. Here, we use mass cytometry to profile NK cell receptor expression on a cohort of ART-suppressed, HIV + donors and healthy controls, to determine how changes in the NK cell repertoire that occur with HIV infection influence HIV-specific NK cell responses.

MATERIALS AND METHODS

Study Subjects and Sample Processing

Cryopreserved peripheral blood mononuclear cells (PBMCs) from HIV-infected patients treated with antiretroviral therapy (ART) were obtained from the Stanford HIV Aging Cohort. This study was approved by the Institutional Review Board of Stanford University. For anonymous healthy HIV uninfected donors, leukoreduction system chambers were obtained from the Stanford Blood Bank. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare), and cryopreserved in 10% DMSO (Sigma Aldrich) and 90% fetal bovine serum (FBS, Thermo Fisher).

CD4 and NK Cell Sorting and Cell Culture

Peripheral blood mononuclear cells were thawed, and stained with a panel consisting of 7-AAD viability staining solution (eBioscience), CD14-BV421 (clone M5E2), CD19-BV421 (clone HIB19), CD16-FITC (clone 3G8), CD3-PE (clone SK7), CD4-BV711 (clone OKT4), and CD56-PE Cy7 (clone HCD56, all antibodies from Biolegend), and sorted for CD4 T cells ($CD14^{-}$ $CD19^{-}$ $CD3^{+}$ $CD4^{+}$) and NK cells ($CD14^{-}$ $CD19^{-}$ $CD3^{-}$ $CD56/CD16^{+}$) using a Sony SH800 sorter. Post-sorting, all cells were cultured in RPMI (Gibco), with 10% FBS (Thermo Fisher), 1% L-glutamine (Hyclone) and 1% penicillin/streptomycin/amphotericin (Thermo Fisher) (RP10). CD4 T cells were plated in RP10 with plate-bound anti-CD3 (clone OKT3, eBioscience), anti-CD28/CD49d (BD Biosciences) and PHA-L (eBioscience) for 48 h. NK cells were separately plated in RP10 with 300 IU/ml recombinant human IL-2 (R&D) for 72 h.

In vitro HIV Infection and NK Co-culture Assays

For all *in vitro* HIV infections, Q23-FL, a clone from early, subtype A infection (26), was used. The Q23-FL virus was produced by transfecting a plasmid encoding a full-length, replication competent clone into 293T cells, harvesting supernatant after 48 h and concentrating by ultracentrifugation. Viral stocks were titrated on TZM-bl cells as previously described (27). Activated CD4 T cells were infected with Q23-FL at an MOI of 25 (based on titrations in TZM-bl cells), using ViroMag magnetofection (OZ Biosciences). HIV-infected cells were used for co-cultures 24 h post infection. NK cells and CD4 T cells were co-cultured at a 1:4 effector:target (E:T) ratio, for 4 h, in the presence of brefeldin A (eBioscience), monensin (eBioscience), and anti-CD107a-APC (Biolegend).

Mass Cytometry

All antibodies were conjugated using MaxPar X8 labeling kits (Fluidigm), except for those purchased directly from Fluidigm; details of all antibodies is given in **Supplementary Table S1**. To maintain antibody stability and consistency in staining, all antibody panels were pre-mixed into separate surface and ICS cocktails (as indicated in **Supplementary Table S1**), aliquoted and frozen at −80°C until use. Palladium (Pd102, Pd104, Pd106,

and Pd108) conjugated CD45 antibodies for barcoding were made as previously described (28).

At the end of co-culture, cells were stained for viability using 25 μM Cisplatin (Enzo) for 1 min and quenched with FBS, and samples were barcoded using palladium-based CD45 barcodes as previously described (28). After barcoding, cells were washed thrice, and all samples from a set of barcodes were combined. Samples were stained with the surface antibody panel for 30 min at 4°C, fixed with 2% paraformaldehyde (PFA, Electron Microscopy Sciences), permeabilized with Permeabilization Buffer (eBioscience), and stained with the intracellular staining (ICS) panel (made in Permeabilization Buffer) for 45 min at 4°C. Cells were suspended overnight in iridium interchelator (DVS Sciences) in 2% PFA, and resuspended in 1x EQ Beads (Fluidigm) before acquisition on a Helios mass cytometer (Fluidigm).

Data Analysis

Bead normalization[1] and debarcoding (using the R package *Premessa*) were performed on all files post-acquisition. All CyTOF data was visualized and gated using FlowJo v10.1 (Tree Star); gated NK cells ($CD3^-$ $CD56/CD16^+$), or $functional^+/functional^-$ cells were exported as fcs files from FlowJo and used in downstream analyses. CD11a was excluded from all downstream analyses due to poor staining. The data supporting this publication is available at ImmPort[2] under study accession SDY1620.

The open source statistical software (29) was used for analyses. For analyses using the generalized linear models as well as the clustering and the Uniform Manifold Approximation and Projection (UMAP) (30), raw channel values were transformed using the inverse hyperbolic sine (asinh) function with a cofactor equal to 5 to account for heteroskedasticity. This transformation was not applied for calculating mean signal intensity values. To compare frequencies of functionally responding cells, as well as frequency of the gated population between HIV+ and HIV− groups, *t*-tests were used. To compare frequencies of responding cells between the gated population and bulk NK cells in each donor, paired *t*-tests were used. To identify markers that are predictors of the HIV+ or HIV− conditions, we used the R package *CytoGLMM* (31) which uses a generalized linear model with bootstrap resampling. This model takes into account the distribution of each marker, and has a donor-specific variable to control for inter-individual variability. For the clustering analyses, the R package *CATALYST* was used (32, 33). This package provides a clustering method which combines the *FlowSOM* algorithm (34) which generates 100 high-resolution clusters, followed by the ConsensusClusterPlus metaclustering algorithm (35) which regroups these high-resolution clusters into metaclusters. Default parameters were used for clustering, and the number of metaclusters (10) was selected based on the delta area plot provided. To test for differential abundance of clusters between groups, the *diffcyt-DA-GLMM* method from the *diffcyt* package (32, 33) was used; the donor IDs were specified as a random effect. The UMAP was run using the *scater* package (36), with default settings.

[1]https://github.com/nolanlab/bead-normalization

[2]https://www.immport.org

RESULTS

HIV+ Donors Have an Altered NK Cell Repertoire Even in the Setting of ART Suppression

To investigate the effect of HIV-1 infection on NK cell responses, we used CyTOF to profile NK cell receptor expression and functional activity in a cohort of 10 ART-suppressed, HIV+ donors (referred to as HIV+), and 10 healthy controls (referred to as HIV−). Patient demographics are given in **Table 1**. We first compared the expression of 28 NK cell receptors between the HIV+ and HIV− donors, in sorted, IL-2 activated NK cells. To look at overall NK receptor expression patterns between the two groups, we used a multidimensional scaling (MDS) plot to visualize all the NK cell samples (**Figure 1A**). NK cells from HIV− and HIV+ donors separated primarily on a diagonal axis; this separation was driven by multiple markers including CD2, NKp30 and NKp46 (as shown in the correlation circle). To further define the NK cell receptors whose expression is altered in HIV+ compared to HIV- individuals, we used a generalized linear model with bootstrap resampling to identify markers predictive of either the HIV+ or HIV− groups. Based on marker distribution, the model generates the log-odds that the expression of a given marker is predictive of either the HIV+ or HIV− group, together with the 95% confidence interval. In purified, IL-2 activated NK cells, the markers NKG2C, CD2, NKp46, and PD-1 were predictive of HIV+ (95% confidence interval does not contain the zero value) while CD244, NKp30, DNAM-1, and NKG2A were predictive of HIV− individuals (**Figure 1B**). To confirm these results, we also compared mean signal intensity (MSI) for the top 4 NK cell markers predictive of either the HIV− or HIV+ groups, and observed an increased trend of MSI for NKG2C, CD2, NKp46 and PD-1 in the HIV+

TABLE 1 | Demographic information of HIV cohort.

	HIV+ (*n* = 10)	HIV− (*n* = 10)
Age in years | Mean (SD)	52.9 (9.7)	54.1 (17.5)
Sex | proportion male	10/10	9/10
Years since diagnosis | Mean (SD)	18.5 (8.6)	N/A
Years on ART | Mean (SD)	15.3 (8.1)	N/A
CD4 count in cells/mm^3 | Mean (SD)	705 (335)*	Not available
Nadir CD4 count in cells/mm^3 | Mean (SD)	249 (129)+	N/A
Type of ART (proportion)	NNRTI (6/10), PI (3/10), Integrase inhibitor (3/10)^	N/A

CD4 counts were only available for 7/10 HIV+ individuals.

+Nadir CD4 counts were only available for 6/10 HIV+ individuals.

^NNRTI, non-nucleoside reverse transcriptase inhibitor, PI, protease inhibitor.

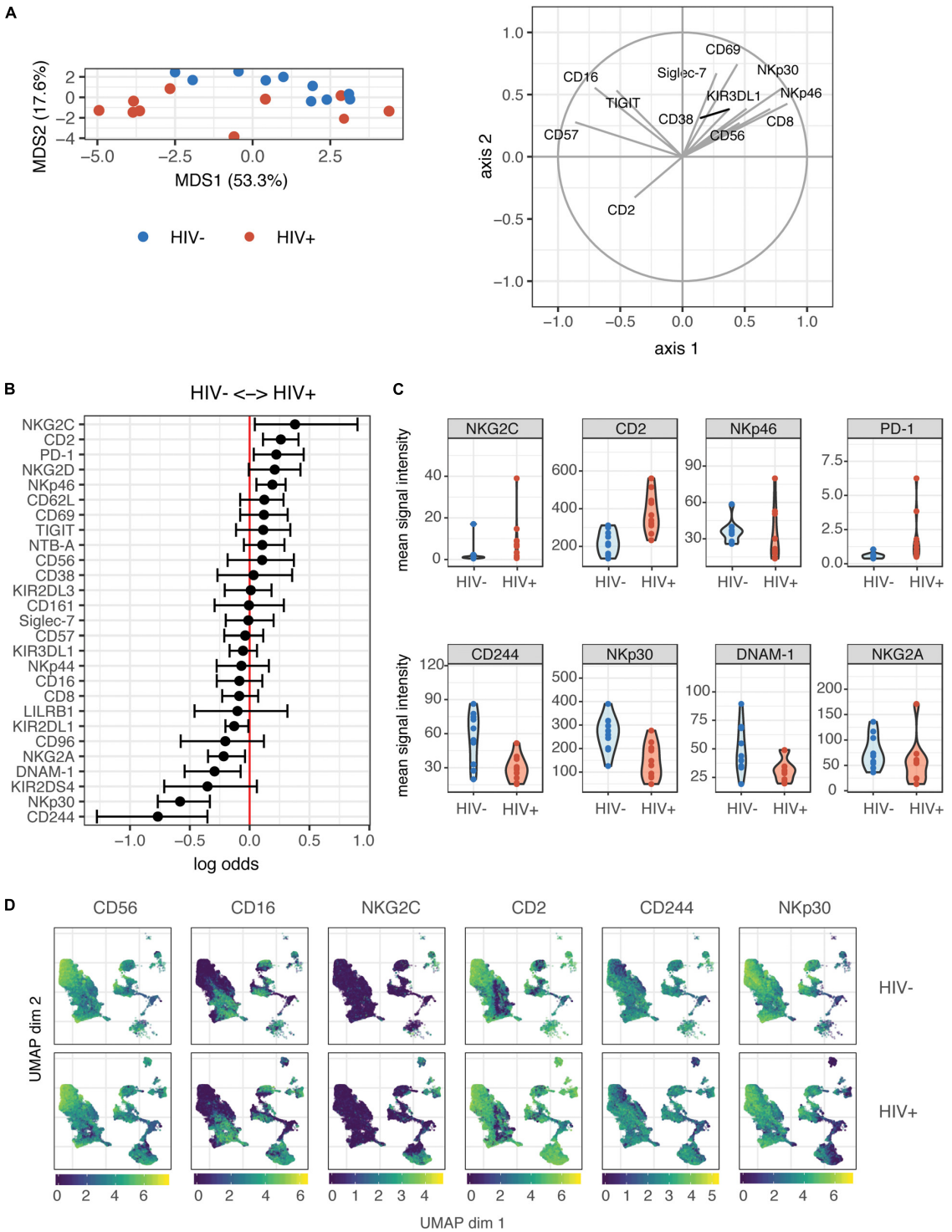

FIGURE 1 | Profiling of NK cell repertoire using CyTOF in both healthy, HIV– and ART-suppressed, HIV+ donors demonstrates alterations in NK cell surface receptor expression. **(A)** Multidimensional scaling (MDS) plot showing separation of NK cells from HIV+ (n = 10) and HIV– donors (n = 10). Only markers whose contributions are greater than 0.25 in both MDS1 and MDS2 are displayed in the marker loadings. **(B)** A generalized linear model with bootstrap resampling was used to find receptors predictive of either HIV+ (right), or HIV– (left) donor NK cells. Log-odds are logarithm of ratios of the probability that a cell belongs to either group. For each marker, the 95% confidence interval is represented by the line surrounding the point estimate; a larger absolute log-odds value of the parameter indicates that the marker is a stronger predictor. **(C)** Mean signal intensity (MSI) of NKG2C, CD2, NKp46, and PD-1 (top 4 predictors of the HIV+ group; top), and CD244, NKp30, DNAM-1, and NKG2A (top 4 predictors of the HIV– group; bottom). **(D)** UMAP visualization of all NK cells from the HIV+ and HIV– groups, colored by expression of CD56, CD16, NKG2C, and CD2 (top 2 predictors of the HIV+ group), and CD244 and NKp30 (top 2 predictors of the HIV– group). Scales show asinh-transformed channel values.

group, and a decreased trend in CD244, NKp30, DNAM-1, and NKG2A (**Figure 1C**).

To further visualize the changes in subsets of NK cells between HIV+ and HIV− groups, we used the Uniform Manifold Approximation and Projection (UMAP) to visualize purified NK cells from both groups (**Figure 1D**). To identify classic NK cell subsets, the expression of the markers CD56 and CD16 are shown, revealing the expected pattern in that the cells with highest expression of CD56 have low expression of CD16, identifying the canonical $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cell subsets. In addition, the expression of the top predictors for each of the HIV− (CD244 and NKp30) and HIV+ groups (NKG2C and CD2) are shown. These plots reveal that greatest differences in both cellular distribution and NK marker expression between HIV− and HIV + donors occurred on the right part of the UMAP plots. Thus, these data demonstrate that even in the setting of long-term virological suppression with ART, the NK cell repertoire following IL-2 treatment remains altered.

NK Cells From HIV+ Individuals Do Not Have an Increased Response Upon *in vitro* Restimulation With Autologous HIV-Infected Cells

Contrary to their classic designation as innate immune cells, NK cells have more recently been shown to demonstrate antigen-specific memory to viral antigens. As such, we were interested in determining whether prior HIV-1 exposure (in the HIV+ individuals) would alter the magnitude of the NK cell response or the phenotypes of responding cells, when restimulated with autologous HIV-infected cells *in vitro*. We co-cultured NK cells from HIV− and HIV+ donors with autologous CD4 T cells infected *in vitro* with the HIV strain Q23-FL (**Figure 2A**); infection levels in CD4 T cells after co-culture were similar between HIV+ and HIV− donors (**Supplementary Figure S1**). To identify NK cell responses, we looked at expression of functional markers on these cells by CyTOF. For each sample, we separately gated on NK cells that were positive for the expression of cytokines IFN-γ, MIP-1β (CCL4), TNF-α or the degranulation marker CD107a (**Figure 2B**), after 4 h of co-culture. All gating was performed based on samples of NK cells in the absence of target cells; these samples had generally low levels of expression of all functional markers. We found that the majority of responding cells were polyfunctional: for example, using the generalized linear model, the top predictors of $CD107a^{+}$ cells included MIP-1β, TNF-α, and IFN-γ (**Supplementary Figure S2**). As such, we used Boolean gating to identify functionally responding cells (positive for *any* of the functional markers above, hereafter named as functional^{+}), or non-functionally responding cells (negative for *all* of the markers above, named as functional^{-}).

To identify differences in the magnitude of NK cell responses to HIV-infected cells between HIV− and HIV+ donors, we gated on cells that were positive for functional markers, and sought to identify differences in frequency of these cells between the two groups. We applied background subtraction (by subtracting the frequency of NK cells expressing each functional marker in the NK alone condition) to account for variations in baseline NK activity. In response to autologous HIV-infected cells *in vitro*, the frequency of NK cells expressing any of the functional markers (IFN-γ, MIP-1β, TNF-α, CD107a) individually, or combined (functional^{+}), was not significantly different between the HIV− and HIV+ groups (**Figure 2C**).

To further understand whether the NK cells that were generating a functional response were phenotypically similar between the HIV+ and HIV− groups, we used the generalized linear model to identify predictors of responding (functional^{+}) and non-responding cells (functional^{-}). Multiple NK cell receptors were strong predictors of functional^{+} or functional^{-} cells, indicating a clearly distinct phenotype of cells that respond to HIV *in vitro*; these cells express higher levels of CD96, NKp30, TIGIT, and Siglec-7, and lower levels of CD62L, CD16, and NKp46 (**Figure 3A**). Notably, the top predictors of responding cells were very similar between the HIV- and the HIV+ group, including CD96, NKp30, and TIGIT. To identify a subset of responding cells, we included cells with high expression of positive predictors (predictors of functional^{+}), and with negative expression of negative predictors (predictors of functional^{-}), that had a log odds greater than 0.2 in both groups; the resultant phenotype was $CD96^{hi}$ $NKp30^{hi}$ $TIGIT^{hi}$ $CD16^{-}$ $CD62L^{-}$ $NKp46^{-}$. CD38, while being a strong predictor of functional^{+} cells in the HIV+ group, was excluded; it was not a predictor of responding cells in the HIV− group, and CD38 expression is increased in the context of HIV infection and remains high even after ART treatment (37). We gated on each marker individually (**Figure 3B**), and then used Boolean gating to generate a gated population that included all the features of the phenotype. Gating on this subset significantly enriched for functional^{+} cells to a similar extent in both the HIV+ and HIV− groups (**Figure 3C**), suggesting that, despite the alterations in NK cell repertoire, the NK cell subsets responding to HIV *in vitro* were not altered in HIV+ individuals. We also compared the frequency of this subset among total NK cells, in both HIV+ and HIV− groups (**Figure 3D**), and noted that, while the frequency was low (between 0.1 and 3%), there was no significant difference between the two groups, indicating that HIV infection does not alter the frequency of this subset.

NK Cells Clusters That Are Altered in HIV+ Individuals Are Distinct From Those That Are Functionally Responsive to HIV *in vitro*

As the expression of many of the markers on responding cells were markers we found to be altered in the HIV+ group compared to HIV− (**Figure 1**), it was surprising that these changes did not seem to have an effect on the magnitude or quality of the NK cell response to HIV *in vitro*. To better understand the populations of cells that are altered in HIV+ individuals, compared to those that are involved in the functional response, we used UMAP to visualize all NK cells from co-cultures with HIV-infected cells *in vitro*, in both groups. We also performed unsupervised clustering and metaclustering, using

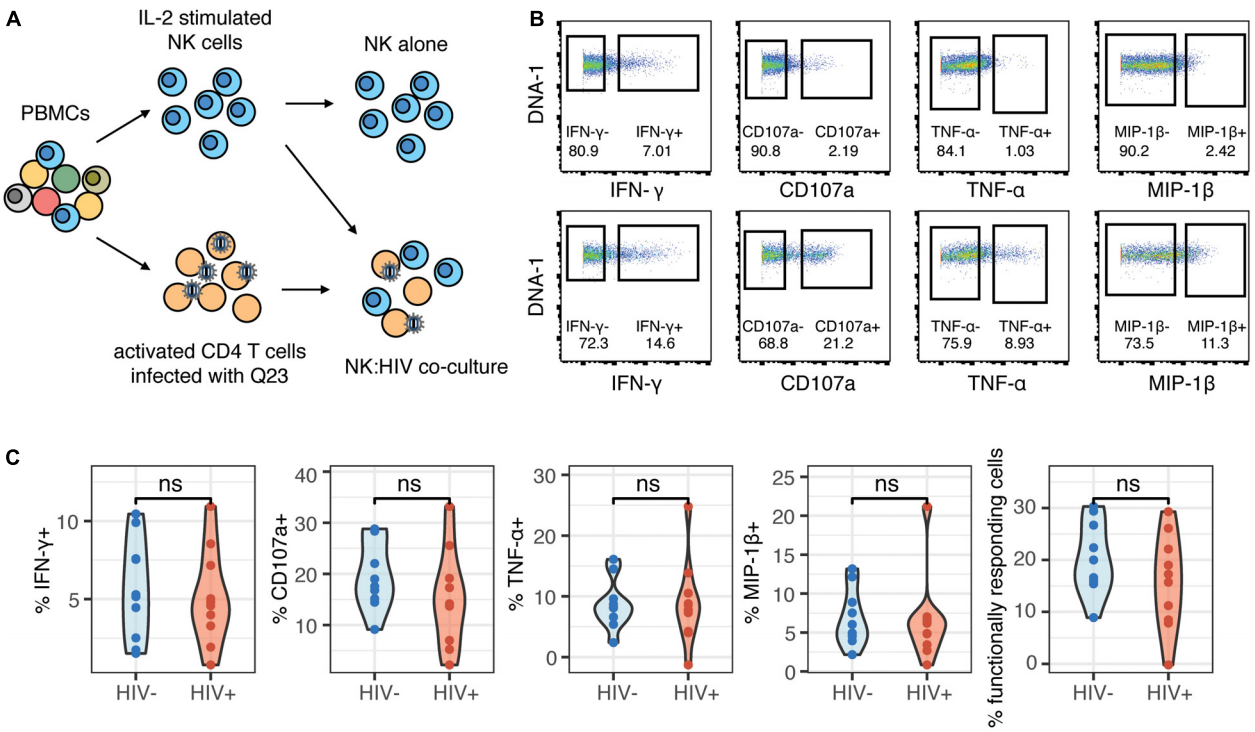

FIGURE 2 | The magnitude of the HIV-specific response does not differ between HIV- and HIV+ donors. **(A)** Schematic of experimental set-up for NK co-culture assays. **(B)** Representative flow cytometry plots of CD107a, IFN-γ, MIP-1β, and TNF-α production (by frequency of positive cells), after 4 h co-culture without (top) or in the presence of HIV-infected autologous $CD4^+$ T cells (bottom). **(C)** Summary data of CD107a, IFN-γ, MIP-1β and TNF-α production (by frequency of positive cells), after 4 h co-culture with HIV-infected autologous $CD4^+$ T cells, in NK cells from HIV– (n = 10) and HIV+ (n = 10) donors. ns, not significant, by t-test.

the *FlowSOM* and *ConsensusClusterPlus* algorithms, to identify 10 metaclusters of NK cells (**Figure 4A**); this included clusters that were shared between HIV+ and HIV− groups, such as cluster 1, but also clusters that were distinct to NK cells from HIV+ individuals (cluster 3), or HIV- individuals (cluster 5). To identify responding NK cells, we overlaid marker expression of functional markers onto the UMAP visualization (**Figure 4B**). All responding NK cells (positive for functional markers) clustered in a similar area on the left part of the plots that was shared between HIV+ and HIV− donors; many of these cells are positive for multiple functional markers, confirming that most responding cells were polyfunctional (**Supplementary Figure S1**). To identify which cluster these functionally responding cells belonged to, we looked at a heatmap of mean marker expression of all NK cell markers across all clusters (**Figure 4C**); this identified clusters 9 and 10 as the predominant clusters expressing functional markers, with cells in cluster 9 expressing high levels of CD107a, TNF-α, and MIP-1β, and cells in cluster 10 also expressing high levels of IFN-γ. In line with our previous analysis of the phenotypes of functionally responding cells, cluster 9 expresses high levels of NKp30, while cluster 10 has relatively high levels of TIGIT expression and low levels of NKp46 expression; both clusters have low CD16 and CD62L expression.

To identify if these clusters were differentially abundant between the HIV+ and HIV− groups, we performed differential abundance tests using the *diffcyt* package, which identified 3 clusters with significantly different abundance between the HIV+ and HIV− groups – clusters 3, 5, and 7 (**Figure 4D**). Notably, neither cluster 9 or 10 were differentially abundant between the HIV+ and HIV− groups, indicating that the functionally responding subsets remain unaltered in abundance or phenotype (**Figure 3**) in the setting of ART-treated HIV infection. Hence, although HIV infection induces a reshaping of the NK cell repertoire *in vivo*, it does so in a way that does not alter HIV-specific responses, as the predominant changes occur in NK cell compartments that do not respond to HIV in *in vitro* restimulation.

DISCUSSION

Chronic HIV-1 infection is known to alter NK cell phenotype and function. To better understand how these changes occur in the setting of virological control with ART, as well as how HIV-specific function is impacted in this setting, we used mass cytometry to profile differences in NK cell receptor expression repertoire in peripheral blood NK cells between ART-treated, HIV+ individuals and healthy HIV- controls. We observed differences in the IL-2 stimulated NK cell repertoire between HIV+ and HIV− individuals, although these differences did not impact the HIV-specific response to an *in vitro* restimulation with HIV-infected CD4 T cells. In addition, we identified a unique

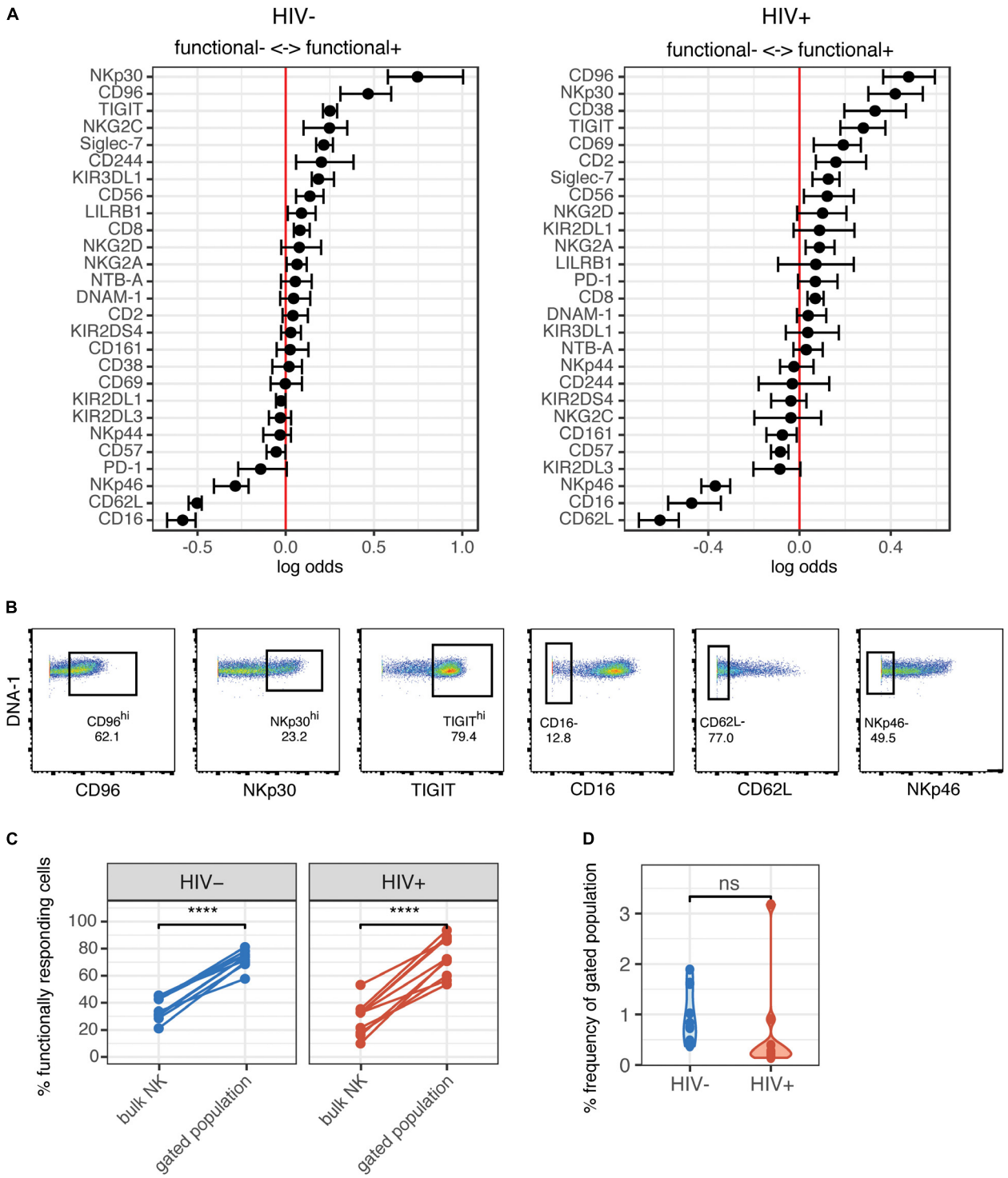

FIGURE 3 | Phenotype of NK cells responding to HIV are similar between HIV- and HIV+ donors, and include many of the markers that are altered in ART-treated HIV infection. **(A)** A generalized linear model with bootstrap resampling was used to identify NK cell receptors predictive of either functionally responding (right), or non-responding (left) NK cells, in HIV– (n = 10) and HIV+ (n = 10) donors. Log-odds are logarithm of ratios of the probability that a cell belongs to either group. For each marker, the 95% confidence interval is represented by the line surrounding the point estimate; a larger absolute log-odds value of the parameter indicates that the marker is a stronger predictor. **(B)** Gating on markers that predict functionally responding cells (NKp30^{hi} CD96^{hi} TIGIT^{hi} CD62L^{-} CD16^{-} NKp46^{-}), with a minimum log-odds threshold of 0.2 in both groups. **(C)** Comparison of functional activity in the gated population, compared to bulk NK cells. Summary data of percentage positive functionally responding (positive for any of CD107a, IFN-γ, MIP-1β, and TNF-α) in HIV– (n = 10) and HIV + groups (n = 10). ****$p < 0.001$, by paired t-test. **(D)** Comparison of the frequency of the gated population, as a percentage of total NK cells, between the HIV– (n = 10) and HIV+ (n = 10) groups. ns, not significant, by t-test.

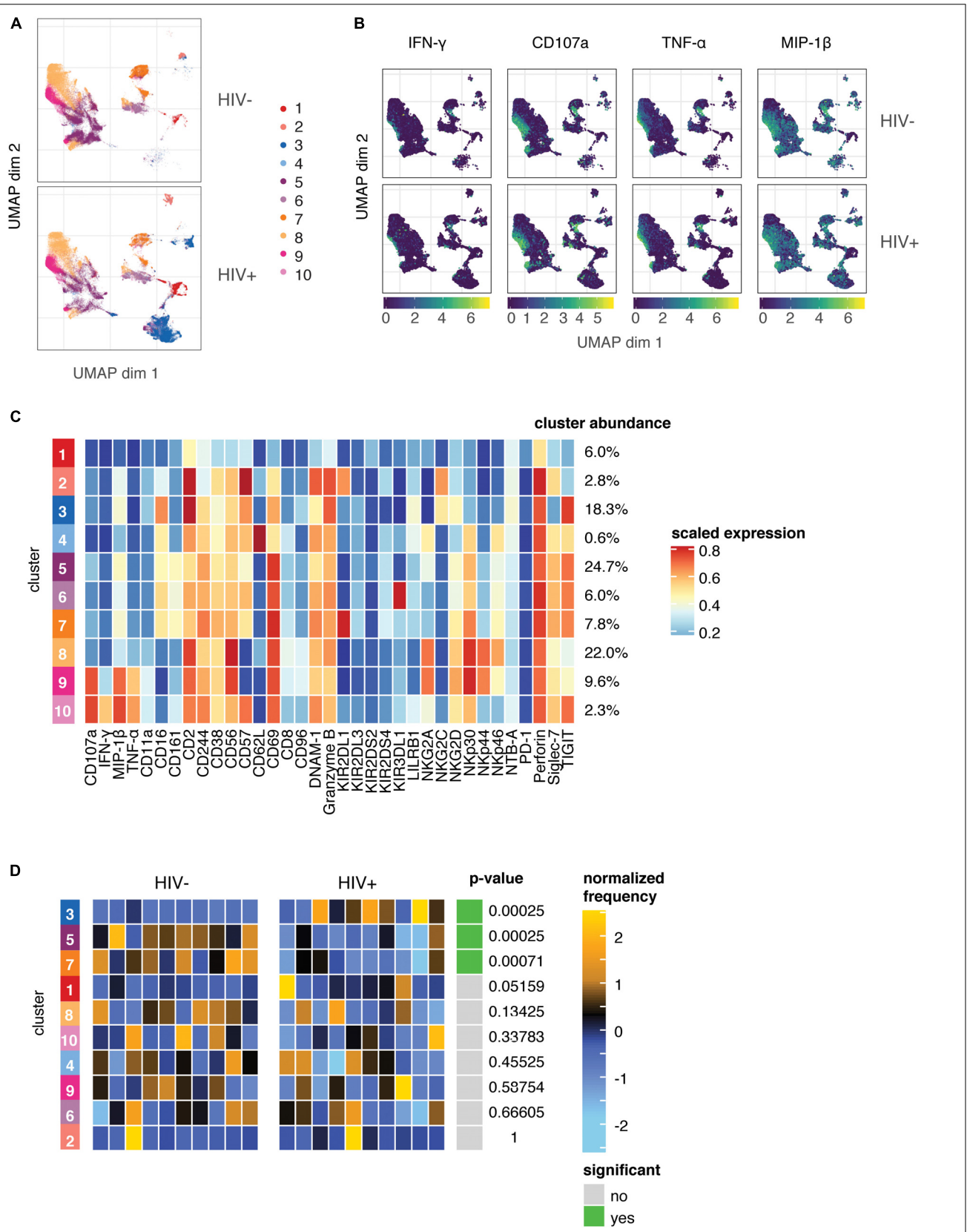

FIGURE 4 | Continued

FIGURE 4 | The predominant changes in the NK cell repertoire of HIV+ individuals occur in NK cell compartments that do not respond to HIV in *in vitro* restimulation. **(A)** UMAP visualization of all NK cells in co-culture with autologous HIV-infected cells in HIV– (n = 10) and HIV+ (n = 10) donors, colored by metacluster identity generated by *ConsensusClusterPlus* metaclustering. **(B)** UMAP visualization of all NK cells from the HIV+ and HIV– groups, colored by expression of functional markers CD107a, IFN-γ, MIP-1β, and TNF-α. Scales show asinh-transformed channel values. **(C)** Heatmap of scaled mean expression of all markers profiled, for each cluster 1 to 10. The abundance of each cluster (% of total cells) is given on the right of the heatmap. Functional markers (CD107a, IFN-γ, MIP-1β, and TNF-α) are on the left. **(D)** Heatmap of the relative abundance of each cluster between the HIV- (left) and HIV+ (right) groups. Each individual column represents a single donor. The heat represents the proportion of each metacluster in each donor, with yellow showing over-representation and blue showing under-representation. These proportions were first scaled with an arcsine-square-root transformation and then z-score normalized in each cluster. Clusters with a statistically significant ($p < 0.05$) difference in abundance between HIV– and HIV+ groups are highlighted in green; adjusted p-values (FDR) are shown beside it.

phenotype of cells that is functionally responsive against HIV; this NK cell subset is shared between both HIV+ and HIV- donors and is similarly responsive in both.

During HIV-1 infection, the NK cell repertoire undergoes significant changes, but even in the setting of virological suppression with ART, the NK repertoire remains altered compared to healthy controls in IL-2 activated NK cells. We found increased expression of NKG2C, CD2, NKp46, and PD-1, and decreased expression of CD244, NKp30, DNAM-1 and NKG2A; many of these markers have been previously described to be altered in HIV infection. The decreased expression of NKp30 and DNAM-1 has been reported for ART-treated HIV-infected patients compared to healthy donors (38, 39); however, these studies also observed lower expression of NKp46 in HIV+ individuals, whereas we saw increased NKp46 expression in the HIV+ group. This discrepancy may be due to the IL-2 activation of NK cells in our study, which is known to increase NKp46 expression (40), and may do so to different extents in the HIV- and HIV+ groups. CD244 expression on NK cells is also known to decrease in HIV infection, although does recover over time after the initiation of ART (41). PD-1 expression is also increased in HIV infection even with ART treatment, and these cells have limited proliferative capacity and may contribute to NK cell dysfunction (42). The increased expression of NKG2C and CD2 may reflect an increase in a subset of NK cells with a more mature, adaptive phenotype that is known to expand during HIV infection, and that persists even during ART treatment (43). Indeed, we observed differential abundance of cluster 3 (**Figure 4**) between the HIV+ and HIV- groups; this cluster, which had greater abundance in donors of the HIV+ group, expressed high levels of CD2 and low levels of Siglec-7, and expressed both NKG2C and CD57, all known features of this subset.

We also identified other clusters of NK cells that are differentially expressed between HIV+ and HIV- groups (**Figure 4**). Clusters 5 and 7 have greater abundance in donors of the HIV- group; cells of these clusters have higher expression of CD244 and NKp30 in cluster 7, which we also identified in our GLM analyses (**Figure 1B**). These differentially abundant clusters may represent the loss of subsets of NK cells that occur after chronic HIV infection or ART treatment. The identification of these phenotypic features of NK cells can provide insight on how chronic, treated HIV infection shapes the activated NK cell repertoire, while the mechanisms and functional consequences of these alterations warrant further investigation.

We identified a unique NK cell subset that has higher functional activity in response to HIV-1-infected cells (**Figure 3**); this subset has the phenotype CD96^{hi} NKp30^{hi} TIGIT^{hi} CD16^{-} CD62L^{-} NKp46^{-}, and its increased functional activity is present in both the HIV+ and HIV- groups. Individually, many of the markers represented in this subset have been previously implicated in immune-mediated control of HIV. For instance, in CD8 T cells, CD96 expression is positively associated with higher CD4 T cell counts in HIV-infected individuals, although CD96^{+} cells produce less perforin upon stimulation with phorbol myristate acetate/ionomycin (PMA/I), compared to CD96^{-} (44). In NK cells, CD96^{+} NK cells from peripheral blood have reduced TNF-α and IFN-γ production following PMA/I stimulation (45). These prior data make it difficult to ascertain whether CD96 contributes to HIV control, but our observation that CD96^{hi} cells have improved functional activity against HIV may reflect different mechanisms of activation between HIV infection and stimulation by PMA/I. In addition, NKp30 expression is induced upon IL-2 stimulation, and is correlated with IFN-γ production and inversely associated with the HIV reservoir, suggesting that cells that upregulate NKp30 upon IL-2 stimulation may have improved activity against HIV (46). This is particularly relevant as our study used IL-2 activated NK cells. TIGIT^{+} NK cells have also been previously implicated in HIV control - TIGIT expansion is markedly enhanced on NK cells in untreated HIV infection (16, 17). We also recently demonstrated that TIGIT expression marks a population of NK cells with an adaptive phenotype with greater functional activity against HIV-infected cells as well as other stimuli (17).

In contrast, the lack of expression of CD16 and NKp46 on these functionally responding cells may reflect downregulation of these receptors after NK cell activation by HIV-infected cells. After stimulation, downregulation of both CD16 and NKp46 have been reported to occur predominantly in activated NK cells that produce IFN-γ and CD107a; this downregulation can occur even in the absence of specific signaling through CD16 or NKp46 (47, 48). As such, these features of this subset may not necessarily reflect their involvement in the HIV-specific response, but instead mark activated NK cells. By using the combinatorial expression of all the markers we have identified, we were able to vastly enrich for cells that were responding against HIV – up to 90% of cells of this phenotype were functionally responding (**Figure 3C**). Although the frequency of this subset is low (0.1-3% of total NK cells, **Figure 3D**), and does not account for all responding cells, the high level of functional activity of these cells suggests that they are important in HIV-targeting activity.

While we did not find evidence of improved memory responses of peripheral blood NK cells in HIV+ individuals upon an *in vitro* re-stimulation with HIV-infected cells, this does not preclude the existence of these memory NK cells in other tissue locations, or in low frequency in the blood. In murine, humanized mice, and non-human primate studies of memory NK cells, memory NK cells were predominantly tissue-resident, particularly in the liver (24, 25, 49). A low frequency of these hepatic phenotype NK cells can be found in peripheral blood (24) and would have been included in our analyses, but may have been at too low a frequency to detect within the bulk of the response to HIV. Indeed, Reeves et al. have previously found that, in non-human primates, memory responses to Gag vaccination by peripheral blood NK cells were much lower in magnitude than splenic or hepatic NK cells, but were still observable (25). Even so, our observation that peripheral blood NK cells did not demonstrate HIV-specific memory responses may be due to the extremely low frequency of these cells, or differences between these memory NK cells in humans compared to mice or non-human primates or with different infections.

There are several limitations to our study. Due to sample availability, we profiled only NK cells in peripheral blood; tissue-resident NK cells may exhibit differences in both phenotype and the ability to generate memory responses, as discussed above. In addition, NK cells were not re-stimulated with the same HIV-1 strain as the primary *in vivo* infection, as we used a separate, *in vitro* infection with a different HIV strain. We used a subtype A strain for all *in vitro* HIV restimulations; however, as all our subjects were recruited in North America, where subtype B strains dominate (50), the mismatch in viral strain used for the secondary challenge may have contributed to the poor memory NK cell responses we observed. Even so, we have shown that infection with HIV-1 viruses across both subtype A and subtype B strains lead to similar patterns of alterations in expression of NK cell ligands on infected CD4 T cells (unpublished data), suggesting that strain-specific recognition is unlikely in NK cells, and the mismatch of strain subtype in primary infection and *in vitro* restimulation would not impair the detection of potential memory responses. Lastly, as our primary interest was in evaluating functional responses to HIV-infected cells, we profiled IL-2 activated NK cells, which may not entirely recapitulate NK cell phenotypes *ex vivo*. IL-2 induces changes in NK receptor expression, including upregulation of the natural cytotoxicity receptors (NCRs) NKp30 and NKp46; however, the expression of most other NK receptors remains unchanged (51). The differences in cytokine-induced upregulation of these NCRs between the HIV+ and HIV− groups can additionally be informative, as upregulation of NCRs have been implicated in control of HIV (46).

In summary, our data demonstrate that phenotypic alterations in peripheral blood NK cells that occur in individuals with ART-treated HIV-1 infection do not result in improved NK-mediated targeting of HIV. These phenotypic changes instead occur in distinct cellular subsets that are not involved in the functional response to HIV. Further work is required to understand whether other tissue-resident NK cells may exhibit differences in phenotypic alterations and functional responses in the course of treated HIV infection.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available at ImmPort (https://www.immport.org) under study accession SDY1620.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Stanford University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NZ and CB designed the experiments. NZ conducted the experiments. NZ and A-MF analyzed the data with statistical analysis input from SH. PG contributed samples to the study. NZ and CB wrote the manuscript. All authors contributed revisions.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Considerations of Antibody Geometric Constraints on NK Cell Antibody Dependent Cellular Cytotoxicity

*Charles D. Murin**

Department of Integrative Structural and Computational Biology, Scripps Research, La Jolla, CA, United States

It has been well-established that antibody isotype, glycosylation, and epitope all play roles in the process of antibody dependent cellular cytotoxicity (ADCC). For natural killer (NK) cells, these phenotypes are linked to cellular activation through interaction with the IgG receptor FcγRIIIa, a single pass transmembrane receptor that participates in cytoplasmic signaling complexes. Therefore, it has been hypothesized that there may be underlying spatial and geometric principles that guide proper assembly of an activation complex within the NK cell immune synapse. Further, synergy of antibody phenotypic properties as well as allosteric changes upon antigen binding may also play an as-of-yet unknown role in ADCC. Understanding these facets, however, remains hampered by difficulties associated with studying immune synapse dynamics using classical approaches. In this review, I will discuss relevant NK cell biology related to ADCC, including the structural biology of Fc gamma receptors, and how the dynamics of the NK cell immune synapse are being studied using innovative microscopy techniques. I will provide examples from the literature demonstrating the effects of spatial and geometric constraints on the T cell receptor complex and how this relates to intracellular signaling and the molecular nature of lymphocyte activation complexes, including those of NK cells. Finally, I will examine how the integration of high-throughput and "omics" technologies will influence basic NK cell biology research moving forward. Overall, the goal of this review is to lay a basis for understanding the development of drugs and therapeutic antibodies aimed at augmenting appropriate NK cell ADCC activity in patients being treated for a wide range of illnesses.

Keywords: antibody, ADCC, NK cell, structural biology, antibody therapeutics, immune synapse, antibody effector functions, immune signaling

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***Correspondence:**
Charles D. Murin
cmurin@scripps.edu

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INTRODUCTION

Antibodies have a bifunctional role within the immune system. This role is physically built into their structure through two parts: the fragment antigen binding (Fab), for recognizing antigen, and the fragment crystallizable (Fc), for recruiting effector immune cells. The process by which antibody-coated cells direct effector cells to attack and kill an opsonized target is known as antibody dependent cellular cytotoxicity (ADCC). This is accomplished through ligation with Fc gamma receptors (FcγRs), which forms a conduit of communication between the target cell (TC) and immune effector cell (1). The FcγRs are an assortment of transmembrane receptors expressed to varying levels on primarily innate, but also some adaptive, immune cells (2).

The ability of antibodies to recruit ADCC is a highly desirable trait for therapeutic and vaccine development, and NK cells are of central focus due to their proclivity for ADCC and as a front-line defense immune cell (3–6). While our understanding of antigen-antibody recognition and Fc-FcγR interaction are each quite extensive in isolation, there is still a gap in knowledge about how these two important aspects of antibodies interplay, especially *in vivo*. Combined with frequent incongruency between available *in vitro* and *in vivo* data regarding antibody effector function as well as the generally complicated nature of the human immune system, we are left with a looming question: what makes an effective antibody for recruiting NK cell ADCC?

Answering the question above requires a much better understanding of the underlying molecular basis of antibody and cellular effector functions. A good place to start is at the point of initial contact between an NK cell and TC, known as the immune synapse (IS). This is the point where activating receptors on the NK cell surface bind to the Fc domain of antigen-engaged antibodies and initialize a cascade of events that lead to NK cell activation and ultimately target-cell death. Extensive studies of the T cell receptor have provided valuable insight into the organization of the T cell IS (7–10), but much less is known about the NK cell immune synapse (NKIS).

Antibodies are necessary for clustering activating receptors in the early stages of ADCC. Structural biology has been instrumental in providing a much more detailed view of this initial interaction of antibody and antigen, especially in the context of viral antigens from HIV, influenza and ebolavirus. Depending on the location of antibody epitopes, the Fc domain of the antibody can differ vastly in how it is presented to a surveying NK cell. Many other variables, including antigen shape, size, and density as well as lipid environment and mobility, can also affect Fc presentation. Further, all these variables can change with antibody isotype, subclass and glycosylation as well as FcγR isotype, cellular subclass, FcγR expression and diversity as well as FcγR glycosylation and alleles (2).

With an increasing number of antibody therapeutics, vaccines and immunotherapies entering the clinical market (11), a greater understanding of NK cell mediated ADCC will guide precision medicine and create more effective drugs. In this review, I will focus on current efforts to understand NK cell ADCC, with a particular focus in the context of virally infected cells. I will explore how advances in microscopy techniques as well as the increasing accessibility of big data technologies such as transcriptomics, proteomics, and metabolomics are challenging our understanding of classical immunology and paving a way to fill the gap between *in vitro* and *in vivo* observations. Such advances will reveal new avenues for vetting therapeutics with the greatest chance of success in patients.

RECEPTORS AND LIGANDS INVOLVED IN ADCC

Humans employ an arsenal of a FcγRs that specifically recognize antibodies via their Fc domains (1, 2, 12). These receptors can be inhibitory or activating for the cells on which they reside, denying or providing the initial spark to perform antibody-based effector functions, respectively. While NK cells almost exclusively utilize a single type of activating FcγR (13, 14), it is important to understand the function of FcγRs more broadly. In this section, I will briefly discuss what is currently known about the receptors and ligands involved in ADCC as well as how their interplay differs among peripheral and tissue resident NK cells.

The FcγRs and Their Antibody Ligands

Each antibody isotype has its own unique Fc receptor, and these have been studied extensively and reviewed elsewhere (1, 12, 15). The receptors include Fc alpha receptor I (FcαRI or CD89) for immunoglobulin (Ig) A (16–18), Fc epsilon receptor I (FcεRI) for IgE (19–21), FcγR for IgG (1, 12) and Fc mu receptor (FcμR) for IgM (22, 23). There is also mixed evidence of a putative receptor for soluble IgD, named Fc delta receptor (Fc∂R) (24, 25). There are additional Ig receptors that reside on other cell types, including the neonatal Fc receptor (FcRn) with a function in recycling antibodies (26, 27), the mixed Fc alpha/mu receptor (Fcα/μR) with a function in endocytosis of IgA/IgM coated microbes (28) and the polymeric Ig receptor with a function in the endocytosis of polymeric IgA and immune complexes (pIgR) (29, 30). Not all antibodies bind to their cognate receptors with equal affinity (31) and each receptor has a unique control over the immune response.

Most antibody therapeutics are overwhelmingly of the IgG class, which is the primary type of antibody formed in response to vaccines and pathogenic threats (32, 33). IgG also makes up a significant portion of the antibodies in human sera to assist the innate immune response in identifying immediate threats and assisting the adaptive memory response. IgGs exist in four known subclasses in humans, including IgG1, IgG2, IgG3, and IgG4 (**Figure 1A**). There are six known IgG receptors, including FcγRI (or CD64), FcγRIIa/b/c (or CD32), and FcγRIIIa/b (or CD16) (**Figure 1B**), and they each display differential binding affinity for these subclasses of IgG (1, 2, 31). Most of FcγRs are activating, signaling through immunoreceptor tyrosine-based activation motifs (ITAMs), with the exception of FcγRIIb, which is an inhibitory receptor and signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRIIIa is the most abundant and important receptor on NK cells for inducing ADCC, and is a prototypic cell marker for mature NK cells in the periphery (34–36). While all the IgGs can bind to FcγRIIIa, IgG1 and IgG3 are the most effective at activating NK cells for ADCC (2, 31). FcγRI has the highest affinity for IgG, particularly IgG1 and IgG3, but is not reported to be found on NK cells (1). Interestingly, there are glycan variants of FcγRIIIa that display affinities close to FcγRI, as I will discuss below (37).

Structural biology has been important in elucidating the molecular nature of the FcγR-Fc interaction and examples exist of every human FcγR both liganded to Fc and unliganded (**Figure 1C**) (38). FcγRs are quite small and are therefore almost exclusively studied by crystallography (12). Small proteins (<100 kDa) are still difficult targets for cryo-electron

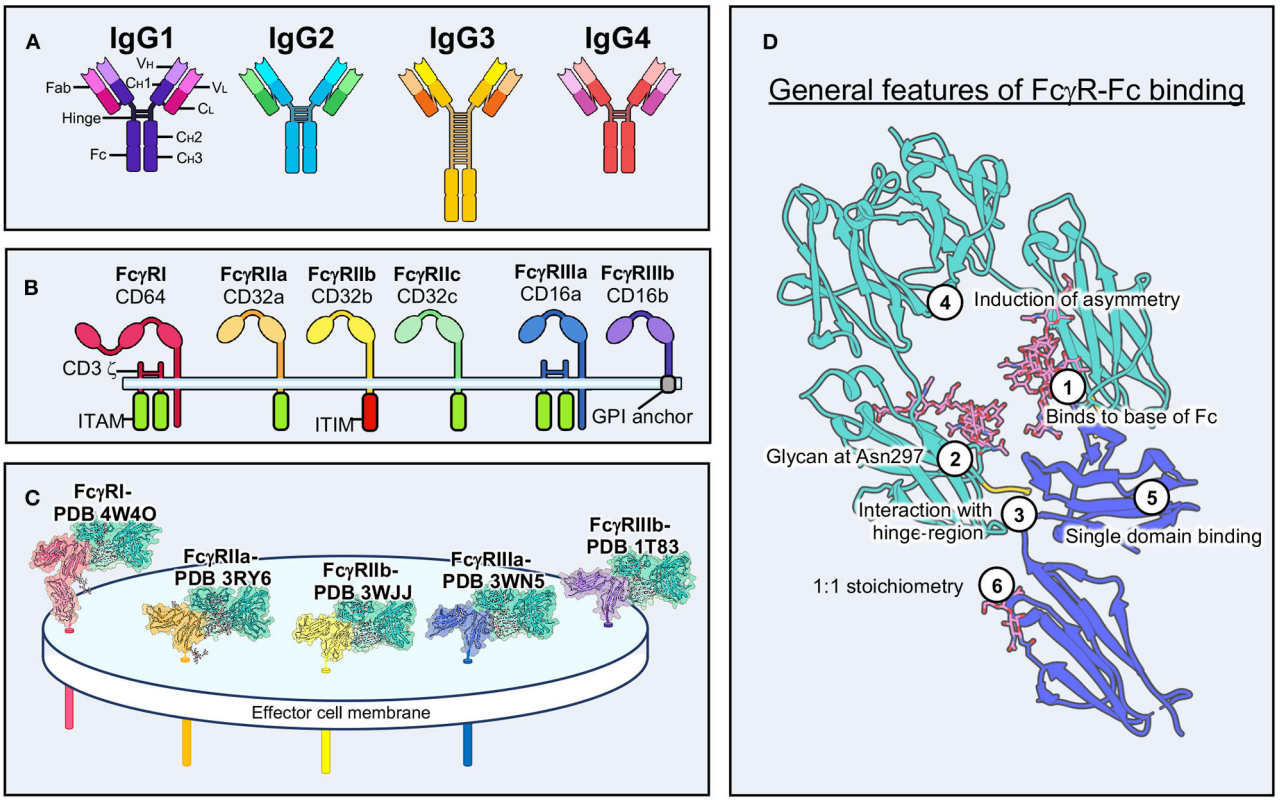

FIGURE 1 | Structural characteristics of antibody-FcγR interactions. **(A)** Schematic of the four IgG antibody isotypes. IgG domains and sub-domains are labeled on the far left. **(B)** Schematic of the six human FcγRs and associated signaling motifs. **(C)** Structural examples of the five main human FcγRs bound to Fc domains with transmembrane domains modeled in the effector cell membrane. **(D)** Conserved features of FcγR-Fc binding. The example shown is of FcγRIIIa (PDB 3WN5). ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; GPI, glycosylphosphatidylinositol.

microscopy (EM) but are becoming increasingly approachable as technology improves (39–41). These receptors adopt an Ig-like fold, similar to antibodies, with two Ig lobes separated by a short elbow (12). Notably, FcγRI has an additional Ig domain, although the function of the third domain is unclear (42).

These structures have shown striking conservation in how IgGs bind to FcγRs (**Figure 1D**). The majority of molecular interactions occur near the hinge-region of IgG, near the base of the Fc, and are heavily reliant upon a glycan at Asn 297 in the Fc domain (1, 42–44). Binding induces asymmetry within the Fc through interaction with a single domain of FcγRs. Despite Fc domains having two equivalent binding sites for FcγRs, binding to IgGs is monovalent, due to this induced asymmetry. The 1:1 stoichiometry of binding is the same for other FcRs, except for FcαR, which is capable of binding IgA as a dimer (18).

IgG glycosylation can take on many different forms and has major implications for the immune response (45, 46). Afucosylated forms of IgG, for example, are capable of a superior ADCC phenotype and structural evidence indicates that this form of IgG allows for a stronger interaction with FcγRIIIa (47–49). FcγRs are themselves glycosylated to varying degrees (50). Glycosylation is often overlooked in the structural context, due to limitations of crystallography, but has a notable influence on activation and affinity and continues to be explored (37).

Cellular and Tissue Distribution of FcγRs

FcγRs exist mainly on immune cells, but have also been found in some neural cells, liver cells and even as part of viral and bacterial defense mechanisms (51). In terms of immunity, the FcγRs clearly dominate in innate immune cells, likely due to their role in first-line defense and surveillance (**Figure 2A**). Conversely, there is little evidence for constitutive FcγR expression within adaptive immune cells such as T cells (although some small subset may express FcγRIIIa) and only the presence of the inhibitory receptor FcγRIIb on B cells (1, 51). Nevertheless, FcγR activation by antibodies can recruit the adaptive immune response and other innate cells, and thus ties both arms of the immune system together (**Figure 2A**). Within the innate cell repertoire, macrophages, monocytes, granulocytes, dendritic cells, and mast cells all express varying combinations of the FcγRs (1, 51). The expression of some receptors can be

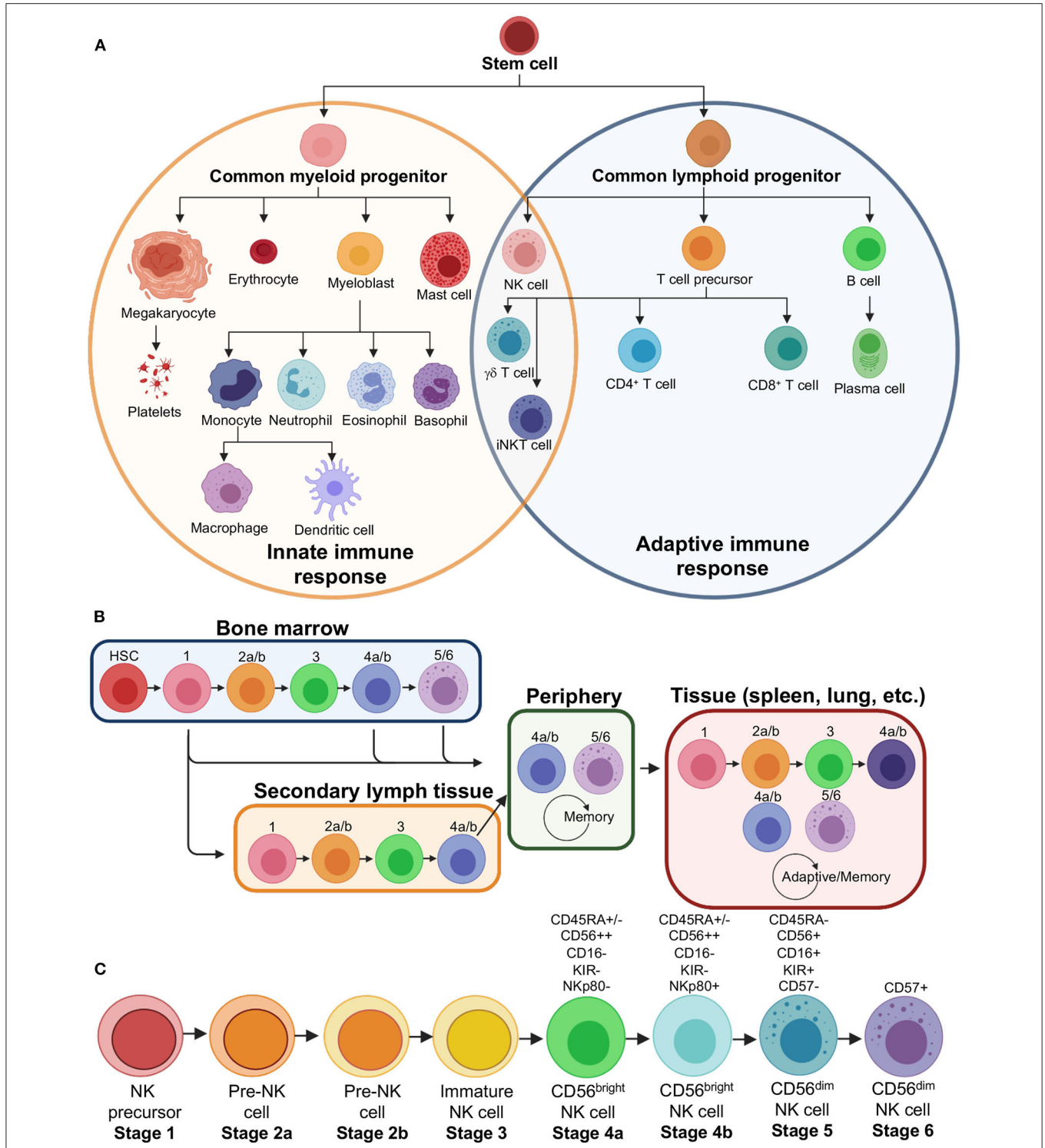

FIGURE 2 | Natural killer cell lineage and development. **(A)** Immune effector cell lineages showing that NK cells derive from a common lymphoid progenitor related to B and T cells, that make up a majority of the adaptive immune response. NK cells, however, share several similarities in function and phenotype to myeloid progenitor cells that make up a majority of the innate immune response. **(B)** Flow chart showing the modern theory of NK cell development, which demonstrates that NK cells may leave bone marrow at various stages and continue development into specialized subsets in the secondary lymph tissue, peripheral blood or become tissue resident NK cells. **(C)** Stages of NK cell development, including distinct sub-stages, with major markers that distinguish mature cell types indicated. HSC, hematopoietic stem cell; iNKT, invariant natural killer T cell.

induced in certain cellular populations, although typically at low levels, or may exist only in a subset of cellular populations. This range of FcγR expression on immune cells is not well-understood but may serve as an advantage to the immune system in being able to quickly respond to a diverse array of insults.

NK cells form a unique cellular subset since they are of the lymphoid lineage, more closely related to B and T cells, but act more like an innate immune cell in their function, are therefore often referred to as innate lymphoid cells or ILCs (**Figure 2A**) (52–54). NK cells are exceptionally diverse, and I will briefly discuss both their presence in peripheral blood (PB) and tissues (**Figure 2B**) (55–59). NK cells form a smaller fraction of lymphocytes within the PB but can vary widely from 5 to 20% or even higher depending on the individual. Typically, peripheral NK cells are defined by a lack of CD3 to distinguish them from T cells, a lack of CD19 to distinguish them from B cells, and the presence of CD45 to distinguish them as lymphocytes. Further, NKs are confirmed by the presence of CD56 and CD16 to varying degrees, leading to so called $CD56^{bright}/CD16^{lo/-}$ and $CD56^{dim}/CD16^{+}$ populations (**Figure 2C**) (60, 61). $CD56^{dim}$ NK cells are thought to be the cellular population that is best at performing ADCC due to a higher constitutive expression of CD16. This makes $CD56^{dim}$ NK cell lines, such as NK-92 cells, particularly desirable for NK cell engineering and use in *in vitro* ADCC assays (62, 63). $CD56^{bright}$ NK cells can respond rapidly to produce cytokines and chemokines in conjunction with the response of other activated cells, including T cells, dendritic cells and monocytes.

The diversity of NK cells extends to tissue resident NK cells (**Figure 2B**) (34, 57, 64). $CD56^{dim}$ NK cells, which predominate the ADCC response in PB, are not found ubiquitously in all tissues and may actually be outnumbered by $CD56^{bright}$ cells overall in the human body (57, 64). The population of $CD56^{dim}$ cells capable of ADCC largely exist in the bone marrow as well as lung, spleen, breast and subcutaneous adipose tissue (57). NK cell diversity is extended by varying degrees of chemokine receptors as well as a huge variety of killer immunoglobulin-like receptors (KIRs) (65). This plasticity specializes NK cells to their environment and makes them functionally distinct (**Figure 2B**). Further, certain NK cells may even develop memory, similar to adaptive immune cells (66, 67).

Limits to Our Current Understanding of Antibody-FcγR Interactions

There are many gaps that prevent a full understanding of antibody-FcγR interactions. First, all of our molecular observations of antibody-FcγR interactions come from fragments. For example, in every structure of FcγRs, the IgG is severed from the antigen recognition domains (**Figure 1C**). Conversely, in every structure of antibody bound to antigen, the Fc fragment is missing. While there are a few structures of IgGs alone, as well as biophysical characterization that demonstrate their range of flexibility and overall architecture, this flexibility has largely restricted their study in complexes due to historical limitations in structural biology (68).

Next, FcγRs are after all membrane glycoproteins, but there are no structures of the full-length receptors. Additionally, many activating receptors use transmembrane adaptors that are necessary for cell surface expression and signaling, proteins that have only limited structural observation in isolation (14, 69, 70). While FcγRs are mostly single pass transmembrane proteins (with the exception of FcγRIIIb, which is a GPI-anchored protein and whose signaling is not well-understood), there are almost certainly higher order assemblies that must form in order for signaling to proceed.

Finally, known molecular observations have not yet been reconciled with the crowded but organized environment of the IS. Most of our understanding of the NKIS has derived from *in vitro* studies outside of a living organism. Although reductionists approaches are necessary as building blocks, these observations must begin to be placed back into a larger context. Below, I will further explore what is involved in the assembly and function of the NKIS in the context of ADCC and how we have amassed this knowledge.

SHEDDING "LIGHT" ON NKIS DYNAMICS

The term "synapse" refers to a junction between cells and is most often used to describe the junction between neurons. This definition has since been expanded to also describe the junction between immune cells and TCs (71–73). While most notably used to describe the T cell IS (72), the term has more recently been expanded to NK cells (74). In both cases, the IS is a delicate ballet of receptors and ligands, cytoskeletal rearrangements and exchange of cytotoxic material in order to specifically destroy a cell deemed a threat. Understanding the players in this immunological dance and how they dynamically move through the process of immunological attack is vital to understanding ADCC and how antibodies affect the process and outcome.

The NKIS can be likened to a busy street corner at rush hour, people and cars crisscrossing and making their way to destinations in a concerted spatio-temporal fashion. Similarly, within the IS, receptors and cell surface molecules must bind to their ligands, signal and move to make way for the next set of molecules to follow suit. Understandably, evaluating the role of an antibody in this context can be extremely challenging. Do we use the detailed approaches of biochemistry and structural biology, medium resolution approaches provided by light microscopy, or more global "omics" types of big data acquisition and analysis? Ideally, details could be obtained equally from any one technique; however, technology has been historically limiting and complete understanding will require integration of all these techniques. In this section, I will provide a general overview of what is known to occur during formation of the NKIS, specifically during ADCC, and how structural biology and light microscopy have brought complementary understanding to this process.

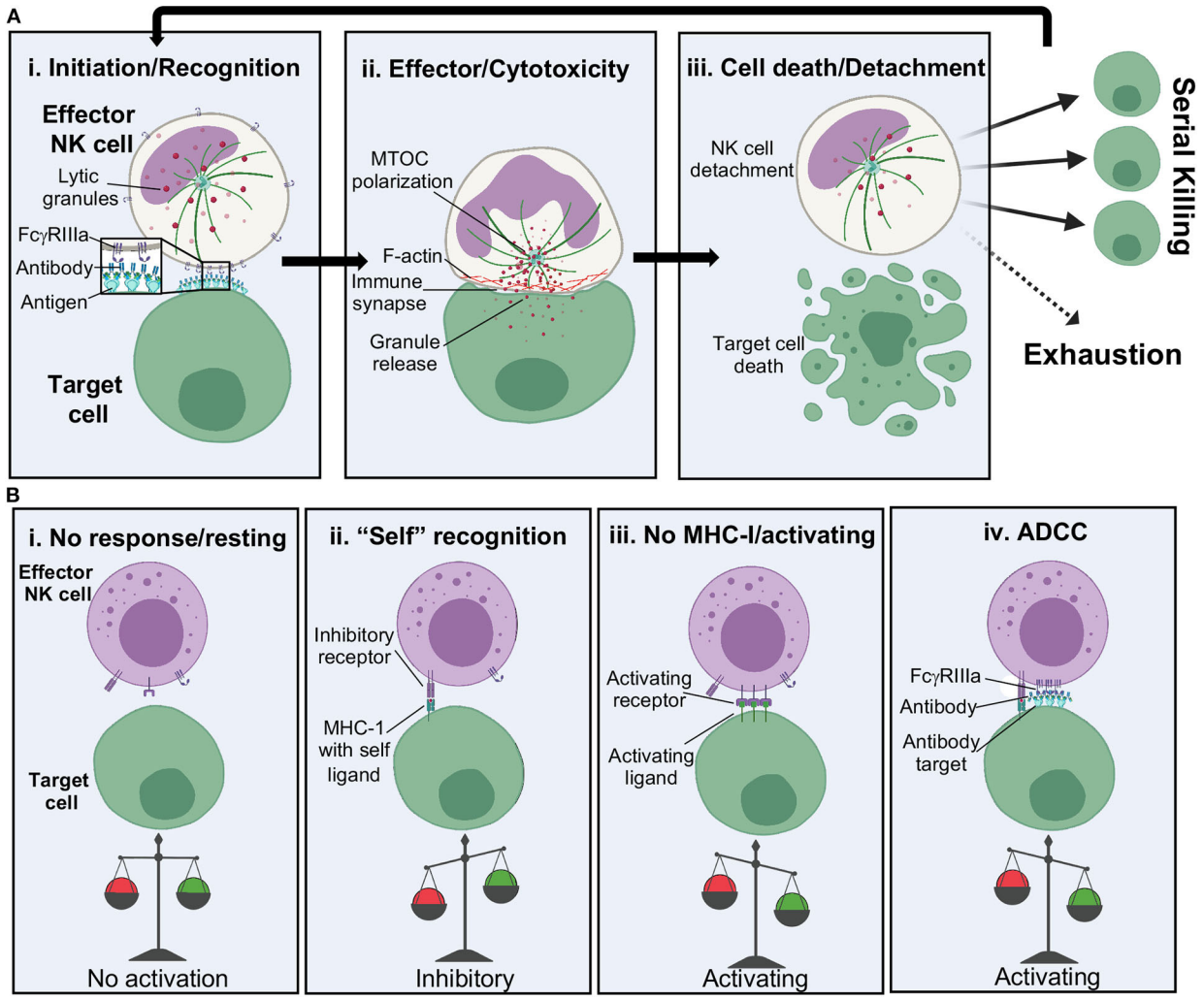

FIGURE 3 | NK cell cytolytic activation. **(A)** Stages of NK cell cytolytic activity including: (i) tethering of effector to target cell and receptor binding, (ii) lytic granule and actin polarization at the immune synapse and release of lytic granules into target cells, and (iii) target cell death and release of the NK cell. NK cells can go on to serially kill or become exhausted. **(B)** NK cell activation relies upon a balance of activating and inhibitory signals. MHC, major histocompatibility complex.

ADCC and the NKIS

The NKIS has been previously studied in detail and the general stages well laid out (**Figure 3A**) (74–77). There are several different types of NKISs (78), but we will focus mainly on the lytic IS here. In every type of synapse, the initial stages are the same, which is that of surveillance (**Figure 3A**i). The process of surveillance involves the tethering of effector to target cell followed by adhesion. Each of these events is not completely well-understood, but likely involves carbohydrate sensing by CD2 (79), selectins like L-selectin (80) and integrins like CD11a/b and CD18 (77), which are upregulated and cluster early in the NKIS (77). These initial steps serve as way to lock effector and target cells together to then proceed to recognition, although some level of pre-activation occurs.

Following attachment to a target cell, it is time for the NK cell to decide: friend or foe? (**Figure 3Ai**) Since NK cells are primed to respond quickly and harshly to threats, their activation relies upon a well-controlled balance of activating and inhibitory signals (**Figure 3B**). Recognition of major histocompatibility complex I (MHC-I) bearing "self" peptides is an important part of this decision but can be overcome by stress signals. For example, certain cancers cause upregulation of stress signals such as MHC class I chain-related protein A/B (MICA and MICB) and UL16-binding (ULBP16) family proteins, which are recognized by the activating NK cell receptor natural-killer group 2, member D (NKG2D), leading to direct killing (81). Further, downregulation of MHC-I can occur during viral infection, also leading to direct killing of infected cells (82, 83). The presence of antibody coated cells can also lead to activation by ADCC. For ADCC to occur, surface expressed FcγRIIIa will recognize antibody bound to the surface of a TC (84), causing the formation of microclusters (85–87). This may be in part aided by concurrent cytoskeletal rearrangements, such as F-actin rearrangement, that is thought to aid in the clustering

of receptors (76, 77, 88, 89) as well as the presence of lipid rafts to assist in fluidity (90, 91). Such rearrangements set the stage for microtububle polarization and delivery of lytic granules present throughout the NK cell cytoplasm to a conduit point (**Figure 3Aii**). Lytic granules bring CD107a to the openings in actin networks and are a tell-tale sign of NK cell activation and cytotoxicity (89, 92).

The release of perforin and granzymes at the synaptic cleft, the point of release of lytic granules, starts to signal the end of ADCC and cytotoxicity (**Figure 3Aiii**) (93). How the NK cell concludes cytotoxicity is still not well-understood, but proteolytic cleavage and shedding of FcγRIIIa ectodomains is thought to contribute to NK cell release (94). Although NK cells have been shown to serially kill multiple targets in a matter of hours, continued stimulation of ADCC via FcγRIIIa can exhaust the NK cell leading to decreased perforin release over time and a slower recovery of FcγRIIIa expression on the surface of NK cells (93–95). Serial killing can proceed until granzyme stores are out, leading to upregulation of CD95L, the ligand for target cell death receptors, resulting in slower apoptosis-mediated killing (96).

In addition to the formation and function of an IS, NK cells also release cytokines and chemokines that can exert effector activity on target cells and help recruit other effector cells, such as macrophages, dendritic cell and T cells as well as the proliferation of additional NK cells (97–99). Such cascades of activity all stem from the initial stages of antibody binding. Thus, elucidating the molecular basis of antibody-based activation of NK cells is fundamental to understanding the regulation of all downstream processes.

Structural Biology as a Tool to Study the IS

Structural biology has been a key driver in our understanding of antibody interactions with both antigen and Fc-receptors. Crystallography has long dominated our understanding of Fab-antigen interactions. More recently, single particle cryo-EM has been increasingly important for determining antibody interactions, including more biologically relevant constructs of antigens and difficult targets. For example, cryo-EM is superbly suited to handle sample heterogeneity, enabling the structural analysis of diverse polyclonal antibody epitopes in a single imaging experiment. This technique has been instrumental in understanding the antibody-based immune response to viral infection as well as novel vaccines (100, 101).

In terms of epitope mapping, the field of infectious disease has exemplary examples of survivor-derived monoclonal antibodies bound to viral entry-associated proteins, which my colleagues and I recently reviewed (102). Such examples include, but are not limited to, HIV, influenza, ebolaviruses, marburgviruses, SARS, MERS, Hepatitis, Chikungunya virus, Zika virus, Dengue virus and Noroviruses, among many others. Antibodies are capable of binding to nearly any epitope presented on enveloped viral antigens (**Figure 4**), however their capacity to induce ADCC varies widely (103–105). The reason for such variance is unknown but may be related to where an epitope is located and the way in which an antibody binds, as well as genetic variation in FcγRs (106). The Fab alone bound to antigen can give clues to how the Fc may be situated and how this relates to receptor binding and macromolecular complex assembly (**Figure 4**).

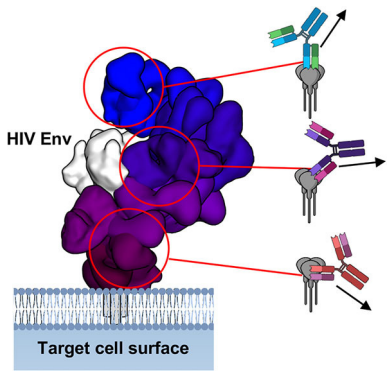

FIGURE 4 | Fab-antigen structures give clues to antibody Fc presentation. Overlay of structures of Fab-HIV Env interaction, demonstrating a wide range of antibody angles-of-approach. On the right are schematics of how the full IgG would bind and the direction in which the Fc may point toward approaching effector cells. Figure adapted from Murin et al. (102).

By far the most important contributor to antibody ADCC activity studied so far is antibody subclass and glycosylation. While the basis of subclass remains somewhat of a mystery, we do have substantial evidence as to the importance of Fc glycosylation (43, 47, 49, 105, 107, 108). Specifically, if the glycan at Asn 297 is fucosylated, then the binding to FcγRIIIa is impaired (43, 47–49, 109). With removal of this core fucose, however, affinity is bolstered to the low nanomolar level. Further affinity can be gained from di-sialylated, complex glycans lacking core fucose, which also have strong anti-inflammatory properties (107, 108, 110). Nuclear magnetic resonance (NMR) studies suggest that the type of glycan attached to the Fc modulates Fc dynamics as well through the C'E loop that contains Asn 297 (111, 112). Conversely, FcγRIIIa glycosylation itself can also influence binding of IgG, hinging primarily upon a single glycan at Asn 162 (107, 108, 110, 113). Indeed, there are vast donor-specific differences in monocyte derived FcγR glycoforms that could influence the effectiveness of antibody therapies as well as donor-derived cell therapies (110). A more complete understanding of glycosylation effects on ADCC, as well as innovative ways to generate uniform and specific antibody glycosylation targeted for donor phenotypes, is an area of active research.

Experimental Setup for Imaging the IS

Fluorescent light microscopy provides the unique advantage of being able to observe live cells, illuminating IS dynamics such as the spatial arrangement of receptors and ligands over time. Work in this area has been pioneered by the study of the T cell IS, which has been extensively reviewed elsewhere (7, 9, 76, 114–119). Study of the T cell IS has been in part driven by a more reductionist approach

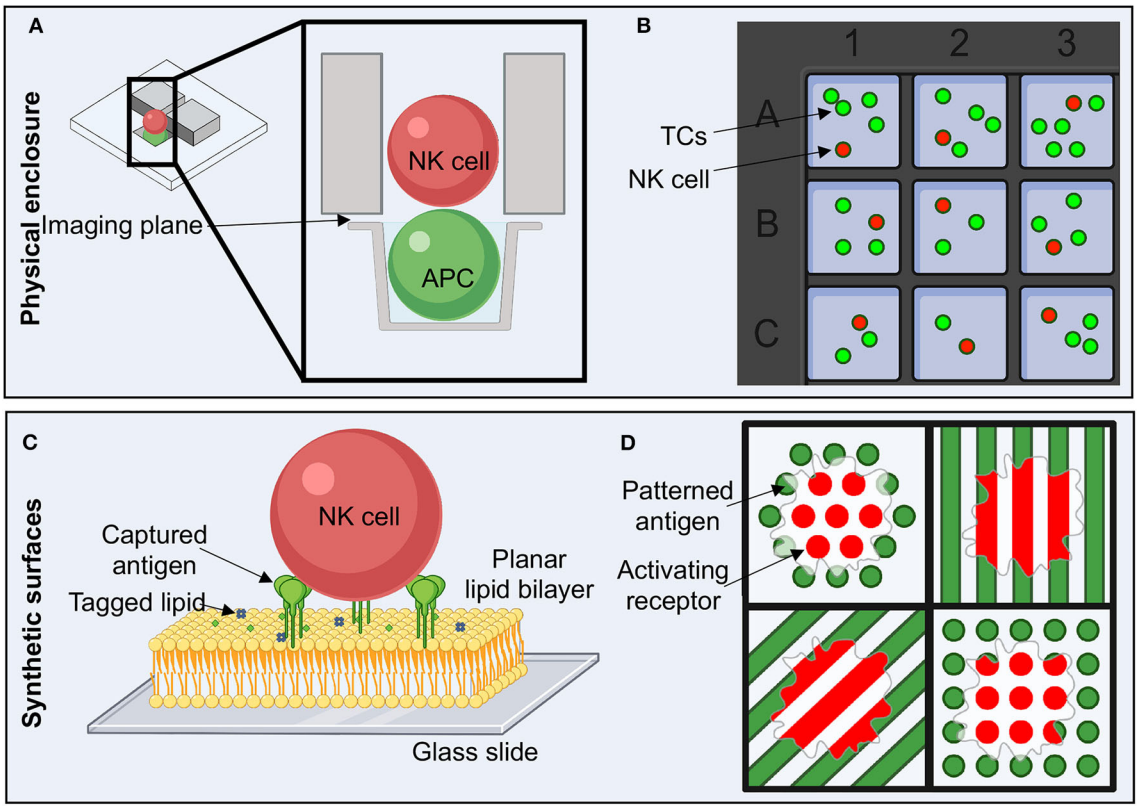

FIGURE 5 | Experimental setup to study NK cell activation and the immune synapse. **(A)** Flow cells with microwells allow the physical isolation of single cell pairs to image the immune synapse in the z plane or *en face*. **(B)** Microwell plates enable the isolation of a small number of TCs and NK cells in order to study live, non-adherent cells. **(C)** The use of planar lipid bilayers on glass slides is a reductionist approach to studying the NK cell immune synapse. Antigens can be attached to chemically modified lipids, enabling ease of titrating antigen density and type as well as lipid composition. **(D)** Activating ligands can be pattered in order to determine how spatial and geometric constraints affect many aspects of NK cell activation. TC, target cell.

to parse the very complex IS into more digestible pieces. These techniques have now been adapted to study the NKIS as well.

The first obstacle to overcome when addressing the question of the role of antibodies within the IS is how to set up and observe single cell interactions. This is especially critical in ADCC since antibodies will influence the earliest stages of IS formation, and therefore timing is crucial. There are several technologies that have been developed to address this issue, but they fall into two major categories: imaging live cells enclosed in a physical space and imaging live effector cells interacting with a synthetic surface representing a TC (114). I will briefly touch on a few examples here.

The first technique of imaging live cells requires physical isolation of these cells (**Figures 5A**,**B**). One approach for this has been the design of microfabricated wells, which are limited in diameter for single cells, but deep enough to allow a second cell to stack on top (120). This allows the imaging of the z-plane between the two cells where all the action of the IS takes place. Similarly, microfluidic chambers can trap pairs of cells and provide both face-on and side views of the IS (**Figure 5A**) (121). Both of these techniques have the advantage of high throughput but suffer from limits in imaging resolutions inherent to the microscopy techniques required for live cell imaging. Optical tweezers, which can capture an TC and present the IS to the focal plane of the microscope, also offer an intriguing solution for examining the NKIS in real time with improved resolution, however, without high throughput (122–124). This technique has been useful in predicting the effectiveness of chimeric antigen receptor (CAR)-modified T cells and may have similar usefulness for CAR-NK cells (75).

Cells can also be trapped within microchambers, which limits the range that non-adherent cells can move (**Figure 5B**) (125). This allows the free movement of effector cells in real time and facilitates tracking of single cell movement. With the additional implementation of acoustic signals, cell to cell interaction is stimulated, which allows for increased observations (126–128). Here we are not looking directly at the IS, but rather looking at whole cell behaviors within the context of a more "real" environment. One can envisage the grafting of tissues into these chambers to observe NK cell infiltration or to add different antibodies into media within chambers to observe the effects on whole cell dynamics within the context of ADCC.

A more reductionist approach to studying the NKIS utilizes synthetic forms of TC surfaces (**Figures 5C,D**). These systems are convenient for varying the type of antigen and also introducing spatial and geometric constraints. Supported lipid bilayers (SLBs) have gained traction in their utility to study the IS, as they allow for maintenance of the type of fluidity that would be encountered in cell membranes (**Figure 5C**) (85, 114, 129–134). There are many different ways in which to assemble SLBs, which have historically also found high utility for studying the electrophysiology of ion channels, pumps and transporters (131, 135, 136). For utility in biology, SLBs are typically formed by generating lipid micelles in solution and then depositing these onto ultra-clean glass slides (85, 137). Bilayers can integrate capture lipids, for example that contain nickel or streptavidin on their head groups, that can subsequently bind tagged antigens (**Figure 5C**) (137–139). In this scenario, antigen density can be titrated, or lipid composition can be easily adjusted.

Antigens or activating ligands can also be deposited directly onto substrate in predefined patterns using printing techniques (**Figure 5D**) (120, 140). This option allows for well-defined spatial constraints that can assist in probing how discrete patterns or geometries influence cellular activation and the organization of the IS, even within a single cell. For example, in an experiment where activating and inhibitory molecules are placed in distinct patterns, NK cell actin cytoskeletal rearrangement is more intense and patterned around activating signal patterns than inhibitory patterns (**Figure 5D**) (141). While this technique suffers from the static nature of the antigen presented, distances may be tightly controlled, and multiple different antigen-antibody complexes could potentially be examined simultaneously.

Imaging the IS With Fluorescent Microscopy

Fluorescent microscopy imaging techniques can provide a range of temporal and spatial resolution (117, 142–144). While some techniques allow dynamic temporal resolution, such as the tracking of events in real time, these often suffer from physical constraints that do not allow high spatial resolution. Wide-field fluorescence microscopy (WFM) gains back spatial resolution from deconvolution methods that allow sharpening of signal post-image acquisition (142). Laser scanning confocal microscopy (LSCM) is also quite often utilized due to ease, but loses temporal resolution due to slow scanning speeds, which impede looking at fast events like those happening in the IS (121). Here, spinning-disk confocal microscopy (SDCM) allows for quicker acquisition (10 to 100-fold over LSCM) with lower photobleaching (117). Total internal fluorescence (TIRF) microscopy has limited z-axis resolution but is quite useful for analyzing the IS, which occurs in a narrow plane (145, 146). Two-photon fluorescence microscopy (TPFM) can complement TIRF by allowing similar resolution but with the ability to look at subcellular properties (117, 147).

Conversely, spatial resolution shines in the realm of super-resolution techniques (76, 85, 89, 115, 142, 148). This type of microscopy is not limited by the wavelength of light like the above examples. Stimulated emission depletion (STED) microscopy uses two lasers to activate and immediately deplete fluorophores, offering the ability to image smaller volumes (115, 149, 150). STED can technically be used for live cell imaging but is still slower than SDCM and has limitations with fluorophores. Single-molecule localization microscopy (SMLM) techniques (151), such as photoactivated localization microscopy (PALM) and stochastic optical reconstitution microscopy (STORM), utilize special photoactivatable probes that can indicate the single XYZ (more resolution in XY and less in Z) location of molecules (152–154). SMLM techniques can be used on live cells but are more practical with higher resolution in fixed samples. More complicated equipment as well as image analysis algorithms have been developed to offer insight into T cell activation in 3D within living cells (148). Lattice light sheet fluorescence microscopy (LLSFM), for example, offers the next generation for studying live cell IS events, with much faster Z slice image acquisition than SDCM along with super resolution (155, 156). However, LLSFM will have increased utility once cost and complexity both go down.

Future Techniques for Understanding the IS

The future of imaging and understanding the NKIS will rely upon two major factors. One will be the marrying of high-resolution techniques, offered by electron microscopy for example, with those of resolution limited techniques, such as light microscopy. The other will be making new technologies more widely available to biologists, which is only a matter of time (157, 158). Such a renaissance has been seen in the field of electron microscopy with the advent of user-friendly microscopes and data analysis software (159). As far as the former, I will highlight a few exciting developments to keep an eye on in the coming years.

In the realm of electron microscopy, the aspirational goal is to achieve sub-nanometer resolution of proteins and complex macromolecular systems *in situ*. Since most high-resolution techniques rely upon averaging, this is not readily possible, but more advanced techniques in cryo-electron tomography (cryo-ET) are quickly closing this gap (160–162). Phase plates, ideal for tomographic techniques to increase image contrast, have been instrumental in solving complex macromolecular assemblies within cells. Additionally, focused-ion beam (FIB) milling instrumentation allows for exquisitely thin and detailed cell sections to be isolated (**Figure 6A**) (163, 164). Cryo-ET examples include cytosolic and mitochondrial structures of actively translating ribosomes (165), complex actin and microtubule network assembly (166, 167), and intriguing views of the neural synapse (168). To examine the nuclear pore complex, detergent solubilization and removal of nucleic acids has enabled thinning samples as much as possible while maintaining 3D structure. Combined with integrative structural biology techniques, we now have the most detailed views of the intact nuclear pore complex ever seen (169). Cryo-ET, however, is labor intensive and requires a high level of expertise that has not become as streamline as single particle analysis. However,

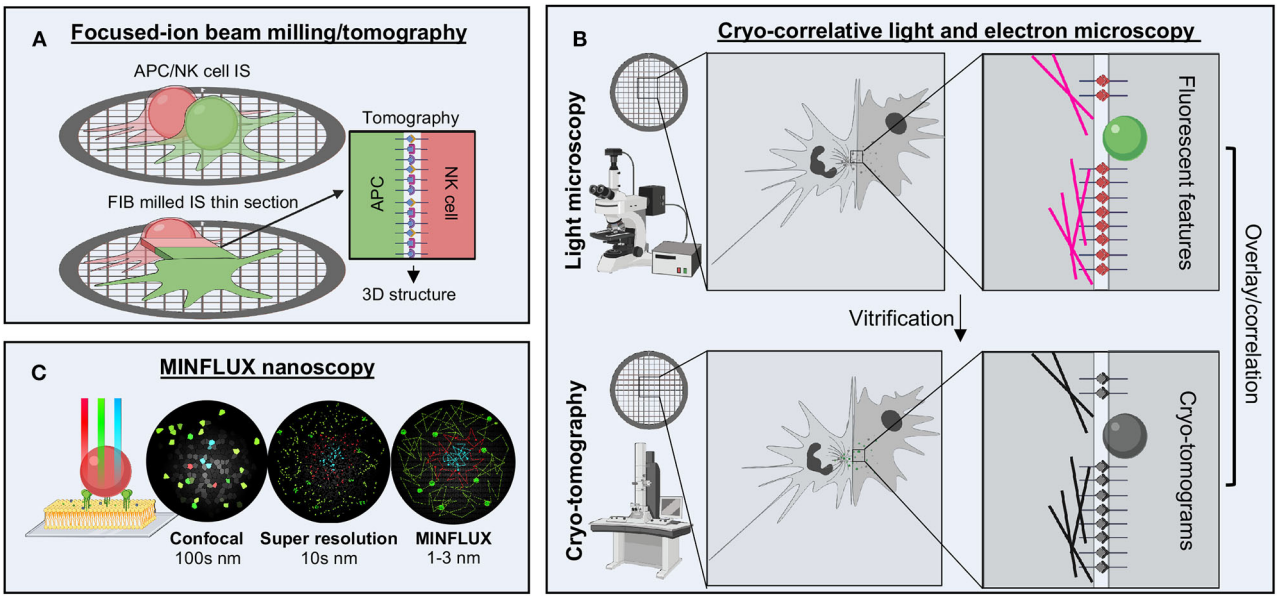

FIGURE 6 | Future techniques for studying the NKIS. **(A)** FIB milling combined with tomography enables the generation of a thin layer to image the molecules bridged between cells. **(B)** Cryo-CLEM enables the localization of several structures within the immune synapse by super resolution microscopy followed by higher resolution structural analysis by tomography, which can be overlaid to provide additional details not offered by either technique alone. **(C)** MINFLUX nanoscopy offers the highest spatio-temporal resolution available of any light microscopy techniques, going beyond the limits of confocal and super resolution, down to the range of 1–3 nm. FIB, focused-ion beam; nm, nanometer.

the field is rapidly moving toward automation and increased sophistication in data analysis of cryo-ET data. This field holds great promise as a tool for examining cell to cell contacts, such as the NKIS (**Figure 6A**).

Cryo-correlative light and electron microscopy, or cryo-CLEM, attempts to fill the gap between light and electron microscopy (**Figure 6B**) (170, 171). In this technique, whole cells are first imaged using fluorescent microscopy techniques to localize and identify features of interest. Next, cells are vitrified and imaged using cryo-ET, allowing for identification of features and subcellular location of the somewhat higher-resolution electron density maps generated. Genetically encoded fluorescent proteins allow the maintenance of cellular integrity and examination of fluorescence post-vitrification, or samples can be fixed, permeabilized and stained prior to freezing. Super resolution techniques are also starting to be combined to provide even more details (172–174). Cryo-CLEM may be a way to more accurately identify the location of signaling proteins within the IS and then extend results to the high-resolution context through electron microscopy.

Within the realm of light microscopy, a new technique has recently broken all the previous barriers associated with resolution limits, including spatial and temporal limitations as well as photobleaching effects. Known as MINFLUX, this revolutionary technique combines the super resolution techniques of PALM/STORM with those of STED by establishing the coordinates of proteins through minimal emission fluxes (**Figure 6C**) (175–177). This allows for nanoscale precision on the order of 1–3 nm spatial resolution. Moreover, this technique is adaptable to both scanning and standing-wave microscopes and can be used on fixed or live samples as well as in 3D. Tracking of single molecules within live *Escherichia coli* cells over long distances as well as highly detailed, multicolor labeling of the nuclear pore complex have been the earliest examples (175, 177). Clearly, this technique could be adaptable to tracking multiple different receptors within the NKIS. The only drawback at this current point is expense and availability.

GEOMETRIC AND SPATIAL CONSIDERATIONS WITHIN THE NKIS

IgGs are highly abundant within the human body at any given time, on the order of 7.5–22 mg/mL. Therefore, for ADCC to be an effective strategy for targeting cellular insults, NK cells must distinguish between free and specifically bound antibodies. This is thought to be achieved by the aggregation and agglutination that antibodies undergo upon antigen binding, whether to cell surface exposed antigens or soluble (178). This brings Fc domains in close proximity, allowing the clustering of cellular receptors. However, this explanation does not account for the sophisticated arrangements that signaling receptors must adopt in order to propagate a real signal, nor does it explain the reason and mechanisms associated with the variety of antibodies, receptors and glycoforms that exist. There is evidence to suggest that antibody arrangement is crucial for effector functions to proceed, that geometry and spacing can tune responses and that antibody allostery may also assist in regulating cellular activation. Below,

I will discuss more detailed current knowledge of the early stages of antibody-based signaling and activation and provide examples that point to the concerted molecular underpinnings of effector functions.

Initial Stages of ADCC

Once an NK cell has docked with a potential target, if opsonized antibodies are present, then FcγRIIIa will subsequently bind to those IgGs. Alone, the binding affinity of FcγRIIIa for IgG is estimated to be in the high nanomolar range (at least *in vitro*) but also depends on the genotype of individuals (31). For activation to proceed, however, the affinity between FcγRIIIa and IgG must be strong enough to allow for sustained interaction. IgG affinity is provided by the aggregation of IgG on immune complexes, increasing antibody avidity. Antibody aggregation is necessary because FcγRIIIa must adapt to a molecular arrangement that allows intracellular phosphorylation of cytoplasmic domains. Such an arrangement provides a platform for kinase binding and activity that is absent in monomeric FcγRIIIa. It stands to reason that there must be discreet forms of FcγRIIIa activation complexes beyond what simple aggregation implies. Indeed, there is evidence to suggest that such a form could be dimeric, as we will discuss more in the next section.

Once antibodies have successfully bound to the α subunit of FcγRIIIa through the ectodomain, this signal must be propagated to the intracellular side of the NK cell. This is achieved by co-stimulatory signal adapter molecules, which for FcγRIIIa is either FcεRI g or CD3 ζ (CD247) (**Figure 1B**) (13, 69, 70). These adapters were first attributed to the FcR for IgE (179, 180) and the TCR complex (181, 182), respectively, but are also adaptable to FcγRIIIa for ADCC. The γ or ζ adapters exist as a single pass transmembrane protein that forms a dimer through a cysteine bond (183–185). There appears to be no preference for either as they are found equally associated with FcγRIIIa (184). Together, the adapter homodimer and FcγRIIIa monomer are thought to form a non-covalent three-helix bundle (184). Mutations that dissociate adapter molecules from FcγRIIIa have been shown to prevent cell surface trafficking and are also thought to prevent FcγRIIIa degradation (184, 186).

ADCC Signaling

Although the overall structural motif of the macromolecular signaling complex has yet to be elucidated, once FcγRIIIa self-associates, downstream signaling can then proceed. The γ or ζ activating adapter molecules contain cytoplasmic tails with ITAMs (187). In the proper conformation, these ITAMs can be phosphorylated at two of 6–8 tyrosine sites, setting up a docking site for Src-family kinases. It may be possible that Src kinases dock and rely on a specific dimeric motif of associated FcγRIIIa and adapters for the kinases to dimerize themselves and auto phosphorylate, structurally similar to what has been shown for the JAK2 kinases (188). Indeed, Src dimerization is predicted to be necessary as its role as a hub for multiple signaling activities (189–191).

Once phosphorylated, the signaling adapters are ready for recognition by Syk or Zap70, for example, through tandem SH2 domains (192–194). Syk or Zap70 interaction with phosphorylated ITAM domains leads to the downstream activation of several signaling pathways. Concurrently, FcγRIIIa cross-linking activates PLC-gamma enzymes to generate inositol 1,4,5-trisphosphate (IP3) and *sn*-1,2-diacylglycerol (DAG), leading to Ca^{2+} release from stores within NK cells, which is required for granule release. FcγRIIIa cross-linking also activates PI-3 kinase, which produces additional signaling molecules to assist in ADCC-associated activation activities. Additional associated signaling pathways include the Ras, ERK2, MAPK, Vav/Rac, and NFAT pathways. Each of these pathways leads to activities such as actin reorganization, cellular proliferation and cytotoxicity. Further, the JAK/STAT pathway can also be secondarily activated, leading to upregulation of cytokines and chemokines, recruiting other cells or enhancing effector functions (195).

Signaling is a highly complex and multicomponent process that can change depending on extracellular stimuli. For example, antibody activation via FcγRIIIa sets up ADCC with a particular response, but that response differs from direct cytotoxicity or activation inhibition (78, 196). Increased understanding of the complex signals that occur during NK cell activation will help us to understand how to modulate ADCC activity, perhaps through new designs of antibodies or a synergistic combination of antibody and small molecule.

Receptor Movement and Lipid Composition

The initial stages of ADCC as well as the formation of the NKIS are both intrinsically linked to composition of the cell plasma membrane. Here is where membrane bound receptors interact both extra- and intracellularly to generate a robust reaction on the cellular level. Once thought to be a somewhat homogeneous environment, the cellular membrane is actually a circus of different elements, composed of a wide range of lipids that tightly control many cellular activities, including immune signaling. While the evidence for how lipid composition of cells is organized and influences cellular activities is not wholly realized, due to the difficulties associated with studying lipid composition *in situ*, there is still some compelling data that warrants discussion, especially in relation to immune signaling and ADCC.

The composition of eukaryotic plasma membranes is primarily of glycerophospholipids, with head groups attached to at least one unsaturated acyl chain (197, 198). While these lipids are sufficient to form a bilayer, it is known that sterols and sphingolipids also make up a large portion of the plasma membrane. The sphingolipids can be further classified into ceramide-based sphingomyelin or carbohydrate-based glycosphingolipids, both which are often saturated in their acyl chains. Of the sterols, cholesterol is the principle component. Both of these additional lipids are of much lower abundance in internal membranes but are made in the ER and Golgi and transferred to the cell surface. The composition of the inner and outer leaflets of the plasma membrane is also known

to be different, with cholesterols and sphingolipids thought to preferentially reside in the outer leaflet.

More than any other component, cholesterol changes the properties of the bilayer by increasing rigidity and reducing permeability, while still allowing free-lateral movement of proteins and lipids. Model lipid studies indicate that cholesterols and sphingolipids form distinct domains within the more fluid background of the plasma membrane, often referred to as lipid rafts, and that transmembrane proteins can be included or excluded from these domains based on their own physical properties (199–202). Within the outer leaflet, GPI-anchored proteins are enriched (203). These are subsequently linked to the inner leaflet by signaling proteins that are preferentially found in this portion of the plasma membrane, forming signaling platforms.

Evidence for such signaling platforms, especially in regard to FcγRIIIa as well as its associated signaling domains, is compelling in NK cells (90, 204–208). Immunoregulatory elements can be preferentially partitioned within different lipid environments, with positive signaling components such as the Src and Lck family kinases being found in cholesterol rich lipid rafts, while negative regulators such as phosphatases are excluded from these regions. By clustering these elements within microdomains, signaling can be more easily and readily achieved. Although NK cell ADCC is not associated with GPI-anchored proteins, many activating immune receptors are indeed GPI-anchored, which also suggests that immune signaling is biased toward lipid rafts.

Evidence suggests that negative regulation of NK cell cytotoxicity results from blocking the association of activating receptors within lipid rafts (**Figure 7A**) (204, 205, 207). It is thought that actin cytoskeletal rearrangement assists in the association of lipid rafts containing positive regulatory components within the immune synapse (208). If negative regulatory components dominate the signaling platform, then actin rearrangements can be blocked, thus limiting the rearrangements of downstream signaling components to lipid rafts containing primarily positive signaling elements. This may explain why actin cytoskeletal rearrangement is one of the earliest and fastest physiological responses to NK cell activation.

Lipid rafts likely alter the way in which signaling components interact once liganded to a target cell. When considering the many ways in which an antibody bound to its target antigen could be presented to FcγRIIIa, it is important to consider how such geometric and physical constraints may be affected by the more rigid confines of a lipid rafts. Indeed, cholesterol enrichment seems to be a harbinger for more efficient NK cell cytotoxicity (209), but there is no evidence to address how this affects ADCC or how the physical arrangement of antibodies within a macromolecular signaling complex may influence activation. Conversely, the lipid environment of a target cell could also affect how antibody-bound antigens are presented within the IS (**Figure 7B**) (210). Much less attention has been paid to the target cell side of NK cell cytotoxicity; however, some evidence suggests that lipid composition is vital to the sensitivity of target cells to attack

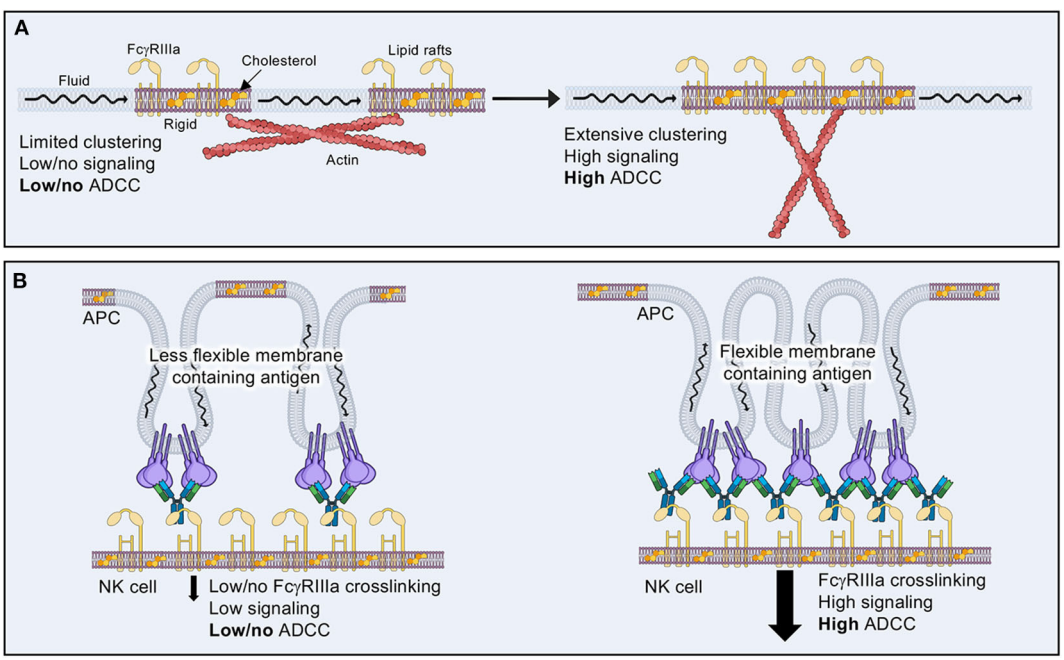

FIGURE 7 | The effects of lipid composition on ADCC. **(A)** Lipid rafts containing activating receptors and higher levels of cholesterol tend to be more rigid and isolated in the plasma membrane of resting or inactive NK cells. Actin cytoskeletal rearrangements are thought to aid in bringing lipid rafts together in order to allow tighter clustering of signaling molecules and increased cytolytic activity. **(B)** Target cell membrane lipid composition may aid or inhibit NK cell ADCC by how well antibody coated antigen is able to cluster, thus promoting FcγRIIIa receptor clustering.

(210). There is much room for exploration in the realm of lipid composition and its influence on ADCC, which may in turn have important implications for the choice of antibody used for immunotherapeutic purposes.

Spatial and Geometric Constraints Within the IS

For NK cell signaling to occur, extracellular signals must be propagated across the cellular membrane. This necessitates some type of unique arrangement of proteins that differentiates a resting cell from a cell that is detecting something in the extracellular environment. In the case of ADCC, this starts with understanding the arrangement of FcγRIIIa. Is there a singular structural motif that must be achieved in order for activation to occur? Or is the arrangement of these receptors more stochastic and tunable to the subtleties defined by extracellular factors? Like most realities of biology, the answer likely lies somewhere in the middle.

In an analogous system, the T cell immune synapse, much work has already been done to answer these questions, ultimately setting a paradigm for lymphocyte-based signaling (181, 183, 185, 211–215). Similar to FcγRIIIa, the T cell receptor (TCR) is composed of extracellular domains that recognize peptide bound MHC-I. These domains, a heterodimer of α and β domains, are single pass transmembrane proteins that must also pair with adapters to propagate signal. Nucleation is accomplished with the CD3 hexamer, comprised of heterodimers of CD3 $\gamma\varepsilon$ and CD3 $\delta\varepsilon$ as well as CD3 $\zeta\zeta$, which is one of the same adapters used by FcγRIIIa. The resultant supramolecular complex is thought to be the basal unit for signaling and requires very tight spatial interaction, which was recently resolved by cryo-EM, revealing a crisscrossing network of transmembrane subunits (185). Ligand binding does not induce any obvious structural changes, with the caveat that this structure utilized glutaraldehyde fixation. There are some single molecule data as well and NMR studies that suggest that reorganization within the TCR signaling complex may occur upon ligand binding still (216, 217). Previous structural data also suggested that a complete signaling complex in solution may be dimeric (211). It is thought that the antigen-bound TCR then interacts with actin and other signals to function as a mechanosensory unit (123, 218).

Studying microcluster formation and dynamics of signaling kinases that are anchored to the plasma membrane has been critical to our understanding of TCR signaling and provides many lessons for studying the NKIS. Studies using PALM revealed that in cells activated on glass coverslips, associated signaling molecules like LAT and SLP-76, which links to actin filaments, form in much smaller nanoscale sized clusters than previously postulated (146, 219, 220). The increased spatial resolution of PALM also revealed that signaling could occur in nanoclusters that may only contain signaling units as small as dimers of the TCR as the minimum requirement for signaling (221–224). Complementary studies using light sheet STORM of activated T cells from mice showed similar types of spatial organization occurs *in vivo* (225).

On the antigen side of immune activation, antigen presentation and spacing seems to be critical for thresholding T cell activation. Several studies using nanoscale spacing of activating molecules suggest that differential activation can be achieved depending on the space provided within the IS (114, 212, 226, 227). One study integrating both lateral and axial spacing of antigen determined that tight 2D clusters with limited axial spacing of <50 nm was an ideal arrangement for T cell activation (212). Such spacing is thought to fortify clustering based activation while excluding CD45, which must exit the IS in order for activation to proceed. Another study concluded individual activated TCRs may contribute more to T cell activation than overall clustering (223). This adds some clout to the idea that there is a necessary arrangement of the TCR that qualifies activation, potentially a dimer as previously suggested.

For ADCC-based activation in NK cells, there is evidence to support a signaling complex may involve FcγRIIIa dimers, which have greater appreciable binding to IgGs (14, 70, 184, 228). Dimers are quite prevalent throughout signaling biology and are thought to generate universal platforms for kinases with broad activity (**Figure 8A**) (181, 191, 213, 229–232). Cytokine mediated signaling provides the richest examples of dimer mediated signaling, with a large diversity of structures induced by cytokine binding (229, 230, 233). There are many additional examples of homo- and heterodimeric complexes that drive signaling, including growth factor receptors, insulin and other hormone signaling receptors and nuclear receptors (**Figure 8A**). In each of these cases, dimerization may occur in several different stoichiometries and can orient dimers in a plethora of ways. Further, toll-like receptors also require extracellular antigen-based dimerization for signaling to occur (**Figure 8A**) (234). Given the diversity of dimerization in signaling, it seems highly likely that ADCC signaling may follow a similar type of organization.

The idea of signaling dimers in ADCC has been previously postulated. Artificial dimers of FcγRIIIa were sufficient to reproduce NK cell activity and conversely increase affinity for IgG (228). Additionally, ectodomains of FcγRIIa within a crystal lattice suggest a possible domain arrangement that may extend to other FcγRs. Mutation of critical residues in this dimeric interface demonstrated reduced cellular activation but not ligand binding (235). Later structures of FcγRIIa demonstrated a new dimer interface potentially that serves as an activating arrangement of signaling as it could reasonably accommodate two opposing Fc domains as well as ligand bound Fabs (**Figure 8B**) (236). The authors proposed a model where constitutive dimers exist on the cell surface in an inactive arrangement that changes upon ligand binding, posing the intracellular signaling domains in an active formation. Perhaps this is similar to the "rotation model" of other signaling motifs, where less flexible intracellular domains are opened up for phosphorylation upon extracellular ligand binding (**Figure 8C**) (233). FcγRIIa, unlike the other FcγRs, does not require adapters for signaling, having its own cytoplasmic ITAMs. It is not clear if

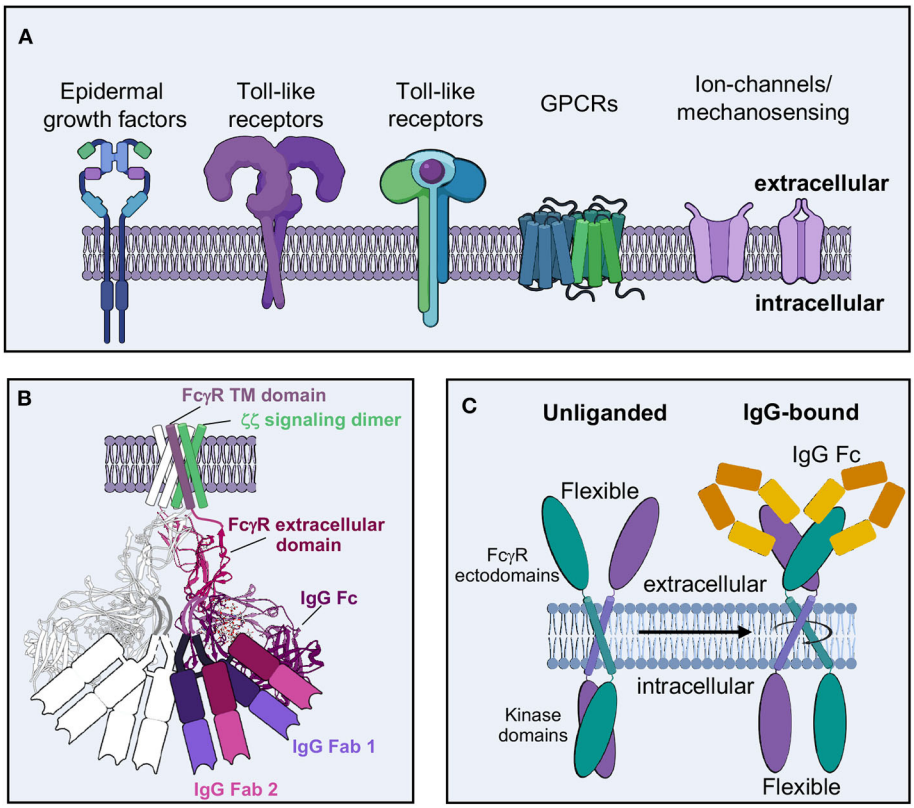

FIGURE 8 | Molecular basis of effector cell activation. **(A)** Dimers are prevalent molecular motifs in cellular activation throughout the immune system. **(B)** Potential dimer model of IgG-FcγR-CD3 ζζ activation complex, based off structures of complex components [FcγRIIb-Fc from PDB-3RY6 overlaid on FcγRIIb crystal contact dimers from PDB-3RY5; model of transmembrane assembly of CD3 ζζ structure from PDB-2HAC and model of FcγRIIIa/adapter assembly from Blazquez-Moreno et al. (184)]. **(C)** Potential model of downstream antibody-mediated signaling based on the "rotation model," which postulates that intracellular kinase domains are exposed for phosphorylation upon proper dimer assembly when extracellular activating ligands bind.

FcγRIIIa or other FcγRs exist as a similar constitutive dimer on the cell surface. Achieving a similar dimeric arrangement of other FcγRs ectodomains may be a critical component for successful ADCC and additional effector activities, as I will discuss in more detail below.

There is evidence for other types of dimeric signaling motifs that suggests that the immune response is not "on or off" in these structural motifs but rather may be tunable. For example, the cytokine Erythropoietin (EPO) and its associated receptor EpoR can be changed in their association topology by diabodies that re-orient their geometry and lead to differences in intracellular signaling (230). Other examples, such as tumor necrosis factor (TNF) signaling as well as some prokaryotic chemoreceptors such as Tar, further demonstrate that dimer formation alone is not always sufficient for signaling and that conformational changes may be additionally necessary (68, 232). Throughout all these examples, there seem to be thresholds and variations in geometries that lead to the idea that cellular activation can be tuned in almost any type of signaling event, possibly even NK cell ADCC.

Complement Dependent Cytotoxicity and Comparison to ADCC

Complement is thought to be a more ancient form of immunity (237); therefore, principles of complement, especially for IgGs, may have important implications for ADCC as well. Complement is another function of the modular antibody that leads to target cell death without the need for effector cell activity. In complement, antibodies binding to cell surface antigen set the stage for additional complement associated proteins to assemble an activation platform ultimately leading to complement deposition and effector cell phagocytosis or assembly of the membrane attack complex (MAC) (238). A plethora of studies have previously set the molecular requirements for complement assembly, along with many isolated structures of complement related proteins, but it was tomography that really started to reconcile these observations (239–241).

Revisiting a fortuitous observation of the first full length structure of a human antibody (242), Diebolder and colleagues tried to understand possible functional consequences of Fc interactions seen in crystal packing, seeding hexameric arrays

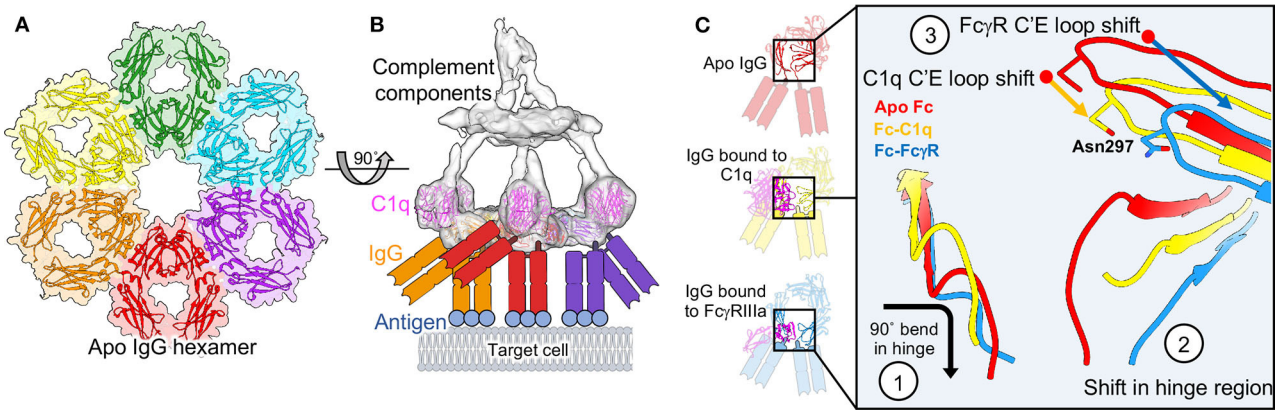

FIGURE 9 | Molecular basis of complement and comparison to ADCC. **(A)** Fc hexamers form the basis for complement deposition (PDB-1HZH). **(B)** Tomography of complement deposition indicates that IgG Fc domains bend parallel to the antigen presenting cell, presenting the binding domain for C1q (EMD-4232, PDB-6FCZ). **(C)** Both C1q and FcγR binding induce similar conformational changes in the Fc domain compared to apo-Fc including (1) a 90 degree bend in the hinge-region loops, (2) a shift in the position of the hinge-region loops and a widening of the base of the Fc and (3) a shift in the position of the C'E loop, including the position of Asn 297 and the associated glycan (Apo Fc from PDB-1HZH, Fc-C1q from PDB-6FCZ and Fc-FcγR/FcγRIIIa from PDB-3WN5).

(**Figure 9A**) (239). These hexamers were reminiscent of evidence indicating that IgG would need to form these types of structures in order for the complement cascade to proceed (243). Mutations that limited Fc-Fc interaction lead to decreases in complement activity while supporting mutations increased activity. Tomographic structures of assembled IgG on antigen presenting liposomes displayed the same type of hexamers seen in crystal packing (**Figure 9B**). Further, it was observed that Fc domains bend into a plane parallel with the antigen presenting plane, presenting the epitope for the first complement protein C1q, which was also observed in their structures when assemblies were made in the presence of complement proteins (**Figures 9B, C**). Interestingly, only a single Fab of IgG was necessary for complement deposition, freeing up the rest of the IgG structure to assemble properly (**Figure 9B**). Bispecific antibodies actually performed better in complement assays.

Later EM observations of pentameric and hexameric IgM also showed very similar structural assembly for complement, with many additional proteins and observations added on (240, 241). In these observations, both Fab arms of the IgM were bound to antigen, which suggests that either IgM and IgG antigen recognition geometries are different, or there is a dependence on antigen type. Previous studies on IgM alone indicated that these antibodies exist in a pre-bent shape that may help to influence complement binding, whereas IgGs tend to be much more flexible in solution (244–246). More recent high-resolution cryo-EM structures of IgM as well as IgA are beginning to give us even more detailed insight into how full-length antibodies operate pre- and post-receptor binding, hopefully providing lessons that can extend to other immunoglobulins (22, 247).

A recent structure of Rituxumab Fab bound to CD20 indicated that the antibody recognizes CD20 dimers and that Fab-Fab interactions are important to antibody binding (248). The authors presented some evidence that these types of Fab-Fab interactions may promote hexameric assemblies important for complement deposition. Fab-Fab and/or Fc-Fc interactions may be a much more prevalent antibody adaptation as there are more examples in the literature, such as the Fab-Fab interactions for some malaria antibodies (249).

Most intriguing is the folded presentation of Fc in both IgG and IgM complement assemblies, that is similar to those seen in Fc-FcγR structures (**Figure 9C**) (42, 239). Although FcγRs can bind free IgG, perhaps a folded Fc domain is required for proper FcγR dimer assembly and signaling. Varying degrees of Fc presentation based on antibody binding angle or the number antigens engaged by an IgG simultaneously may modulate FcγR dimerization, thus tuning the immune response and leading to the differential innate activities observed by antibodies with identical phenotypes but varying levels of ADCC.

Antibody Allostery

The idea of antibody allostery or "intramolecular signaling" has been discussed for many decades and remains a debated topic in the antibody field (**Figure 10**) (250–253). This hypothesis proports that the Fab and Fc domains of an antibody communicate through structural properties inherent to antibodies. To that end, antigen binding in the variable domains would change structural conformations of constant regions, priming them for ideal FcR binding that would be lacking in the apo form of the antibody. However, the reigning theory behind antibody structure-function relationships is that Fab and Fc operate independently of each other, with the former binding to antigen and the later binding to FcRs without one affecting the other directly (178). Antigen-induced aggregation of IgG is thought to increase the relatively low affinity of receptor binding affinity through avidity and induce receptor aggregation that is required for downstream signaling. Without antigen, the 1:1 interaction of IgG and FcγR remains weak and/or does not induce receptor clustering even in high affinity interactions like FcγRI. While there is an ample amount of evidence to support

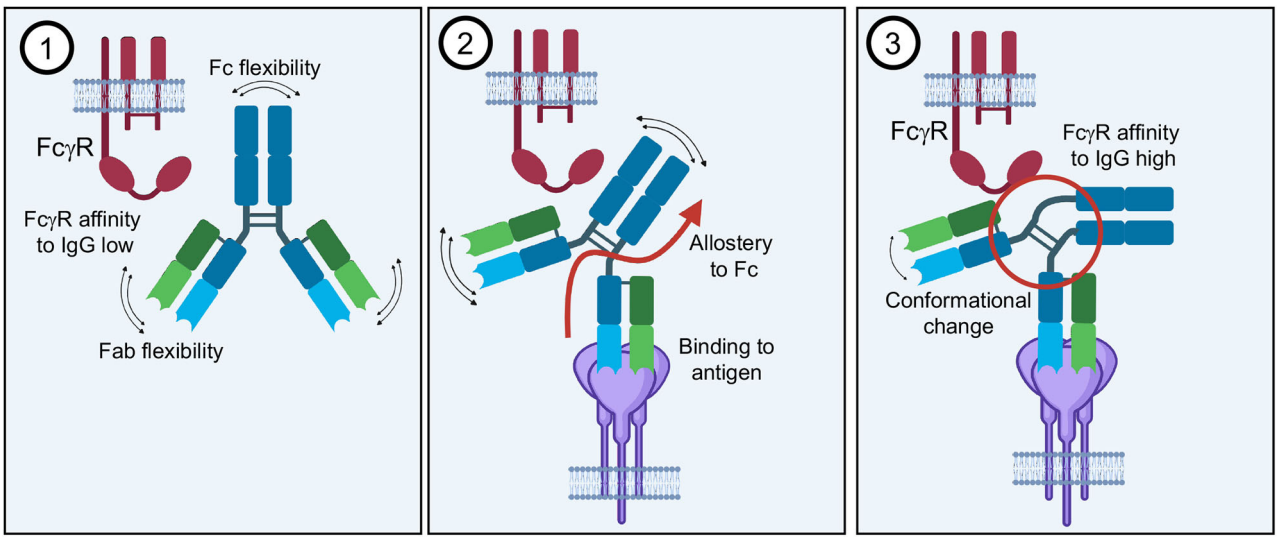

FIGURE 10 | Antibody allostery. The theory of antibody allostery or "intramolecular signaling" asserts that (1) an apo-IgG has flexibility that impedes its affinity to FcγRs. (2) Antigen binding generates an allosteric signal from the Fab to Fc domain. (3) Allostery causes a conformational change in the Fc as well as decreased flexibility that facilitates FcγR binding with higher affinity.

this type of associative cooperativity, there continue to be models and complementary evidence that antibody allostery may play a subtle but significant role in Fc-mediated effector functions.

Studies of antibodies in solution demonstrate that there is a large degree of flexibility associated with the antibody structure, mostly likely imparting a wider sampling space for a diversity of target antigens (**Figure 10**) (244, 245). Furthermore, crystal structures of intact IgGs also indicate that the hinge-regions are largely responsible for this flexibility, although these regions tend to be poorly resolved and in the confines of a crystal lattice (68). However, detailed dynamics studies of antibody behavior in solution provide computational evidence that antigen binding changes the sampling space of Fc as well as the flexibility, providing a type of "rigidity" that is not found in unbound antibodies, which may lead to greater FcγR affinity in the presence of antigen (254).

There is some direct evidence to suggest that Fab binding also has consequences for the constant region of an antibody, at least within in the Fab domain. Comparison of several crystal structures of native and liganded Fab demonstrate changes that occur within the Fab hinge as well as elsewhere in structures (255). Although earlier studies comparing bound and unbound Fab run contrary to these observations, it appears that there are some antigen/antibody specific properties that may account for variability. This may also point to an explanation for differing innate properties of antibodies depending on the epitope.

Moving toward the Fc end of the antibody, there are several studies that indicate that perturbations to the Fc region can affect binding to antigen, for example with Pertuzumab (as an IgA1 and IgA2) and Her2 binding (256). Similarly, many other studies have shown that using identical variable regions with different constant regions (i.e., isotype or subclass switching) leads to variable antigen binding as well (257). Further, an antibody's ability to neutralize viruses is possibly linked to its ability to bind to FcγR and perform effector functions (258). Although indirectly, the above evidence suggests that Fc and Fab are intrinsically linked.

Looking back to complement, we now know that antigen binding is indeed required for the formation of IgG hexamers, leading to a bending of Fc and Fc-Fc interactions (239). Since these hexamers do not form stochastically in solution, and since membrane bound antigens likely do not stochastically form the hexameric shape required for complement deposition, a system of allostery seems a likely explanation for how antigen binding leads to complement associated macromolecular assembly (259). Higher resolution studies of the complement activation complex, perhaps using single particle studies, may be required to confirm this and to piece together what intermediate IgGs look like moving into the complement-bound state (259, 260).

Detailed structures of full IgGs alone will also provide critical data showing the structure of Fc in solution when not influenced by neighbors within a crystal lattice. Indeed, comparing structures of free Fc and Fc bound to FcγR show that FcγR binding induces a change in Fc from symmetric to asymmetric, which precludes a second FcγR from binding (**Figures 1D**, **9C**) (12). It would be most intriguing to determine if antigen binding pushes Fc toward a more asymmetric shape, perhaps facilitating FcγR binding. The increasing capacity of cryo-EM to solve structures of very small, Fc-sized molecules as well as to deal with the type of sample heterogeneity associated with IgGs will prove to be a valuable tool toward these efforts.

Together, these data collectively fit into a model of leukocyte variability in relation to effector functions and suggest a mechanism that inherently must include allostery. This is not to suggest that antigen induced aggregation is not necessary, but

that there are likely complementary mechanisms that lead to effector functions.

Reconciling Antibody-Antigen and Antibody-FcγR Structures

Given the above discussions, we are led to the idea that antibody angle-of-approach and Fc presentation may indeed play an important role in how well an antibody performs NK cell ADCC. Many structures of antibodies in complex with viral antigens show that antibody Fab can bind at multiple angles-of-approach (**Figure 4**) (102). This may suggest that the Fc domains of these antibodies are differentially displayed to the immune system as well. How close antigens are to each other, as well as their individual size and shape, could influence how Fc domains are presented as well as the level of avidity experienced (**Figure 11A**). There is some evidence to suggest that where an antibody binds on antigen may influence innate effector activity for HIV (261), influenza (262, 263) and Ebola viruses (104, 264), among other examples, but there is not yet convincing molecular data to provide a general model that ties these observations together.

Given the space requirements for receptor signaling to occur between cells, along with the details of structures of both Fab-antigen and Fc-FcγR complexes, there is some suggestion that antibody Fc and upper-hinge must be presented in a specific way in order for FcγRs to recognize them, with the Fc folded over parallel to the effector cell surface (**Figures 8B**, **9B,C**) (2, 12). This provides space for every type of FcγR so far described to dimerize in the extracellular region without clashing with bound Fc (**Figure 9B**). This type of binding is well-suited to antibody structure, which is a unique ligand because of flexibility between antigen and receptor binding domains provided by hinge-regions. The degree of flexibility can vary depending on subclass and may correlate with FcγR binding and activation activity. In this model, antibodies could be quite effective at assembling the ADCC activation complex over a wide range of antibody binding angles but may be inhibitory in certain situations (**Figure 11B**). Additionally, this model also provides for the tuning of activation that could result from less than ideal but still permissive geometries of FcγR dimer assembly. However, there are currently no structures of full-length antibody bound to antigen and/or FcγRs that would provide the types of details required to reconcile structures of isolated domains in the context of the IS. This field is ripe for discovery and will benefit from future studies that reflect those already accomplished in the T cell field.

NEXT-GENERATION APPROACHES TO STUDYING NK CELL ADCC

Much of our understanding of NK cell ADCC, as well as antibody effector function in general, is based on individual biochemical studies or fragmented structural biology. Tying these data together is proving to be difficult and is only beginning to paint a picture that seems pervasive throughout immunology, which is that the immune system is dynamic and variable as well as extensively complex. Therefore, many scientists are beginning to rethink altogether how they approach such difficult questions as those regarding antibody effector function. The next wave of impactful research toward defining realistic and meaningful hypotheses about NK cell function and how to design better therapeutics will almost certainly derive from taking a "bigger picture" approach, such as offered from omics-type studies. Below, I will describe what these approaches are and how they are being used to answer questions regarding NK cell activation.

Transcriptomics, Proteomics, and Metabolomics

The term "omics" refers to the collective, encompassing and complete study of a particular aspect of biology (265). The major contributors to integrative omics techniques, now referred to as "systems biology," are genomics, transcriptomics, proteomics and metabolomics. These techniques refer to, respectively, the collective and unbiased study of the genes, RNA, proteins and metabolites that make up single cells, tissues or whole organisms (266, 267).

Historically, these fields have been quite niche owing to the immense amount of time, expertise and expense associated with using any one of them for analyzing a biological question. For example, sequencing the first human genome is estimated to have cost $2.7 billion US dollars and took nearly 15 years to complete. However, advances in technology have not only made these techniques much cheaper and faster, but they are now accessible to nearly any scientist in any field. Much of this success has stemmed from advances in computing, automation and bioinformatics that can handle the massive amounts of samples and data needed to be analyzed. Only recently have these techniques found a foothold in the service-based scientific industry as well as core technologies at many academic institutes. We are now beginning to see immunologists exploit omics as a means of hypothesis generation and an exciting new way to tackle the study of disease, donor immune response variability and mining for distinct cellular subsets (268).

Omics Studies on NK Cell Activation

Historically, NK cells are distinguished by cell surface markers which tend to lump NK cells into two populations as cytotoxic and regulatory, $CD56^{bright}$ and $CD56^{dim}$, respectively (35). However, transcriptomic profiles of NK cells are beginning to demonstrate a wide range of heterogeneity within NK cell populations. For example, one study utilized single cell RNA sequencing (scRNA seq) to determine transitional populations of NK cells within bone marrow and PB that exist between these two examples (269). Another group demonstrated that NK cells exhibit organ-specific transcriptional profiles (270). Overall, these studies indicate an ability of NK cells to adapt to their location and to maintain plasticity during development. Additionally, transcriptomics has been able to distinguish up to 29 different immune cells types based off of their gene expression profiles, offering a useful tool to study NK cell activation in the context of multiple immune cells or within a whole organism (271, 272).

NK cell activation has also been shown to induce unique transcriptional profiles depending on the type of activation

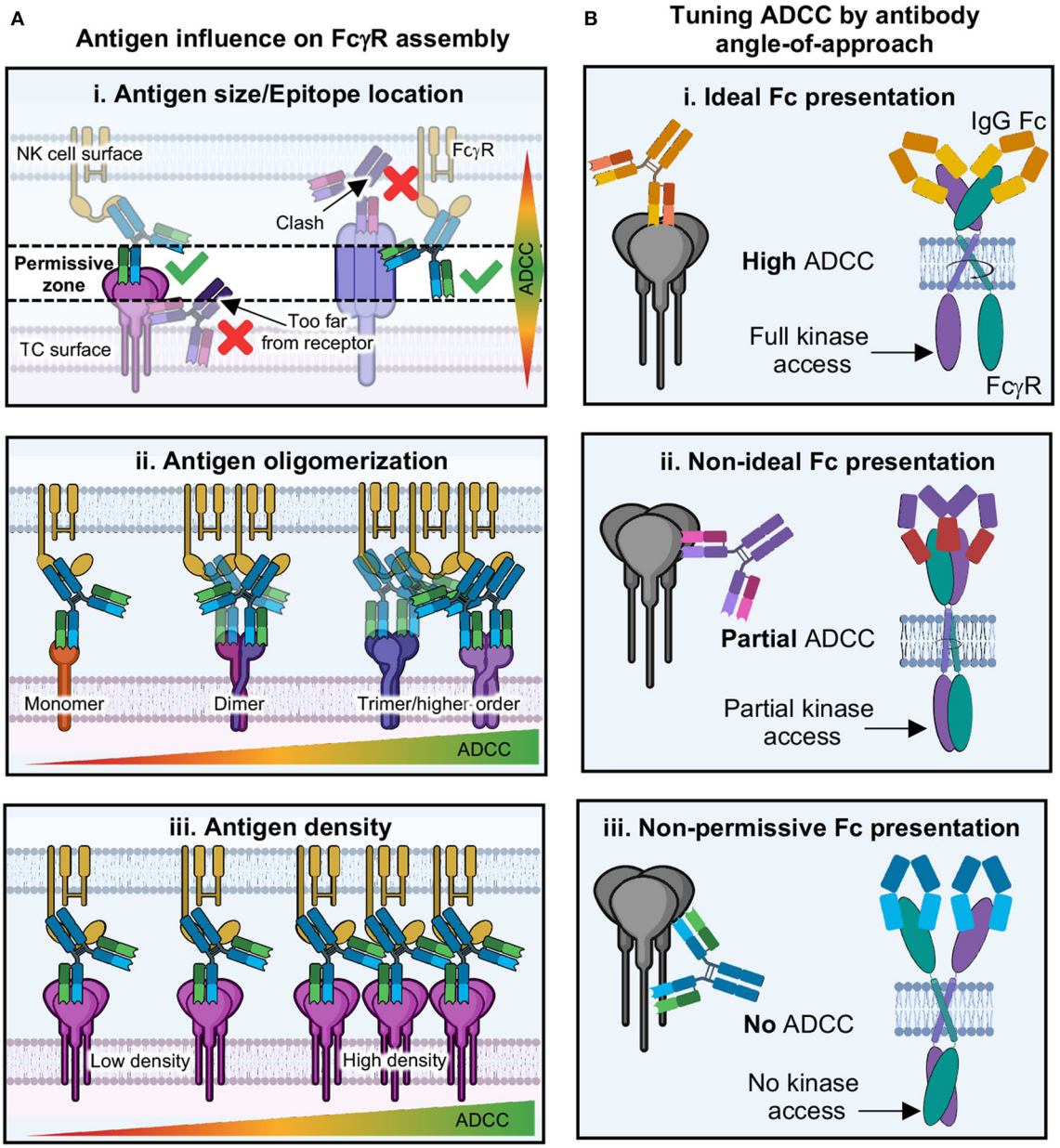

FIGURE 11 | Proposed model of antibody tuning ADCC through activation complex geometry. **(A)** The immune synapse involves a finite distance between target and effector cell; therefore, the spacing requirements present a "permissive zone" in which antibodies can bind. (i) antigens that are short (i.e., Ebola GP) require antibodies to bind to epitopes on the top of the antigen, while antigens that are longer (i.e., influenza HA), require antibodies to bind lower down on the antigen in order for proper binding to FcγRs. (ii) Antigens that are monomeric may be less likely to facilitate FcγR dimerization/aggregation, while those with higher oligomers present multiple epitopes that increase avidity and chances for FcγR clustering. (iii) Antigens that are of low density may prevent ADCC due to the inability for enough FcγRs to cluster. Conversely, those with higher antigen density present more opportunities for antibodies to induce FcγR clustering and thus higher levels of ADCC. **(B)** (i) If antibodies bind to antigen in an epitope that presents Fc domains in a way that allows FcγRs to dimerize in an ideal way, then intracellular domains can be properly phosphorylated and ADCC is potent. (ii) Potentially, antibodies could bind at non-ideal epitopes, but this may still allow FcγR dimerization, although non-ideally thus preventing full intracellular domain signaling motifs from forming, resulting in partial ADCC. (iii) Some epitopes may present antibody Fc in geometries that physically will not allow FcγR dimerization, thus blocking kinase access and resulting in no ADCC.

stimulus (97). For example, comparison of ADCC, cytokine and direct activation of primary NK cells showed unique gene expression profiles and differential expression of genes commonly associated with NK cell activation (196). Further, HIV infected individuals have NK cells that differ in their activation profiles from healthy donors, indicating that viral infection can alter NK cell activity. This has similarly been demonstrated for CMV infected individuals, where NK cells can adapt over time

and act more like an adaptive immune cell (273). These type of RNA seq studies expose the subtleties in NK cell activity that can be distinguished by gene expression data.

Proteomic analysis serves as an important way to understand the relationships to gene expression data. The earliest studies of proteomes in NK cells utilized gel electrophoresis to identify membrane enriched proteins, differences in proteins found in activated vs. resting NK cells as well as the identity of microvesicle enriched proteins (274, 275). As technology has advanced, larger numbers of proteins have been identified in an unbiased manner, demonstrating the growing utility of proteomics (276, 277). A more recent study used proteomic analysis to identify proteins important for NK cell proliferation and pointed a pathway toward increasing the activity of NK cells in a tumor model through therapeutic blockade (278).

Metabolic studies have also revealed a new aspect of NK cell biology and differentiation based on the influence of metabolic factors outside of traditional routes of cellular influence, and these studies have been well-reviewed recently (279–282). Indeed, large differences in metabolic processes have been identified within NK cell subsets. These differences can help distinguish the regulatory, cytotoxic, and memory functions of NK cells. Robust metabolism is essential for efficient cytotoxicity, but metabolic evidence suggests that activated NK cells use alternative routes for oxidative phosphorylation (279, 280). Generally, our knowledge of NK cell metabolism is quite limited and has left many questions unaddressed, such as the role of metabolism in organ specific and tissue resident NK cells or whether metabolism can be used to modulate immunotherapies. Further, this field has not tapped into the robust tools of metabolomics yet, which could provide much broader *in vivo* based knowledge. Although, the field of discovery-based metabolomics is becoming a more accessible technique, it unfortunately still requires large amounts of starting material, which can be inhibitory in certain experimental setups.

CLOSING REMARKS

So, what does make an effective antibody for recruiting NK cell ADCC? Unsurprisingly, the answer to that question remains incomplete, but what is clear is that fully understanding the underlying mechanisms of antibody effector functions is a complex and difficult task. While this review was certainly not meant to be comprehensive, it was meant to set a stage for understanding the more subtle roles of the molecular underpinnings that seed NK cell activation in the context of ADCC, as well as to provide a perspective from the point-of-view of structural biology. The roles of structural and biophysical constraints entailed in antibody-based cellular activation have historically been overlooked, and subsequently poorly explored and understood. When developing an antibody with therapeutic potential for NK cell recruitment, one must ask many questions beyond simply "what is the target?" For example, where *exactly* does this antibody bind? What does the target *look* like? How will the antibody coordinate activating ligands as a full IgG? How will

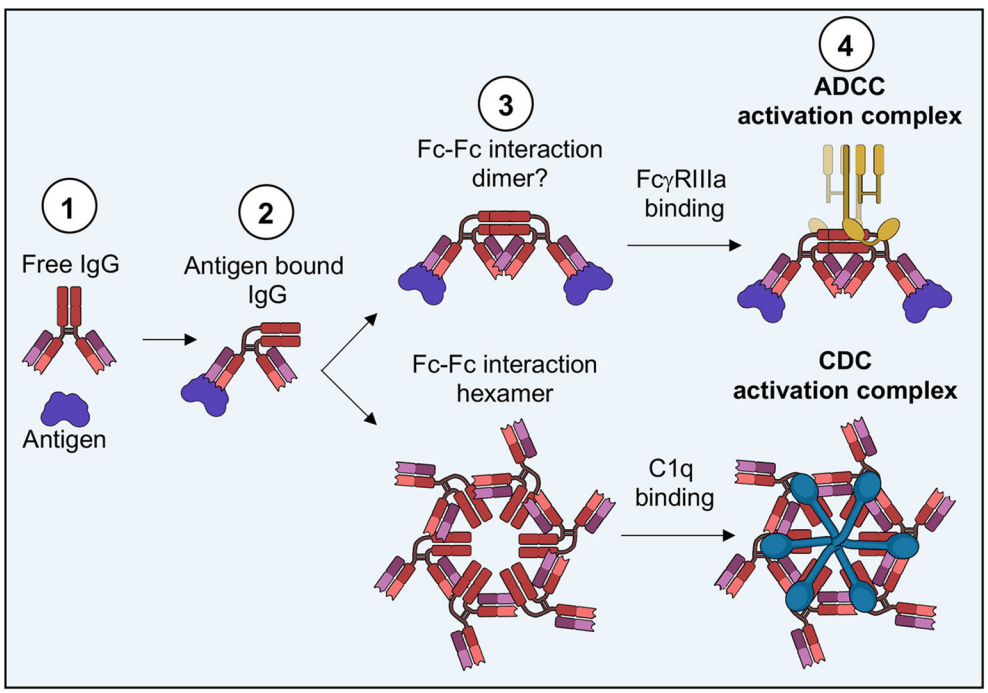

FIGURE 12 | Potential model for IgG effector function fate. (1) Free IgG binds to antigen, (2) causing a change in presentation of the IgG Fc domain. (3) Depending on the arrangement of antigen and/or antibody phenotype, Fc-Fc interactions are facilitated in different oligomeric arrangements. (4) Specific arrangements of Fc-Fc domains influence the binding of FcγRs or C1q, thus resulting in ADCC, CDC or potentially other effector functions.

this interplay with the rest of the IS? How will these factors affect the dynamics and coordination of activation?

These types of considerations are important because we have learned from experience that *in vitro* activity and validation have not always translated well to the clinic. While brute force in evaluating many different potential targets and antibodies is certainly a way to address the gap in treatment for many diseases, a far more cost effective and long-term solution is to rethink how to approach antibody therapeutic research. While comparing autoimmune disease, genetic disorders, cancer and pathogenic infection is difficult to do, the human immune system is designed to be a one stop shop for handling all of these conditions. Therefore, tackling how to harness that power should be, in theory at least, straightforward to do once we gain a better understanding of how it operates.

By returning to the basic biology of NK cell activity, and understanding the molecular nature of activation, we can develop more broadly applicable principles of antibody function and correlates of protection that can be consistently relied upon. Already, the many advances pointed out in this review begin to paint a picture of a more concerted mechanism for how antibodies function post-antigen binding, offering an exciting potential model for antibody effector fate (**Figure 12**). By combining this simple model with increasing knowledge regarding antibody/receptor glycosylation, as well as the more complex functions of tissue resident immune cells, engineering antibody function may become much more straightforward.

In the future, access to many of the more complex applications discussed here, such as single cell transcriptomics and MINFLUX live cell nanoscopy, may become more commonplace as technology becomes cheaper and computer processing power become more powerful and widely available. With access to such technologies, immunologists can add new layers to their understanding of how molecular perturbations affect higher order cellular functions. This density of information will make understanding antibody function *in vivo* much easier, by building a basis of understanding across biological scales up to the organismal level.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Characterization of Rhesus Macaque Liver-Resident CD49a$^+$ NK Cells During Retrovirus Infections

*Daniel R. Ram[1], Christian F. Arias[2], Kyle Kroll[1], Brady Hueber[1], Cordelia Manickam[1], Rhianna A. Jones[1], Scott T. Smith[1], Spandan V. Shah[1], Valerie H. Varner[1] and R. Keith Reeves[1,3]**

[1] Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States, [2] Boston University School of Medicine, Boston, MA, United States, [3] Ragon Institute of Massachusetts General Hospital, MIT, and Harvard, Cambridge, MA, United States

CD49a$^+$ tissue resident NK cells have been implicated in memory-like NK cell responses, but while this population is well-characterized in mice and in humans, they are poorly described in non-human primates (NHP) which are particularly critical for modeling human viral infections. Others and we have shown that memory-like NK cells are enriched in the liver and because of the importance of NHP in modeling HIV infection, understanding the immunobiology of CD49a$^+$ NK cells in SIV-infected rhesus macaques is critical to explore the role of this cell type in retroviral infections. In this study mononuclear cells isolated from livers, spleens, and peripheral whole blood were analyzed in acutely and chronically lentivirus-infected and experimentally-naïve Indian rhesus macaques (RM). NK cells were then identified as CD45$^+$CD14$^-$CD20$^-$CD3$^-$NKG2A/C$^+$ cells and characterized using multiparametric flow-cytometry. Our data show that in RM, CD49a$^+$ NK cells increase in the liver following retroviral infections [median = 5.2% (naïve) vs. median = 9.48% (SIV+) or median = 16.8% (SHIV+)]. In contrast, there is little change in CD49a$^+$ NK frequencies in whole blood or spleens of matched animals. In agreement with human and murine data we also observed that CD49a$^+$ NK cells were predominantly Eomes^{low} T-bet^{low}, though these frequencies are elevated in infected animal cohorts. Functionally, our data suggests that infection alters TNF-α, IFN-γ, and CD107a expression in stimulated CD49a$^+$ NK cells. Specifically, our analyses found a decrease in CD49a$^+$ CD107a$^+$ TNFα$^+$ IFNγ$^-$ NK cells, with a simultaneous increase in CD49a$^+$ CD107a$^+$ TNFα$^-$ IFNγ$^+$ NK cells and the non-responsive CD49a$^+$ CD107a$^-$ TNFα$^-$ IFNγ$^-$ NK cell population following infection, suggesting both pathogenic and inflammatory changes in the NK cell functional profile. Our data also identified significant global differences in polyfunctionality between CD49a$^+$ NK cells in the naïve and chronic (SHIV+) cohorts. Our work provides the first characterization of CD49a$^+$ NK cells in tissues from RM. The significant similarities between CD49a$^+$ NK cells from RM and what is reported from human samples justifies the importance of studying CD49a$^+$ NK cells in this species to support preclinical animal model research.

Keywords: natural killer, HIV, non-human primate, macaque, SIV

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***Correspondence:**
R. Keith Reeves
rreeves@bidmc.harvard.edu

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INTRODUCTION

Natural killer (NK) cells are considered as the prototypic innate immune effector cell capable of rapid and broad (non-specific) responses to several agents—including viral infections and cancerous cells. NK cells are generally thought to function through engagement of either activating or inhibitory molecules on the cell surface, leading to activation, or repression of NK cell function depending on the ratio of receptor engagement (1–3). Recently, NK cells have been identified as also having peptide-specificity and memory-recall potential, once previously thought to belong only to adaptive immune cells, like B cells or T cells (4–6). Adaptive NK cells have been shown to be enriched in the livers of mice (7) and non-human primates (NHP) (5), and recently in human livers from BLT mice (8).

The α1β1 integrin CD49a (also VLA-1) has been shown to be associated with liver-resident lymphocytes and is further described as one of several markers for adaptive NK cells that accumulate in the liver (7, 9, 10). CD49a expression on uterine NK cells (uNK) and other tissue-resident NK cells, may also delineate adaptive-like properties (11–13). CD49a may play a functional role in NK cell responses in tissues by regulating migration, or perhaps influencing proliferation in the tissues (14). In humans it has been shown that CD49a$^+$ NK cells are enriched in liver cirrhosis and further that CD49a$^+$CD25$^+$ NK cells have enhanced proliferative capacity *ex vivo* (15). Further, ligation of CD49a has been shown to influence tyrosine kinase signaling leading to IL-2 dependent NK cell activation (16). CD49a has been shown to have many binding partners, but is predominantly thought to interact with collagens (I, IV, IX, and XVI) (17–19) and laminins (111 and 112) (20). Additionally, CD49a has been shown to interact with Galectins 1, 3, and 8 (21, 22) and semaphorin 7A (23), which has been implicated in cytokine-induced NK cell memory responses (24).

In contrast, CD49b$^+$ (DX5 in mice, also α2β1) NK cells have been characterized as more migratory, and show greater similarity to conventional spleen NK cells in mice (11, 25), providing a more direct comparison for tissue-resident vs. trafficking NK cells. CD49b may also play a role in binding the complement molecule C1q, although whether this occurs in NK cells is still unclear (26). Recent mouse studies have shown that CD49b is not required for NK cell effector responses in the spleen or liver, but may play a role in the proliferation of NK cells in response to ectromelia virus (ECTV) and mouse CMV (MCMV) infection (27). The role of CD49b on human NK cells is not as clear, though it likely also plays a role in NK cell migration (28).

While there have been several studies characterizing CD49a$^+$ NK cells in mice, humanized mice, and humans, to date these cells remain unexplored in NHP. Given the role that NHP play for modeling several human diseases, like HIV/AIDS, ZIKA, influenza, and tuberculosis (29–36), it is critical to characterize this population of NK cells in relevant NHP models.

MATERIALS AND METHODS

Ethics Statement

All animals were housed at Biomere Inc. (Worcester, MA) or the New England Primate Research Center (Southborough, MA). All study blood samplings were reviewed and approved by the local Institutional Animal Care and Use Committee. All animal housing and studies were carried out in accordance with recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with recommendations of the Weatherall report; "The use of non-human primates in research." Animals were fed standard monkey chow diet supplemented daily with fruit and vegetables and water *ad libitum*. Social enrichment was delivered and overseen by veterinary staff and overall animal health was monitored daily. Animals showing significant signs of weight loss, disease, or distress were evaluated clinically and then provided dietary supplementation, analgesics, and/or therapeutics as necessary. Animals were euthanized with an overdose of pentobarbital, followed by necropsy. Liver and spleen samples were then processed as detailed below.

Animals

Samples from sixteen necropsied Indian origin rhesus macaques (*Macaca mulatta*) were analyzed in this study: four experimentally naïve animals, seven animals that were infected with SIV_{mac251}/SIV_{mac239} for 7–14 days, and five chronically infected with SHIVSF162P3. All experiments were performed with approval from the local Institutional Animal Care and Use Committee (IACUC). All animals were group housed until the start of the study and then infected animals were housed under BSL2 conditions.

Macaque Samples

Liver and spleen mononuclear cells were isolated using standard isolation protocols (5). Briefly, after *ex vivo* excision the liver was flushed and then liver mononuclear cells were isolated using mechanical disruption followed by density-gradient centrifugation layered over 60% Percoll. Splenic mononuclear cells were isolated by mechanical disruption. Contaminating red blood cells were lysed using an ACK lysis buffer (Gibco, Cat. No. A1049201). Cell aliquots were immediately cryopreserved in 90% FBS, 10% DMSO (Sigma) and stored in liquid nitrogen vapor. Whole blood samples were collected in EDTA blood collection tubes and following lysis of red blood cells an aliquot was immediately used for flow cytometry analysis.

Functional Assay

Cryopreserved liver and spleen mononuclear cells were cultured in R10 medium (RPMI + 10% FBS) only or stimulated with phorbol myristate acetate (PMA, 2.2 μg/mL, Sigma) and Ionomycin (5 μg/mL, Sigma) for 4 h in the presence of monensin (GolgiStop) and Brefeldin A (GolgiPlug; BD Biosciences, concentrations as recommended by manufacturer). Cells were then processed for flow cytometry.

Flow Cytometry

All antibodies were purchased from BD Biosciences unless specified otherwise and their clone information is in parentheses. For the liver phenotypic panel, antibodies against the following cell antigens were used: Eomes-FITC (WD1928, Life Technologies), CD150-BB630 (A12), CD195-BB700 (3A9),

SYK-BB790 (4D10), CD49a-PE (SR84), CD49b-PECF594 (AK-7), CD49e (NKI-SAM1, Biolegend), CD336-PE Cy5 (Z231, Beckman Coulter), CD20-PE Cy5.5 (2H7, Life Technologies), T-bet-PE Cy7 (4B10, Life Technologies), DAP12-Alexa405 (405288, Novus), CD69-BV510 (FN50, Biolegend), CD14-BV570 (M5E2, Biolegend), CD337-BV605 (p30-15), CD366-BV650 (F38-2E2, Biolegend), PD-1-BV750 (EH12.1), Zap70-BV786 (1E7.2), CD3-BUV395 (SP34.2), CD16-BUV496 (3G8), CD8α-BUV563 (RPA-T8), CD45-BUV615 (D058-1283), HLA-DR-BUV661 (G46-6), CD56-BUV737 (NCAM16.2), CD62L-BUV805 (SK11), CD159a-APC (Z199, Beckman Coulter), FcεRI-A700 (rabbit polyclonal, Millipore, conjugated in-house). For the liver functional panel antibodies against the following cell antigens were used: CD45-FITC (D058-1283), CD49a-PE (SR84), CD49b-PECF594 (AK-7), CD159a-PE Cy7 (Z199, Beckman Coulter), CD3-BV450 (SP34.2), TNF-α-BV650 (MAb11), IFN-γ-BV711 (B27), CD107a-BV786 (H4A3), CD20-BUV395 (L27), CD16-BUV496 (3G8), CD56-BUV563 (NCAM16.2), CD14-BUV737 (MφP9), HLA-DR-Alexa700 (G46-6), CD8α-APC Cy7 (SK1). Flow cytometry data was acquired on a BD LSRII or BD FACSymphony A5 (BD Biosciences, La Jolla, CA) and analyzed with FlowJo software (version 10.2, Tree Star, Ashland, OR).

For the spleen phenotypic panel, antibodies against the following cell antigens were used: Eomes-FITC, CD49a-PE, CD49b-PECF594, CD336-PERCP Cy5.5, CD3-V450, CD56-BV570, CD337-BV605, CD366-BV-650, CD14-BV711, CD45-BV786, CD20-BUV395, CD16-BUV496, CD159a-APC, HLA-DR-A700, and CD8α-APC Cy7. For the spleen functional assay, the antibodies used were against the following antigens: MIP-1β-FITC, CD49a-PE, Granzyme B-ECD, CD107a-PERCP Cy5.5, IFNγ-PE Cy7, CD3-V450, CD56-BV570, CD14-BV711, CD45-BV786, CD20-BUV395, CD16-BUV496, CD159a-APC, TNF-α-A700, and CD8α-APC Cy7. All antibody clones were consistent between spleen and liver samples. The spleen flow cytometry data was acquired on an LSRII (BD Biosciences, La Jolla, CA) and analyzed with FlowJo software (version 10.2, Tree Star, Ashland, OR).

Statistical Analyses

Statistical and graphing analyses were performed with GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA). Non-parametric Mann-Whitney *U*-or Wilcoxon tests were used where indicated, and a *p*-value of $p < 0.05$ was considered to be statistically significant. Permutation analyses were carried out in SPICE (37) in order to compare the polyfunctional data plots.

RESULTS

Frequencies of CD49a$^+$ NK Cells Are Elevated Following Retroviral Infection

Liver NK cells from naïve, acute SIV-infected or chronically SHIV-infected rhesus macaques were identified using the following previously defined criteria: CD45$^+$CD14$^-$CD20$^-$CD3$^-$NKG2AC$^+$ (38, 39). This co-expression analysis of NKG2A and NKG2C (CD159a and CD159c) identifies the majority of NK cells in rhesus macaque blood and tissues. These NK cells were then further characterized by the expression of CD49a and CD49b (**Figure 1A**). Quantification of CD49a$^\pm$b$^\pm$ NK cells revealed that the majority of NK cells in the liver did not express CD49a or CD49b, but interestingly there was a significant increase in the frequencies of CD49a$^+$b$^-$ NK cells from acute SIV+ (median = 9.48%) or chronic SHIV+ (median = 16.8%) infected animals as compared to the naïve group (median = 5.2%; **Figure 1B**). Statistical comparisons between acute and chronic infection groups are not shown for most analyses given the different challenge viruses. For a subgroup of SIV+ animals we had the opportunity to longitudinally monitor expression of CD49a and CD49b in the blood, and though we observed minor animal-to-animal variability we did not observe any significant changes in the frequencies of CD49a$^+$ or CD49b$^+$ NK cells over either 7 or 14 days following challenge with SIV (**Supplementary Figure 1**). We also assessed frequencies of CD49a and CD49b in the spleen and observed a reduction in CD49a$^+$ NK cells in the chronic (SHIV+) cohort relative to naïve, albeit not statistically significant (**Supplementary Figure 2**). Liver resident NK cells also do not generally express CD49e, as this integrin is an indicator of cells in circulation (40). For this reason we also assessed CD49e on NK cells from the naïve and acute (SIV+) cohorts and found that, as expected, CD49a$^+$ and CD49e$^+$ NK cells were generally mutually exclusive (**Supplementary Figure 3**). Further, we observed that the frequency of CD49a$^-$e$^+$ NK cells showed a small but non-significant ($p > 0.05$) increase in livers of the acute (SIV+) infection cohort relative to the naïve group (**Supplementary Figure 3**).

Liver Resident CD49a$^+$ NK Cells Are Phenotypically Distinct

Multiparametric flow cytometry analysis revealed several phenotypic changes following infection in CD49a$^+$ NK cells from livers (**Figure 2A**) and spleens (**Figure 2B**). Interestingly, in livers we saw significant changes in several proteins, including altered frequencies Eomes, FcεRI, Syk, CD62L, and PD-1 NK cells in the infected groups relative to naïve (**Figure 2A**, **Supplementary Data Sheet 1A**). In the retrovirus-infected cohorts we also observed several changes (at or approaching $p \leq 0.05$) between CD49a$^+$ and CD49a$^-$ NK cells, including CD16, CD56, CD62L, CD69, CD150, CD336 (NKp44), CD366 (Tim-3), Eomes, NKG2AC^{high}, and NKG2AC^{low} and T-bet (**Figure 2A**, **Supplementary Data Sheet 1A**). Though the cell frequencies are low it is interesting to note elevated levels of CD336 (NKp44) on CD49a$^+$ NK cells relative to CD49a$^-$ NK in the infected cohorts as NKp44$^+$ NK cells are potent antiviral effectors (41). It is well-established that the currently available antibodies to detect NKG2A cross react with NKG2C in NHP (42) and thus the convention is to term the cell population identified by the anti-NKG2A antibody as NKG2AC$^+$. As a result, we have developed an RNA flow-based approach to discriminate between NKG2A and NKG2C (39, 43). However, some observations suggest that the NKG2AC^{high} population corresponds to a population that predominantly expresses

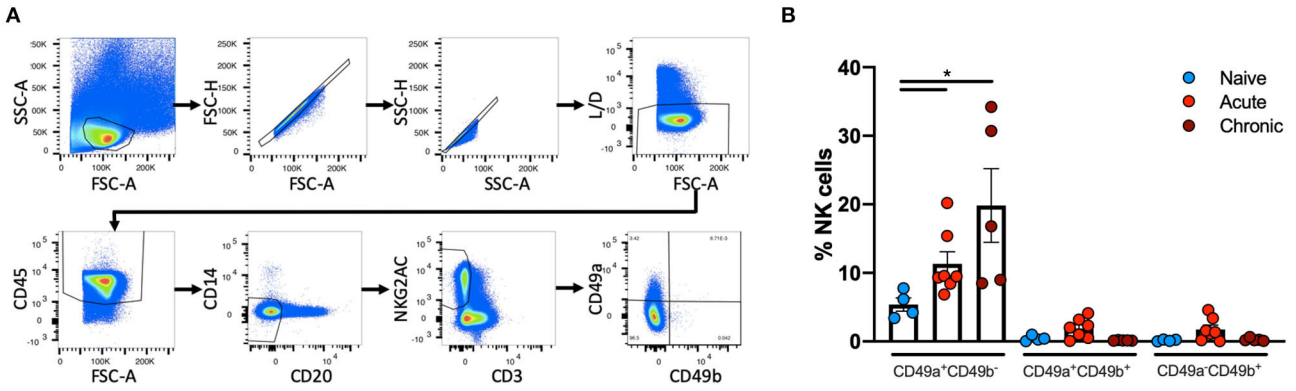

FIGURE 1 | CD49a+ NK frequencies vary in in Liver following infection. **(A)**, Representative, gating strategy showing the identification of CD49a±CD49b± NK cells. **(B)** Quantification of frequencies of CD49a±CD49b± cells in livers of naïve ($n = 4$), acute SIV+ ($n = 7$), or chronic SHIV+ ($n = 5$) animals. Mann-Whitney *U*-test was used to determine statistical significance, $^*p < 0.05$.

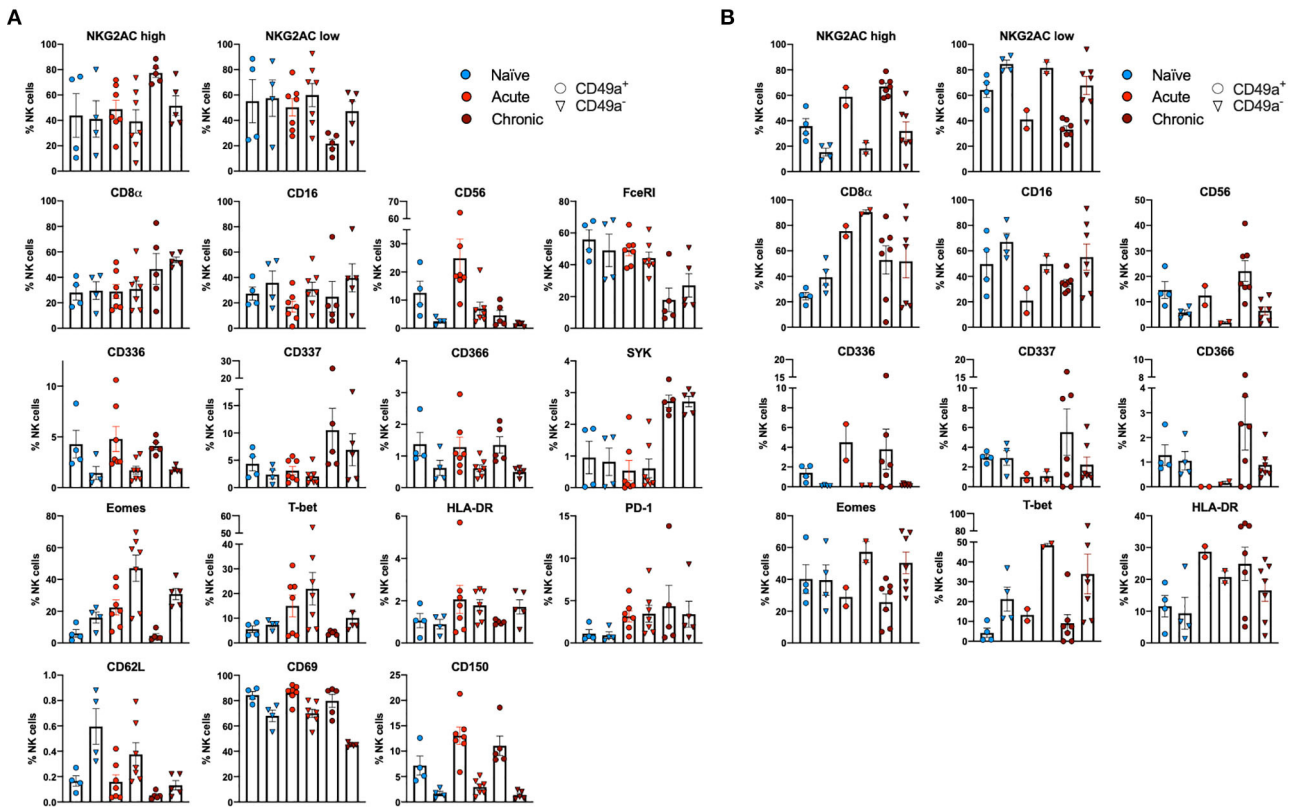

FIGURE 2 | CD49a+ NK cells exhibit tissue-specific phenotype. **(A)** Phenotypic characterization of Liver CD49a± NK cells from naïve macaques ($n = 4$) or macaques acutely infected with SIV ($n = 6$) or chronically infected with SHIV ($n = 5$). **(B)** Phenotypic characterization of Spleen CD49a± NK cells from naïve macaques ($n = 4$) or macaques acutely infected with SIV ($n = 2$) or chronically infected with SHIV ($n = 7$). Mann-Whitney *U*-test or Wilcoxon test was used as indicated in **Supplementary Data Sheets 1A,B** to determine statistical significance.

NKG2A (relative to NKG2C), whereas the NKG2AC^{low} population corresponds predominantly NKG2C expressing cells (39)—shown in **Supplementary Figure 4** where gene expression of KLRC1 (NKG2A) is elevated in the NKG2AC high population, whereas gene expression of KLRC2 (NKG2C) is elevated in the NKG2AC ^{low} population in peripheral blood mononuclear cells from both experimentally naïve animals (CMV+) and an acute SIV+ cohort (39). These observations thus suggest that in the livers of the chronic (SHIV+) group there is an elevation of NKG2A+ CD49a+ NK cells. **Figures 2A,B** also illustrate

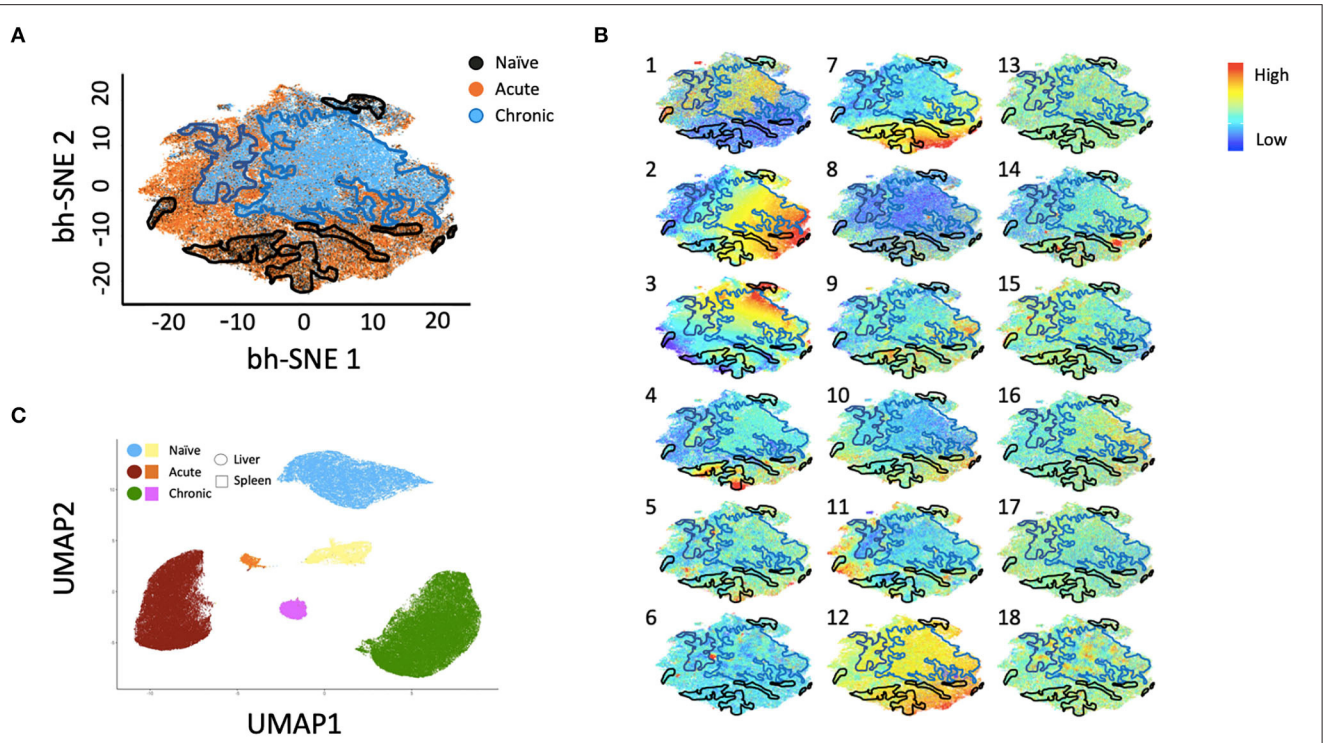

FIGURE 3 | *bh*-SNE reveals infection-specific expression profiles in Liver CD49a$^+$ NK cells. **(A)** *bh*-SNE representations illustrating the distribution of various phenotypic markers on CD49a$^+$ NK cells from naïve (enclosed by black boundaries), chronic SHIV+ (enclosed by blue boundaries), or acute SIV+ samples (remaining unbound orange space). **(B)** Normalized expression of various phenotypic markers are superimposed on **(A)** showing distribution of: (1) CD8α, (2) CD159AC (NKG2AC), (3) CD49b, (4) Eomes, (5) T-bet, (6) PD-1, (7) FcεRI/FcRγ, (8) CD16, (9) CD337 (NKp30), (10) CD336 (NKp44), (11) CD150, (12) Dap12, (13) CD62L, (14) CD69, (15) CD195, (16) CD366 (Tim-3), (17) HLA-DR, and (18) Zap70. **(C)** UMAP clustering reveals relationships between liver and spleen CD49a$^+$ NK cells.

several differences between liver and spleen CD49a$^+$ NK cells: elevated CD56 in liver CD49a$^+$NK cells from the acute (SIV+) cohort relative to spleen, as well as elevated CD8α and HLA-DR in spleen CD49a$^+$ and CD49b$^+$ NK cells following infection relative to the liver. There were several significant changes in the CD49a$^+$ vs. CD49a$^-$ NK populations and these are highlighted in **Supplementary Data Sheet 1B**. We also utilized UMAP in order to assess the multiparametric relatedness of the various CD49a$^+$ NK cell populations between liver and spleen, and in the naïve, acute (SIV+) and chronic (SHIV+) cohorts (**Figure 3C**). We observed that while the populations clustered into distinct groups, the spleen and liver samples appeared to generally localize according to infection status. Using *bh*-SNE we also observed the clearest overall phenotypic differences between naïve and chronic (SHIV+) samples as opposed to naïve and acute (SIV+) animals shown by the distinct clustering in the chronic (SHIV+) relative to naïve samples (**Figure 3**). Interestingly, there was a consolidation/reduction of distinct CD49a$^+$ NK populations from the naïve samples (outlined in black) to a smaller number of clusters in both the acute (SIV+) and chronic (SHIV+) infected cohorts (outlined in orange and blue, respectively, **Figure 3A**). By overlaying the normalized expression of several phenotypic markers, we were able to see their relative expression on the naïve and infection cohorts (**Figure 3B**). There were several phenotypic markers that seemed to drive the overall differential clustering, including CD8α, CD16, CD56, NKG2A, CD366, CD337, CD336, T-bet, and Eomes.

Liver CD49a$^+$ NK Cells Display Preferential IFNγ Production During Infection

Following stimulation of liver mononuclear cells, CD49a$^+$ NK cells upregulated CD107a and production of TNFα and IFNγ (**Figure 4**). Interestingly, relative to the naïve cohort, cells from the chronic SHIV+ cohort produced reduced levels of TNFα ($p = 0.016$; **Figure 4A**). TNFα levels were also reduced in the acute SIV+ cohort relative to naïve ($p = 0.067$). While all groups showed elevated frequencies of IFNγ in CD49a$^+$ relative to CD49a$^-$ NK cells, the differences were most robust in retrovirus-infected cohorts. We also assessed functional properties of spleen CD49a$^+$ NK cells in naïve, acute (SIV+) and chronic (SHIV+) and observed significant elevation of IFNγ, Granzyme B (GZB), and MIP1β in CD49a$^+$ vs. CD49a$^-$ NK cells in the chronic infection cohort (**Supplementary Figure 5**). Analysis of polyfunctionality in liver samples revealed a significant loss of CD49a$^+$ CD107a$^+$ IFNγ$^-$ TNFα$^+$ NK cells following retroviral infection, whereas there was a significant increase in the CD49a$^+$ CD107a$^+$ IFNγ$^+$ TNFα$^-$ only in the acute SIV+ cohort (**Figures 4B,C**). Further, we observed an increase in the CD49a$^+$ CD107a$^-$ TNFα$^-$ IFNγ$^-$ population in the chronic infection

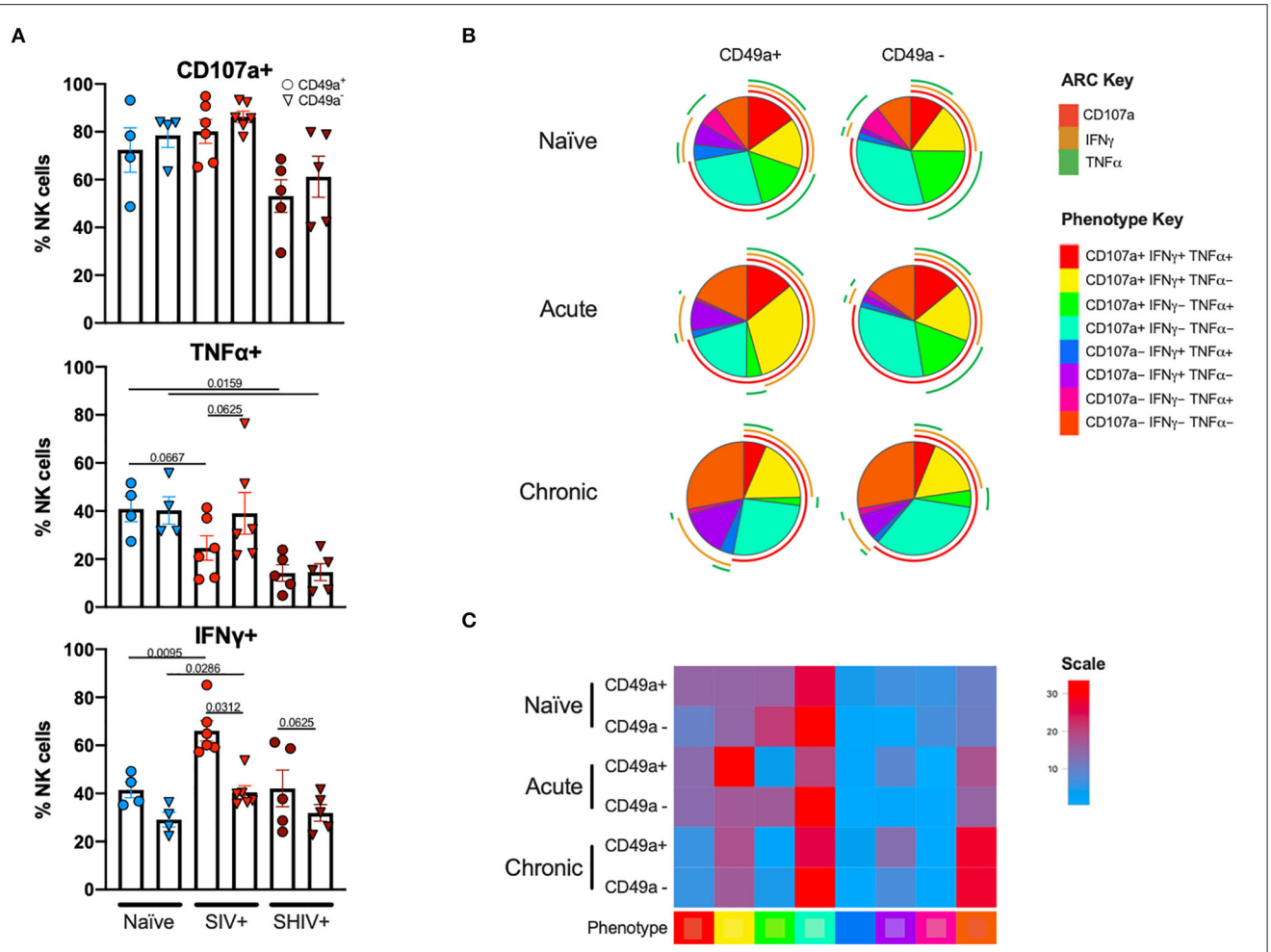

FIGURE 4 | CD49a$^+$ NK cells exhibit enhanced IFNγ during infection. **(A)** Bar graphs showing quantification of IFNγ, TNFα, and CD107a expression following 4 h stimulation with PMA and ionomycin in liver CD49a$^\pm$ NK cells from naïve macaques ($n = 4$) or macaques acutely infected with SIV ($n = 6$) or chronically infected with SHIV ($n = 5$). **(B)** Pie charts illustrating the proportion of NK cells that exhibit polyfunctional characteristics following 4 h stimulation with PMA and ionomycin. **(C)** Heatmap showing comparison of polyfunctional phenotype across the infection groups. The scale shows population frequencies of NK cells. Mann-Whitney *U*-test or Wilcoxon test was used to determine statistical significance.

group relative to naïve animals. In order to compare the various polyfunctional populations we also carried out a permutation test with 1,000,000 permutations. This analysis revealed significant differences between naïve and chronic (SHIV+) CD49a$^+$ NK cells ($p = 0.0492$, **Supplementary Data Sheet 1C**).

DISCUSSION

CD49a$^+$ NK cells are still poorly characterized in any tissue from NHP. Given their association with liver-residence and that liver-resident CD49a$^+$ NK cells have been thought to play a role in the adaptive NK cell response (5, 7, 9, 10), understanding CD49a$^+$ NK cells may provide a potential novel avenue for vaccine or immunotherapy design. It is therefore crucial to evaluate the impact of HIV and SIV infections on this population. In human livers CD49a$^+$ NK cells have been shown to also express high levels of NKG2C (10). Here we also show elevation of NKG2AC^{high} CD49a$^+$ NK cells following chronic infection (with SHIV). This suggests that the resulting NK cells may possess greater inhibitory properties, since NKG2A is an inhibitory molecule and has been suggested to play a role in diminution of the NK response in the liver of humanized mice (44). This may provide an opportunity for NKG2A blockade therapy in order to restore NK cell function (44, 45). The concurrent observation of increased frequencies of the putatively non-functional CD49a$^+$CD107a$^-$TNFα$^-$IFNγ$^-$ NK cell following retroviral infection may suggest a diminished NK cell response following retroviral infection that may be different from what is seen in human livers, albeit in the context of cancer (10). While the expansion of the polyfunctional CD49a$^+$CD107a$^+$IFNγ$^+$TNFα$^-$ population was not necessarily surprising, given the role of IFNγ in antiviral responses, it was surprising to see a loss of the CD49a$^+$ CD107a$^+$ IFNγ$^-$ TNFα$^+$ population in CD49a$^+$ NK cells from the acute (SIV+) cohort

and both CD49a$^+$ and CD49a$^-$ NK cells in the chronic (SHIV+) cohort. It is unclear why the frequency of CD107a$^+$ IFNγ^- TNFα^+ polyfunctional NK cells were unchanged in the CD49a$^-$ NK cells from acute (SIV+) cohort as compared to the naïve cohort. Whether or not acute or chronic infection result in altered responses requires further investigation as this study was not designed to specifically resolve this possibility.

Our phenotypic characterization has also highlighted several populations of interest, including CD49a$^+$ NK cells expressing CD56 or CD150 (SLAM) during acute or chronic retrovirus infections. While the expression of certain proteins like Eomes and T-bet appear low in our naïve samples, overall the ranges fall within observed values from our work and from others as well (5, 8, 46). We also see several differences in CD49a$^+$ NK cells between the liver and the spleen, particularly in their differential expression of HLA-DR following retroviral infection. The role of HLA-DR on NK cells is still unclear. HLA-DR expression has been posited as a marker of NK cell activation but it has also been shown to play a role in immune modulation (47), though it has also been suggested that while HLA-DR+ NK cells are less phenotypically mature they still display high functional activity (48). While we did not see a statistically significant increase in frequencies of CD49a$^-$e$^+$ NK cells in the livers of acute SIV-infected macaques relative to naïve animals, the small increasing trend may be interesting to explore in further studies with a larger animal cohort. Regardless, our multiparametric phenotypic and functional characterization of CD49a$^+$ NK cells provides the first investigation of CD49a$^+$ NK cells in livers of both naïve and infected RM cohorts. Understanding how CD49a$^+$NK cells are modulated in the liver following infection may provide clues to how we can best engage this liver-resident NK cell population and possibly improve responses to SIV/HIV infections.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by Harvard IACUC and Biomere IACUC.

AUTHOR CONTRIBUTIONS

DR and RR conceived the experiment and wrote the manuscript. DR, CA, KK, BH, CM, RJ, STS, SVS, and VV carried out experiments. DR, CA, KK, and RR analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01676/full#supplementary-material

Supplementary Figure 1 | Frequencies of peripheral blood CD49a$^+$ NK cells are unchanged during acute SIV infection. **(A)** Frequencies of NK cells in whole blood of acute-SIV infected animals. Frequencies of **(B)** CD49a$^+$ or **(C)** CD49b$^+$ NK cells in the whole blood following acute SIV infection.

Supplementary Figure 2 | Frequencies of CD49a$^\pm$b$^\pm$ in spleens. Quantification of frequencies of CD49a$^\pm$CD49b$^\pm$ cells in spleens of naïve ($n = 4$), acute SIV+ ($n = 2$), or chronic SHIV+ ($n = 7$) animals. Mann-Whitney *U*-test was used to determine statistical significance, $^*p \leq 0.05$.

Supplementary Figure 3 | Frequencies of CD49a$^\pm$e$^\pm$ NK cells in livers. **(A)** Representative flow plot showing identification of CD49a$^\pm$e$^\pm$ NK cells from liver samples. **(B)** Quantification of CD49a$^\pm$e$^\pm$ NK cell populations from livers of naïve ($n = 3$) and acute SIV+ ($n = 3$) animals.

Supplementary Figure 4 | NKG2AC^{high} and NKG2AC^{low} populations exhibit unique KLRC1 and KLRC2 gene expression patterns. **(A)** Gating strategy showing identification of gene expression of KLRC1 and KLRC2 in NKG2AC high and NKG2AC low populations. **(B)** Quantification of KLRC1$^+$KLRC2$^+$ (K1+K2+) and KLRC1$^-$KLRC2$^+$ (K1–K2+) populations in NKG2AC^{high} (H) or NKG2AC^{low} (L) NK cells from CMV+ and SIV-infected animals as per (39). Mann-Whitney *U*-test was used to determine statistical significance, $^*p < 0.05$, $^{**}p < 0.01$.

Supplementary Figure 5 | Functional characterization of spleen CD49a$^+$ NK cells. **(A)** Bar graphs showing quantification of CD107a, TNFα, and IFNγ expression in spleen CD49a$^\pm$ NK cells from naïve macaques ($n = 2$) or macaques acutely infected with SIV ($n = 2$) or chronically infected with SHIV ($n = 5$). Wilcoxon test was used to determine statistical significance, $^*p < 0.05$.

Supplementary Data Sheet 1 | Statistics tables. *p*-value tables showing comparisons in **(A)** liver and **(B)** spleen phenotype data (from **Figure 2**) as well as **(C)** permutation testing of polyfunctional populations (from **Figure 4B**). For **(A,B)** Wilcox test was used when comparing CD49a$^+$ and CD49a$^-$ from the same infection group whereas Mann-Whitney *U*-test was used when comparing across infection cohorts. $p \leq 0.05$ is shown by bold font. 1,000,000 iterations were used in order to carry out the permutation test in **(C)**. $p \leq 0.05$ is shown in cells highlighted in pink as generated by SPICE (37). Statistical comparisons between acute and chronic infection groups are not shown for these analyses given the different challenge viruses.

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Non-human Primate Determinants of Natural Killer Cells in Tissues at Steady-State and During Simian Immunodeficiency Virus Infection

*Nicolas Huot[1], Philippe Rascle[1,2], Caroline Petitdemange[1], Vanessa Contreras[3], Jean-Louis Palgen[3], Christiane Stahl-Hennig[4], Roger Le Grand[3], Anne-Sophie Beignon[3], Beatrice Jacquelin[1] and Michaela Müller-Trutwin[1]**

[1] Unité HIV, Inflammation et Persistance, Institut Pasteur, Paris, France, [2] Université Paris Diderot, Sorbonne Paris Cité, Paris, France, [3] CEA-Université Paris Saclay-Inserm, U1184, Center for Immunology of Viral, Auto-immune, Hematological and Bacterial Diseases, IMVA-HB/IDMIT, Fontenay-aux-Roses, France, [4] Deutsches Primatenzentrum, Göttingen, Germany

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United States

***Correspondence:**
Michaela Müller-Trutwin
mmuller@pasteur.fr;
michaela.muller-trutwin@pasteur.fr

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Natural killer (NK) cells play essential roles in immunity to viruses and tumors. Their function is genetically determined but also modulated by environmental factors. The distribution and functional regulation of these cells vary depending on the tissue. NK cell behavior in lymphoid tissues is so far understudied. Non-human primate (NHP) models are essential for the development of therapies and vaccines against human diseases, and access to NHP tissues allows insights into spatial regulations of NK cells. Here, we investigated tissue-specific parameters of NK cells from NHP species, i.e., cynomolgus macaque (*Macaca fascicularis*), African green monkey (*Chlorocebus sabaeus*), rhesus macaque (*Macaca mulatta*), and baboon (*Papio anubis*). By comprehensive multi-dimensional analysis of NK cells from secondary lymphoid organs, intestinal mucosa, liver, and blood, we identified tissue- and species-specific patterns of NK cell frequencies, phenotypes, and potential activity. Also, we defined the tissue-specific characteristics of NK cells during infection by the simian immunodeficiency virus. Altogether, our results provide a comprehensive anatomic analysis of NK cells in different tissues of primates at steady-state and during a viral infection.

Keywords: SIV, HIV, NK cells, lymph node, gut, tissue resident, CXCR5, NKp44

INTRODUCTION

In recent years, it has become increasingly clear that human natural killer cells (NK cells) are far more diverse (1). Some of the diversity is genetically determined, whereas substantial diversification seems to be also determined by environmental factors, including vaccinations and age (1–4). NK cells are frequent in organs such as the liver and the uterus but are probably present in all tissues to various degrees, including bone marrow, lymph nodes, spleen, kidneys, skin, lung,

and gut (2, 5–9). NK cells in secondary and tertiary lymphoid organs are still understudied, given their low frequencies in these lymphoid tissues (10–12). A proportion of them express molecules known as tissue-resident markers for T cells, such as CD69 and CD103 (5). The local tissue environment impacts NK cell diversity and function (5, 13). The role of tissue localization in human NK cell development and function and how circulating NK cells relate to those in different sites are, however, not yet sufficiently well understood. Also, markers for the identification of distinct tissue-resident NK cell subpopulations are still scarce. Characterization of NK cells is particularly challenging in tissues, mainly due to lack of hallmark lineage markers for tissue-derived innate lymphoid cells (ILCs) and NK cells in humans. In the context of viral infections, access limitations to organs in humans are an additional hurdle to fully understand the role of NK cells in tissues.

Non-human primates (NHPs) are essential models for the development of therapies and vaccines against human diseases and allow insights into spatial cellular regulations of NK cells. African green monkeys (AGMs), macaques, and baboons are the most largely used NHP models in pre-clinical research. They have contributed essentially to the research or development of vaccines against viral diseases, including severe acute respiratory syndrome coronavirus 2, HIV, influenza, yellow fever, and Ebola virus (14–18). Efforts to identify subpopulations of NK cells in NHP were complicated by incomplete definitions of them, mainly because the antibody against CD56 does not stain all NK cell subpopulations in NHP species (19). Alternatively, NHP NK cells in the blood have been defined as $CD3^{-}CD8\alpha\alpha^{+}CD20^{-/dim}NKG2a/c^{+}$, which is considered the most effective inclusive definition for NK cells from Old World monkeys, such as rhesus and pig-tailed macaques, sooty mangabeys, and AGMs (20–23), whereas this definition does not fully characterize neotropical primate blood NK cells (24).

Some of the NK cell diversity driven by environmental factors is linked to their maturation and differentiation. In humans, the modulation of NKG2a/c expression among CD56dim cells defines a crucial step of their differentiation (25, 26). Immature and mature NK cells can be respectively defined as $CD56^{bright}NKG2a/c^{hi}CD62L^{hi}CD57^{-}$ and $CD56^{dim}NKG2a/c^{-}CD62L^{-}CD57^{+}$. Their distribution varies according to the tissue. NK cells in tissues also share several markers with ILCs. The natural cytotoxicity receptors (NCRs) NKp46 and NKp44 have been described on both NK cells and ILCs (27). NKp46 is a major determinant of NK cell function and is implicated in tumor immune surveillance in acute myeloid leukemia (28, 29). NKp44 is expressed on activated human NK cells and is important for tumor target cell recognition by NK cells during natural cytotoxicity responses, whereas, in the adult intestine, NKp44 + ILCs are the main ILC subset producing IL-22 (30, 31). NKp44 + CD103 + CD69 + NK cells have been shown to persist in adult intestine, corresponding to previously described intraepithelial ILC1s (32). The recent identification of the NKp44 receptor, moreover, indicates an important role of Nkp44 in viral infections (33). NKp30 is another interesting NCR in viral infections due to its capacity to interact with dendritic cells (DCs) and impact on the NK-DC cross-talk (34–36).

There is an urgent need for a better characterization of these NK cell subpopulations in lymphoid tissues. Until now, it has been very difficult to relate our knowledge of human NK cell biology to that of NHP and translate this information into pre-clinical practice. Among the different NHP models, African NHPs, such as AGM, have been a natural host for simian immunodeficiency viruses (SIVs) for probably more than one million of years (37–39). In contrast to people living with HIV (PLH), natural hosts for SIV typically do not progress toward disease despite persistent high viremia (40). At the opposite, Asian macaques are not natural hosts of SIV, and experimental infection with SIVmac induces AIDS preceded by rapid loss of $CD4^{+}$ T cells in intestinal mucosa and chronic systemic inflammation as in PLH. We have previously shown that in contrast to HIV-1 infected humans and SIVmac-infected macaques, AGMs infected with SIVagm are free of chronic inflammation and efficiently control viral replication in secondary lymphatic tissue (SLT) (41). The viral control in SLT of SIVagm-infected AGMs is mediated predominantly by NK cells in a CXCR5-dependent manner (41). Paradoxically, no viral control is observed in the intestine of SIVagm-infected monkeys (42). AGMs, therefore, represent a model of tissue-specific NK cell-mediated viral control.

In this study, we comprehensively determined tissue-specific frequencies, phenotypes, and potential activity profiles of NK cells in major NHP models. We investigated NK cells in blood, liver, lymph nodes, spleen, ileum, jejunum, colon, and rectum from uninfected animals and after infection with SIV. This work provides clues to understand better the behavior of tissue NK cells and delivers novel tools for the study of therapeutic and vaccinal approaches in NHP models for human diseases.

MATERIALS AND METHODS

Monkeys and Simian Immunodeficiency Virus Infections

Samples from a total of 36 NHPs were used in this study distributed across four different species. The number of animals per species was as follows: 13 AGMs (Caribbean *Chlorocebus sabaeus*), 12 CMs (*Macaca fascicularis*), 7 RMs (*Macaca mulatta*), and 4 baboons (*Papio anubis*). Seven CMs and seven AGMs were infected with SIVmac251 and SIVagm.sab92018, respectively, as previously described (23, 41). The viremia levels are shown in **Supplementary Figure S2**. All infected monkeys were highly viremic. All the animals were free of simian retrovirus type D and simian T-lymphotropic virus type 1.

The AGMs, CMs, and baboons were housed at the IDMIT Center (Fontenay-aux-Roses, France), and the RMs at the German Primate Center (DPZ). All experimental procedures were conducted in strict accordance with the International European Guidelines 2010/63/UE on the protection of animals used for experimentation and other scientific purposes and with the national laws (French Decree 2013-118). The IDMIT Center complies with the Standards for Human Care and Use of the Office for Laboratory Animal Welfare (United States) under Office for Laboratory Animal Welfare Assurance number

A5826-86. Monitoring of the monkeys at IDMIT was under the supervision of the veterinarians in charge of the animal facilities. The Ethical Committee of Animal Experimentation (CETEA-DSV, IDF, France) (notification 12-098 and A17-044) approved experimental animal protocols. The DPZ is allowed to breed and house NHPs under license number 392001/7 approved by the local veterinary office and conforming with § 11 of the German Animal Welfare act. The Lower Saxony State Office for Consumer Protection and Food Safety with the project license 33.9-42502-05-10A041 granted the collection of blood samples at the DPZ. In compliance with the principles of the three Rs (replacing, reducing, and refining), all samples used here were from animals that were shared with other studies. Indeed, the animals have been purchased or infected for other programs. For instance, we used tissues that were collected from animals that have been killed for other studies, and some of these have already been published (4, 41, 81).

Monkeys were sedated with ketamine chlorhydrate (Rhone-Mérieux, Lyons, France) before handling. Sample collection was performed in random order, according to the tripartite harmonized International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline on Methodology (previously coded Q2B). The investigators were not blinded, whereas the animal handlers were blinded to group allocation.

Tissue Collections and Processing

Whole venous blood was collected in ethylenediaminetetraacetic acid tubes. Ficoll density-gradient centrifugation isolated peripheral blood mononuclear cells. Biopsies of pLNs were performed by excision. Other tissues were collected at autopsy. After careful removal of adhering connective and fat tissues, pLNs and spleen cells were dissociated using the gentlemacS^{TM} Dissociator technology (Miltenyi Biotec, Germany). The cell suspension was subsequently filtered through 100- and 40-μm cell strainers, and cells were washed with cold phosphate-buffered saline. Cells were either immediately stained for flow cytometry or cryopreserved in 90% fetal bovine serum and 10% dimethyl sulfoxide and stored in liquid nitrogen vapor.

Viral RNA Quantification

The viral RNA copy numbers in plasma of the animals were quantified as previously described (23, 41).

Polychromatic Flow Cytometry

For flow cytometric analysis, cells were stained in U-bottom tubes in the dark. Cells were analyzed using 11-parameter flow cytometry. For surface staining, cells were incubated in phosphate-buffered saline with the appropriate monoclonal antibodies and Zombie Aqua^{TM} for 30 min at 4°C, washed and fixed with 4% paraformaldehyde. The following monoclonal antibodies were used for surface and intracellular staining (clone, suppliers): CD3 (SP34-2, *BD Biosciences*), CD8 (BW135/80; Miltenyi Biotec), CD20 (2h7; eBioscience), CD16 (3G8,Coulter), NKG2A (Z199, Coulter), NKP30 (AF29-4D12, Miltenyi Biotec), CD45 (D058-1283, *BD Biosciences*), CD69 (FN50, *BD Biosciences*), NKP46 (BAB281,Coulter), CXCR5 (MU5UBEE, Thermo), CD279 (PD-1) (EH12.2H7, Biolegend), CXCR3 (1C6/CXCR3, *BD Biosciences*), IFN-γ (4S.B3,*BD Biosciences*), GzmB (GB11, *BD Biosciences*), TNF-A (MAB11, *BD Biosciences*), NKP44 (REA1163, Miltenyi Biotec), and CD14 (TÜK4, Miltenyi Biotec). Reference listing the cross-reactivity of each antibody is listed in **Supplementary Table S1**. The anti-NKG2A antibody used recognizes both NKG2A and NKG2C on simian cells. Due to the lack of information on the KIR polymorphism in African NHP and the rare anti-KIR antibodies specific for NHP, the KIR expression has not been analyzed. Flow cytometry acquisitions were performed on an LSRII (BD Biosciences). The data were further analyzed using FlowJo 10.4.2 software (FlowJo, LLC, Ashland, OR, United States). Intracellular staining was performed using BD Cytofix/Cytoperm^{TM}. Uniform manifold approximation and projection was performed with Flojow, using 1,000 iterations and perplexity of 0.25.

Statistics

The GraphPad Prism 7 (GraphPad Software, San Diego, CA) was used to analyze data and to perform statistical analyses. Statistical significance of differences was assessed using non-parametric Mann–Whitney *U* tests (**Figures 1A**, **3B,C**) or Wilcoxon matched-pairs signed-rank test for paired samples (**Figures 1E,F,H**). All group comparisons were carried out using a one-way analysis of variance and followed by a Tukey's multiple-comparison test. The latter is a *post hoc* test based on the studentized range distribution (**Figures 2E,F**, **4E,F**, **5A,B,E,F**). Values of $p < 0.05$ were considered significant. NK cell populations were analyzed by the uniform manifold approximation and projection method provided by the FlowJo plugin version 3.1. Heatmap of the Log2(FC) of the marker expression was compared with the median across all samples. It was color-coded with blue for lower expression and red for higher expression. Dendrograms present the clustering of samples (columns) and markers (row), which is based on hierarchical clustering with Euclidean distance metric and average linkage.

RESULTS

Tissue-Intrinsic Distribution of Non-human Primate Natural Killer Cell Subsets

To analyze NK cell distribution and phenotype profiles across different anatomic sites in Asian and African NHPs, we collected blood, secondary lymphoid organs (spleen and peripheral lymph nodes [pLNs]), liver, and gut (small and large intestines) from cynomolgus macaques (CMs) and AGMs (**Figure 1A**). NK cells were defined as previously reported by us and others (20–22, 41) as $CD45^{+}CD3^{-}CD14^{-}CD20^{-}NKG2_{a/c}{}^{+}$ cells. The tissue distribution was analyzed first in healthy CM and AGM. The distribution was consistent between animals for a given species with blood and liver having significantly higher frequencies of NK cells (5–40%) compared with low frequencies (<0.1–5%) of NK cells in the pLNs and gut (**Figure 1B**).

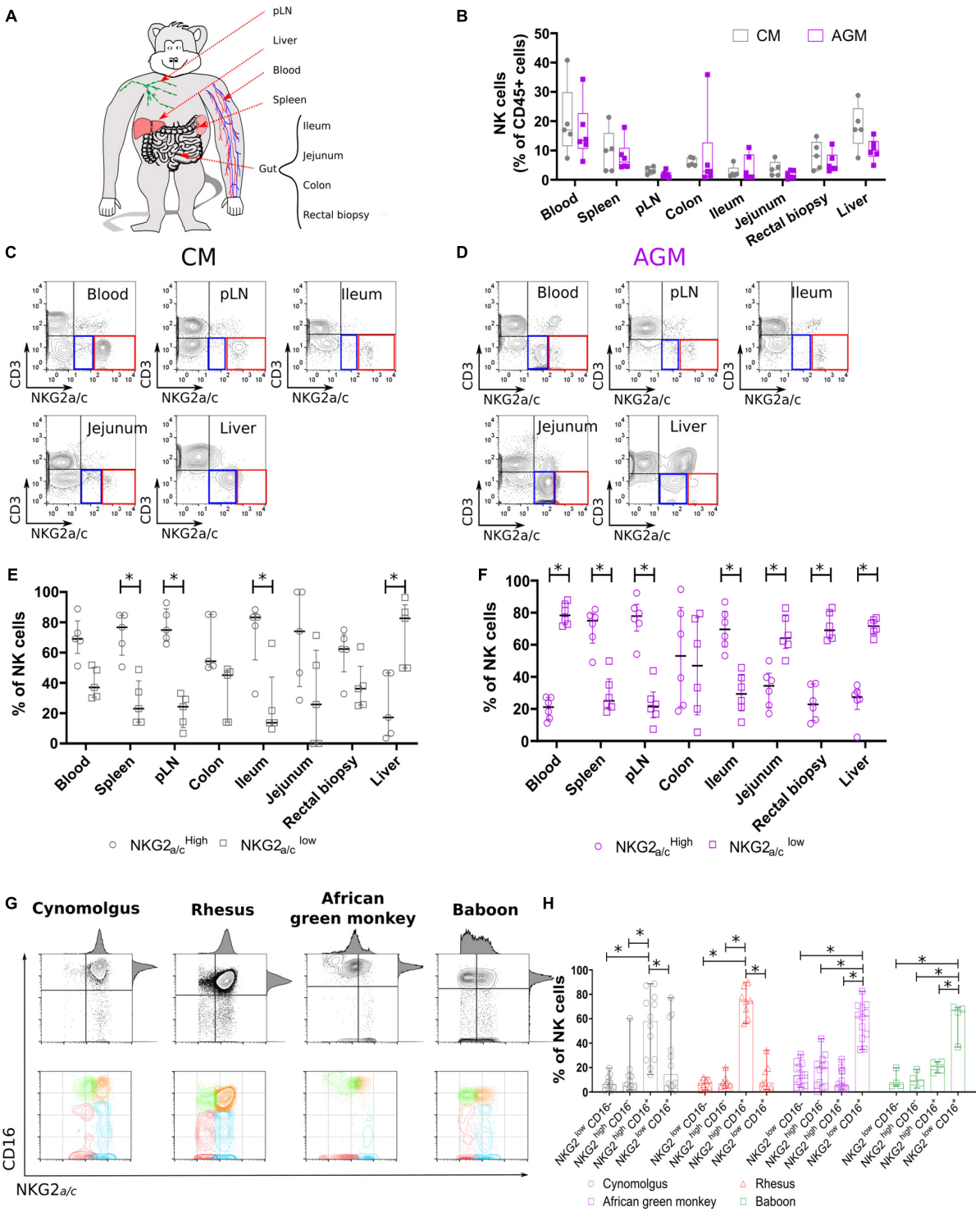

FIGURE 1 | Non-human primate NK cell subset distributions in tissues. **(A)** Scheme of the NHP tissues obtained and experimental workflow presented in this study. **(B)** NK cell distribution in distinct tissue compartments. Frequencies are shown in boxplots and correspond to compiled data from 5 SIV negative CMs (gray) and 6 SIV negative AGMs (purple). **(C,D)** Representative flow cytometry plots for NKG2a/c^{LOW} (blue square) and NKG2a/c^{HIGH} (red square) NK cell subsets in multiple tissue sites obtained from **(C)** CM and **(D)** AGM. **(E,F)** Distribution of NKG2a/c^{LOW} (empty circle) and NKG2a/c^{HIGH} (empty square) NK cell subsets in multiple tissue sites obtained from **(E)** five CMs and **(F)** six AGMs. **(G)** Representative dot plots showing blood NK cell subset distributions according to NKG2a/c and CD16 in four NHP species as indicated. **(H)** Frequency of blood NK cell subsets in four SIV negative NHP species. Each symbol depicts an individual animal among the 13 AGM, 12 CM, 7 RM, and 4 baboons. Median and standard error bars are shown. Statistical significance of differences was assessed using non-parametric Mann–Whitney *U* tests **(B)**, or Wilcoxon matched-pairs signed-rank test, for paired samples **(E,F,H)**. Asterisks indicate a *p*-value <0.05.

NK cells were further delineated into two NK cell subsets, NKG2a/c^{HIGH} and NKG2a/c^{LOW} (**Figures 1C,D** and **Supplementary Figure S1**). The distribution of NKG2a/c^{HIGH} and NKG2a/c^{LOW} NK cell subsets was also dependent on the tissue site (**Figures 1C,D**). NKG2a/c^{HIGH} NK cells predominated in pLNs, spleen, and ileum, whereas NKG2a/c^{LOW} NK cells comprised the majority of NK cells in liver independently of the species (**Figures 1E,F**). Thus, NKG2a/c^{HIGH} NK cells were more prevalent in sites known for containing an abundance of immature NK cells such as pLN, spleen, and gut, and in the liver, potentially mature NK cells were predominant.

We also observed a difference between the species, as NKG2a/c^{HIGH} NK cells were the main population in blood, jejunum, and rectum of CM in contrast to AGM where NKG2a/c^{LOW} NK cells dominated in these three compartments (**Figures 1E,F**). To clarify the difference observed between these two species, we analyzed a higher number of animals and more species (**Figure 1G**). Thus, blood NK cells were analyzed in 12 CMs, 11 AGMs, 8 rhesus macaques (RMs), and 4 baboons. The NKG2a/c^{HIGH} NK cells were again the major population in the blood of the two Asian NHPs (CM and RM) and not in the two African NHPs (AGM and baboon) (**Figure 1H**).

To summarize, the NK cell distributions across tissues varied, similar to the ones described in humans (5). The frequencies of total NK cells in different anatomical sites did not differ between NHP species. The frequency of NKG2a/c^{LOW} NK cells in blood was higher in the NHP species from Africa compared with that in the macaque species.

Evaluation of Natural Killer Cell Diversity in Tissues From Non-human Primates

To further analyze the difference between the tissues, we implemented a high-dimensional flow cytometry analysis of simian NK cells, allowing notably the measurement of NCRs, homing receptors, and cellular exhaustion marker (PD-1). We included, as homing receptors, CXCR3 and CXCR5, which are, respectively, a major tissue homing receptor for NK cells (43) and a B cell follicle homing receptor for lymphocytes (44–46). The NK cells from six anatomical sites (blood, pLN, spleen, liver, ileum, and jejunum) collected from five healthy CMs and four healthy AGMs were analyzed. A heatmap based on the mean fluorescence intensity for each marker at the cluster level was generated. Force-directed clustering analysis resulted in 11 distinct NK cell clusters for both AGM and CM (**Figures 2A–D**). In both species, blood NK cells segregated into three distinct subclusters that did not reveal overlaps with NK cells from tissues. Either the liver NK cells formed only one cluster or the clusters grouped together, generally separate from other tissues. Spleen and pLN NK shared some clusters. The jejunum and ileum NK cells not only appeared to be closely linked but also sometimes were found in clusters with other tissue NK cells (**Figures 2A–D**).

When comparing the two species, NK cells from pLN and spleen in AGM showed elevated levels of CXCR5 expression compared with other tissues. In contrast, for CM, the highest CXCR5 expression was observed in the gut (**Figures 2B,D**). CM also showed high frequencies of CXCR5 + NK cells in the liver (**Figures 2E,F**). Another distinct feature of the CM was a high frequency of NK cells with lower CD8 expression in tissues (**Figure 2E**).

In summary, NK cells showed tissue-specific clusters with LN and spleen NK cells closely related. Tissue NK cells in CM rarely expressed CD8. In the intestine and liver, NK cells in CM frequently expressed CXCR5.

Tissue Natural Killer Cells Are Differentially Impacted in Pathogenic Compared With Non-pathogenic Simian Immunodeficiency Virus Infection

We evaluated if and how SIV infection modulates NK cell distribution and phenotype among tissues. We included in the analysis of tissue homing markers and compared the distribution of NK cell subpopulations between the tissues. Seven CMs and seven AGMs were followed during SIVmac and SIVagm infection, respectively (**Supplementary Figure S2**). NK cells from blood, pLN, and rectal biopsy were longitudinally monitored during the time course of infection (**Figure 3A**). To have access also to liver, ileum, and jejunum during acute infection, we analyzed tissues collected at necropsy from three monkeys of each species at day 9 post-infection (p.i.) and at the time of peak viremia and from four animals of each species during the chronic phase of infection (day 240 p.i.). Assessment of absolute NK cell counts in blood did not reveal statistically significant differences during SIV infection, even if a trend for the decrease was observed in each species during an acute infection—up to day 3–4 p.i. (**Figure 3B**). In pLN, the frequency of NK cells rapidly decreased in CM during acute infection to never reach the level observed before infection throughout chronic infection, whereas no statistically significant change was observed in AGM (**Figure 3C**, left graph). In contrast, AGM NK cells in rectal biopsies showed a constant decrease during infection, whereas CM NK cells tended to drop at day 7 p.i. but only transiently and remained normal after that (**Figure 3C**, right graph).

Force-directed clustering analysis of NK cells from six tissues obtained at day 9 p.i. generated seven distinct NK cell clusters per species (**Figures 3D–G**). NK cells clustered again according to tissues as before infection, except some overlap between liver and blood or pLN. The highest density of expression was observed for CXCR5 on NK cells from SLT in AGM, whereas it was also elevated in some liver NK cells in CM. CXCR3 expression was high on NK cells from blood and SLT in AGM, whereas high on intestinal and blood NK cells for CM, suggesting that CXCR3 might be involved in the distinct tissue distribution of NK cells after SIVmac and SIVagm infection.

We then performed the same analysis on chronically infected animals. Due to the decrease and few numbers of NK cells in gut tissues from AGM during SIVagm infection (**Figure 3C**, right graph), we included an additional intestinal compartment (the colon) at the expense of the liver in the analyses (**Figures 4A–E**). Whereas, in healthy animals, CXCR3 and CXCR5 were found in the same clusters (**Figures 2B,D**), they clustered

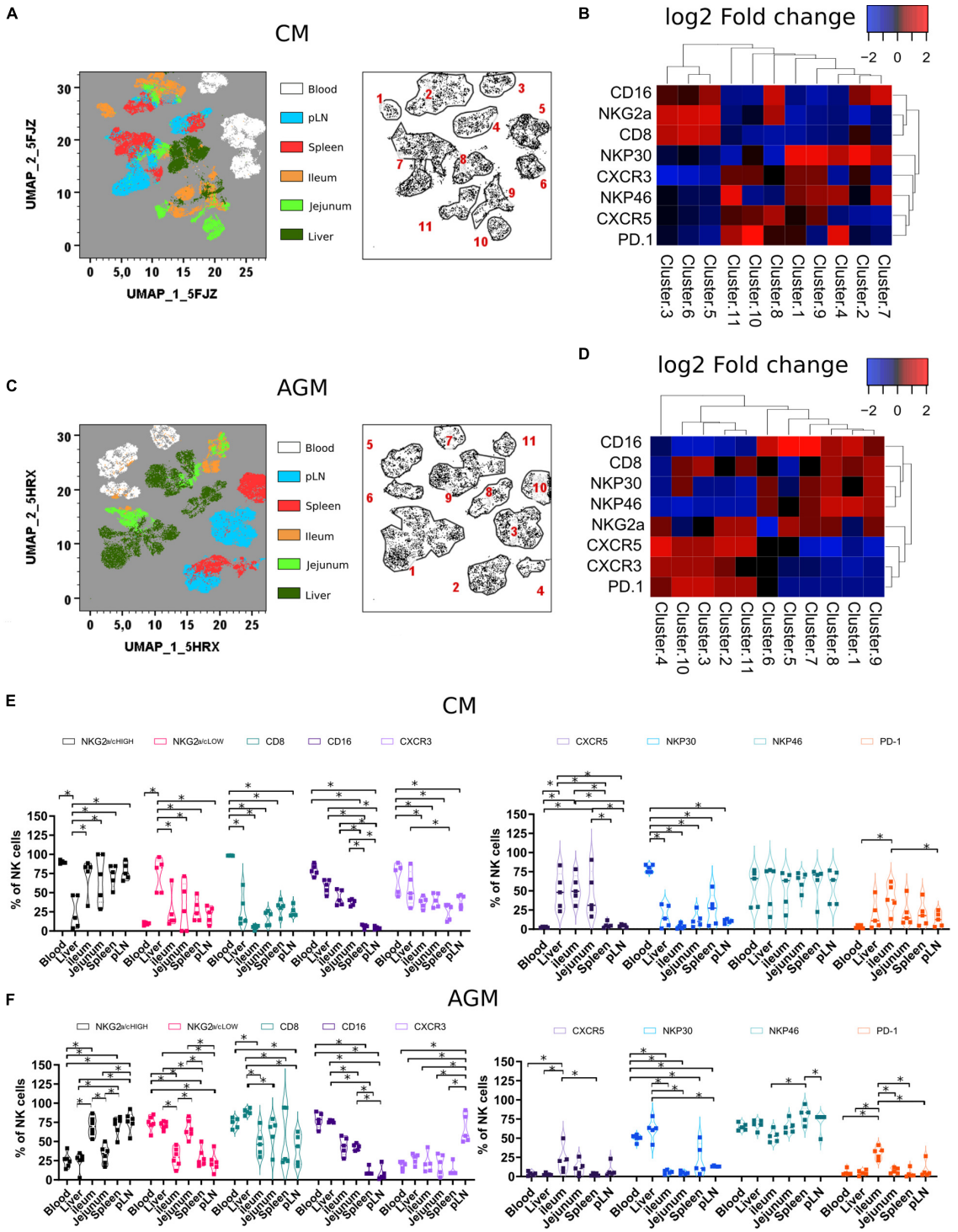

FIGURE 2 | Tissue localization shapes the phenotype of NK cells **(A)** Force-directed plot showing the relationship between NK cells from distinct tissues identified by multidimensional analysis (data compiled from five healthy CM). Each node is an aggregation of phenotypically similar cells. Resulting from several steps, including downsampling, merging, and overall dimension reduction followed by gating (left panel), and collection of similar nodes forms 11 distinct NK cell clusters (right panel). **(B)** Heatmap showing the differential expression of cell surface markers used for clustering and community identification in the multidimensional analysis in panel **(A)**. **(C)** Same analyses as in panel **(A)** performed on six healthy AGM. **(D)** Heatmap showing the differential expression of cell surface markers used for clustering and community identification in the multidimensional analysis in panel **(C)**. **(E,F)** Violplots showing the distribution of markers on bulk tissue NK cells from **(E)** healthy CMs and **(F)** healthy AGMs. Each marker is labeled with a unique color. All comparisons were carried out using a one-way analysis of variance (ANOVA) and followed by a Tukey's multiple-comparison test. The latter is a *post hoc* test based on the studentized range distribution. Median and standard error bars are shown. Asterisks indicate a *p*-value <0.05.

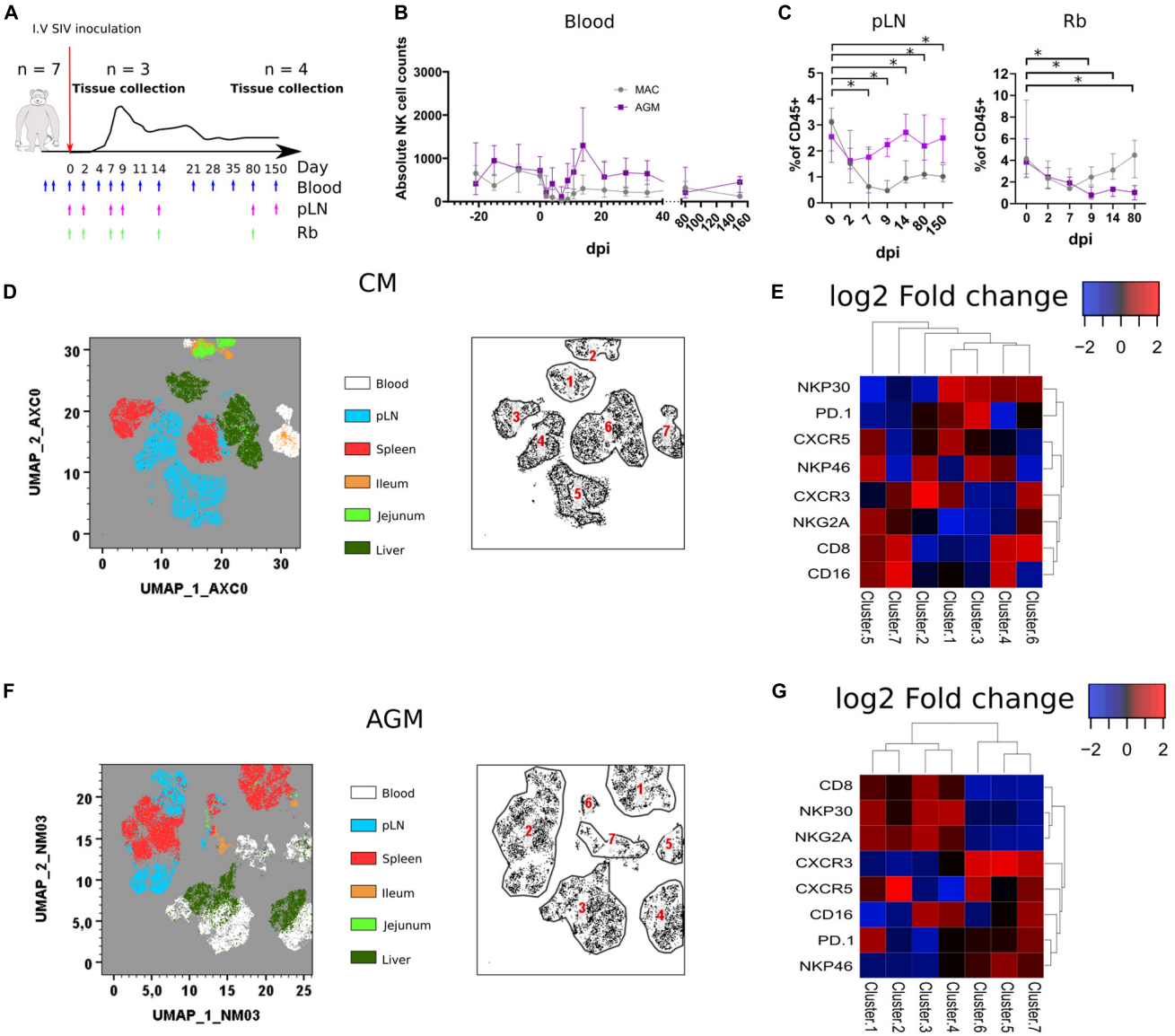

FIGURE 3 | Rapid change in tissues NK cell phenotypes following SIV infection. **(A)** Schematic representation of the SIV infection schedule used in this study. Seven CMs and seven AGMs were intravenously injected with SIV (see section *Method*). Blue arrows indicate blood sampling. Purple and green arrows indicate pLN and rectal biopsy (Rb) samplings, respectively. Three monkeys per species were euthanized at day 9 p.i., and the remaining animals were euthanized during the chronic phase (day 240 p.i.). **(B)** Follow-up of blood NK cell absolute counts before and during SIV infection in CM (gray line) and AGM (purple line). **(C)** Frequency of NK cells among CD45 + cells in pLN (left panel) and Rb (right panel) during SIV infection in CM (gray line) and AGM (purple line). **(D)** Force-directed plot showing the relationship between NK cells from distinct tissues at day 9 p.i. identified by multidimensional analysis (data compiled from three CMs). Each node is an aggregation of similar cells resulting from the downsampling of data (left panel), and the collection of similar nodes forms six distinct NK cell clusters (right panel). **(E)** Heatmap showing the differential expression of cell surface markers used for clustering and community identification in the multidimensional analysis in panel **(D)**. **(F)** Same analyses as in panel **(A)** performed on three AGMs at day 9 p.i. **(G)** Heatmap showing the differential expression of cell surface markers used for clustering and community identification in the multidimensional analysis in panel **(F)**. Median and standard error bars are shown. Asterisks indicate a *p*-value <0.05.

separately in infected animals (**Figures 4A–D**), indicating that the CXCR3 + and CXCR5 + NK cells evolved differentially after SIV infection. In SIVmac infection, the frequencies of CXCR5 + NK cells in the gut decreased (**Figure 4E**) as compared with those before infection (**Figure 2E**). CXCR5 expression on NK cells was also low in SLT of CM compared with AGM in chronic SIV infection (**Figures 4A–D**), correlating with previous data on the higher levels CXCR5 + NK cells in SLT during chronic SIVagm infection as compared with chronic SIVmac infection (41).

PD-1 is known to be expressed on exhausted CD8 + T cell lymphocytes. However, PD-1 expression is also high on CXCR5 + lymphocytes residing in the B cell follicles of SLT (T_{FH} and B cells). On NK cells, as shown here, PD-1 was often expressed on the same cell subset of CXCR5 + NK cells

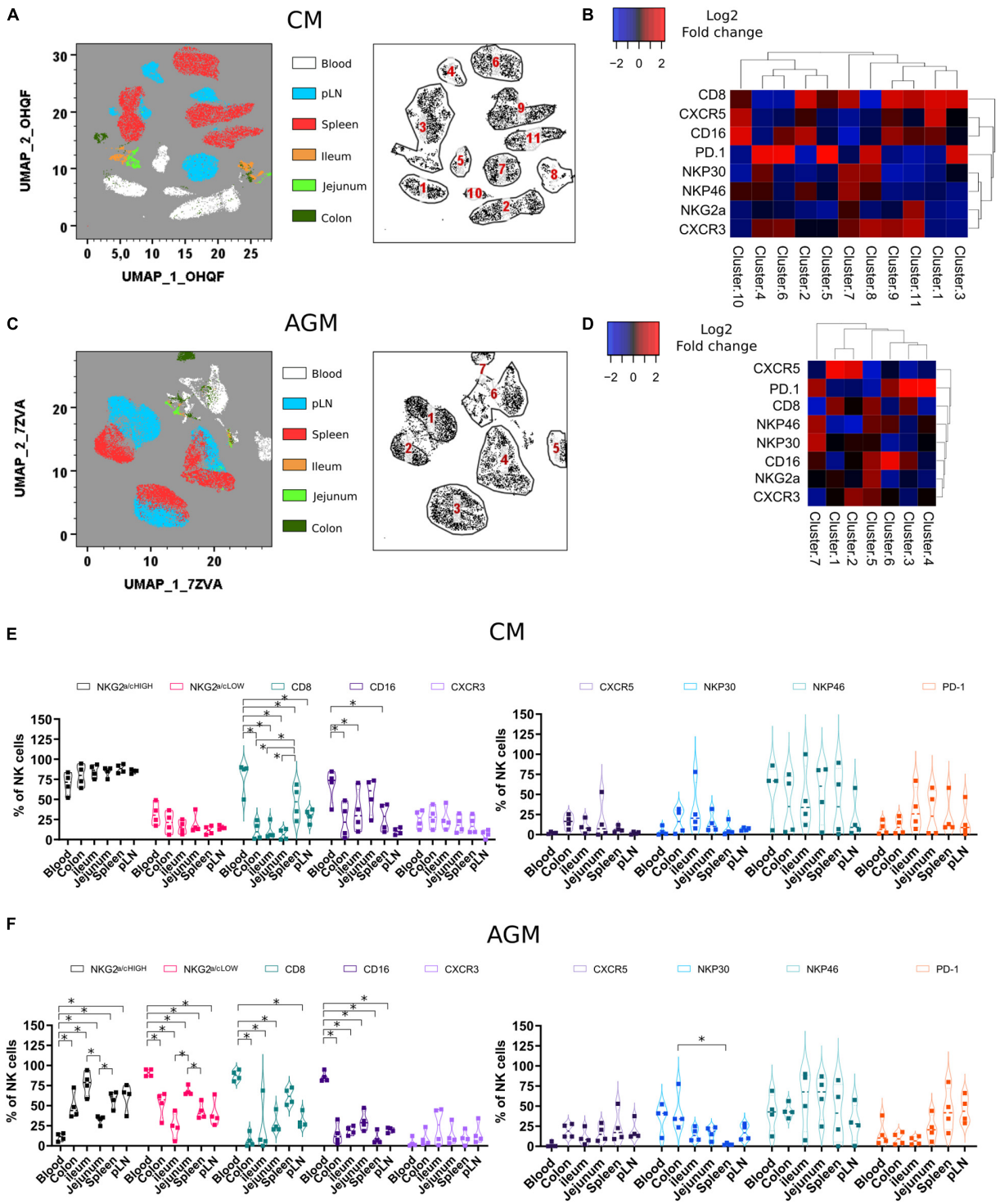

FIGURE 4 | Phenotypic analysis of NK cells during chronic SIV infection across different tissue sites. **(A)** Force-directed plot showing the relationship between NK cells from distinct tissues identified by multidimensional analysis (data compiled from five chronically infected CMs). Each node is an aggregation of similar cells resulting from the downsampling of data (left panel), and the collection of similar nodes forms 11 distinct NK cell clusters (right panel). **(B)** Heatmap showing the differential expression of cell surface markers used for clustering and community identification in the multidimensional analysis in panel **(A)**. **(C)** Same analyses as in panel **(A)** performed on four chronically infected AGMs. **(D)** Heatmap showing the differential expression of cell surface markers used for clustering and community identification in the multidimensional analysis in panel **(C)**. **(E,F)** Violplots showing the distribution of markers on bulk tissue NK cells from four chronically SIV-infected CMs **(E)** and four chronically SIV-infected AGMs **(F)**. Each marker is presented with a unique color. All comparisons between NK cells were carried out using a one-way analysis of variance (ANOVA) and followed by a Tukey's multiple-comparison test. The latter is a *post hoc* test based on the studentized range distribution. Median and standard error bars are shown. Asterisks indicate a p-value <0.05.

before infection but mostly on distinct NK cell subsets after SIV infection (**Figures 4B,D**). PD-1 + NK cells were increased in SLT during SIVagm infection (**Figure 4F**) as compared with healthy animals (**Figure 2F**), which might be related to their accumulation in B cell follicles during chronic SIVagm infection (41).

All over, we demonstrate a distinct tissue distribution of NK cells after SIV infection as compared with healthy animals. Also, the dynamic of NK cell frequencies were opposite between AGM and CM: decrease in SLT for CM and decrease in the gut for AGM after SIV infection.

Resident Tissue Natural Killer Cells Express a Cytotoxic Phenotype in the Natural Host of Simian Immunodeficiency Virus Infection

Next, we analyzed the phenotype of the NK cells in distinct tissues. We performed the first analysis on total NK cells and then on NK cells that express markers that have been associated with tissue residency of T cells, such as CD69, and those present on innate mucosal lymphocytes, such as NKp44. We assessed the distribution of CD69 + and NKP44 + NK cells in distinct tissues from SIV-infected animals (**Figures 5A,B**). CD69 + NK cells were generally more frequent in tissues than in blood, in particular in the colon of CM (34 to 85%) (**Figure 5A**) and in all analyzed tissues in AGM (pLN, spleen, ileum, jejunum, and colon) (**Figure 5B**). As for CM, the highest frequencies of CD69 + NK cells in AGM were observed in the intestine compared with secondary lymphoid organs and blood (**Figure 5B**). NKP44 + NK cells were rare in blood and LN, whereas frequent in the intestine (**Figures 5A,B**). CM also harbored NKp44 + NK cells in the spleen. Altogether, CD69 + and NKp44 + NK cells were generally observed more often in tissues, and Nkp44 + NK cells were more frequent in the gut than in SLT, in particular in AGM, suggesting that these markers can be used in a similar way as in humans.

We evaluated the cytotoxic phenotype of the NK cells between the distinct tissues and combined the analysis with effector molecules: granzyme B (GzmB), interferon-gamma (IFN-g), and tumor necrosis factor-alpha (TNF-a). We performed *ex vivo* intracellular staining for GzmB, IFN-g, and TNF-a in the healthy AGMs and CMs (**Figures 5A,B**). We thus avoided any potential bias of *in vitro* cultures and provided profiles the closest to the physiological patterns *in vivo*. We observed high levels of IFN-g + NK cells in all compartments (**Figures 5A,B**). In contrast, the frequencies of TNF-a + NK cells and GzmB + NK cells varied according to the tissues. The frequencies of GzmB + NK cells were high in blood from both CMs (65–80%) and AGMs (72–96%) and low in SLT (**Figures 5A,B**). In the intestine, GzmB + NK cells were sometimes as frequent as in blood, in particular in CM, and sometimes at intermediate levels between SLT and blood. Similar to GzmB + NK cells, TNF-a + NK cells were rare in SLT and sometimes frequent in the gut. There was, however, a high interindividual variability, for example, for TNF-a + NK cells in the blood. Altogether, NK cells with a cytotoxic phenotype were seen more often in effector sites (blood and gut) than in inductive sites (pLN and spleen). IFN-g + NK cells were observed in all compartments, including in inductive sites (SLT).

Finally, we studied if there was a link between tissue-specific residency of NK cells and their effector profiles. We analyzed, therefore, the *ex vivo* expression of IFN-g and GzmB according to the expression of the potential tissue residency marker CD69 in the distinct lymphoid compartments of both species (**Figures 5C,D**). In SIV-infected CMs, most of the GzmB + cells did not express CD69, irrespective of the tissue studied (**Figure 5E**). These $CD69^{-}$GzmB + NK cells in CM were more frequent in the intestine (colon and ileum) than in SLT. In AGM, the GzmB + NK cells in blood were CD69- but in tissues CD69 + (**Figure 5F**). Similar to GzmB, the IFN-g + NK cells in SIV-infected CM were mostly CD69-, whereas in the SIV-infected AGM, many IFN-g + NK cells were CD69 + . In the SIV-infected CM, most NK cells were IFN-g + in all lymphoid tissues analyzed, in contrast to the SIV-infected AGM, where most NK cells were IFN- g-, in particular in the intestine. Altogether, most GzmB + and IFN-g + NK cells in the SLT and intestine of SIV-infected CMs were CD69-, whereas CD69 + in AGMs. Given that NK cells were increased in the intestine of CM in chronic SIV infection as compared with AGM (**Figure 3C**, right panel), this could suggest that most of the NK cells in the intestine during chronic SIVmac infection were infiltrating and not resident NK cells.

DISCUSSION

In this study, we set out to investigate the tissue distribution, phenotype, and function of NK cells in tissues of healthy and SIV-infected NHPs. Moreover, we analyzed tissue-residence markers combined with effector molecules inside tissues. This study allowed to identify tissue- and species-specific patterns of NK cell frequencies and phenotype in lymphoid and non-lymphoid tissues of primates.

Studies on NK cells in lymphoid tissues are scarce because NK cell frequencies are low in these tissues. However, it is important to gather more information about NK cells in such tissues, as their numbers and functions can be modulated by viral infections and can also be given the importance of the NK-DC cross-talk in LN for shaping the adaptive immune responses (47, 48). Although we did not analyze all possible tissues, we focused the study on lymphoid tissues where there is little information and that are key for the study of vaccine responses, i.e., mucosa as a site of viral entry and replication and SLT for the education of B and T cell responses. We compared here the NK cells present in such anatomic sites with body compartments where NK cells are more frequent and more often described (blood and liver).

We performed these analyses in representative species from Asia and Africa. We, thus, studied four NHP species and only two of them extensively: one Asian (CM) and one African (AGM) species. Of note, these two species are among the most used NHP

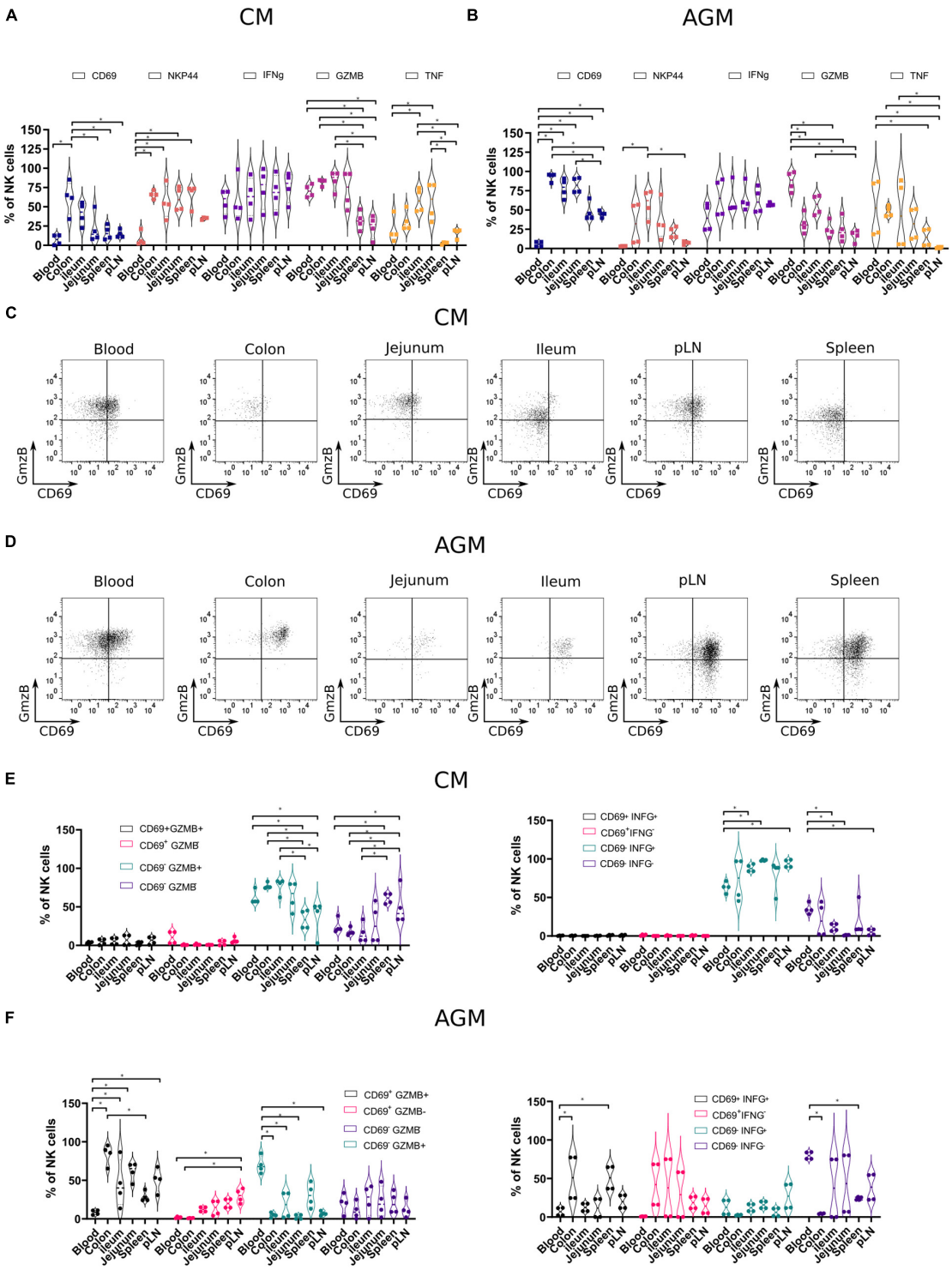

FIGURE 5 | Functional profiles of NK cells in tissues from chronically SIV-infected non-human primates. NK cells from four SIVmac-infected CMs and four SIVagm-infected AGMs were analyzed. **(A,B)** Vioplots showing the distribution of markers on bulk tissue NK cells. Each marker is labeled with a unique color. All comparisons between NK cells were carried out using a one-way analysis of variance (ANOVA) and followed by a Tukey's multiple-comparison test. The latter is a *post hoc* test based on the studentized range distribution. **(C,D)** Representative dot plots showing NK cells according to CD69 surface expression and intracellular expression of GzmB in different tissues. Dot plots for a randomly chosen animal per species are shown. **(E,F)** Graphics showing frequencies of NK cells subsets in different tissues according to CD69 and Grzm B expression (left) or CD69 and IFN-g expression (right). All comparisons were carried out using a one-way ANOVA and followed by a Tukey's multiple-comparison test. The latter is a *post hoc* test based on the studentized range distribution. Median and standard error bars are shown. Asterisks indicate a *p*-value <0.05.

models in biomedical research. Also, the data observed here for CM might be similar in related species, such as RMs.

Here, we performed a multiparameter flow cytometry. Even if we did not use next-generation sequencing technology, such analysis allowed us to focus on specific markers and to analyze a wide range of tissues (blood, liver, spleen, lymph nodes, ileum, jejunum, colon, and rectum) concomitantly. We also performed a longitudinal analysis in two distinct tissues (SLT and intestine) in response to a viral infection. Most importantly, we provide *ex vivo* data for NK cell functionality in these tissues.

Our data demonstrate that NK cells of CM were rarely CD8 + in tissues. Previous reports have shown that CD8 engagement on NK cells delivers an activating signal that increases the synthesis and secretion of IFN-g (49). CD8 may deliver an activating signal only when it is expressed at a high density (50). KIR and/or C-type lectin inhibitory receptors such as NKG2a/c can downregulate CD8-mediated triggering (49). The downregulation of CD8-dependent IFN-g production exerted by inhibitory receptor superfamily members may represent a mechanism to limit NK cell responses, as it has been proposed for the inhibiting signal delivered through inhibitory receptors to avoid lysis of autologous cells expressing the appropriate human leukocyte antigen (HLA)-I ligand of a given inhibitory receptor superfamily member (51). Other NK cell surface receptors, including NKp30 and NKp44 (52–54), could thus rather be involved in recognition of target cells and activation of NK cell-mediated lysis in CM tissues. Our data also imply that studies using anti-CD8a antibodies to deplete NK cells need to consider the low frequency of CD8 + NK cells in tissues of some species. Indeed, our study indicates that in CM, NK cells will not get efficiently depleted in tissues when using anti-CD8 antibodies.

CXCR5 is a chemokine receptor mainly expressed on B cells, as well as DCs and T cell subsets such as T_{FH} cells (55, 56) present in B cell follicles. We have recently provided evidence that the capacity to control SIV viral replication in B cell follicles of AGM was associated with the presence of $CXCR5^{+}$ NK cells (41), showing that CXCR5 could be also be frequently expressed on NK cells under some circumstances. The role of CXCR5 in the gut is less studied (57). Nevertheless, in the gut, CXCR5 is known to support solitary intestinal lymphoid tissue (SILT) formation and B cell homing (58). Here, we show that CXCR5 is expressed to high frequency by ileum NK cells in healthy CM and AGM. This high frequency of CXCR5 + NK cells was also noticed in CM jejunum and colon. Strikingly, after SIV infection, the frequency of CXCR5 + NK cells strongly decreased in the gut of CM for unknown reasons. It could be related to the increase of infiltrating, CXCR5- NK cells. Microbiota and other external stimuli can foster the formation of aberrant SILT distinguished by impaired development of B cell follicles in CXCR5-deficient mice (58–60). In this context, it could be interesting to investigate if the decrease of CXCR5 + NK cells in the intestine during SIVmac infection, concomitant with the well-known induction of microbial translocation, may be associated with the formation of SILT in NHPs and humans during HIV and SIVmac infections.

Although CD69 has long been viewed as an activation marker of both T and NK cells, it is now clear that it has an important role in retaining immune cells in tissues by inhibiting sphingosine-1-phosphate receptor 1 (61, 62). CD69 is now considered a tissue-resident marker for T cells (63, 64). For NK cells, it is less clear if tissue-resident NK cells can be characterized based on the expression of CD69. It has recently been shown that the CD69 expression displays subset- and site-specific variations by tissue NK cells in humans (5). Here, we show that during SIV infection in NHP, NK cells in peripheral blood largely lack expression of CD69. Our data in the tissues argue in favor of CD69 as a tissue residence marker for NK cells in NHP. The main difference between the pathogenic and non-pathogenic SIV infections in the two species (CM and AGM) came from the observation that the frequency of CD69 cells in tissues during pathogenic infection was relatively low, whereas in the natural host, a high frequency of CD69 + NK cells was observed in tissues. The high frequency of CD69- NK cells in SIVmac infection might be the result of infiltrating NK cells. The combined analysis of CD69 with GzmB + and IFN-g indicates that most of resident NK cells harbored a cytotoxic profile, whereas potentially infiltrating (CD69-) NK cells also expressed the pro-inflammatory IFN-g cytokine. It is not clear though if these changes are the result of blood NK cell infiltration or whether there are phenotypic changes in resident NK cell populations during SIV infection.

The role of tissue localization in NK cell development and function and how circulating NK cells relate to those in different sites are not well understood during HIV/SIV infection (22, 65, 66). Only a few data on NK cells in tissues are available during HIV/SIV infections (41, 65, 67). Immediately after HIV/SIV infection, changes occur in NK cell populations in the gut-associated lymphoid tissue. Thus, the frequency of both intraepithelial and lamina propria NK cells was increased in PLH with an incomplete blood CD4 + T cell recovery (CD4 < 350 cells/μl) despite several years of effective antiretroviral treatment (68). Similarly, an increase in total and NKG2a/c + NK cells in rectum RM has been shown during SIVmac infection (66, 69). In contrast, the NKp44 + lymphocytes were significantly depleted in acute HIV and SIV infections in the mucosa and continued to decline in frequency during chronic phase (70). Antiretroviral therapy significantly increased the frequency of mucosal NKG2a/c + NK cells (71). SIV infection in pathogenic models drives trafficking away from secondary lymphoid organs toward the intestine (22, 72, 73). This is related to the upregulation of the gut-homing marker $\alpha_4\beta_7$ on NK cells, as shown in chronic SIVmac infection coupled with downregulation of the LN-trafficking marker CCR7 (66, 74). Our data in the CM are in line with these reports. Strikingly, we observed opposite tissue dynamics in heterologous and natural hosts of SIV. In the natural host, NK cells were not directed toward the intestine, and the frequencies of total NK cells in the gut were decreased in SIVagm infection in contrast to SIVmac infection. This could explain the lack of NK cell-mediated viral control in the intestine of SIVagm-infected AGMs. In CM, NK cells decreased in the LN but increased in the gut in contrast to SIVagm infection in AGM. These distinct NK cell tissue distributions were associated with CXCR3 expression, supporting

its role as an inflammatory tissue homing marker for NK cells. The higher levels of the CXCR3 ligand (IP-10) in the intestine of the SIVmac-infected macaques as compared with those of the SIVagm-infected AGMs (75, 76) might explain in part, together with previously reported upregulations of other receptors such as $\alpha_4\beta_7$, the higher levels of NK cells in the intestine of the SIVmac-infected macaques as compared with the natural host. The fact that NK cells seem to be redirected to the intestine in SIVmac infection raises the question of their inefficient activity against the cells replicating the virus in the gut. In fact, an increase in these cells in this compartment should favor a better control of the viral replication. However, beyond the mechanisms established by the virus to escape NK cell lysis, the inflammatory environment, as well as the loss of regulatory cells (i.e., ILCs, TH17, CD4 T cells), might profoundly and durably alter NK cell functions. It has also previously been reported that NKG2a/c + NK cells accumulate in vaginal mucosa during acute SIVmac infection and that this occurred concomitant with an increase in HLA-E that could induce an inhibitory signal to the NKG2a/c + NK cells (77). Alternatively, it is not excluded that NK cells directed to the gut contribute to tissue damage in the intestine (78). It has indeed been demonstrated in the macaque that microbial translocation increased the recruitment of cytotoxic NK cells in the liver (78). This increase in cytotoxic NK cells resulted in accelerated destruction of this organ. Recently, NKp44 + NK cells have been implicated in autoimmune diseases, such as inflammatory bowel disease or Crohn disease (79); however, whether they participate in pathologic or protective processes of chronic inflammation *in vivo* remains controversial. HLA-DP molecules have recently been shown to bind NKp4433 directly. HLA-DP is constitutively expressed on antigen-presenting cells, including B cells. HLA-DP molecules are upregulated on non-hematopoietic tissues in response to inflammation (80). Thus, it might be relevant to investigate the impact of NKP44 + NK cells on gut homeostasis in the context of gut inflammation, such as HIV infection. The potential contribution to tissue damage and homeostasis by NK cells during chronic HIV/SIVmac infections will need to be addressed in future studies.

Given the remaining necessity in the use of NHP in biomedical research, as shown by the recent severe acute respiratory syndrome coronavirus 2 outbreak, these data are highly relevant for establishing the validity and utility of such models for studying the role of NK cells in human diseases. More and more studies raise the question of the utility of NK cells in antitumor and antiviral immunotherapies. Our data on the NK cell tissue distribution and functional profiles in the NHP contribute to the understanding of their role in lymphoid tissues during HIV infection as well as to efforts in the development of vaccines and therapies against human diseases.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of Animal Experimentation (CETEA-DSV, IDF, France) (Notification 12-098 and A17-044 and license number 392001/7).

AUTHOR CONTRIBUTIONS

NH and MM-T designed the study. NH designed the experiments. NH, PR, CP, and BJ performed the experiments. CS-H and A-SB provided the samples. NH performed the statistical analyses. NH, PR, CP, J-LP, A-SB, BJ, and MM-T analyzed the data. VC, RL, and BJ coordinated the animal studies. MM-T obtained the funding. NH and MM-T wrote the manuscript. All co-authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02134/full#supplementary-material

FIGURE S1 | Gating strategy used to define NK cell subsets in the NHP.

FIGURE S2 | Viremia profiles during SIV infections. Seven CM and seven AGM were respectively infected with SIVmac251 and SIVagm.sab92018. The SIV copy numbers in the plasma over time are indicated.

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REVIEW
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Airway Natural Killer Cells and Bacteria in Health and Disease

*Maud Theresine†, Neha D. Patil† and Jacques Zimmer**

CG I Group, Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg

Natural killer (NK) cells are innate lymphoid cells at the interface between innate and adaptive immunity and mostly studied for their important roles in viral infections and malignant tumors. They can kill diseased cells and produce cytokines and chemokines, thereby shaping the adaptive immune response. Nowadays, NK cells are considered as a strong weapon for cancer immunotherapy and can for example be transduced to express tumor-specific chimeric antigen receptors or harnessed with therapeutic antibodies such as the so-called NK engagers. Whereas a large body of literature exists about the antiviral and antitumoral properties of NK cells, their potential role in bacterial infections is not that well delineated. Furthermore, NK cells are much more heterogeneous than previously thought and have tissue-characteristic features and phenotypes. This review gives an overview of airway NK cells and their position within the immunological army dressed against bacterial infections in the upper and predominantly the lower respiratory tracts. Whereas it appears that in several infections, NK cells play a non-redundant and protective role, they can likewise act as rather detrimental. The use of mouse models and the difficulty of access to human airway tissues for ethical reasons might partly explain the divergent results. However, new methods are appearing that are likely to reduce the heterogeneity between studies and to give a more coherent picture in this field.

Keywords: natural killer cells, bacteria, infection, lungs, airways, chronic obstructive pulmonary disease, pathogenesis

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Ana Stojanovic, Heidelberg University, Germany

***Correspondence:**
Jacques Zimmer
jacques.zimmer@lih.lu

† These authors have contributed equally to this work

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INTRODUCTION

Historically, human natural killer (NK) cells have mostly been harvested from and studied in peripheral blood (PB), which is an easy way to access them, and where they usually represent 5–20% of all lymphocytes (1–3). Two different subsets have been initially described, called $CD56^{bright}CD16^{-}$ (up to 10% of PB NK cells) and $CD56^{dim}CD16^{bright}$ (at least 90% of PB NK cells). Phenotypic and functional (cytokine production for the former and cytotoxic activity for the latter) characteristics distinguish both populations (1–3). However, things are not that simple, as four additional subpopulations have been identified, which are (i) $CD56^{bright}CD16^{dim}$, (ii) $CD56^{dim}CD16^{-}$, (iii) $CD56^{-}CD16^{bright}$ and finally (iv) $CD56^{dim}CD16^{dim}$ (4), the latter still being almost systematically overlooked in the literature (5). Human NK cell functions are governed by a balance between the messages transmitted through inhibitory receptors (IR), such as KIR, CD94/NKG2A, ILT2, TIGIT, and activating receptors (AR), such as particularly NKG2D and the natural cytotoxicity receptors (NCR) NKp46, NKp30, and NKp44 (6). When stimulated, NK cells exert natural cytotoxic activity against tumor cells and virally infected cells, antibody-dependent

cellular cytotoxicity (ADCC) toward antibody-coated target cells via the Fcγ receptor CD16, and cytokine and growth factor production (2, 6).

Most of the ligands of the IR are represented by Human Leukocyte Antigen (HLA) class I molecules, so that target cells lacking those molecules in part or in total, become killed by the NK cells. The IR nevertheless have another important function, as they are responsible for NK cell education. Indeed, before a developing NK cells becomes functional, its self-specific IR must interact with their ligands expressed by cells in their micro-environment (7, 8). NK cells without such IR, which can represent up to 20% of all PB NK cells, remain uneducated, and hyporesponsive (7, 8). However, they can be activated under certain conditions, such as some viral infections (9).

A hot topic in the NK cell field is of course their potential use as immunotherapeutic anticancer agents. To reach this aim, several approaches are studied, and for example the chimeric antigen receptor (CAR) NK cells, which allow the specific targeting of a tumor antigen (10), or the use of multi-specific antibody constructs directed simultaneously at several NK cell AR and tumor surface molecules (6), appear as particularly promising. It has also been discovered that NK cells, which had been previously considered as exclusively innate immune cells, can develop a memory-like behavior (11). Finally, NK cell metabolism, which appears to be different between educated and uneducated cells, is extensively studied (12, 13).

Another aspect that has changed our view on NK cells in recent years is the observation of a broad heterogeneity of this population. Not only are there many subsets in PB based on the clonal distribution of several IR, immature, partly mature and completely mature fractions based on the relative expression of CD56, CD16 and the IR NKG2A and KIR (14), conventional and adaptive NK cells (14, 15), but there are also heterogeneous aspects between PB and various tissues (15, 16). Very recent data by Dogra et al. (17) suggests a model in which the phenotype, the degree of maturity and the functions of NK cells are closely dependent on the anatomic location, with no influence of age and gender.

NK CELLS IN THE UPPER AIRWAYS

It is quite difficult to find a substantial amount of references regarding upper airway NK cells. In human, the articles were mostly reporting on the investigation of NK cells in chronic rhinosinusitis, an inflammatory state of the mucosa of the nose and the sinuses (18) with a significant impact on quality of life. Two different forms, one with nasal polyps and one without nasal polyps, are distinguished (19, 20). Bacterial pathogens are considered as one of the etiological factors in this disease (18). However, as the bacteriology of ethmoidal biopsies was the same regardless of the presence or absence of polyps, Niederfuhr et al. questioned the bacterial role in the pathogenesis of the polyps as well as a systematic antibiotic treatment (19). In a study of 18 patients, further subdivided into those responding and those resistant to treatment, and 19 healthy controls, Kim et al. investigated exclusively PB NK cells. The authors demonstrated that the PB NK cells from the patients had decreased effector functions compared to the healthy controls, with the treatment-resistant individuals being most severely affected (18). The recent manuscript by Kaczmarek et al. (20) reported not only on PB NK cells, but also on those from nasal mucosa and from nasal polyps. However, the exploitation of the material was limited to $CD3^{-}CD56^{+}CD16^{+}$ events, which excluded the population of $CD56^{bright}CD16^{-}$ NK cells that might be numerically well represented in these tissues. The phenotypic investigations of this subset in the nose revealed a predominance of relatively immature, $CD27^{+}$ NK cells. Furthermore, the AR NKG2D was expressed at lower frequencies (lower percentages of $NKG2D^{+}$ cells) and lower density of expression in the nasal mucosa and the polyps compared to PB (populations negative for NKG2D were identified in the tissues). Finally, the percentage of NK cells among lymphocytes (mean: 33%) was significantly higher in the polyps than in PB (20).

Okada et al. published a paper about NK cells in the nasal mucosa of the mouse on the C57BL/6 background (21), in which they showed that these NK cells were more immature (according to the relative levels of expression of CD27 and CD11b) and phenotypically more activated (reduced expression of CD62L, higher percentage of $CD69^{+}$ cells) than those from spleen and lung. Around 12% expressed the tissue residency and activation marker CD69 and one third of those also CD103. The pattern of expression of the Ly49 receptor family was different between the three tissues. Functionally [CD107a staining and interferon (IFN)-γ production], nasal NK cells appeared to be hyporesponsive compared to their spleen, but not their lung counterparts (21), which might be related to the possibility that the fraction of $CD69^{+}$ NK cells was not sufficient to significantly activate the global NK cell population in the chosen experimental readouts.

Although this dataset is interesting *per se*, it should not be ignored that Casadei and Salinas (22), in a review about different animal models of nasal infections and immunity, cited several anatomic (functional vomeronasal organ in contrast to human, no Waldeyer's ring) and physiologic (macrosmatic, no coughing-sneezing reflex, lower sensitivity to human viruses) limitations of the mouse in this context, so that such results should always be considered with care before extrapolating to the human situation.

NK CELLS IN THE LUNGS

Lung NK cells have recently been extensively reviewed in this journal (23, 24), so that a summary of their most important features might be sufficient. Lungs are constantly exposed to microparticles from the environment. Particularly, as the mucosal lung epithelium is at the interface between the outside world and the organism, it can become the entry site for infectious pathogens, be they bacterial, fungal, or viral in nature. Therefore, an extensive and sophisticated local immune response is waiting to be triggered at this anatomic location, and human NK cells, which represent around 5–20% of lung lymphocytes (24), are a part of it. The work of Marquardt et al. has established that most human lung NK cells represented the circulating

subset and had the mature $CD56^{dim}CD16^{bright}$ phenotype (25). They expressed more frequently the differentiation marker CD57 as well as educating KIR than blood NK cells from the same donors but were relatively hyporesponsive upon stimulation with HLA class I-negative target cell lines. In addition, however, a putative tissue-resident subset (around 20% of all lung NK cells), further subdivided into relatively immature $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{-}$ cells (24, 25), expressed the tissue residency markers CD69, CD49a, and CD103. These cells were characterized in detail again by Marquardt et al. (26), who showed that they were functional, especially after stimulation with the cytokine interleukin (IL)-15 and displayed a unique transcriptional profile. Several subpopulations could be distinguished based on the relative expression of CD49a and CD103 (24, 26).

Natural killer cells have likewise been investigated in mouse lungs, particularly by Wang et al. (27) and Michel et al. (28). Both groups found that lung NK cells were more mature than those from the spleen (28) or other organs (27) according to the relative expression of CD27 and CD11b. Whereas the former authors described a higher expression level of the IR CD94/NKG2A and a lower level of the AR NKG2D, the second paper could confirm this data only regarding NKG2D in terms of mean fluorescence intensities. Lung NK cells proliferated less, degranulated less (CD107a assay) and were less cytotoxic than splenic NK cells (28), but these functions were rapidly up-regulated upon bacterial lung infection (27). This suggests that at homeostasis, lung NK cells are inhibited to avoid damage to normal autologous cells, but that they can quickly intervene in case of an infectious insult (27). Michel et al. showed in *in vitro* co-culture systems that both spleen and lung macrophages could significantly up-regulate the cytotoxic activity of lung NK cells through a contact-dependent mechanism (28).

Regarding the homeostatic situation, research in recent years has revealed that in contrast to the older view of the lungs as sterile organs, a lung microbiota is present in the lower airways which exerts significant effects in health and disease, although it is not as abundant as in the gut (29–32). The term "microbiota" refers to all the microorganisms present, namely bacteria, fungi, protozoans, and viruses (29), but here we will only consider the role of bacteria. Six phyla are predominantly represented in the lower airways: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria (31, 32), Acidobacteria, and Fusobacteria (32). This microbiota is supposed to be transient in healthy donors and to be established from micro-aspiration and inhalation (32) and its composition at any given time point submitted to the parameters of bacterial arrival, bacterial removal, and local immune responses (32, 33). In this way, an equilibrium state is reached that depends also strongly on the gut microbiota through various bacterial metabolites and contributes to the maintenance of homeostasis in the lower airways (gut – lung axis) (32–34). Everything that disturbs this balance, such as some medications and particularly antibiotics, increases in nutrients (high fat diet, low fiber diet), cigarette smoke, infectious agents, chronic inflammation, can disturb the gut as well as the lung microbiota and lead to a state of dysbiosis, characterized by an increased number of airway bacteria and a change in its composition. The dysbiosis is profoundly linked to several severe lung diseases [asthma, chronic obstructive pulmonary disease (COPD), infections, cancer] (29–35).

Natural killer cells have, to our knowledge at least, not been investigated in detail in the context of a normal lung microbiota to date. As most lung NK cells are not activated nor tissue-resident (as illustrated by their negativity for CD69), they might not react very strongly to a normal microbiota. However, as they are expressing several bacteria-specific toll-like receptors (TLRs) that signal in the presence of bacterial pathogens (36), it might be conceivable that they could also mount an immune response toward microbiota components and that this would contribute to homeostasis. The overall immunosuppressed state of lung NK cells at baseline would help to avoid aggression of harmless and useful bacteria and of uninfected autologous cells (31). Yang et al. (31), as well as Fuchs and Colonna (37), discuss data claiming that at steady state, alveolar macrophages secrete immunosuppressive cytokines which keep NK cells in respect. This is in contrast with the results of Michel et al. (28), discussed above. However, the macrophages and dendritic cells (DC) switch to pro-inflammatory cytokine production in case of a bacterial or viral infection and thereby activate the NK cells.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

This entity is the third cause of mortality in the United States of America (3) and worldwide (38) and is in most cases the consequence of prolonged cigarette smoking (39). It is characterized by airflow obstruction, emphysema, recurrent infections (24, 39), chronic inflammation, and overproduction of mucus (40). Acute exacerbations significantly limit the quality of life of the patients (38, 39). The exacerbations are in principle caused by viral or bacterial infections, the latter most frequently due to *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (39). *Pseudomonas aeruginosa* is another bacterium frequently involved and one of the most dangerous ones, based on its highly pathogenic properties (39), and its remarkable level of resistance to antibiotics.

Natural killer cells have been investigated in human COPD as well as in animal models of this disease. Motz et al. demonstrated that exposure of pulmonary leukocytes to viral pathogen-associated molecular patterns (PAMP) induced higher functional properties (degranulation measured with the CD107a assay, and IFN-γ production) *ex vivo* in chronic cigarette smoke exposed than in non-exposed C57BL/6 mice (40). Interestingly, bacterial PAMP appeared to be less efficient in this model, as among five molecules tested, only FSL-1 (bacterial lipopeptide, TLR2/6 agonist) and lipopolysaccharide (LPS, TLR4 ligand) increased the percentage of IFN-γ^{+} NK cells above the one of the non-exposed mice. In contrast, other papers reported that NK cell functions are diminished in COPD (41).

It has been further repeatedly demonstrated that in COPD or relevant animal models, NK cell cytotoxic activity is increased relative to non-COPD smokers and healthy individuals (23,

24). Based on the model of lung NK cell hypo-responsiveness at baseline, cigarette smoke and even more the inflammatory state of the lower airways in COPD would activate the alveolar macrophages and induce their production of pro-inflammatory cytokines. These would, in turn, unleash the NK cells and increase their cytotoxic activity, cytokine and chemokine expression, leading to a further aggravation of the inflammation and the clinical status of the patients.

Indeed, in accordance with this concept, Freeman et al. (42) showed that $CD56^+$ cells (in fact a mixture of NK cells and $CD56^+$ T lymphocytes) isolated from lung parenchymal samples of non-COPD smokers and COPD patients with a smoking history, although similar in terms of frequencies between the cohorts, had a different cytotoxic activity toward autologous lung epithelial cells. The $CD56^+$ lymphocytes from the COPD patients were more cytotoxic than the cells from the non-COPD smokers, in an experimental setup without additional stimulation. The target cells were supposed to be mostly epithelial cells based on their positivity for CD326, their size, and their negativity for the hematopoietic cell marker CD45. The cytotoxicity was measured as the percentage of Annexin V^+ target cells after the co-culture with the effectors and was around 10% in most samples. This was not a lot, but the NK cells and $CD56^+$ T cells were not otherwise activated. Most of the parenchymal lung NK cells were $CD56^+CD16^+$ and the minor rest $CD56^+CD16^-$ (42).

Another study was provided by the same group in 2018 (43). It showed that isolated, purified lung NK cells induced apoptosis in autologous epithelial cells. This time, the mean level of cytotoxicity was rather high compared to the previous paper, and it was very significantly stronger in COPD patients than in non-COPD smokers. The NK cells, but not the target cells, determined this increased cytotoxic activity, because K562, a HLA class I-negative myeloid leukemia cell line used as the standard NK cell target, was also lysed more efficiently by COPD NK cells than by their non-COPD counterparts. The authors confirmed their data in a mouse model and then showed that the NK cells were primed by DC *via trans*-presentation of IL-15 (43), a phenomenon first described in 2007 by Andreas Diefenbach and his group (44). This would nicely explain the higher level of NK cell cytotoxicity observed in COPD.

Along the same line, Okamoto et al. administered IL-2 and IL-18 to normal mice and observed a lethal effect within 4 days, selectively involving the lungs, with a profound interstitial infiltration of lymphocytes dominated by NK cells (45). High levels of various cytokines and chemokines were found in serum and lungs. Depletion of the NK cells by antibodies completely abrogated the lethal injury, which is a convincing demonstration of the potentially destructive power of NK cell-activating cytokines and NK cells themselves (45). This work was intended as a contribution to the elucidation of the pathogenesis of interstitial pneumonia, but similar mechanisms, in the presence of high levels of pro-inflammatory cytokines in bacterial infections, might contribute to COPD. In human cancer patients, administration of high dose IL-2 induced a vascular leakage syndrome where the so-called lymphokine activated killer cells (equivalent to highly activated NK cells) destroyed endothelial cells, causing a generalized edema, and damaging several organs (46).

Hodge et al. demonstrated a higher number of NK cells in the bronchoalveolar lavage fluid (BALF) of COPD patients (the cohort was composed of current smokers and of ex-smokers) than in healthy smokers (47), a higher content of the cytolytic molecule granzyme B and, most importantly, a significantly increased cytotoxic activity against K562. They also found a reduction in the percentage of BALF NK cells expressing CD94 (which they consider as IR, although it is more a chaperone molecule for the true IR NKG2A). Nevertheless, this indirect measure of a down-regulation of NKG2A could indicate that it contributes to the higher NK cell cytotoxic activity observed in COPD (47).

Recently, Osterburg et al. presented a multiparameter flow cytometry study of PB NK cells from COPD patients compared with smokers and never smokers (38). In contrast to those, COPD patients and smokers highly expressed the maturation marker CD57 as well as the AR NKp46 and NKp44 (normally only present on activated but not on baseline NK cells), but lower levels of CD56. Certain NK cell subpopulations were indicative of prior exacerbations (38).

The AR NKG2C, which is significantly present only on adaptive NK cells from human cytomegalovirus (CMV)-infected individuals, was not differentially expressed in PB of COPD patients with a smoking history and healthy volunteers, but present on a higher percentage of NK cells in the patients with the most frequent exacerbations and the most reduced lean mass (48). A relationship with the bacterial burden cannot be excluded in this context, as there might be a correlation between the viral reactivations and the bacterial colonization, contributing together to the higher number of exacerbations.

Most of the papers discussed above investigated the NK cell cytotoxicity toward autologous cells or conventional NK target cells, but what about a potential direct bacterial killing? NK cells, upon appropriate stimulation, release cytolytic molecules called perforin, granzymes and, in human but not in mice, granulysin, which have an additive or synergistic cytolytic effect toward bacteria (49). They can form pores in the target cell walls and thereby eliminate the microorganisms, but in addition they are able to eliminate some types of eukaryote cells infected by bacteria (41, 49, 50). Furthermore, in addition to the direct effect, NK cells are embedded in the immunological network and react (through an increased cytotoxic activity and cytokine production) to the immune cells and the cytokines/chemokines in their environment (50), which is strongly shaped in case of a bacterial infection ["cellular crosstalk" (50)].

Data about chronic rhinosinusitis, nasal polyposis and COPD are summarized in **Table 1**.

Pseudomonas aeruginosa

As mentioned above, this ubiquitous Gram-negative pathogen is part of those colonizing the lower airways in COPD, but it is also a major problem in cystic fibrosis and in nosocomial infections, with a high morbidity and mortality (51). The role of NK cells in the host defense against this bacterium has been quite extensively studied by the team of Michael T. Borchers (51, 52) in

TABLE 1 | Natural Killer (NK) cells in airway diseases.

Disease	Species	Origin of NK cells	Effect on NK cells	References
Chronic rhinosinusitis	Human	Peripheral blood	↓ Effector functions	(18)
Nasal polyps	Human	Nasal mucosa	↓ NKG2D; ↑ CD27	(20)
COPD model	Mouse	Lung	↑ IFN-γ	(40)
COPD	Human	Lung	↑ Cytotoxicity	(43)
COPD	Human	BALF	↑ Cytotoxicity	(47)
			↑ Number	(47)
COPD	Human	Peripheral blood	↑ CD57, NKp46, NKp44	(38)

Consequences of the indicated diseases on NK cell phenotype and functions. COPD, chronic obstructive pulmonary disease; BALF, bronchoalveolar lavage fluid; and IFN-γ, interferon-gamma.

mouse models. In the chronologically first work, outbred CD-1 mice were intranasally infected with the *P. aeruginosa* laboratory strain PAO1 (52) and evaluated 24 h later. The findings can be summarized as follows: (i) the infection increased the expression of ligands for the AR NKG2D, present on almost all NK cells but also on a subpopulation of $CD8^+$ T lymphocytes, *in vivo*; (ii) similarly, these ligands increased in an infected human lung epithelial cell line *in vitro*; (iii) the inhibition of the AR NKG2D with a monoclonal antibody significantly reduced the clearance of *P. aeruginosa* from the lungs; (iv) antibody-mediated NKG2D blockade down-regulated the amount of the cytokines IL-1β, IFN-γ and tumor necrosis factor (TNF)-α and in addition of nitric oxide; and finally, (v) the same experiment also revealed a threefold reduction of epithelial cell damage, measured as shedding of these cells into the BALF (52). The latter point brings us again to the recurrent theme of lung cell damage that can be induced by activated NK cells, whereby it would have to be determined if this is beneficial (elimination of infected cells by NK cells) or deleterious (exaggerated damage to the epithelium).

The follow-up paper (51) then presented a conditional mouse model with an inducible expression of NKG2D ligands on lung epithelial cells. Here, the bacterial clearance was significantly higher in those mice that overexpressed the NKG2D ligands. Moreover, the survival up to 96 h post-infection and the level of phagocytosis were significantly increased in the latter group. Similarly, in *in vitro* experiments, where the NK cells were stimulated with LPS, the percentage of NK cells producing IFN-γ was much higher in the mice with the increased expression of NKG2D ligands. As expected, this percentage dropped (but was not completely abolished) in NK cells from infected mice treated with an anti-NKG2D antibody (51).

However, the *P. aeruginosa*-derived exotoxin A, which in combination with IL-1α may induce a dangerous inflammatory state with tissue damage in the host, has also been shown to inhibit NK cell cytotoxic activity against K562, even in the presence of usually stimulating cytokines such as IL-2 (53). The inhibition was almost complete with a high dose of the toxin and still partial with a low dose (53). The effector cells were not purified NK cells but peripheral blood mononuclear cells (PBMC), so that an indirect effect on the NK lymphocytes might play a role in this readout.

Furthermore, Pedersen and Kharamzi described already in 1987 that the *P. aeruginosa*-derived alkaline protease and elastase inhibited NK cell cytotoxic activity against K562, presumably due to a reduction in the effector-target conjugate formation (54). In addition, these molecules strongly reduced the binding of an anti-CD16 (called Leu-11 at that time) antibody (54).

Burkholderia cepacia Complex

This group of pathogenic Gram-negative bacteria is composed of several species, of whom some are dangerous for cystic fibrosis patients, as they are highly resistant to multiple antibiotics (55). Li et al. (55) investigated the interaction between *Burkholderia cenocepacia* and NK cells, and first demonstrated that the NK-like leukemia cell line YT (56), as well as primary purified human NK cells, significantly reduced the number of living bacteria (measured as CFU) after a co-incubation of 2 to 4 h. The results were confirmed with life cell imaging techniques and bacterial uptake of propidium iodide (PI). The authors then wanted to know if the killing activity was contact-dependent or not, and first showed that YT cells bound the fluorochrome-labeled bacteria. Then, they could demonstrate that a direct contact was needed for the killing activity, as nothing happened to the bacteria when they were separated from the NK cells by a porous membrane, allowing passage of soluble molecules but not of cells (55). Most bacteria remained extracellular and were not taken up by the YT cells. Killing was almost completely abrogated after treatment with strontium chloride ($SrCl_2$), which is known to deplete NK cells from their cytotoxic granules (57). Finally, it was established that Src family kinases were activated in YT cells after the contact with *B. cenocepacia* (55). This is a very nice demonstration that NK cells are able to directly kill certain extracellular bacterial species through NK cell – bacteria contact, although the precise mechanism is still unknown. Other possible mechanisms of NK cell-mediated elimination of bacteria are the lysis of intracellular pathogens within the infected cells and the activation of other immune cells, and particularly of macrophages, via NK cell-derived cytokines (such as IFN-γ) (55), and most likely also the killing of bacteria-infected cells expressing ligands for NK cell AR.

Klebsiella pneumoniae

This is another Gram-negative pathogen which poses a major problem due to its frequent causative involvement in nosocomial infections (particularly in pneumonia) and the steady increase of strains multi-resistant to antibiotics (58). Chalifour et al. (59) demonstrated that the outer membrane protein A (KpOmpA) from this microorganism, known to signal via TLR2, induced

IFN-γ and α-defensin (an antimicrobial peptide) synthesis and release in human NK cells. In the mouse, both NK cell-derived IFN-γ (58) and IL-22 (60) have been described to be necessary for bacterial clearance (58, 60). In the paper from Xu et al., it was nicely shown with genetic controls and depletion experiments that the immune defense against this pathogen indeed deeply involved NK cells and that a subset of them produced IL-22. The NK cells had a conventional and mature phenotype (less $CD27^+$, more $KLRG1^+$) distinct from other innate lymphoid cells (ILC) (60). Ivin et al. focused on the fact that IFN-γ production by NK cells, likewise necessary for the elimination of the bacteria through a network with alveolar macrophages, was dependent on the NK cell-intrinsic stimulation by type I IFN, in turn induced by *K. pneumoniae* (58). In contrast to the crucial role of NK cell-derived cytokines, their granzymes (A and B), one of the constituents of the lytic granules, did not seem to play a major role in this model (61). However, this does not rule out that in human, granulysin and perforin together might have a cytotoxic effect on these bacteria.

OTHER GRAM-NEGATIVE BACTERIA

In the case of *Helicobacter pylori*, responsible for chronic gastric inflammation with the potential to lead to ulcers or cancer, pre-incubation with fixed bacteria increased the cytotoxic activity of NK cell-enriched PBMC toward K562 and other tumor target cells, as well as the release of IFN-γ (62). Furthermore, Rudnicka et al. showed that the bacterial glycine acid extract induced NK cell expansion and IFN-γ production, whereas the LPS from the same bacteria inhibited these parameters, and instead favorized the apparition of IL-10-producing NK cells (63). Although this might just marginally be relevant for NK cells in the lungs, it nevertheless shows to which extent these cells can react to bacteria and how the latter try to manipulate them.

Legionella pneumophila, the agent of Legionnaires' disease, is replicating intracellularly in macrophages. Here again, NK cell production of IFN-γ, induced probably through direct TLR messages (64), IL-12 [produced by DC (65)], and IL-18 [produced by neutrophils (66)], was crucial for bacterial clearance from the lungs. In addition, Blanchard et al. had already observed in 1988 in a mouse model that this pathogen stimulated NK cells *in vivo* and *in vitro* to produce IFN-γ and to increase their cytotoxic activity to tumor cell lines, the highest levels having been measured in the lungs (67).

GRAM-POSITIVE BACTERIA

One of the most frequent culprits in community-acquired pneumonia is *S. pneumoniae*. Regarding the role of NK cells in this infection, their beneficial or detrimental action depended on the pathogen's serotype (68). Thus, the control of serotype 1 depended on NK cells, as demonstrated by Baranek et al. in a mouse model (68). These authors investigated the consequences of a defect in the transcriptional cofactor Four-and-a-half LIM-only protein 2 (FHL-2) on NK cells in general and on pneumococcal infection particularly. It had been previously established that IFN-γ was, once more, the crucial factor in host defense in this context, and that NK cells were one of its major producers (69). In the spleen and the lungs of FHL-2 knockout (KO) mice, the number of NK cells and their expression of the AR NKG2D and NK1.1 (CD161c) were down-regulated and a negative effect of the deficiency on NK cell maturation was observed. Mortality to *S. pneumoniae* lung infection was strongly increased in the KO mice but could be rescued by the adoptive transfer of wildtype NK cells. Finally, the authors showed that IFN-γ production by NK cells was severely reduced and that less neutrophils were recruited to the lungs of the KO animals (68).

The role of the mostly immunosuppressive cytokine IL-10 in dampening the immune response to pneumococcal infection was shown in 1996, when van der Poll et al. administered the pathogen intranasally together with IL-10 and observed early mortality and reduced levels of the pro-inflammatory factors IFN-γ and TNF. Conversely, all this was restored when the mice were pre-treated with an anti-IL-10 antibody (70).

These results were very recently confirmed by Clark et al. (71), who worked with IL-10 reporter and IL-10-KO mice to observe that *S. pneumoniae* induced IL-10 production by NK cells (around 50% of total lung NK cells) with a negative effect on animal survival, and that the bacterial burden was diminished in the lungs of the KO mice compared to wildtype animals. NK cell depletion in the latter induced a strong reduction in the bacterial lung counts and in IL-10. Furthermore, IL-10-deficient mice had significantly more neutrophils and monocytes in the infected lungs. Finally, the virulence protein Spr1875 from *S. pneumoniae* was identified as the IL-10-inducing factor (71).

None of these papers investigated the potential balance between the pro-inflammatory and anti-inflammatory effects of IFN-γ and IL-10, respectively, on the outcome of this infection, which would anyhow have been technically challenging. One might suppose that IL-10 is there to down-regulate an overwhelming immune response that would damage lung tissues, but on the other hand, it might also be counterproductive to dampen it too much and thus to lose control over the pathogens (72). Other groups have described that human as well as mouse NK cells could produce and release IL-10 (73, 74), although, according to Perona-Wright et al., this only occurred in the case of a systemic, but not a localized, pulmonary infection (with the Gram-negative bacterium *Yersinia pestis*) (74). In the case of systemic infections with *Listeria monocytogenes* and *Y. pestis*, approximately 50% of blood NK cells became $IL\text{-}10^+$, and the cytokine was produced by a NK cell subset circulating in blood prior to the infection (74).

Before studying *S. pneumoniae* (71), Clark et al. had already shown that *L. monocytogenes* elicits IL-10 production by NK cells via the virulence factor p60 (with, as a consequence, an inhibition of the recruitment and the activation of myeloid cells) in a mouse model of systemic infection, where the lungs were not further investigated (75).

Another frequently encountered nosocomial and multi-resistant infectious agent is *Staphylococcus aureus*. Small et al. could demonstrate the fundamental role of NK cells in the response to these bacteria in the case of mouse lung infections

(76), as (i) NK cell numbers in the airways increased; (ii) *in vitro* contact with products from the pathogens activated NK cells; (iii) co-culture of NK cells with alveolar macrophages increased the phagocytic activity of the latter, (iv) IL-15-KO mice were much more susceptible to the infection than wildtype mice, whereas they had much more neutrophils and macrophages in the lungs; and (v) NK cell depletion rendered even wildtype mice highly sensitive, despite a conserved IL-15 production (76). These findings demonstrate indeed once again the important role of NK cells in immune defense against extracellular bacteria.

In accordance with this model, Zhao et al. showed that particular matter, associated epidemiologically with enhanced numbers of lung infections, diminished the amount of NK cells migrating to rat lungs in case of infection with *S. aureus*, whereas adoptive NK cell transfer restored a vigorous NK cell response (77). In *ex vivo* experiments, NK cells improved, as in the previous study, the phagocytosis of the pathogens by alveolar macrophages.

It is well known that after influenza, recovering patients are very susceptible to bacterial superinfection, notably by *S. pneumoniae* and *S. aureus* (78). The contribution of NK cells to this phenomenon was demonstrated in a mouse model of H1N1 influenza virus infection followed by intratracheal instillation of *S. aureus*. The sequentially double-infected mice were much more susceptible to the infection (weight loss, survival rate) than those receiving PBS or bacteria only. This went hand in hand with severe changes in the histopathological aspect of the lungs and a marked reduction of local NK cell numbers and TNF-α^+ NK cells. Furthermore, the concentrations of TNF-α and of the chemokines IP-10 and MIP-1α were diminished in the BALF. Adoptive transfer of naive NK cells could restore the immune response. The NK cells needed TNF-α to perform their antibacterial effect and this was organized via an interaction with alveolar macrophages and increased phagocytosis (78).

The conclusion that might be drawn from all these papers is that NK cells are very important, at least in mouse models, for the immune response to and the defense against pulmonary infections due to Gram-positive bacteria, with, on the other hand, a detrimental influence of these lymphocytes in case they produce too much immunosuppressive factors [the same old story (72)].

Mycobacterium tuberculosis and Other Mycobacteria

Mycobacterium tuberculosis is the agent of tuberculosis (TB), an infectious disease that puts a high burden on the populations in developing but also in developed countries and increasingly shows resistance to conventional antibiotics. It latently infects about 25% of the total population and becomes clinically apparent in ten million patients per year, according to estimations from the World Health Organization (WHO) (79, 80), rendering it a major public health issue. As recently reviewed by Cong and Wei (23), NK cells could interact with this intracellular pathogen through the AR NKp46, NKp44, and NKG2D, as well as TLR2. Although they became activated under these conditions, they seemed to play only a negligible protective role, according to Junqueira-Kipnis et al. (81). These authors showed in a mouse model that lung NK cells augmented in number within the first 3 weeks after exposure to aerosols containing the mycobacteria and up-regulated CD69, IFN-γ and perforin. However, their depletion did not at all change the kinetics of the infection. Human PB NK cells likewise up-regulate IFN-γ after contact with *M. tuberculosis* (23).

Barcelos et al. (82) compared PB NK cells in cohorts of patients with active TB, isolated tuberculin$^+$ skin tests, and tuberculin$^-$ healthy donors. They found a different subset distribution according to the cohorts, with putative TB-exposed but-resistant individuals (defined as those with a positive tuberculin test) having overall less NK cells but an increased percentage of $CD56^-CD16^+$, $CD56^+CD16^-$ and especially $CD56^{bright}CD16^{-/+}$ NK cell subsets compared to the other two donor groups. In contrast, TB patients displayed lower frequencies of $CD56^+CD16^+$ cells. The authors speculated that this different subset distribution might have been related to the resistance or sensitivity to active TB, but of course, as the cells stem from PB, this dataset might have to be interpreted with some caution.

Surprisingly, however, Roy Chowdhury et al. (80), by following a cohort of adolescents from an endemic region in South Africa, could demonstrate with mass cytometry and functional experiments, that latent TB was associated with increased responses of PB NK cells, with a particular role for the AR CD16. Indeed, the percentages of NK cells among total living cells, of total $CD16^+$ cells, of granzyme B^+ and of perforin$^+$ cells were significantly higher in patients with latent TB than in healthy, non-infected donors. In addition, ADCC (mediated via CD16) against P815 target cells was also higher in latent TB. By following further cohorts, the authors found that the percentage of PB NK cells was dynamically regulated during latency, progression of the disease and responses to antibiotic medication. This level of NK cells in PB even correlated inversely with inflammation in the lungs of patients with active TB (80). Such observations push NK cells again at the forefront of immune defenses in TB and at a possible role in the maintenance of latency.

With a similar cohort-based approach, Harris et al. (79) evaluated NK cell phenotype and functions in individuals with latent TB compared with healthy controls. Furthermore, participants were separated in infected and non-infected in a TB-endemic region in Kenya and a healthy volunteer cohort from the United States. Among the three groups, the persons from the United States had the significantly lowest percentage of $CD56^-$ cells, which are known to expand in chronic human immunodeficiency virus (HIV) infection and other viral diseases and to be dysfunctional (83). The Kenyan volunteers displayed, among $CD56^{dim}$ NK cells, a higher expression of granzyme B and of the non-MHC class I-specific IR TIGIT, with the highest levels found in the healthy cohort. Furthermore, these individuals had an increased expression of the AR NKp46. Within the $CD56^{bright}$ subpopulation, the Kenyan participants showed increased expression of NKG2D but again decreased levels of NKp46, compared to the cohort from the United States. Functionally, degranulation (CD107a assay), IFN-γ production (intracellular flow cytometry) and CD69 expression

were compared between the three cohorts after co-culture with K562 cells (evaluation of natural cytotoxicity) and P815 mouse cells plus anti-mouse antibody (evaluation of ADCC). Whereas for the latter parameter, no significant differences were observed, frequencies of $CD69^+$, $CD107a^+$, and IFN-γ^+ NK cells turned out to be significantly higher in the United States study population, such as if an environment endemic for TB would impact the "missing self"-recognition capacities of NK cells (79). The same regional discrepancies were observed after stimulation of total PBMC with three different antigen extracts from *M. tuberculosis*, and the reactivity to these antigens was shown to be at least partially dependent on the presence of IL-12 and IL-18, supposed to be derived from accessory cells.

Conradie et al. (84) described that the level of activation of PB NK cells (frequency of $CD69^+$ and $CD69^+HLA\text{-}DR^+$ events) allowed, among other parameters, to discriminate between *M. tuberculosis*-induced immune reconstitution syndrome, HIV infection and co-infection with both pathogens.

Although these three papers suggest some influence of *M. tuberculosis* on PB NK cells, it is not clear yet to which extent NK cells really intervene in the immune defense against this pathogen that persists in the lungs. An investigation on tuberculous pleurisy (85) revealed a large predominance of $CD56^{bright}CD16^-$ NK cells in the pleural fluid, and an apoptotic effect of soluble factors from this environment predominantly on $CD16^+$ NK cells. *M. tuberculosis* induced IFN-γ production from $CD56^{bright}$ NK cells in the absence of monocytes, T cells and B cells, leaving open the possibility of a direct productive interaction between the bacteria and the NK cells.

Lai et al. (86) presented a work on nontuberculous mycobacterial lung infections, which means due to other mycobacterial species, such as *Mycobacterium abscessus* and *Mycobacterium kansasii*. As the latter become more and more prevalent in developed countries, these authors performed a study in C57BL/6 mice that were infected intratracheally with *M. kansasii*. They found that NK cell depletion increased bacterial burden, mortality, and pathogenetic postinfectious changes (macrophage phagocytosis, DC activation, cytokine production, and development of granuloma). The same observations were made in IFN-γ-KO animals and restored after transfer of wildtype NK cells. These cells were also the most important producer of IFN-γ in this model (86). Lai et al. further cited papers that had demonstrated a similar protective effect of IFN-γ produced by NK cells in the infections with *Bordetella pertussis*, *Francisella tularensis*, and *Chlamydia muridarum* in mouse models of respiratory infection.

Previous publications by the same group had shown that NK cells can directly lyse *M. tuberculosis* and *M. kansasii* via the cytotoxic proteins granulysin and perforin in a contact-dependent manner disrupting mycobacteria cell wall integrity (87), and that in some patients with mycobacterial infections, anti-IFN-γ autoantibodies were detected (88). The killing process involved signaling through NKG2D and NCR as well as MAP kinases, suggesting that similar mechanisms are involved for the killing of bacteria and of eukaryotic target cells (87). This is potentially a very important observation, as it strongly suggests that both conventional cytotoxic mechanisms and cytokine production might be relevant in anti-mycobacterial defense.

Some studies were also performed on *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), an attenuated mycobacterial strain used as an anti-tuberculous vaccine (89). For example, it was demonstrated *in vitro* that $CD56^{bright}$ NK cells reacted to this microorganism by proliferation and IFN-γ production, whereas their $CD56^{dim}$ counterparts better up-regulated the cytolytic proteins perforin and granzyme A (90), all of which was largely expected based on what is known about the functional specialization of these two NK cell subsets (1–3). In a mouse *in vivo* model, where BCG was directly administered (intratracheally) into the lungs, NK cell-mediated production of IFN-γ rapidly increased in the first days after infection, similarly to the number of lung NK cells (89). After NK cell depletion, the reduction of body weight was less pronounced compared to non-depleted mice, whereas the bacterial load remained identical. Importantly, inflammation and injury of the pulmonary structures was much less pronounced in the NK cell-depleted animals, suggesting a pathogenic role for these lymphocytes. Indeed, the level of pro-inflammatory cytokines and chemokines was also reduced in the absence of NK cells, and the percentages of IFN-γ^+CD4^+ and IFN-γ^+CD8^+ T cells was significantly increased in these mice. Bacillus Calmette-Guérin-infected macrophages up-regulated NKG2D ligands, which induced their lysis via this receptor-ligand interaction. Finally, the blocking of NKG2D with a monoclonal antibody restored the survival of the macrophages and the T cell-mediated immune response (89).

It is difficult to make a coherent synthesis of all these observations on NK cells and mycobacteria, but it is nevertheless quite appealing that again positive, negative and neutral aspects are described, which may vary according to the models and the experimental setup. This shows that NK cells still hide a lot of secrets regarding their function in anti-mycobacterial infections as well as in bacterial pathogenesis overall. A summary of the relationships between the bacteria discussed and NK cells is presented in **Table 2**.

CHLAMYDIA

These are obligate intracellular pathogenic bacteria that are responsible for several types of human and mouse diseases. Various studies dedicated to this type of microorganisms illustrated the concept that NK cells usually do not respond as a pure population as may be the case for *in vitro* experiments, but that *in vivo* they are part of a tightly controlled immune network composed of cells, cytokines, chemokines and exosomes.

Thus, in mouse models, NK cells influenced the interaction between DC, T helper (h)1 and Th17 T lymphocytes in *C. muridarum* lung infection (91), modulated the balance between Th1 and Th17 T cells and T regulatory cells (Treg) in the same type of infection (92), and again positively regulated the interactions between DC and T lymphocytes against *Chlamydophila pneumoniae* (93). In all these situations, NK cells exerted a protective and disease-controlling effect via

TABLE 2 | Natural Killer (NK) cells and different bacteria.

Bacteria	Sp. infected	Origin of NK cells	Effects on/of NK cells	References
Pseudomonas aeruginosa	Mouse	Lung	↑ Clearance via NKG2D	(51)
P. aeruginosa exotoxin A	Human	Peripheral blood	↓ Cytotoxicity (K562)	(53)
Burkholderia cenocepacia	Human	Peripheral blood	Killing of bacteria	(55)
Klebsiella pneumoniae	Human	Peripheral blood	↑ IFN-γ, ↑ α-defensin	(59)
Klebsiella pneumoniae	Mouse	Lung	↑ IFN-γ, ↑ IL-22	(60)
Legionella pneumophila	Mouse	Lung, spleen	↑ cytotoxicity, ↑ IFN-γ	(67)
Streptococcus pneumoniae	Mouse	Lung	↑ IFN-γ, ↑ IL-10	(71)
Staphylococcus aureus	Mouse	Airways	↑ Number/activation	(76)
Mycobacterium tuberculosis	Mouse	Lung	↑ Number, ↑ IFN-γ	(81)
Mycobacterium tuberculosis	Human	Peripheral blood	↑ IFN-γ	(23)
Mycobacterium kansasii	Mouse	Lung	↑IFN-γ	(86)
Mycobacteria	Human	Peripheral blood	Killing of bacteria	(87)
Mycobacterium bovis BCG	Mouse	Lung	↑ IFN-γ, ↑ number	(89)
Mycobacterium bovis BCG	Human	Peripheral blood	↑ IFN-γ, ↑ PF, ↑ GZM A	(90)

Summary of the effects of and/or on NK cells in infections with the listed bacterial pathogens. Sp., species; IFN-γ, interferon-gamma; BCG, bacillus Calmette-Guérin; IL, interleukin; PF, perforin; and GZM A, granzyme A.

their influence on the bridge between innate and adaptive immune responses.

With the ambitious aim to experimentally investigate the famous "hygiene hypothesis," Han et al. (94) studied mice infected with *C. muridarum* and rendered allergic to ovalbumin (OVA). They observed that prior infection could inhibit at least certain parameters of allergy. However, NK cell depletion partly suppressed the "beneficial" effect of the lung infection. Adoptive transfer of NK cells from infected mice inhibited partially the development of an allergic response in non-infected recipients. NK cell-devoid mice coherently produced more Th2 type cytokines ("pro-allergic" Th2 cytokines, IL-4, and IL-5) than IFN-γ ("anti-allergic" Th1 cytokine).

A detrimental effect of NK cells had been shown for the immune response to the respiratory rodent pathogen *Mycoplasma pulmonis*, related to the human infectious agent *Mycoplasma pneumoniae*. Indeed, in a quite complicated experimental setup, Bodhankar et al. demonstrated that NK cell depletion interfered positively with the development of a protective adaptive immunity after nasal-pulmonary immunization with bacterial antigens (95). This could be explained because NK cells shaped the T cell cytokine response toward more IL-4, IL-13, and IL-17 but away from IFN-γ production.

NATURAL KILLER CELLS AS CLINICAL INDICATORS IN RESPIRATORY INFECTIOUS DISEASES IN CHILDREN

Wurzel et al. presented large cohort studies of children with protracted bacterial bronchitis (PBB) and mild bronchiectasis, associated or not with human adenovirus co-infection (96, 97). Besides typical socio-economic and clinical factors, an elevated NK cell number relative to the values of healthy children of the same age was observed in the PB of diseased children in general and with adenovirus species C particularly. NK cell phenotype and function were not further investigated.

A HUMAN KIR RECOGNIZES A CONSERVED BACTERIAL EPITOPE

Recently, Sim et al. (98) made the important discovery that the HLA-C-specific activating KIR2DS4 did recognize a conserved bacterial peptide presented by HLA-C, and more precisely by HLA-C*05:01. The sequence of the peptide required for this recognition was a "rare" self-peptide, but the epitope of interest is conserved in the recombinase A (RecA) of many bacterial species (more than 1000 according to the authors' claims), most of them belonging to serious human pathogens, such as *Helicobacter pylori*, Brucella, *Campylobacter jejuni,* and *Chlamydia trachomatis* (98). Interestingly, activation of resting NK cells via KIR2DS4 alone was sufficient to induce degranulation and cytokine production, whereas all other known AR, except CD16, need at least one co-activating molecule engaged at the same time (99). There was, furthermore, an inverse correlation between the frequency of the KIR2DS4 full length gene and the HLA-C*05:01 allele. Thus, it appears that the KIR family is not only involved in NK cell licensing and in multiple disease associations, but also, most likely, in antibacterial defense. This paper received an accompanying Commentary by Peter Parham, which places the findings in the broader context of KIR and HLA class I molecules (100).

To sum up, NK cells might be directly activated by various bacteria via contact-dependent mechanisms whose modes of functioning are still unknown, via TLR, via KIR2DS4, or more indirectly via the up-regulation of ligands for their AR, such as NKG2D, by infected cells. They might also react to cytokines released into the microenvironment by antigen-presenting cells (macrophages, DC).

STATE-OF-THE-ART METHODS FOR INVESTIGATION OF THE LUNGS

Dietert et al. published a plea for the histopathological evaluation of the consequences of different infectious lung diseases in

mouse models and described the pathogen-specific features characteristic for each of them (101). Indeed, many variations were observed between the infecting microorganisms, be they bacterial or viral in nature. The authors emphasized that histopathology remains the "most conclusive and practical read out" for the evaluation of the effects of the various infectious models on mouse lungs.

Although this is true, more "modern" and state-of-the-art methods are being developed and are about to enter the laboratories, as a consequence of general scientific and technical progress but also of the "3R" approach regarding experiments with animals.

In 2019, the team of Hans Clevers described the generation of human airway organoids derived from surgical material or from BALF (102). These were long-term proliferating structures that recapitulated a normal airway with different types of cells that are physiologically present *in vivo*. The beauty of the system *per se* was already an accomplishment, but it could be used for the study of various lung diseases, such as cystic fibrosis, cancer, or viral infections (102). Therefore, it is likely that bacterial infections could similarly be investigated in this system, and the data obtained would probably be more relevant to human pathology than the mere mouse models (and save the life of many mice by the way).

The same year, Ross et al. (103) published a review on the "*ex vivo* human lung." They worked with donor lungs not retained for transplantation, extracted primary cells from them and developed an "*ex vivo*-perfused single human lung" that would allow the investigation of different lung diseases. The system seems at first sight less elegant than the lung organoids and is maybe also more limited in the spectrum of possible pathologies that can be investigated. The advantage would be that an entire, complete organ is available and not just an organoid.

Yet another option is the "alveolus-on-a-chip," developed by Deinhardt-Emmer et al. (104). It was a three-dimensional structure with an air phase and a liquid phase, where endothelial cells, epithelial cells and macrophages could be co-cultured. In the presented work, a primary influenza virus infection, followed by a *S. aureus* superinfection, were investigated, and it was shown that the endothelium was seriously damaged under these conditions.

Likewise, single cell transcriptomics is a powerful tool that can reveal huge amounts of details about all kinds of immune cells, and among them NK cells, as exemplified by lung cancer-infiltrating immunocytes in human and mouse (105).

NATURAL KILLER CELLS AS A THERAPEUTIC OPTION FOR AIRWAY INFECTIONS? CAVE CANEM!

One aspect of NK cells is their putative potential for a dual role as "pro-inflammatory" and "regulatory" effectors, which might be mediated by different subsets (106). Our group has previously touched the problem that NK cells are in fact a double-edged sword, meaning that they might have sometimes beneficial but sometimes rather deleterious effects (72). This has again become clear throughout this review, although the models and studies presented and discussed were all but homogeneous. It might be expected that this will change in the coming years if more and more teams will use the organoid and organ on-a-chip technologies and go into various "omics." Overall, given the current and justified hype for NK cells as efficient agents for cancer immunotherapy, it would be difficult to convince the NK community that their favorite cells might also have a dark side. We emphasize that several methods to improve NK cell antitumoral efficiency, such as particularly CAR-NK cells (10) and NK cell engagers, recently described by the Vivier group (107), should be sufficient to stand up for the use of NK cells in this indication.

However, what about the therapeutic indication of NK cells in infectious diseases in general and in the lungs particularly? Due to the current COVID-19 pandemic, this question has gained increased interest (108, 109), and in addition, most of the papers discussed here that describe an influence of NK cells on the disease course in the airways conclude with the statement that NK cells should be targeted in respiratory infections. But it has clearly been shown that these lymphocytes can have detrimental side effects and cause significant damage to the airways, at least in viral diseases (108, 109). Available literature does not give clear indications regarding bacterial pathogenesis, but the issue was already discussed in 2012, with the question if NK cells are angels or devils in bacterial infectious diseases (50). This problem is, in our opinion, not yet resolved and a lot of research work will be necessary in the field, keeping in mind that the number of multi-resistant bacterial strains is increasing at a terrifying rate and that alternatives to antibiotics must be discovered and developed.

Finally, a general problem in the field and a caveat to many of the presented studies is the difficulty of distinguishing NK cells reliably form ILC1, which also produce IFN-γ as a signature cytokine and have a partially overlapping phenotype. A high plasticity within the ILC family renders even possible the conversion of NK cells, in certain microenvironments, into ILC1-like cells (110–112). However, whereas both NK cells and ILC1 require the transcription factor T-bet for their development and function, NK cells need and express Eomes in addition. ILC1 are preferentially located in tissues and are very rare in peripheral blood, in contrast to NK cells. Thus, one can be confident that the studies discussed here that worked with blood (human) and blood or spleen (mouse) NK cells really investigated NK cells and not ILC1. For tissue-based studies, the differences might be more blunted, although ILC1 are considered as non-cytotoxic cells (110–112). These difficulties are in line with the increasing number of "new" cell types that are currently discovered [for example MR1 T cells (113)], as a consequence of the ever growing diversification and performance of the experimental tools in immunology.

AUTHOR CONTRIBUTIONS

MT and NP conceived the article, participated to bibliographic research, and critically reviewed the final revised manuscript. JZ conceived the article and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Antigens of *Mycobacterium tuberculosis* Stimulate CXCR6+ Natural Killer Cells

*José Alberto Choreño-Parra[1,2], Luis Armando Jiménez-Álvarez[1,2], Marcela Muñoz-Torrico[3], Gustavo Ramírez-Martínez[2], Luis Antonio Jiménez-Zamudio[1], Citlaltepetl Salinas-Lara[4], Ethel Awilda García-Latorre[1] and Joaquín Zúñiga[2,5]**

[1] Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico, [2] Laboratory of Immunobiology and Genetics, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico, [3] Tuberculosis Clinic, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, Mexico, [4] Department of Neuropathology, Instituto Nacional de Neurología y Neurocirugía "Manuel Velasco Suárez", Mexico City, Mexico, [5] Tecnologico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Mexico City, Mexico

Natural killer (NK) cells participate in immunity against several pathogens by exerting cytotoxic and cytokine-production activities. Some NK cell subsets also mediate recall responses that resemble memory of adaptive lymphocytes against antigenic and non-antigenic stimuli. The C-X-C motif chemokine receptor 6 (CXCR6) is crucial for the development and maintenance of memory-like responses in murine NK cells. In humans, several subsets of tissue-resident and circulating NK cells with different functional properties express CXCR6. However, the role of CXCR6+ NK cells in immunity against relevant human pathogens is unknown. Here, we addressed whether murine and human CXCR6+ NK cells respond to antigens of *Mycobacterium tuberculosis* (Mtb). For this purpose, we evaluated the immunophenotype of hepatic and splenic CXCR6+ NK cells in mice exposed to a cell-wall (CW) extract of Mtb strain H37Rv. Also, we characterized the expression of CXCR6 in peripheral NK cells from active pulmonary tuberculosis (ATB) patients, individuals with latent TB infection (LTBI), and healthy volunteer donors (HD). Furthermore, we evaluated the responses of CXCR6+ NK cells from HD, LTBI, and ATB subjects to the *in vitro* exposure to CW preparations of Mtb H37Rv and Mtb HN878. Our results showed that murine hepatic CXCR6+ NK cells expand *in vivo* after consecutive administrations of Mtb H37Rv CW to mice. Remarkably, pooled hepatic and splenic, but not isolated splenic NK cells from treated mice, enhance their cytokine production capacity after an *in vitro* re-challenge with H37Rv CW. In humans, CXCR6+ NK cells were barely detected in the peripheral blood, although slightly significative increments in the percentage of CXCR6+, CXCR6+CD49a−, CXCR6+CD49a+, and CXCR6+CD69+ NK cells were observed in ATB patients as compared to HD and LTBI individuals. In contrast, the expansion of CXCR6+CD49a− and CXCR6+CD69+ NK cells in response to the *in vitro* stimulation with Mtb H37Rv was higher in LTBI individuals than in ATB patients. Finally, we found that Mtb HN878 CW generates IFN-γ-producing CXCR6+CD49a+ NK cells. Our results demonstrate that antigens of both laboratory-adapted and clinical Mtb strains are stimulating factors for murine and human CXCR6+ NK cells. Future studies evaluating the role of CXCR6+ NK cells during TB are warranted.

Keywords: tuberculosis, *Mycobacterium tuberculosis*, natural killer cells, CXCR6, innate immunity

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***Correspondence:**
Joaquín Zúñiga
joazu@yahoo.com

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INTRODUCTION

Natural killer cells participate in immune responses against viral and bacterial pathogens (1). In some cases, such as the infection of mice with murine cytomegalovirus (MCMV), the function of NK cells is crucial for the development of protective immunity (2). This protective role of NK cells is mediated by their cytotoxic capacity, allowing the elimination of intracellular reservoirs of infection. Furthermore, NK cells produce a wide range of inflammatory cytokines that shape the effector activities of other innate and adaptive immune cells (1).

Recently, novel functional characteristics that resemble immune memory have been described in NK cells (3–5). These memory-like properties are triggered by antigenic and cytokine priming, allowing NK cells to enhance their effector functions during recall responses. The phenotype of NK cells mediating immunological memory against a particular stimulus is, in some cases, very specific. For instance, Ly49H+ NK cells are protective against MCMV infection (3), whereas liver resident CD49a+ NK cells mount recall responses against haptens in mice (6). In humans, CD94/NKG2C+ NK cells expand during human cytomegalovirus (HCMV) infection (7–9). Similarly, the molecules CD45RO and CD27 have been identified as potential markers of memory-like NK cells among individuals infected with *Mycobacterium tuberculosis* (Mtb) (10–12). Despite this, the relevance of other memory-like NK cell subsets in infectious disorders remains undetermined.

The C-X-C motif chemokine receptor 6 (CXCR6) plays a pivotal role in memory-like responses of murine NK cells (13). The expression of this molecule increases in NK cells from *Rag1*-/- mice after the exposure to non-infectious virus-like particles (VLP) containing antigens from influenza A virus, vesicular stomatitis virus (VSV), and human immunodeficiency virus (HIV). In VLP-treated *Rag1*-/- mice, CXCR6 mediates the recruitment of primed NK cells to the liver, where they reside, maintaining their adaptive properties. During secondary antigenic challenges, hepatic CXCR6+ NK cells re-expand and are able to protect *Rag2*-/-*Il2rg*-/- mice from a lethal infection with influenza A virus and VSV upon adoptive transfer (13). These data suggest that the liver is a reservoir of memory-like CXCR6+ NK cells that may play an important role in protective immunity against infections. In humans, different subsets of circulating and hepatic NK cells also express CXCR6 (14–16). However, there is little evidence about the role of human CXCR6+ NK cells in relevant infectious diseases.

In the current study, we evaluated the responses of memory-like CXCR6+ NK cells to the exposure to Mtb antigens in mice. In addition, we characterized the immune phenotype and expression of CXCR6 in circulating NK cells from humans with distinct clinical forms of tuberculosis (TB). Our results demonstrate that murine CXCR6+ NK cells can respond to Mtb antigens both *in vivo* and *in vitro*. Remarkably, the repetitive administration of mycobacterial preparations to mice enhances the capacity of NK cells to produce cytokines after the *in vitro* re-challenge with the same stimulus. Furthermore, the phenotype and responses of human CXCR6+ NK cells differed between individuals with distinct degrees of immunity to Mtb. Collectively, our findings demonstrate that antigens of Mtb are stimulating factors for murine and human CXCR6+ NK cells.

MATERIALS AND METHODS

Human Samples

We obtained blood samples from individuals with active pulmonary tuberculosis (ATB) and latent tuberculosis infection (LTBI) that attended the TB clinic of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (INER), in Mexico City. The ATB group included symptomatic patients with positive results in sputum smear microscopy, sputum/bronchoalveolar lavage (BAL) culture, and GeneXpert MTB/RIF test (Cepheid, Sunnyvale, CA, United States). The LTBI cohort included asymptomatic individuals in close-contact with ATB patients that tested positive in the QuantiFERON®-TB Gold Plus (QFT®-Plus) test (QIAGEN, Hilden, Germany). Individuals with primary or acquired immunosuppression, as well as ATB patients receiving anti-TB drugs during the last week before enrollment, were ineligible. A group of ten healthy volunteer donors (HD) was recruited and served as control. These healthy individuals did not report any relevant comorbidity nor history of contact with TB patients and were examined by two independent physicians, which ruled out symptoms of acute illness. Also, HD were subjected to a complete laboratory workup and screened for LTBI by QuantiFERON®-TB Gold Plus.

Clinical and demographic data from study participants were retrieved by direct clinical interview, physical examination, and review of their medical records. All participants or their legal guardians provided written informed consent to participate in the study. Blood samples were processed and stored according to the Mexican Constitution law NOM-012-SSA3-2012, which establishes criteria for the execution of clinical research projects in humans. The current study was reviewed and approved by the Institutional Review Board of the INER (project number B04-15).

Mice

Male C57BL/6 (B6) mice were bred at the INER animal facility. Experimental mice were used between the ages of 6–8 weeks, in accordance with the Institutional Animal Care and Use guidelines at INER in Mexico City, approved under the protocol B04-15.

Mtb Exposure in Mice

B6 mice were exposed to a cell wall (CW) extract from Mtb H37Rv administered by subcutaneous injection at a concentration of 1 mg/ml diluted in 100 μl of 1× phosphate-buffered saline (PBS). Some mice received subcutaneous PBS and were considered as controls.

Mouse NK Cell Isolation

At given time points, spleen and liver were collected from B6 mice, and single-cell suspensions were prepared as follows. Spleens were mechanically homogenized by passage through a 40 μm pore size nylon tissue strainer (Falcon; BD Biosciences, San Jose, CA, United States) using a 3 ml syringe plunger. Spleen cell suspensions were treated with ACK buffer to

lyse erythrocytes, washed twice, and counted by Trypan's blue exclusion method. For hepatic single-cell suspensions preparation, livers were perfused with 1× PBS through the portal vein, placed on Petri dishes with complete Dulbecco's modified Eagle's medium (cDMEM) containing glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, and cut into 1 mm pieces using sterile razor blades. Dissected livers were then homogenized by passage through a 70 μm pore size nylon tissue strainer (Falcon; BD Biosciences, San Jose, CA, United States). Homogenates were re-suspended in 40% Percoll solution (Percoll™, GE Healthcare, Sigma-Aldrich, St. Louis, MO, United States), placed over a layer of 70% Percoll, and centrifuged for 25 min at 850 *g*. Hepatic leukocytes were recovered from the 40%/70% Percoll interface, washed twice with cDMEM, treated with ACK solution, and counted. NK cells were enriched from liver and spleen single-cell suspensions using a commercial kit of magnetic beads (NK Cell Isolation Kit II mouse, Miltenyi Biotec, Germany).

In vitro Stimulations

Isolated mouse liver and spleen NK cells were pooled and plated at a density of 2.5×10^6 cells per ml in cDMEM supplemented with 10% fetal bovine serum (FBS). Pooled liver/spleen NK cells were cultured with 25 μg/ml Mtb H37Rv CW at 37°C, 5% CO_2. After 48 h of Mtb CW exposure, NK cells were collected for flow cytometry and supernatants stored for analysis of IFN-γ production by ELISA (Mouse IFN gamma ELISA Ready-SET-Go!®, Affymetrix eBioscience, San Diego, CA, United States). Spleen NK cells were cultured alone in the same conditions and served as controls.

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation gradient using Ficoll-Paque PLUS (GE Healthcare-Life Sciences, Bensalem, PA, United States). PBMCs from HD, LTBI, and ATB individuals were plated at a density of 2.5×10^6 cells per ml in complete Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 2 mM L-Glutamine and 10% FBS, and cultured with 25 μg/ml Mtb H37Rv CW at 37°C, 5% CO_2, during 48 h. In addition, PBMCs isolated from buffy coats of six HD obtained from the blood bank of INER were stimulated with 25 μg/ml of an Mtb HN878 CW preparation as described above. The H37Rv and HN878 CW preparations were gently provided by Dr. Shabaana A. Khader, from the Department of Molecular Microbiology, Washington University School of Medicine in St. Louis, MO, United States.

Flow Cytometry

Mouse liver and spleen cell suspensions, and pooled liver/spleen murine NK cells were incubated with fluorochrome-labeled anti-mouse CD3, CD14, NK1.1, and CXCR6 antibodies. Freshly isolated human PBMCs were stained with two different panels of flow cytometry anti-human antibodies: (A) CD3, C14, CD19, CD56, CXCR6, CD49a, CD69, and IFN-γ; (B) CD3, CD14, CD56, and CD16 (**Supplementary Table 1**). For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ kit (BD Biosciences, United States). Cells were acquired using a BD FACS™ Aria II cytometer (BD Biosciences, United States) and gated based on their forward and side scatter characteristics, as well as on fluorescence minus one (FMO) controls for each specific marker using the FACSDiva software. Mouse NK cells were defined as CD3−, CD14−, and NK1.1+, whereas human NK cells were defined as CD3−, CD14/CD19−, and CD56+. The frequency of specific cell types was calculated using Flow Jo (Flow Jo, LLC, Ashland, OR, United States).

Statistical Analyses

Descriptive statistics were used to characterize the study population clinically. Statistical analyses were performed using GraphPad Prism 8.4.2 (La Jolla, CA, United States). Specific tests are mentioned in figure and table legends. Values of $p \leq 0.05$ were considered as significant: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

RESULTS

Murine CXCR6+ NK Cells Respond to Mtb Antigens

The chemokine receptor CXCR6+ is a marker of murine memory-like NK cells that mediate recall responses against viruses and haptens (6, 13). To address whether these cells also respond to Mtb, B6 mice were exposed to a CW extract from Mtb H37Rv administered by consecutive subcutaneous injections, as shown in **Figure 1A**. Four weeks after the last administration, NK cells were quantified in liver, and spleen cell suspensions by flow cytometry (**Figure 1B**). As compared to naive B6 mice, a significant reduction in the number of total NK cells was observed in the spleen of animals treated with Mtb H37Rv CW. Conversely, mice treated with the mycobacterial CW preparation exhibited an increase in the total amount of hepatic NK cells with respect to the number of NK cells in the liver of naïve B6 mice (**Figure 1C**). Notably, the proportion of hepatic CXCR6+ NK cells augmented after the *in vivo* exposure to Mtb H37Rv CW in treated animals with respect to naïve B6 mice (**Figures 1D,E**). These data suggest that the systemic administration of Mtb antigens promotes the expansion of murine CXCR6+ NK cells in the liver.

To address functional changes induced by the *in vivo* exposure to Mtb H37Rv antigens in murine NK cells, we enriched splenic and hepatic NK cells from mice treated with the CW preparation, as well as from naïve animals. However, the amount of hepatic NK cells obtained from each mouse was not enough for *in vitro* assays. Thus, pooled splenic and hepatic NK cells were incubated with the same mycobacterial preparation. After such stimulation, we measured levels of IFN-γ in the supernatants. The results were compared with levels of IFN-γ in supernatants from splenic NK cells isolated from control and treated mice and cultured alone. Interestingly, we found that pooled splenic and hepatic NK cells from mice receiving injections of Mtb H37Rv CW produced higher amounts of IFN-γ after the *in vitro* exposure to the same antigens, as compared to NK cells from naïve mice (**Figure 1F**). This effect was not observed in splenic NK cells from both groups of animals cultured alone. In fact, the levels of IFN-γ in the supernatants from splenic NK cells were lower

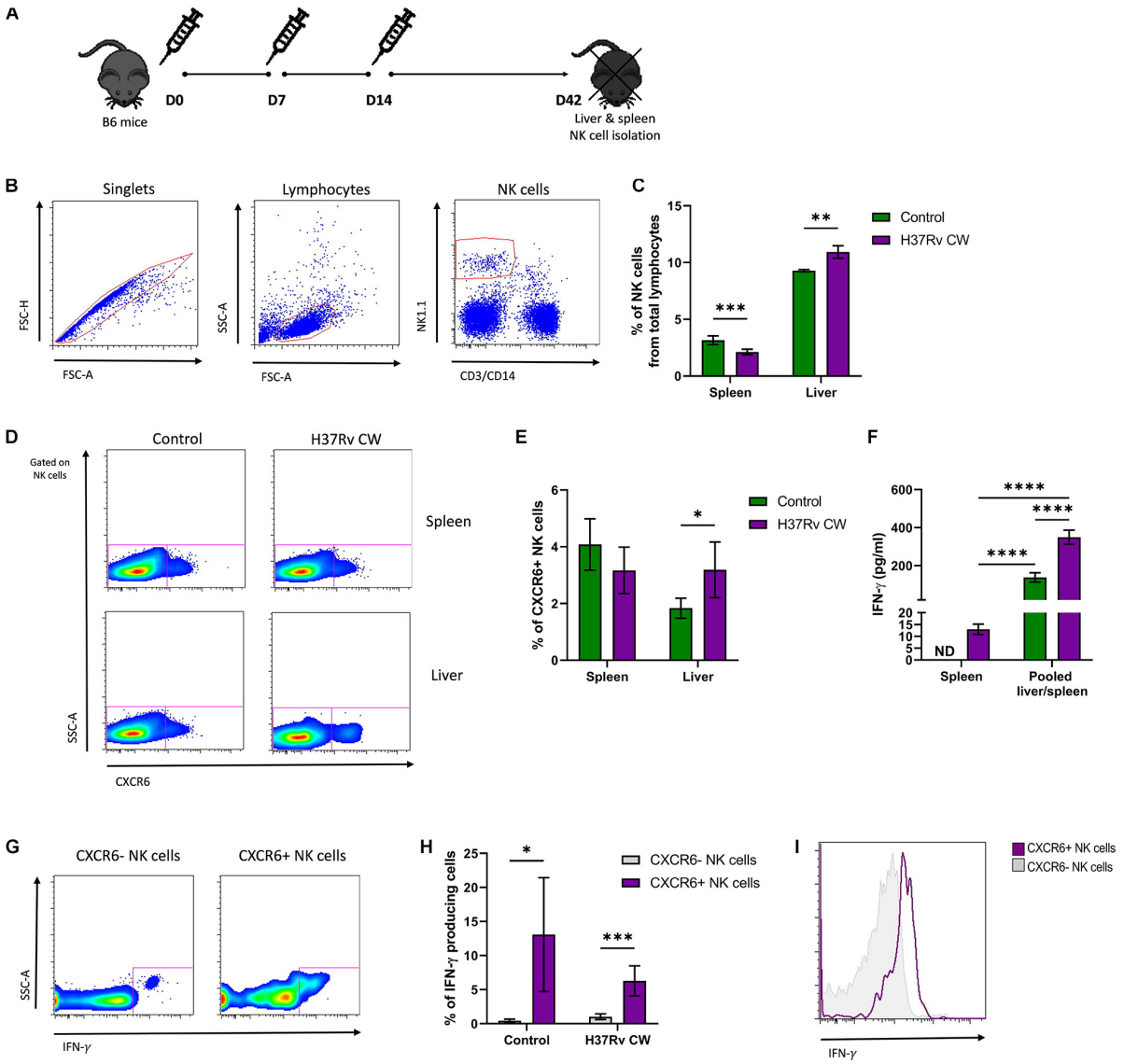

FIGURE 1 | Murine CXCR6+ NK cells respond to Mtb antigens. **(A)** B6 mice were treated with an Mtb H37Rv cell wall (CW) extract administered by subcutaneous injections at days 0, 7, and 14. Another group of mice received 1× phosphate-buffered saline (PBS) and were considered as controls. Thirty days after the last administration, the spleens, and livers from both groups of animals were harvested (n = 5 per group). **(B,C)** Percentage of NK cells in total leukocytes from spleen and liver were determined by flow cytometry. **(D,E)** Percentage of splenic and hepatic CXCR6+ NK cells from Mtb H37Rv CW-treated mice and controls were also determined. **(F)** Splenic and hepatic NK cells were isolated from B6 mice treated with the Mtb H37Rv CW and from control animals using antibodies coupled to magnetic beads. Pooled splenic and hepatic NK cells were stimulated *in vitro* with the CW preparation of Mtb H37Rv. After 48 h, levels of IFN-γ in supernatants were quantified by ELISA. The results were compared with the levels of IFN-γ determined in the supernatants of splenic NK cells from treated and control animals that were cultured alone. **(G–I)** Expression of IFN-γ in CXCR6+ NK cells was also determined by flow cytometry. Differences between groups were analyzed using the unpaired Student t-test at each tissue and experimental condition. The data shown represent mean (±SE) values from two independent experiments per experimental condition. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$. ND, not detectable.

than in supernatants from pooled splenic and hepatic NK cells (**Figure 1F**). This finding suggests that hepatic, but not splenic NK cells, improve their functional capacity after consecutive encounters with Mtb antigens. Notably, we found that a higher proportion of CXCR6+ NK cells participate in the production of such cytokine and express more IFN-γ in response to the CW extract with respect to their CXCR6− counterpart in both treated and control animals (**Figures 1G–I**). Together, these findings indicate that murine CXCR6+ NK cells actively respond to Mtb antigens.

CXCR6+ NK Cells in Humans With Pulmonary TB

Using flow cytometry, we characterized the immune profile of circulating NK cells in HD (n = 10), individuals with LTBI

(n = 10), and ATB (n = 17) patients. Their main clinical characteristics are summarized in **Table 1**. The gating strategy used to identify different human NK cell subsets is illustrated in **Figure 2A**. We observed that LTBI and ATB groups had elevated proportions of circulating lymphocytes as compared to HD (**Figure 2B** and **Supplementary Figures 1A,B**). Nonetheless, no differences in the percentage of lymphocytes from total PBMCs were observed between LTBI and ATB patients. Strikingly, NK cells were increased among LTBI individuals as compared to HD and ATB patients (**Figure 2C** and **Supplementary Figure 1C**). Also, the proportion of $CD56^{bright}$CD16− NK cells was lower in peripheral lymphocytes from ATB patients as compared to HD and LTBI individuals (**Supplementary Figure 1D**). This coincides with previous reports (17, 18). No differences in peripheral $CD56^{dim}$CD16+ NK cells were observed between groups (**Supplementary Figure 1E**).

In humans, CXCR6 marks a subgroup of liver-resident NK cells with a tolerant phenotype that is barely found in peripheral blood under normal conditions (14). As such, we observed little amounts of CXCR6+ NK cells in blood samples from HD and LTBI. Similarly, CXCR6+ NK cells represented a minor proportion of total circulating NK cells in ATB patients, although this latter showed a slight significant increase in the percentage of CXCR6+ NK cells as compared to HD (**Figures 2D,E**). Indeed, higher percentages of circulating CXCR6+ NK cells were observed in three (∼18%) ATB patients (**Figure 2E**). CD49a identifies another subset of human intrahepatic NK cells that produce high levels of cytokines upon stimulation (16, 19). Although this subset is almost undetectable in peripheral blood, we found high percentages of NK cells with the phenotype CXCR6-CD49a+ in two HD (20%), three LTBI individuals (30%), and six ATB patients (35%), with no differences between groups, indicating that this population of NK cells are not relevant for TB (**Supplementary Figure 2**). Conversely, after excluding for CD49a expression, we found that single positive CXCR6+CD49a− NK cells remained increased among ATB patients as compared to the other participant groups (**Figure 2F**). A third subset of double-positive hepatic CXCR6+CD49a+ NK cells exist and are thought to represent the human counterpart of murine memory-like hepatic CXCR6+ NK cells (15). These cells are immature but have a high capacity to produce inflammatory cytokines like single positive CD49a+ NK cells (15). As for total CXCR6+ NK cells, double-positive CXCR6+CD49a+ NK cells were elevated among a fraction of ATB patients but not in HD and LTBI individuals (**Figures 2G,H**).

Although the differences described above were not robust, our findings suggest an increased circulation of CXCR6+ NK cells in a minor proportion of ATB patients. Thus, we evaluated the expression of the activation and tissue-homing marker CD69 in CXCR6+ NK cells. Interestingly, a higher percentage of CXCR6+ NK cells expressed CD69 as compared to their CXCR6− counterparts in all participant groups (**Figure 2I**). However, CXCR6+CD69+ NK cells were increased only in the circulation of ATB patients, but not LTBI individuals and HD (**Figure 2J**). Finally, CXCR6+ NK cells from LTBI and ATB groups showed a higher relative expression of CD69 as compared to HD (**Figure 2K**).

In vitro Responses of Human CXCR6+ NK Cells to Mtb Antigens

We evaluated the responses of circulating CXCR6+ NK cells to the *in vitro* exposure with the Mtb H37Rv CW extract using total PBMCs from the last five consecutive enrolled individuals of each participant group. After such antigenic priming, CXCR6+ NK cells represented a higher percentage from total NK cells and showed a more significant fold increase with respect to the baseline percentage only in the LTBI group (**Figures 3A–C**). Little expression of IFN-γ was observed in stimulated CXCR6+ NK cells, and the percentage of IFN-γ+CXCR6+ NK cells was not different between groups (**Supplementary Figure 3**). Furthermore, the proportion of NK cells that produce such cytokine was similar in the CXCR6+ and CXCR6− subsets from all participant groups (**Supplementary Figure 3**).

The antigenic stimulation with the CW preparation of Mtb H37Rv did not induce significant changes in the proportions of double-positive CXCR6+CD49a+ NK cells in the cultures of PBMCs from any group of individuals (**Figures 3D–F**). Conversely, after excluding for CD49a+ cells, we found that Mtb antigens induced a significant increase in the proportion of single-positive CXCR6+CD49a− NK cells among LTBI individuals, but not HD and ATB patients (**Figures 3G,H**). The

TABLE 1 | Participant characteristics.

Characteristic	HD (n = 10)	LTBI (n = 10)	ATB (n = 17)
Median age (range)	35 (24–51)	41 (19–80)	43 (18–64)
Female, n (%)	4 (40)	6 (60)	9 (52.94)
BMI, mean (SD)	26.59 (3.39)	28.54 (5.92)	19.92 (3.4)
Diabetes, n (%)	0 (0)	0 (0)	8 (47.05)
BCG vaccination	10 (100)	8 (80)	17 (100)
Drug resistance			
MDR, n (%)	NA	ND	4 (23.52)
Sensitive, n (%)	NA	ND	12 (70.58)
Undetermined, n (%)	NA	ND	1 (5.88)

ATB, active pulmonary tuberculosis; BCG, bacillus Calmette-Guerin; HD, healthy volunteer donors; LTBI, latent tuberculosis infection; MDR, multi-drug resistant; NA, not applicable; ND, not determined; SD, standard deviation.

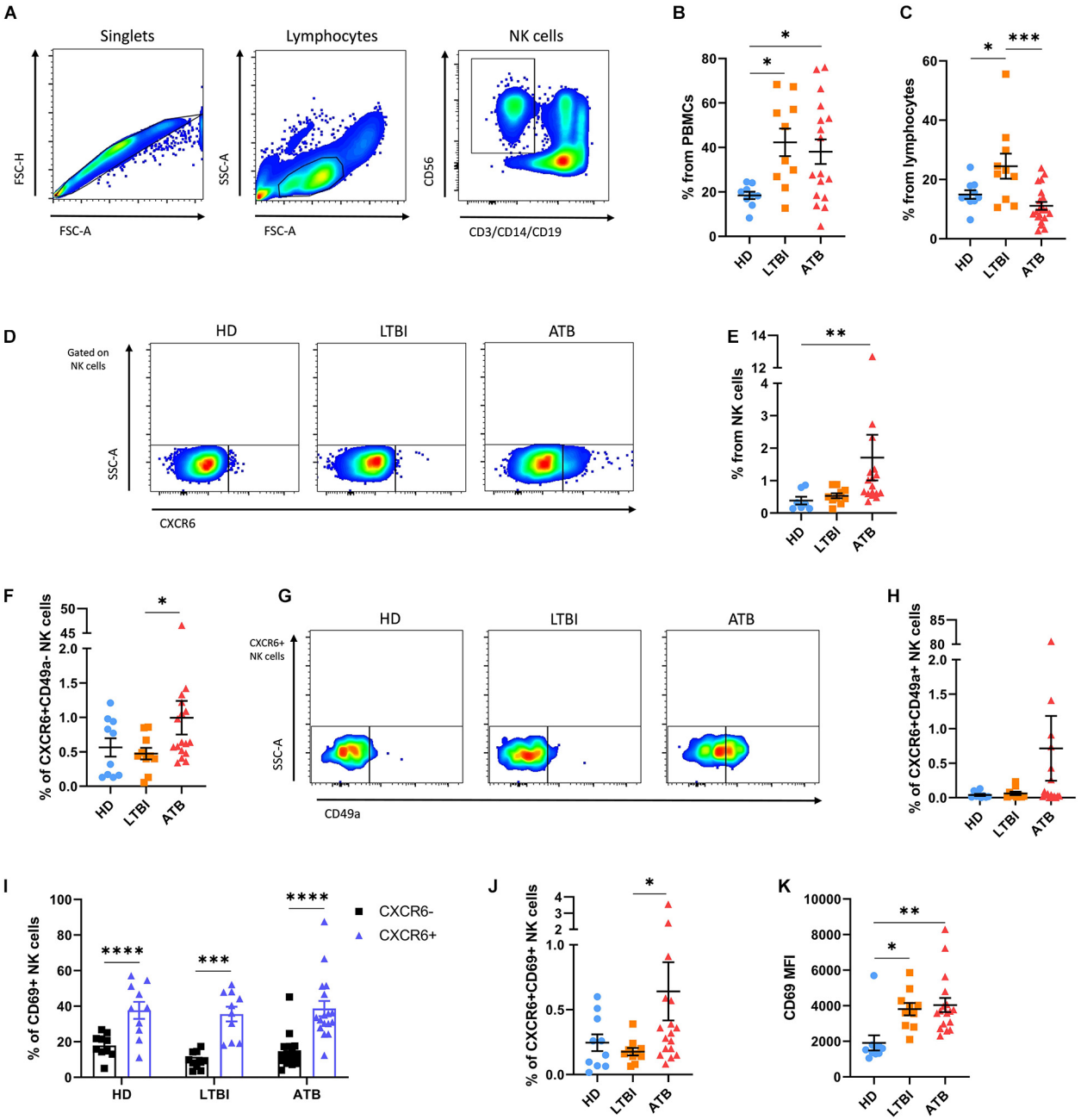

FIGURE 2 | Expression of CXCR6 in NK cells from humans with TB. The immune phenotype of circulating NK cells was analyzed in peripheral blood mononuclear cell (PBMC) samples from healthy donors (HD, *n* = 10), individuals with latent TB infection (LTBI, *n* = 10), and patients with active pulmonary TB (ATB, *n* = 17) by flow cytometry. **(A)** Gating strategy used for the analysis of the immune phenotype of human NK cells. **(B)** Percentage of lymphocytes from total PBMCs. **(C)** Percentage of NK cells from total lymphocytes. **(D,E)** The percentage of CXCR6+ NK cells from total lymphocytes was compared between groups. We also determined the percentage of **(F)** CXCR6+CD49a– NK cells and **(G,H)** double-positive CXCR6+CD49+ NK cells in all participant groups. **(I)** The proportion of cells expressing CD69 were compared between CXCR6+ and CXCR6– NK cells from each group. **(J,K)** Also, the percentage of CXCR6+CD69+ NK cells and mean fluorescence intensity (MFI) values for CD69 in CXCR6+ NK cells were determined in HD, LTBI, and ATB individuals. Differences between groups were analyzed using the one-way ANOVA test and the *post hoc* Tukey's for multiple comparisons test. Comparisons between cells from the same group were analyzed with the Student *t*-test and *p* values corrected for multiple comparisons using the Holm method. The data shown represent mean (±SE) values. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

same pattern was observed for CXCR6+CD69+ NK cells, which were elevated only in the PBMCs from LTBI participants after the antigenic priming (**Figures 3I–K**). Despite this, CXCR6+ NK cells were more responsive to the mycobacterial CW preparation independently of their source as they expressed more CD69 than CXCR6− NK cells in all groups of participants (**Figure 3L**).

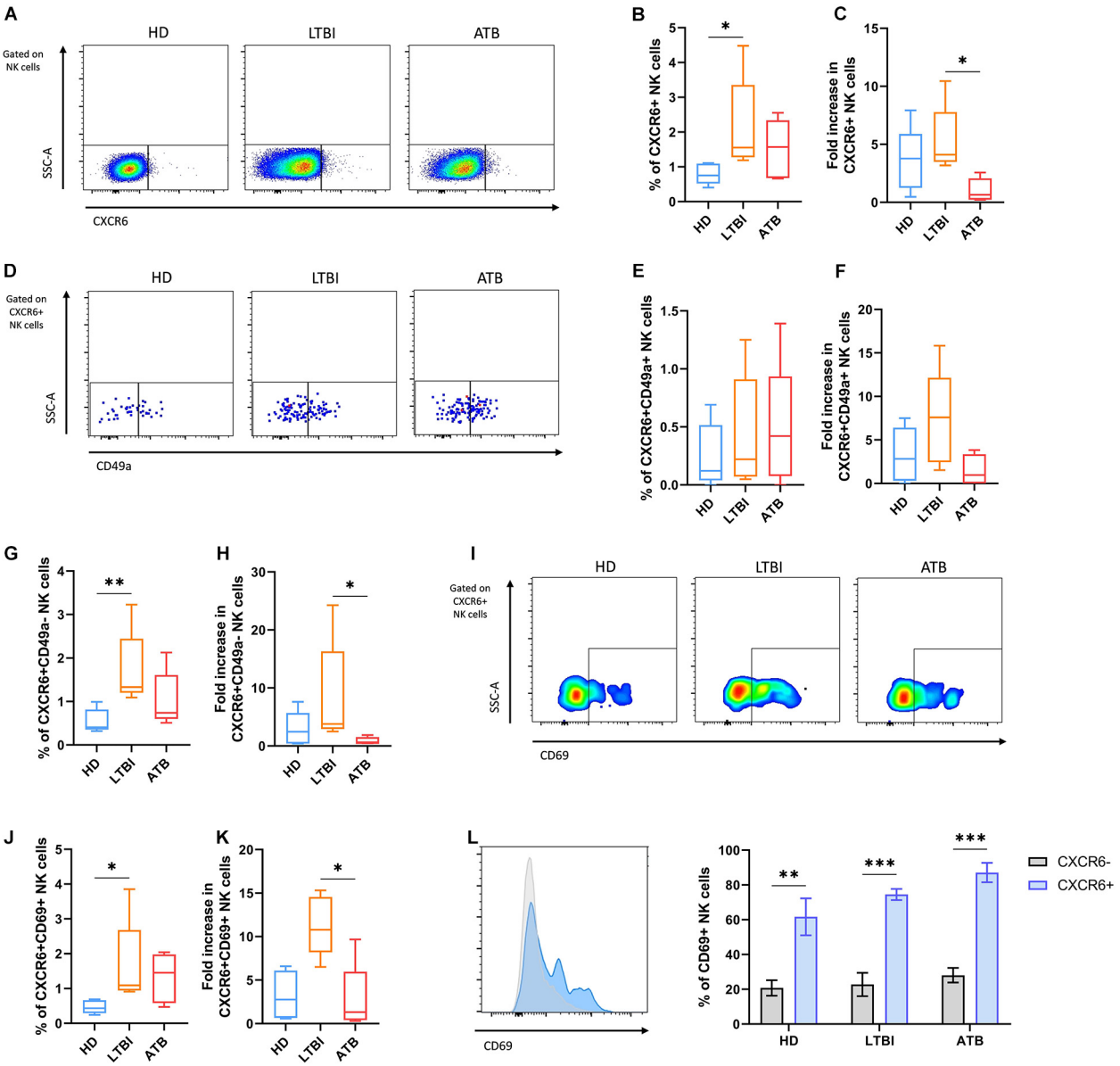

FIGURE 3 | Responses of circulating CXCR6+ NK cells from individuals with TB after the *in vitro* exposure to Mtb antigens. Peripheral blood mononuclear cells (PBMC) from healthy donors (HD), individuals with latent TB infection (LTBI), and patients with active pulmonary TB (ATB) were cultured with a cell wall (CW) extract of Mtb H37Rv for 48 h (n = 5 per group). After the *in vitro* stimulation with Mtb antigens, cells were characterized by flow cytometry. Total NK cells were gated as shown in **Figure 2A**. **(A)** Flow cytometry analysis of CXCR6+ NK cells after the *in vitro* stimulation. **(B)** The percentage and **(C)** fold increase of CXCR6+ NK cells were determined by flow cytometry. **(D,E)** We also determined the percentage and **(F)** fold increase of CXCR6+CD49a+ NK cells. **(G,H)** Comparisons of the percentage and fold increase of CXCR6+CD49a– NK cells between groups were also performed. **(I,J)** Percentage and **(K)** fold increase of CXCR6+CD69+ NK cells determined by flow cytometry. **(L)** The relative expression of CD69 and the proportion of CD69+ cells were compared between CXCR6+ and CXCR6– NK cells at each group. Fold increases were calculated as follows: the percentage of a specific cell subpopulation after the stimulation of PBMCs with Mtb antigens was divided by the percentage of the same cell subset before such stimulation. Differences between groups were analyzed using the Kruskal-Wallis test and the *post hoc* Dunn's test for multiple comparisons. Comparisons between cells from the same group were analyzed with the Student *t*-test and *p* values corrected for multiple comparisons using the Holm method. The data shown represent mean (±SE) values. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Finally, we evaluated whether human CXCR6+ NK cells respond not only to the stimulus with antigens from the lab-adapted Mtb H37Rv strain but also to a CW preparation from a clinically relevant Mtb isolate. Thus, we cultured PBMCs from six healthy individuals with antigens of the prototype W-Beijing lineage Mtb strain, hypervirulent HN878. Our results showed that the Mtb H878 CW extract triggered an increase in the percentage of NK cells expressing CXCR6 (**Figure 4A**), as well as in the percentage of total- and IFN-γ-producing CXCR6+CD49a+ NK cells (**Figures 4B–D**). Such a stimulus

also resulted in higher expression of CD69 in CXCR6+ NK cells, augmenting the percentage of CXCR6+CD69+ NK cells (**Figures 4E–G**). This increase in the expression of the tissue-homing marker CD69 was significatively higher in CXCR6+ than in CXCR6− NK cells (**Figures 4H,I**). Collectively, these data indicate that CW antigens from laboratory-adapted and clinical Mtb strains are stimulating factors for human CXCR6+ NK cells.

DISCUSSION

Tuberculosis is a leading cause of death worldwide. The incomplete understanding of the mechanisms implicated in protective immunity against Mtb is a barrier to the development of novel and more effective TB vaccines. For many years, the TB vaccine field has relied on the paradigm that IFN-γ-mediated T cell responses are the chief mechanism of protection against Mtb. However, novel insights into the role of innate immune cells in TB are motivating innovative strategies to target several components of the innate immune system and improve Mtb control (20–23). In this regard, NK cells are group 1 innate lymphoid cells (ILC1s) that play a role in the defense against this pathogen (20, 24). Studies in humans have shown that NK cells infiltrate the lung of patients with ATB and localize within tubercle granulomas (25). Also, phenotypical deficiencies of circulating NK cells have been observed among ATB patients with respect to individuals with LTBI. For instance, there is a reduced frequency of $CD56^{bright}$ accompanied by decreased expression of the activating receptors NKp30 and NKp46 in peripheral blood NK cells from ATB patients but not LTBI individuals (17, 18). Also, a higher prevalence of inhibitory killer-immunoglobulin like receptors (KIR) has been documented among patients with ATB as compared with resistant individuals (26–29). NK cells also produce a broad range of soluble immune mediators that are known to be crucial for anti-Mtb immunity. These mediators include IFN-γ, TNF-α, IL-17, IL-22, and GM-CSF (2, 30–34). In murine TB models, the production of IL-22 by NK cells is crucial for protective immune responses induced by *Mycobacterium bovis* bacillus Calmette—Guérin (BCG) vaccination (35). Also, in *Rag1*−/− mice infected with Mtb, NK cells can mediate a certain degree of protection against the infection *via* the production of IFN-γ (36). Finally, *in vitro* assays have revealed that NK cells are capable of recognizing several components of Mtb and exert effector activities (37), including cytokine production and cytotoxicity (37–39). Moreover, NK cells can promote the development of protective responses of CD8+ T cells (40), eliminate CD25+ T regulatory cells (41), and even lyse extracellular Mtb (42).

The memory-like properties of NK cells make them an attractive candidate target for TB vaccine development programs (20–23). In this regard, several lymphoid and non-lymphoid tissues are thought to harbor subpopulations of NK cells capable of mediating memory-like immune responses against Mtb (20). For instance, the pleural fluid contains a subgroup of NK cells that express the molecule CD45RO, a marker classically used to identify memory T cells (10, 11). These pleural CD45RO+ NK cells have increased cytotoxic capacity and produce higher levels of inflammatory cytokines in response to IL-12 and BCG as compared to CD45RO- NK cells (10, 11). Similarly, the vaccination of mice with BCG induces an IL-21 dependent expansion of splenic and lymph node NKp46+CD27+KLRG1+ NK cells that mediate protective memory-like responses against Mtb (12). Thus, the memory of NK cells might be an active mechanism of defense during TB (20). However, our knowledge of the mechanisms regulating the function of memory-like NK cells in the context of TB is limited. Moreover, other yet unrecognized subsets of NK cells with specific phenotypes and adoptive properties against Mtb may exist.

In this context, the results of the current study indicate that the repetitive exposure of mice to CW extracts from Mtb H37Rv promote functional changes in murine NK cells, as they improve their cytokine production capacity after consecutive encounters with such mycobacterial components. This coincides with previous reports describing a BCG-induced training of NK cells in humans (43). Nonetheless, our results additionally demonstrate that memory-like NK cell properties induced by mycobacterial components are independent of the persistence of the antigen, as we inoculated mice with CW extracts rather than live Mtb bacilli. Interestingly, the effect described here seems to be restricted to hepatic NK cells from treated mice, as we found enhanced cytokine production only in cultures of pooled hepatic and splenic, but not isolated splenic NK cells re-challenged with Mtb H37Rv CW. This is in agreement with previous studies that revealed that the BCG vaccination does not enhance the capacity of murine splenic NK cells to produce IFN-γ after a re-challenge with BCG *in vitro* (44). Thus, our findings suggest that the liver is an additional reservoir of NK cell subsets with the ability to mount recall responses against Mtb antigens in mice.

One of such hepatic memory-like NK cell subpopulations could be represented by CXCR6+ NK cells, as we found that murine hepatic CXCR6+ NK cells expand *in vivo* after the administration of the Mtb H37Rv CW. This expansion was also independent of the persistence of the antigen. The effects of Mtb antigens are long-lasting, as the phenotypical changes observed in splenic and hepatic NK cells were observed 4 weeks after the last administration of the Mtb H37Rv CW extract. In addition, we demonstrated that CXCR6+ NK cells are capable of responding to direct exposure to Mtb antigens *in vitro*. This subpopulation of murine NK cells has shown memory-like properties in the past (13). Based on our results, these cells also seem to possess an intrinsic higher capacity to produce IFN-γ in response to Mtb antigens as compared to CXCR6− NK cells. These findings may indicate that vaccination with components of Mtb could enhance the functional capacity of CXCR6+ NK cells. However, the effect of the priming of CXCR6+ NK cells on the protective immunity induced by vaccines against Mtb infection was not addressed. Also, changes in murine lung CXCR6+ NK cells in response to the administration of the Mtb H37Rv CW were not evaluated.

To our knowledge, there is no evidence of the role of memory-like CXCR6+ NK cells in respiratory infections. However, the chemotactic axis involving CXCR6 and its ligand CXCL16 might be implicated in defense of the lungs against pathogens. CXCL16 is highly expressed in the sinusoids of the liver and is responsible for the tissue-homing pattern of hepatic CXCR6+ NK cells

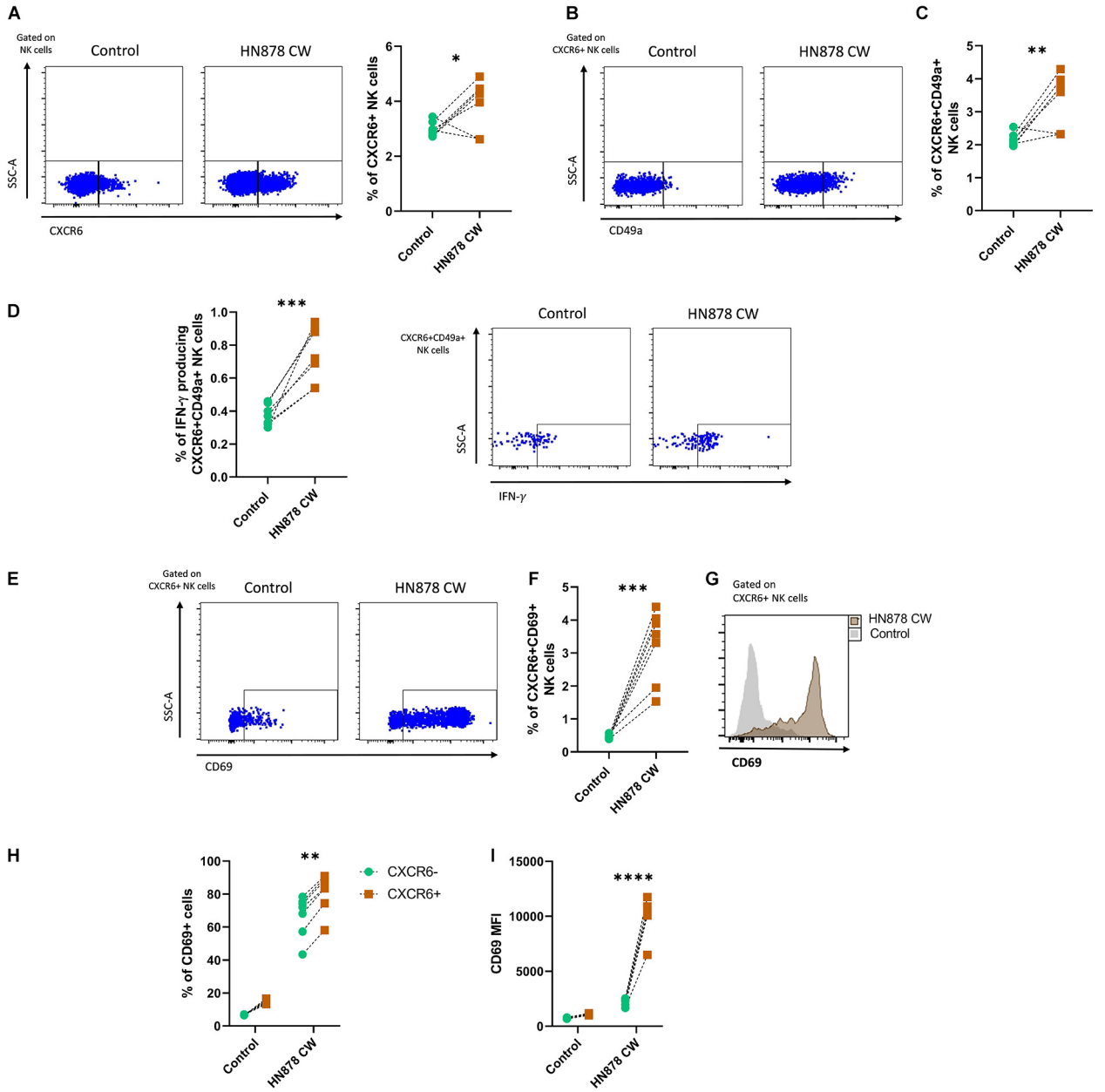

FIGURE 4 | Responses of human CXCR6+ NK cells to the *in vitro* infection with the hypervirulent clinical Mtb strain HN878. Peripheral blood mononuclear cell (PBMC) samples from healthy volunteer donors (HD) were obtained from the blood bank and cultured with a cell wall (CW) extract of the hypervirulent clinical Mtb HN878 for 48 h ($n = 6$). Some PBMCs from the same donors received sterile culture medium and were considered as controls. After the *in vitro* stimulation, cells were characterized by flow cytometry. Total NK cells were gated as shown in **Figure 2A**. **(A)** The percentage of CXCR6+ NK cells was compared between stimulated and non-stimulated PBMCs. **(B)** Expression of CD49a in CXCR6+ NK cells and **(C)** percentage of CXCR6+CD49a+ NK cells. **(D)** Percentage of IFN-γ producing CXCR6+CD49a+ NK cells. **(E)** Flow cytometry dot-plot showing the expression of CD69 by CXCR6+ NK cells. **(F)** Percentage of CXCR6+CD69+ NK cells and **(G)** relative expression of CD69 in CXCR6+ NK cells. **(H)** The percentage of CD69+ cells and **(I)** mean fluorescence values intensity (MFI) for CD69 were compared between CXCR6+ and CXCR6– NK cells in both stimulated and non-stimulated PBMCs. Differences between groups and between cells from the same group were analyzed with the unpaired Student *t*-test and Mann-Whitney *U* test as appropriate. The data shown represent mean (±SE) values. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

(45). Interestingly, CXCL16 has also been detected in high concentrations in human BAL specimens. This chemokine is highly expressed by alveolar macrophages, bronchial epithelial cells, airway smooth muscle cells, and lung fibroblasts, and mediates the recruitment of CXCR6+ T cells to the lungs under inflammatory conditions (46, 47). The expression of CXCR6 is upregulated in T cells after pulmonary infection with *Pneumocystis jirovecii* in mice (48). CD4+ and CD8+ T-cells

also increase the expression of CXCR6 after mucosal vaccination with antigens from Mtb (49), as well as during pulmonary Mtb infection in murine models of TB (50). Notably, the expression of this chemokine receptor in lung T lymphocytes is associated with vaccine-induced protective immunity against Mtb (49).

Conversely, the role of CXCR6+ T cells is redundant for TB control in unvaccinated mice infected with Mtb. Indeed, CXCR6−/− naïve mice display a lower bacterial burden in the lungs after Mtb infection, as compared to wild type animals (50). This resistant phenotype is also associated with lower IFN-γ production in the lungs of CXCR6−/− mice with pulmonary TB, suggesting that IFN-γ-producing CXCR6+ T cells play pathogenic roles during Mtb infection. In this context, we observed that several subsets of peripheral NK cells (CXCR6+, CXCR6+ CD49a−, and CXCR6+CD49a+) are present at slightly higher frequencies in the blood of a proportion of patients with ATB as compared to LTBI individuals. This may reflect an active mobilization of these cells to the sites of infection during pulmonary TB. Indeed, CXCR6+ NK cells expressing the tissue-homing marker CD69 were found to be increased in the circulation of ATB patients but not HD and LTBI individuals. CD69 is also a marker of activation in lymphocytes (51). Interestingly, the relative expression of this molecule was also increased in both ATB and LTBI subjects. As such, the higher expression of CD69 might be a reminiscent readout of a previous activation in CXCR6+ NK cells from LTBI individuals, whereas it may reflect an ongoing process of activation and tissue-homing in CXCR6+ NK cells from ATB patients. Importantly, we found that the expansion of CXCR6+ NK cells after the *in vitro* exposure to a CW extract from the laboratory-adapted Mtb H37Rv strain was higher in LTBI individuals as compared to ATB subjects. These responses were explained by the expansion of CXCR6+CD49a− NK cells but not double-positive CXCR6+CD49a+ NK cells in LTBI individuals. The former cells are known to display an immature and tolerant phenotype and possess a reduced ability to produce IFN-γ after the antigenic stimulations (14). Meanwhile, double-positive CXCR6+CD49a+ NK cells produce high quantities of IFN-γ upon stimulation (15).

Together, these data indicate that: (a) despite being slightly increased in the circulation of a minor proportion of ATB patients, the subpopulations of circulating CXCR6+ NK cells are less capable of responding to Mtb antigens *ex vivo* as compared to LTBI individuals. Nonetheless, it is possible that memory-like NK cells with enhanced functions against Mtb could be depleted from the circulation and mobilized to the infected lungs. Thus, future studies should look for the presence of CXCR6+ NK cells in lung biopsy or autopsy specimens from ATB patients. (b) Different types of immune cells expressing CXCR6 and producing IFN-γ may play contrasting roles in immunity against murine and human TB. Based on our results and previous studies (50), it is likely that subsets of memory-like NK cells with tolerant phenotypes and reduced capacity of producing IFN-γ, such as hepatic CXCR6+CD49a− NK cells, may play protective roles against Mtb in humans. Similarly, IFN-γ-producing CXCR6+ T cells might be pathogenic during murine TB (50). Unfortunately, the role of other cytokines produced by murine and human memory-like CXCR6+ NK cells in response to Mtb antigens was not addressed in our study. Furthermore, we did not evaluate the cytotoxic capacity of these cells.

Finally, previous studies showed that priming of peripheral blood NK cells with the cytokines IL-12 and IL-15 induces the upregulation of both CD49a and CXCR6 (15). CXCR6+CD49a+ NK cells generated *in vitro* after cytokine priming display phenotypic and functional features similar to liver-resident CD49a+ NK cells, including enhanced IFN-γ expression (15). In line with these findings, we demonstrated that the *in vitro* exposure to clinical strains of Mtb, such as the hypervirulent HN878, induces an increase in the expression of CXCR6 and CD49a in circulating NK cells from healthy volunteer individuals. This stimulation can generate double-positive CXCR6+CD49a+ NK cells with the capacity to produce IFN-γ. Moreover, the CW of Mtb HN878 induces the expression of the activation and tissue-homing marker CD69 in CXCR6+ NK cells. These data add to the evidence about the capacity of different antigenic and non-antigenic stimuli to activate peripheral NK cells, which then acquire tissue-resident properties (15).

The current study has several limitations that must be considered when interpreting our findings. First, the differences in the immunophenotype of human CXCR6+ NK cells between participant groups were not robust. Thus, the protective or pathogenic role of different subsets of CXCR6+ NK cells needs to be elucidated in future studies. Despite this, we found that the responses against the stimulation with Mtb H37Rv CW differed in LTBI and ATB patients. Thus, the main contribution of our manuscript is that we demonstrated that antigens of Mtb stimulate both murine and human CXCR6+ NK cells. Second, even though we found that murine and human CXCR6+ NK cells respond to Mtb antigens, we did not evaluate their mechanisms of activation. In this regard, it is possible that NK cells could have directly recognized Mtb antigens since previous studies have demonstrated the direct activation of NK cells triggered by the toll-like receptor 2 (TLR-2) recognition of CW components from *Mycobacterium bovis* BCG (52). Our results also support this hypothesis, as at least in the case of murine NK cells, we excluded other immune cells from our NK cell cultures using antibodies coupled to magnetic beads. Another possibility is that the responses induced by Mtb antigens in CXCR6+ NK cells could have resulted from the effect of cytokines produced by other immune cells. Indeed, it has been shown that the function of CD4+ T cells is crucial to sustaining the effector activities of NK cells during Mtb infection (53). Thus, T cell-derived cytokines might prime NK cells to respond to Mtb antigens, and even promote the development of cytokine-induced memory-like NK cells in a similar manner to the bystander mechanisms of induction of non-specific memory CD8+ T cells (54). This might apply to the *in vivo* expansion of CXCR6+ NK cells that we observed in mice treated with the Mtb H37Rv CW extract, and to the *in vitro* activation of human CXCR6+ NK cells with the same stimulus, which were cultured as total PBMCs rather than isolated NK cells.

Although our results suggest that hepatic NK cells might develop memory responses against Mtb in mice, we were unable to isolate a sufficient amount of such cells to directly evaluate their functions instead of using pools of splenic and hepatic

NK cells. Also, our observations should have been confirmed in RAG1 deficient mice to exclude the interference of T cell memory responses. Despite this, our study constitutes the first evaluation of the capacity of murine and human CXCR6+ NK cells to respond to Mtb antigens and should motivate future investigations to confirm a possible role for these cells in TB.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants and animal study were reviewed and approved by Institutional Review Board of the Instituto Nacional de Enfermedades Respiratorias "Ismael Cosio Villegas," Mexico City, Mexico. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JC-P participated in the conception of the study, collected clinical data and biological samples from human participants, performed the experiments in mice, and wrote the manuscript. LJ-Á and GR-M participated in experiments with mice and performed flow cytometry. MM-T and CS-L collected clinical and biological samples from human participants and participated in the discussion of the manuscript. LJ-Z, EG-L, and JZ conceived the idea of the study, participated in the analysis of results, and revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.582414/full#supplementary-material

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Umbilical Cord Blood and iPSC-Derived Natural Killer Cells Demonstrate Key Differences in Cytotoxic Activity and KIR Profiles

Benjamin H. Goldenson[1†], Huang Zhu[1†], YunZu Michele Wang[1†], Naveen Heragu[1], Davide Bernareggi[1], Alessa Ruiz-Cisneros[1], Andres Bahena[1], Eivind Heggernes Ask[2], Hanna Julie Hoel[2], Karl-Johan Malmberg[2,3,4] and Dan S. Kaufman[1]*

[1] Division of Regenerative Medicine, Department of Medicine, University of California, San Diego, San Diego, CA, United States, [2] Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway, [3] The KG Jebsen Center for Cancer Immunotherapy, Institute of Clinical Medicine, University of Oslo, Oslo, Norway, [4] Department of Medicine Huddinge, Center for Infectious Medicine, Karolinska Institutet, Stockholm, Sweden

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***Correspondence:**
Dan S. Kaufman
dskaufman@ucsd.edu

[†] These authors have contributed equally to this work

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Natural killer (NK) cells derived or isolated from different sources have been gaining in importance for cancer therapies. In this study, we evaluate and compare key characteristics between NK cells derived or isolated from umbilical cord blood, umbilical cord blood hematopoietic stem/progenitor cells, peripheral blood, and induced pluripotent stem cells (iPSCs). Specifically, we find $CD56^+$ NK cells isolated and expanded directly from umbilical cord blood (UCB56) and NK cells derived from $CD34^+$ hematopoietic stem/progenitors in umbilical cord blood (UCB34) differ in their expression of markers associated with differentiation including CD16, CD2, and killer Ig-like receptors (KIRs). UCB56-NK cells also displayed a more potent cytotoxicity compared to UCB34-NK cells. NK cells derived from iPSCs (iPSC-NK cells) were found to have variable KIR expression, with certain iPSC-NK cell populations expressing high levels of KIRs and others not expressing KIRs. Notably, KIR expression on UCB56 and iPSC-NK cells had limited effect on cytotoxic activity when stimulated by tumor target cells that express high levels of cognate HLA class I, suggesting that *in vitro* differentiation and expansion may override the KIR-HLA class I mediated inhibition when used across HLA barriers. Together our results give a better understanding of the cell surface receptor, transcriptional, and functional differences between NK cells present in umbilical cord blood and hematopoietic progenitor-derived NK cells which may prove important in selecting the most active NK cell populations for treatment of cancer or other therapies.

Keywords: natural killer cells, cancer, stem cells, umbilical cord blood, KIRs

INTRODUCTION

Natural killer (NK) cells are innate immune lymphocytes with the ability to rapidly recognize and exhibit cytotoxicity toward tumor and virus infected cells in a HLA-independent manner (1). These unique properties have led to growing use of different allogeneic NK cell adoptive transfer approaches to be used for cancer treatment (2). Sources of primary NK cells used in these clinical trials to treat both hematologic malignancies and solid tumors include peripheral blood

(PB)-derived, umbilical cord blood (UCB)-derived, and induced pluripotent stem cell (iPSC)-derived NK cells (3–12). However, despite clinical use of these diverse NK cell populations, there is relatively little known about key phenotypic, genotypic, and functional comparison between these NK cell populations that may lead to difference in clinical efficacy (10, 13–16).

NK cells were first described as a lymphocyte population able to detect and rapidly kill tumor cells or viral infected cells without prior sensitization (17, 18). A key observation that NK cells could kill tumor cell lines that had lost expression of MHC class I surface molecules, and the finding that MHC class I expressing cells were resistant to lysis by NK cells, led to the formulation of the "missing self-recognition hypothesis" which states that NK cells are able to detect and kill target cells that do not express MHC class-I molecules (19, 20). The missing self-recognition hypothesis has been refined following the discovery of multiple germline encoded inhibitory and activating NK receptors (21–23). In humans, the main inhibitory receptors are inhibitory killer Ig-like receptors (KIRs), which recognize groups of HLA class-I alleles and the CD94/NKG2A heterodimer which is specific for the HLA-E molecule (24–26).

Inhibitory KIRs contain two (KIR2D) or three (KIR3D) polymorphic extracellular immunoglobulin (Ig)-like domains followed by long (L) cytoplasmic tails harboring two immunoreceptor tyrosine-based inhibition motifs (ITIMs). Four major inhibitory KIRs specific for epitopes found on distinct groups of HLA class I allotypes include KIR2DL1 which recognizes the HLA-C2 epitope, KIR2DL2/L3 which recognizes the HLA-C1 epitope, KIR3DL1 which recognizes HLA-B, or HLA-A epitopes and KIR3DL2 which recognizes HLA-A*03 and -A*11 epitopes (22, 24). NK cells also express germ-line encoded activating NK cell receptors and co-receptors that induce NK cell activation through interactions with various ligands that are expressed on tumor-transformed or virus-infected cells (27–29). Activating KIRs differ from inhibitory KIRs in that the intracellular cytoplasmic tail of activating KIRs is shorter, lacks ITIMs, and interacts with the signaling adaptor protein DAP12 that contains immunoreceptor tyrosine-based activation motifs (ITAMs) (30–32). Among the activating KIRs, KIR2DS1, and its ligand HLA C2 is the best defined receptor-ligand pair with an influence on NK cell function (33, 34).

KIRs have been shown to be clinically significant in allogeneic hemopoietic stem cell transplant (HSCT) treatments for acute myeloid leukemia (AML) in cases of KIR profile mismatches where the inhibitory KIRs on the NK cells derived from the stem cell donor do not encounter their cognate ligands on the transplant recipient's AML blasts. In these cases, NK cells become activated and kill their targets more effectively leading to a survival benefit to AML patients (35, 36). These findings have been adopted in practice, as when otherwise similarly HLA-matched allogeneic or haploidentical HSCT donors are available, HSCT donor selection algorithms consider KIR profiles as a donor selection criteria (35, 37–39). However, selection of KIR ligand mismatches in HSCT remains an active area of investigation, as a recent study found that optimization of donors based on KIR2DS1 and KIR3DL1 expression did not provide a survival benefit (40).

Expression and function of KIRs in PB-NK, UCB-NK, and iPSC-derived-NK cells have not been well-defined. UCB provides an internally genetically-controlled model to compare the $CD56^+$ NK cells that are present in a cord blood unit (when collected at birth) to genetically identical NK cells derived from cord blood $CD34^+$ hematopoietic stem/progenitor cells from the same cord blood unit. We find the expression of NK cell surface antigens, activating and inhibitory receptors between the different NK cell types are relatively similar, but with variable KIR expression linked to expression of markers for NK cell differentiation. To better understand the functional importance of this difference in KIR expression, we analyzed the cytotoxic function of the cells against targets expressing or lacking specific KIR ligands and demonstrate that the NK cells different KIR profiles are a significant mediator of the difference in the cells killing function.

MATERIALS AND METHODS

Derivation and Expansion of NK Cells From Umbilical Cord Blood and Peripheral Blood

Umbilical cord blood units were purchased from the San Diego Blood Bank. Donor consent was obtained and the study followed guidelines of the UCSD Institutional Review Board. UCB56 NK cells were isolated directly from each unit using CD56 positive selection beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The UCB56 NK cells were then expanded under the culture conditions described previously and cryopreserved. UCB56 cells were then thawed prior to use (41). CD34 expressing hematopoietic stem cells were also isolated from the same units using CD34 positive selection beads (Miltenyi). Using previously published protocols, the CD34+ cells were differentiated into UCB34 NK cells from each unit (10, 42). In brief, CD34+ cells were plated on irradiated OP9-DLL4 cells in medium containing a 2:1 mixture of Dulbecco modified Eagle medium/Ham F12 (Thermo Fisher Scientific, Waltham, MA, 11965092, 11765054), 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA, 25030081), 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, 15140122), 25 μM β-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, 21985023), 20% heat-inactivated human serum AB (Corning, NY, U.S., MT35060CI), 5 ng/mL sodium selenite (Merck Millipore, Burlington, MA, S5261), 50 μM ethanolamine (MP Biomedicals, ICN19384590), 20 mg/mL ascorbic acid (Merck Millipore, Burlington, MA, A4544), interleukin-3 (IL-3; R&D Systems Minneapolis, MN, 203-IL); for first week only), stem cell factor (SCF; R&D Systems Minneapolis, MN, 7466-SC), interleukin-15 (IL-15; R&D Systems, 247-ILB), Fms-like tyrosine kinase 3 ligand (FLT3L; R&D Systems Minneapolis, MN, 308-FK), and interleukin-7 (IL-7; R&D Systems Minneapolis, MN, 207-IL). The cells were cultured in these conditions for 28–35 days receiving weekly media changes until they developed into $CD45^+CD56^+CD33^-CD3^-$ cells as determined by flow cytometry (43). Peripheral blood NK cells were isolated and expanded in culture as previously described. Briefly, CD56+ NK cells were positively selected from a buffy coat prepared

from peripheral blood obtained from the San Diego Blood Bank by Ficoll gradient centrifugation (Miltenyi). PB-NK were then co-cultured with irradiated K562 cells in NK cell media (41).

HLA and KIR Genotyping

HLA and KIR genotypes were determined by the UCSD Health Clinical Laboratories. HLA and KIR Luminex-based tissue typing assays using sequence-specific oligonucleotides (SSO) Genotyping Kits were used (One Lambda, Thermo Fisher). HLA typing was performed by direct DNA sequence analysis using next-generation DNA sequencing technology in conjunction with allele sequence-specific primer PCR. DNA sequence data was analyzed by alignment and comparison with IMGT database of known HLA DNA sequences. Allele types unresolvable with available reagents were assigned NMDP G group codes. KIR ligands were assigned based on the amino acid residues at position 77 and 80 of each HLA B and HLA C protein (Bw4 with N/D/S at residue 77 and I/T at residue 80; Bw6 with G/S at residue 77 and N at residue 80; C1 with N at residue 80; and C2 with K at residue 80) (44, 45). For HLA serological typing, serological equivalents were derived from the molecular typing results.

Cell Lines and Cell Culture

Neuroblastoma cell lines SK-N-AS, IMR32, and NBLS were provided by Dr. Peter Zage (UCSD Moores Cancer Center), and their identities were validated by American Type Culture Collection (ATCC, Manassas, Virginia, U.S.). The 721.221 lymphoblastic cell line (221.wt) that was transfected with HLA cw3 (C1) and HLA cw4 (C2) was generously provided by Peter Parham. K562, NK-92, Jurkat, and Raji cell lines were obtained from ATCC. Raji, K562, 721.221.wt, and neuroblastoma lines were cultured in RPMI 1640 media (Thermo Fisher Scientific, Waltham, MA, 11875085) supplemented with 2 mM glutamine (Sigma) and with 10% heat-inactivated fetal bovine serum (FBS) (Sigma). 221.C1/C2 lines were maintained with Geneticin (Thermo Fisher Scientific). NK-92, K562, Raji and Jurkat cells were sub-cultured following ATCC recommendations.

Derivation and Expansion of NK Cells From iPSCs

The derivation of NK cells from iPSCs has been previously described (9, 11, 43). Briefly, 8,000 TrypLE-adapted iPSCs were seeded in 96-well round-bottom plates with APEL2 media (Stem Cell Technologies, Vancouver, BC, Canada) containing 40 ng/ml human Stem Cell Factor (SCF), 20 ng/ml human Vascular Endothelial Growth Factor (VEGF), and 20 ng/ml recombinant human Bone Morphogenetic Protein 4 (BMP-4). After day 8 of hematopoietic differentiation, spin embryoid bodies were then directly transferred into each well of uncoated 24-well plates under a condition of NK cell culture. Cells were then further differentiated into NK cells using 5 ng/mL IL-3 (first week only), 10 ng/mL IL-15, 20 ng/mL IL-7, 20 ng/mL SCF, and 10 ng/mL flt3 ligand for 28–32 days. iPSC-NK cells were expanded using irradiated K562-IL21-41BBL cells, also termed artificial antigen presenting cells (aAPCs) (41).

Flow Cytometry

Flow cytometry was done on a BD FACS Calibur, BD LSRII or NovoCyte 3000, and data were analyzed using FlowJo or NovoExpress.

Antibodies

The following antibodies were used for flow cytometry (all anti-human): CD16-PE (BD Biosciences, 560995, clone 3G8), CD16-APC (BD Biosciences, 561248, clone 3G8) NKG2D-PE (BD Biosciences, 557940, clone 1D11), NKp44-PE (BD Biosciences, 558563, clone p44-8), NKp46-PE (BD Biosciences, 557991, clone 9E2), TRAIL-PE (BD Biosciences, 565499, clone YM366), FAS ligand-PE (BD Biosciences, 56426, clone NOK-1), NKG2A-PE (Beckman Coulter, IM3291U, clone Z199), CD158a,h (KIR2DL1, KIR2DS1)-PE (Beckman Coulter, A09778, clone EB6B), CD158b1/b2,j (KIR2DL2, KIR2DL3, KIR2DS2)-PE (Beckman Coulter, IM2278U, clone GL183), CD158e1(KIR3DL1)-BV421 (BioLegend, 312713, clone DX9), CD158a(KIR2DL1)-APC (Miltenyi, 130-120-584, clone REA284), CD158b1/b2,j (KIR2DL2, KIR2DL3, KIR2DS2)-PE-Cy5.5 (Beckman Coulter, A66900, clone GL183), CD158a,h(KIR2DL, KIR2DS1)-PE-Cy7 (Beckman Coulter, A66899, clone EB6B), CD158b2(KIR2DL3)-PE, (R&D systems, FAB2014P, clone 180701), CD155-PE (BioLegend, 337609, Clone SKII.4), HLA E-PE (BioLegend, 342603, Clone 3D12) MICA/MICB-PE (BD Biosciences, 558352, Clone 6D4), CD112-PE (BD Biosciences, 551057, Clone R2.525), ULBP2/5/6-PE (R&D Systems, FAB1298P, Clone 16590).

Mass Cytometry

For viability assessment, cells were stained with Cell-ID Intercalator-103Rh (Fluidigm, San Francisco, CA, 201103B) in complete medium for 20 min at 37°C. Maxpar Cell Staining Buffer (Fluidigm, 201068) was used for all antibody staining and subsequent washing. Samples were incubated with Fc receptor binding inhibitor (Thermo Fisher Scientific, 14-9161-73) for 10 min at room temperature, before adding surface antibodies and incubating for 30 min at 4°C. Subsequently, cells were fixed in Maxpar PBS (Fluidigm, 201058) with 2% formaldehyde, transferred to methanol and stored at −20°C. The day after, cells were stained with an intracellular antibody cocktail for 40 min at 4°C and labeled with Cell-ID Intercalator-Ir (Fluidigm, 201192B). Samples were supplemented with EQ Four Element Calibration Beads (Fluidigm, 201078) and acquired on a CyTOF 2 (Fluidigm) equipped with a SuperSampler (Victorian Airship, Alamo, CA) at an event rate of <500/s. Antibodies were either obtained pre-labeled from Fluidigm or conjugated with metal isotopes using Maxpar X8 antibody labeling kits (Fluidigm) (**Supplementary Table 1**). FCS files were normalized using Helios software (Fluidigm) and gated on CD45+ CD19- CD14- CD32- CD3- viable single cells using Cytobank (Cytobank Inc., Santa Clara, CA). For subsequent analysis, data was imported into R (R Core Team, 2019) using the *flowCore* package, and transformed using *arcsinh(x/5)*. Ten thousand events were randomly sampled from each file and concatenated. t-Distributed Stochastic Neighbor Embedding (t-SNE) was then performed using the *Rtsne* R package with default settings and results were

visualized using the *ggplot2* R package. The following markers were used for the clustering shown in **Figure 1**: 2B4, CD2, CD8, CD16, CD161, CD27, CD34, CD38, CD45, CD56, CD57, CD94, DNAM-1, Granzyme B, ILT-2, Ki-67, KSP37, NKG2A, NKG2C, NKG2D, NKp30, Perforin, Siglec-7, SYK, TIGIT, and TIM-3. The clustering in **Figure 2** was based on the following markers: KIR2DL1, KIR2DL1/S1, KIR2DL3, KIR2DL2/L3/S2, KIR2DS4, KIR3DL1, and KIR3DL2. t-SNE plots showing the number of expressed KIRs per cell were created by manually gating on positive cells in 5 KIR stains (KIR2DL1/S1, KIR2DL2/L3/S2, KIR2DS4, KIR3DL1, KIR3DL2) and determining for how many of these gates each cell is positive. t-SNE plots showing "any gated KIR" indicate which cells express at least one KIR, according to the described manual gating.

Cytotoxicity Assays

CellEvent™ Caspase-3/7 Green Flow Cytometry Assay

Target cells were pre-stained with CellTrace™ Violet (ThermoFisher Scientific, C34557) at a final concentration of 5 μM in PBS for 15 min at 37°C. After staining, the cells were washed in complete culture medium prior to being mixed with NK cell cultures at the indicated effector to target (E:T) ratios. After a brief centrifugation, co-cultures were incubated at 37°C for 3.5 h. Afterwards, CellEventR Caspase-3/7 Green Detection Reagent (Thermal Fisher Scientific, C10423) was added for an additional 30 min of culture for a total incubation time of 4 h. During the final 5 min of staining, SYTOX™ AADvanced™ dead cell stain solution (Thermal Fisher Scientific, S10349) was added and mixed gently. Cells were then analyzed by flow cytometry.

IncuCyte Caspase-3/7 Green Apoptosis Assay

Target cells were labeled with CellTraceTM Far Red (ThermoFisher, C34564). Adherent target cells were seeded in a 96-well plate at a density of 4,000 cells/well 24 h before addition of IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (Essen Bioscience, 4440) to each well-diluted by a factor of 1,000. Non-adherent target cells were seeded in fibronectin coated 96-well plates at a density of 30,000 to 50,000 cells/well and further incubated at room temperature for 30 min before the addition of IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent. After incubation, NK cells were added at various E:T ratios and monitored on the IncuCyte ZOOM to acquire images every 1 h for adherent cells and every 30 min for non-adherent cells. Experiments were performed with 3 independent biological triplicates. The cytotoxicity of target cells was analyzed by quantifying red cell number and/or overlay of Caspase 3/7 (green) within the red cells.

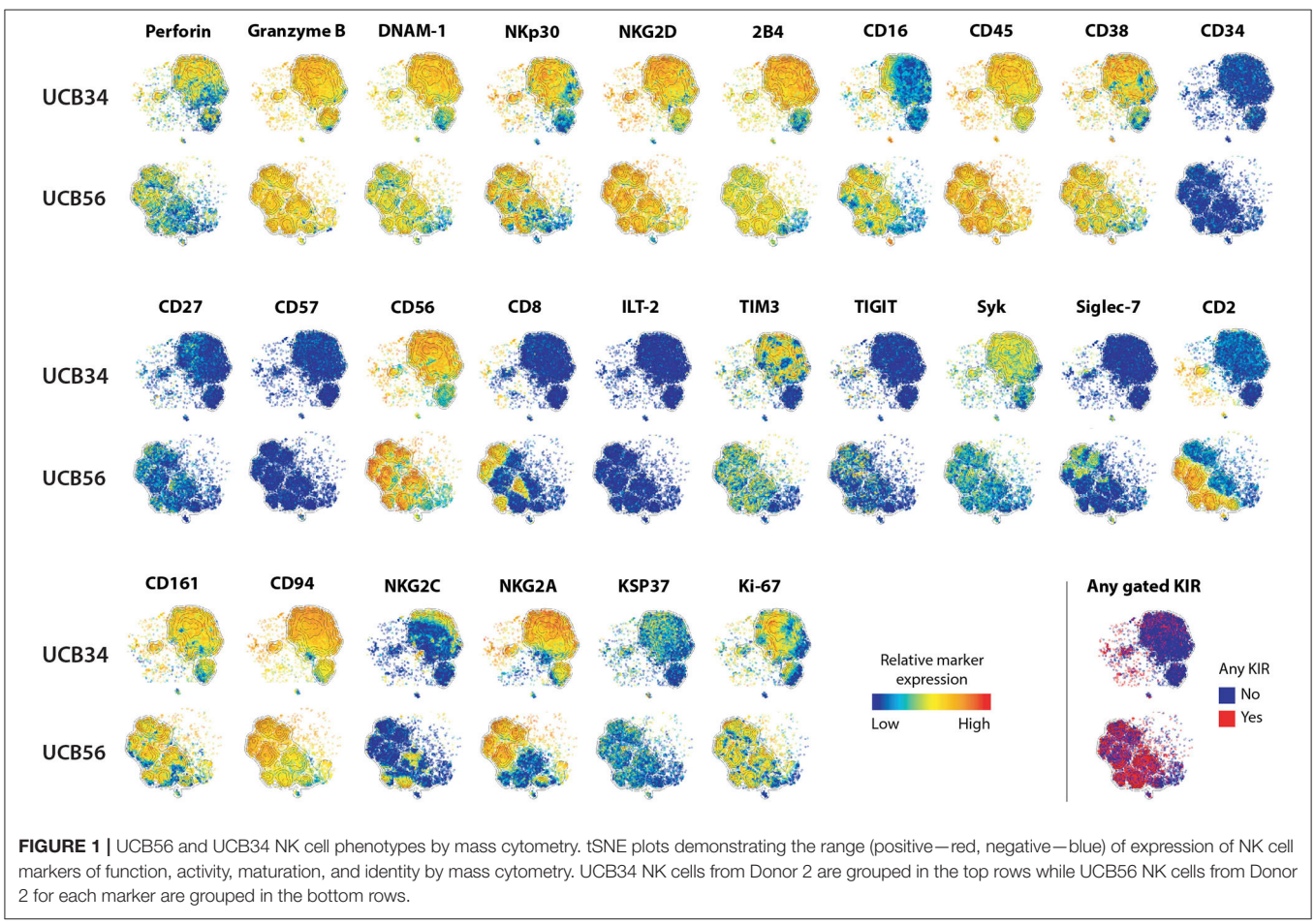


FIGURE 1 | UCB56 and UCB34 NK cell phenotypes by mass cytometry. tSNE plots demonstrating the range (positive—red, negative—blue) of expression of NK cell markers of function, activity, maturation, and identity by mass cytometry. UCB34 NK cells from Donor 2 are grouped in the top rows while UCB56 NK cells from Donor 2 for each marker are grouped in the bottom rows.

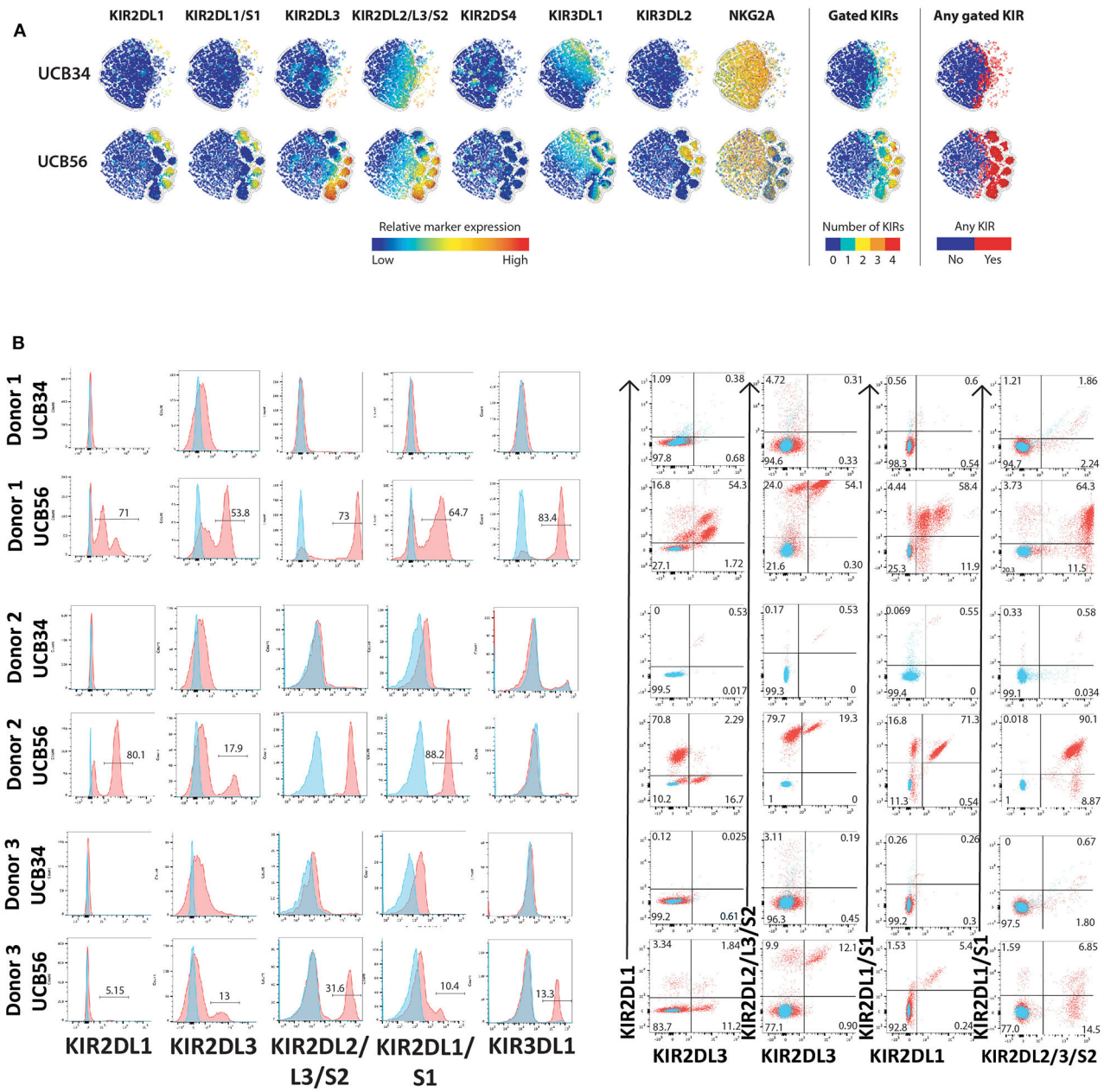


FIGURE 2 | KIR Expression of UCB56 and UCB34 NK cells **(A)** KIR phenotypes by mass cytometry. tSNE plots demonstrating the range (positive—red, negative—blue) of expression of KIR markers. UCB34 NK cells from Donor 2 are grouped in the top rows while UCB56 NK cells from Donor 2 for each marker are grouped in the bottom rows. **(B)** NK cell KIR markers by flow cytometry of Donor 1, 2, and 3 UCB34 and UCB56 NK cells (red) compared to isotype controls (blue). Representative panels are shown from $n = 3$ replicates.

RNA Sequencing and Quantitative Real Time PCR

Total RNA was isolated from cells using the RNeasy mini Kit (Qiagen, 74104) according to the manufacturer's protocol. RNA quality control, library construction, sequencing and data analysis were performed by Novogene Inc. Briefly, RNA quantification and qualification were performed using Nanodrop for checking RNA purity (OD260/OD280), agarose gel electrophoresis and Agilent 2100 for checking RNA integrity. mRNA was purified from total RNA using poly-T oligo attached magnetic beads. A cDNA library was synthesized using random hexamer primer and MMuLV Reverse Transcriptase and sequencing using Illumina Hiseq 2000. Colors descending from red to blue in heatmap of differential expression genes indicated log10 (FPKM+1) from largest to smallest. ClusterProfiler software was used for all enrichment analysis, including Gene

Ontology (GO) enrichment, Disease Ontology (DO) enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG, http:www.kegg.jp/). $p_{adj} < 0.05$ was considered as significant enrichment. RNAseq data was deposited at Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) with reference numbers GSE150806 and GSE150363.

For qRT-PCR complementary DNA (cDNA) was reverse transcribed from RNA isolated as above using the iScript gDNA clear cDNA Synthesis Kit (Bio-Rad, 1725034) according to the manufacturer's instructions. cDNA was used as a template in qPCR experiments using specific primers (500 nM) and Sso Advanced Universal SYBR Green Super Mix as the detection chemistry (Bio-Rad, 1725270). The thermal profile included one phase at 95°C for 3 min, second phase of 40 cycles at 95°C for 15 s, 60°C for 30 s. Melt curve analysis was run after every experiment. The experiments were carried out and analyzed with Bio-Rad CFX 96 and software (Bio-Rad). Cycle threshold (Ct) values were determined and relative mRNA contents were inferred from normalization of the gene of interest expression to that of the housekeeping gene GAPDH (ΔCt). Relative expression results were plotted as ($2^{-\Delta Ct}$). Forward and reverse primer sequences are: SOS1-F: CAAATCATGGGCAGCCAAG A, SOS1-R: TCTCTTCAGCTGACTTGGCA, PRF1-F: ACCAG GACCAGTACAGCTTC, PRF1-R: GGGTGCCGTAGTTGGAG ATA, GZMB-F: CTTCAGGGGAGATCATCGGG, GZMB-R: T CGTCTCGTATCAGGAAGCC, IFNG-F: TGAATGTCCAACG CAAAGCA, IFNG-R: TACTGGGATGCTCTTCGACC, CSF2-F: GCGTCTCCTGAACCTGAGTA, CSF2-R: CAGTGCTGCT TGTAGTGGC, SH2D1A-F: CAGTGGCTGTGTATCATGGC, SH2D1A-R: TCAGCACTCCAAGAACCTGT, GAPDH-F: GTC TCCTCTGACTTCAACAGCG, GAPDH-R: ACCACCCTGTTG CTGTAG CCAA.

Statistics

Unpaired student's *t*-test and ANOVA were used to quantify statistical deviation between experimental groups. Asterisks denote significant differences $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for comparisons as indicated in figure legends.

RESULTS

NK Cell Surface Antigen Expression on UCB34 NK Cells and UCB56 NK Cells

To investigate the differences between NK cells derived *in vitro* from HSCs and NK cells that develop *in utero* while controlling for genetic heterogeneity between individuals, we isolated $CD56^{+}$ UCB NK cells (termed UCB56 NK) and $CD34^{+}$ UCB hematopoietic stem/progenitor cells from the same donors. The UCB34 cells were then differentiated into NK cells using standard methods (termed UCB34 NK) (10, 42) (**Supplementary Figure 1A**). UCB34 NK cells and UCB56 NK cells were derived from 3 separate UCB units (labeled donors 1–3) and NK cells were first analyzed by mass cytometry for 37 cell surface antigens (**Figure 1**, **Supplementary Table 1**). Both the UCB34 and UCB56 cell populations expressed similar levels of NK cell activating receptors, such as NKp30 (**Figure 1**). Many other similarities in cell surface receptors were present between the UCB34 and UCB56 NK cells including comparable expression of markers of NK cell activity and activation including Granzyme B, Perforin, and NKG2D (**Figure 1**). Some key differences are notable such as UCB56 NK cells expressing higher levels of CD16, the FcgRIII receptor important to mediate NK cell antibody mediated cellular cytotoxicity (6, 15, 46) and CD2, an adhesion molecule that is tightly linked to terminal NK cell differentiation (47). These results were also investigated by flow cytometry on UCB34 and UCB56 cells from donors 1–3 (**Supplementary Figure 2**). Overall results were similar between flow cytometry and mass cytometry, with low NKG2A expression by flow cytometry an exception. NKp44 expression, a marker of activation, was positive in all NK cell populations examined.

UCB34-NK Cells Lack KIR Expression

The most striking phenotypic difference between the UCB56 NK cells and UCB34 NK cells was that UCB34 NK cells had very low expression of KIRs, measured by pan-KIR antibodies (**Figure 1**). Expression of individual KIRs on UCB56 NK and UCB34 NK cell populations was further interrogated by mass cytometry and flow cytometry (**Figures 2A,B**). UCB56 NK cells from all of donors 1–3 expressed KIR2DL3 and donor 1 and 2 expressed KIR2DL1 while the UCB34 NK cells from these same donors demonstrated minimal expression of individual KIRs by mass cytometry and flow cytometry (**Figures 2A,B**, **Supplementary Table 2**). Apart from the common expression of KIR2DL3 and KIR2DL1 in the UCB56 cells, there were expected differences in KIR expression between the donors. For example, Donor 3 UCB56 cells express KIR3DL1, while Donor 2 UCB56 NK cells express KIR2DL3 and KIR2DL1 in addition to the shared KIRs (**Figure 2B**). Each donor expressed a slightly different KIR profile, but across all donors the lack of KIR expression in the UCB34 NK cells was consistent. The NKG2A/CD94 heterodimer, that belongs to the C-lectin receptor family, is another important inhibitory NK cell receptor that recognizes HLA-E (26, 30). Both UCB34 and UCB56 NK cells express similar levels of NKG2A by mass cytometry (**Figure 1**).

Improved Cytotoxic Activity of UCB56 NK Cells Cytotoxic Activity Is Independent of Target Cell HLA Expression

We next addressed whether the difference in KIR expression noted between UCB34 and UCB56 NK cells leads to differences in cytotoxic activity between the KIR^{+} UCB56 NK cells and the KIR^{-} UCB34 NK cells. To determine the functional impact of the differences noted in KIR expression, 721.221 WT lymphoblastic cells lacking HLA A, B, and C, were engineered to express HLA proteins that are KIR ligands, specifically Cw3 (HLA C1), and Cw4 (HLA C2). Class I MHC negative K562 leukemia cells were used as positive control target cells in standard cytotoxicity assays (24). KIR^{+} PB NK cells were included as a positive control effector cell population (**Figure 3**). Importantly, the UCB56 NK cells from each of donors 1–3 express KIR2DL3 (corresponding ligand HLA C1) and donor 1 and donor 2 UCB56NK cells also express KIR2DL1 (corresponding ligand HLA C2). Cytotoxicity assays demonstrated higher (**Figure 3A**, donor 1 and donor 3) or equal (**Figure 3A**, donor 2) activity of the KIR^{+} UCB56 NK

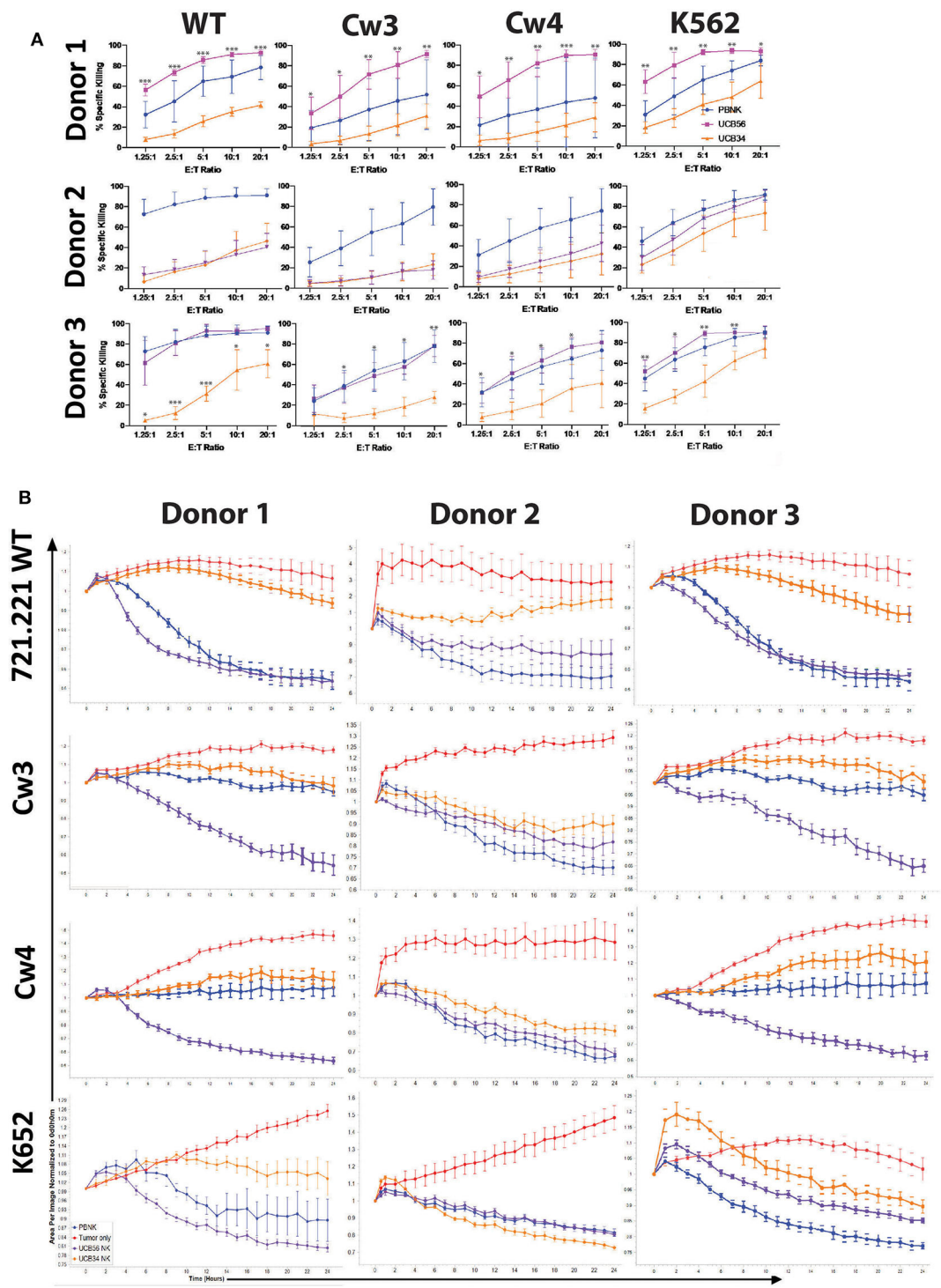


FIGURE 3 | UCB56 NK and UCB34 NK cell killing activity against lymphoblastic 721.221 and myeloid K562 tumors **(A)** Cell death and apoptosis by caspase 3,7 activation, and 7-AAD staining of PBNK cells, UCB56 NK cells, and UCB34 NK cells with 721.221 WT, cw3, cw4, and K562 cells at Effector:Target ratios from 1.25:1 up to 20:1 in 4-h co-culture. Experiments were completed in triplicate and representative panels are shown from $n = 3$ replicates. All statistical analysis is of the comparisons between UCB56 and UCB34 NK cells. **(B)** Tumor cells alone (red) and tumor cell killing by PB-NK (blue), UCB56 (purple), and UCB34 NK cells (orange) measured by Incucyte live-imaging system over 24 h. Experiments were completed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

cells compared to KIR^{-} UCB34 NK cells. This was true across all donors and for all target cell populations (**Figure 3A**). The addition of HLA C1 and HLA C2 expression to the 221.Cw3 and 221.Cw4 lines inhibited killing by both the UCB56 and UCB34 NK cells, though, the UCB56 NK cells maintained an improved killing ability compared to the UCB34 NK cells (**Figure 3A**).

There were no differences in killing of the 221.Cw3 or 221.Cw4 lines, even amongst the donor 3 UCB34 and UCB56 NK cells, where the UCB56 cells expressed KIR2DL3, KIR2DL2/L3/S2, KIR2DL1/S1, and KIR3DL1 (**Figure 3A**). Additional long-term (24 h) cytotoxicity assays demonstrated similar or superior killing of all tumor targets by KIR^+ UCB56 NK cells compared to the KIR^-UCB34 NK cells (**Figure 3B**).

The cytotoxic activity of the KIR positive UCB56 and KIR negative UCB34 NK cells was also tested against three pediatric neuroblastoma solid tumor cell lines with different HLA haplotypes (**Supplementary Figure 3A**). The neuroblastoma line SK-N-AS has high expression of HLA and is HLA genotype C1/C1, the IMR32 cell line does not express HLA A, B, or C and is C1/C1, and the NBLS line has high expression of HLA and is C2/C2 (**Supplementary Figure 3A**). These short-term (4 h) cytotoxicity assays did not demonstrate significantly different killing by the KIR^+ UCB56 NK cells compared to the KIR^- UCB34 NK cells for donors 1 and 2. Donor 3 UCB56 NK cells had significantly increased killing of each neuroblastoma cell line and the control K562 cells (**Supplementary Figure 3B**). Longer-term (24 h) live-imaging killing assays confirmed that KIR^+ UCB56NK cells had equivalent cytotoxicity against the three neuroblastoma lines, consistent with the results obtained with 721.221 cells (**Supplementary Figure 3C**).

iPSC-Derived NK Cell Populations That Differ in KIR Expression Show Similar Cytotoxic Activity

iPSCs are another important source of NK cells for clinical application (11, 48, 49). Given the differences that were noted in KIR expression and killing ability of UC34 and UCB56 NK cells, we extended our analysis to include iPSC-derived NK cells (**Supplementary Figure 1B**). We found that iPSC-derived NK cells derived from different iPSC lines demonstrated significant variability in their KIR expression (**Figure 4A**). iPSC-NK cells lacking KIR expression (iPSC-KIRNeg) expressed minimal KIRs while the iPSC NK cell populations expressing KIRs (iPSC-KIRPos) expressed KIR2DL3, KIR2DL1, KIR2DL2, and KIR2DL1 (**Figure 4A**). iPSC-KIRPos and iPSC-KIRNeg cells expressed similar levels of other activating receptors, including FasL, TRAIL, NKp44, NKp46, and NKG2D, except for CD16, which was expressed higher in iPSC-KIRPos cells (**Figure 4B**). In contrast to the UCB34 and UCB56 NK cells, both the iPSC-KIRPos and iPSC-KIRNeg cells did not express NKG2A (**Figure 4B**). Cytotoxicity assays using iPSC-KIRPos and iPSC-KIRNeg NK cells against lymphoma and neuroblastoma cell lines demonstrated that against a majority of the cell lines the iPSC-KIRNeg cells have similar killing compared to the iPSC-KIRPos NK cells (**Figure 4C**). Similarly, with neuroblastoma line target cells and K562 target cells in long-term (24 h) cytotoxicity assays, iPSC-KIRPos NK cells had no significantly increased killing compared to iPSC-KIRNeg NK cells (**Figure 4D**). Despite the difference in KIR expression, overall the iPSC-KIRPos and iPSC-KIRNeg NK cells had similar cytotoxic ability.

Transcriptome Analysis of UCB and iPSC NK Cell Populations Show Similarity Between the NK Cells Derived From Different Sources

Given the differences we observed between the UCB34, UCB56, and iPSC-NK cells in KIR expression and cytotoxic function, we analyzed gene expression by RNA sequencing to determine how the transcriptomes of the cell populations compared. A gene expression correlation analysis of differentially expressed genes on the whole transcriptome level between the UCB NK cells (specifically UCB56 NK cells post-APC), KIR^+ iPSC NK cells, and PB-NK cells did not differ significantly (**Figure 5A**). Interestingly, when comparing the similarity of the NK cells populations by the number of differentially expressed genes to iPSC NK cells, there were the fewest differentially expressed genes in the comparison with the UCB NK cells, followed by the PB-NK cells, followed by the H9 embryonic stem cell derived-NK cells and lastly by the NK92 cell line (**Figure 5B**). Cluster analysis of differentially expressed genes in each NK cell population and NK-92, Jurkat, Raji, and K562 cell lines showed that the four different NK cells populations (PB-NK, UCB-NK, H9-NK, iPSC-NK) cluster together with iPSC-NK cells having the closest relation with PB-NK in terms of genome-wide gene expression (**Figure 5C**). In the other clusters, the lymphoid NK-92, Jurkat, and Raji cells lines group together, while the myeloid K562 cells were in a separate cluster (**Figure 5C**). A separate clustering analysis focused on 124 genes involved in NK cell mediated cytotoxicity pathways (KEGG PATHWAY: hsa04650) to better parse out the differences in NK cell killing activity. Here, the NK cell populations clustered together separate from the non-NK cells. The UCB NK cells clustered with the H9 NK cells, while the NK92 cells, PB-NK and iPSC NK cells clustered separately (**Figure 5D**). Reverse-transcription quantitative real time PCR (qRT-PCR) analysis confirmed gene expression changes seen in the RNA sequencing analysis. The separate clustering of the iPSC NK cells is defined by lower expression of signaling genes in the NK cell cytotoxicity pathway such as *SOS1* and higher expression of *SH2D1A* compared to UCB and PB NK cells. These gene expression changes were seen in both the RNA sequencing and qRT-PCR experiments (**Supplementary Figure 4**). Differential expression of genes in the NK cell cytotoxicity pathway such as Perforin *(PRF1)*, Granzyme B *(GZHB)*, Interferon gamma *(IFNG)*, and GM-CSF *(CSF2)* had a more variable expression pattern between the three cell types that was consistent between both the RNA sequencing and qRT-PCR analyses and was unlikely to drive the clustering (**Supplementary Figure 4**).

A second RNA sequencing analysis explored the transcriptional differences between the UCB34 NK cells, UCB56 NK cells and iPSC NK cells. UCB34 and UCB56 cells were analyzed both pre- and post- expansion (using irradiated aAPCs) while iPSC NK cells were expanded prior to analysis (41). Cluster analysis of differentially expressed genes in each NK cell population showed that the pre-expansion UCB34 and UCB56 cells were distinct populations (**Figure 5E**). However, post-expansion the UCB34 and UCB56 cells clustered together while the iPSC NK cells were more closely related to the post-APC

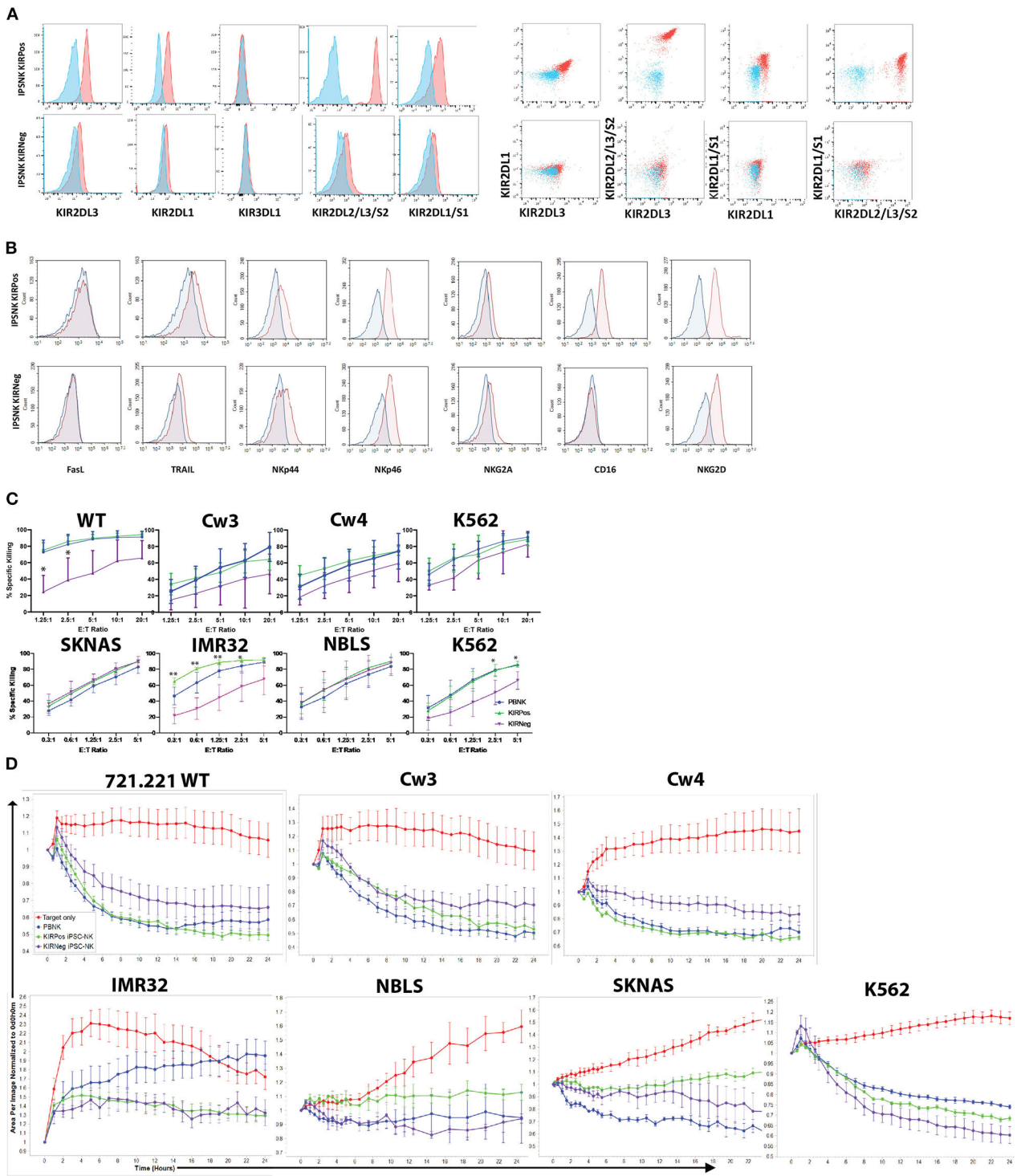
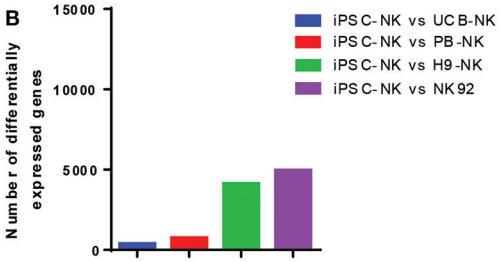
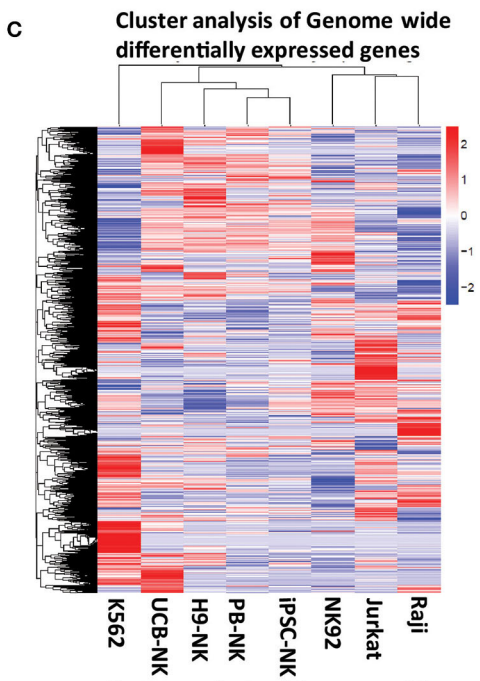


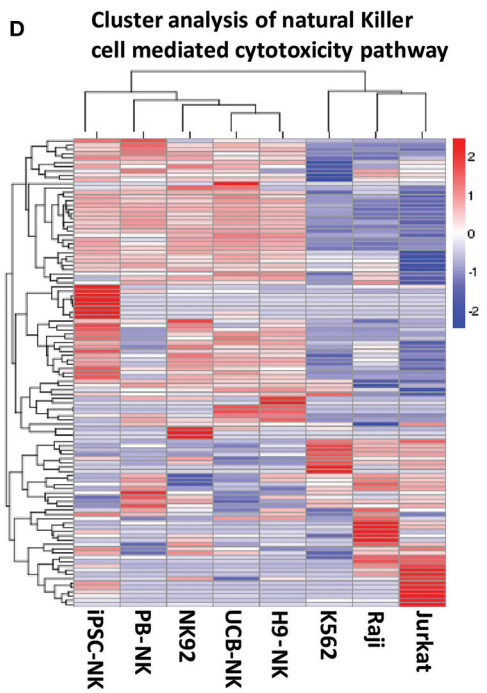
FIGURE 4 | KIR expression and cytotoxic activity of iPSC-derived KIRPos and KIRNeg NK cells. **(A)** NK cell KIR markers by flow cytometry of iPSC-KIRPos and iPSC-KIRNeg NK cells (red) compared to isotype controls (blue). Representative panels are shown from $n = 3$ replicates. **(B)** NK cell markers by flow cytometry of iPSC-KIRPos and iPSC-KIRNeg NK cells (red) compared to isotype controls (blue). **(C)** Cell death and apoptosis by caspase 3,7 activation and 7-AAD staining of 721.221 WT, cw3, cw4, SK-N-AS, IMR32, NBLS, and K562 cells with PBNK cells (blue), iPSC-KIRPos NK cells (green), and iPSC-KIRNeg NK cells (purple) after 4-h co-culture at effector:target ratios from 1.25:1 up to 20:1 (721.221 cells) or 0.3:1 up to 5:1 (neuroblastoma cells). Representative panels are shown from $n = 3$ replicates. All statistical analysis is of the comparisons between KIRPos and KIRNeg iPSC-NK cells. **(D)** Tumor cells alone (red) and tumor cell killing by PBNK (blue), iPSC-KIRPos (green), and iPSC-KIRNeg NK cells (purple) measured by Incucyte live-imaging system over 24 h. Experiments are completed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

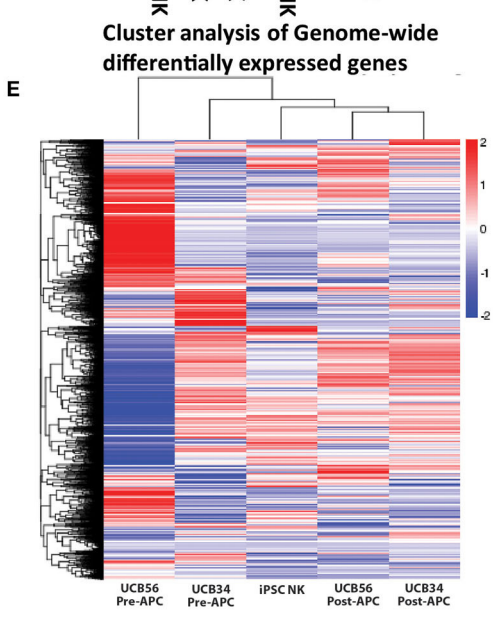
A

	PB-NK	iPSC-NK	UCB-NK	H9-NK	NK92
PB-NK	1	0.84225	0.864065	0.887	0.82175
iPSC-NK	0.84225	1	0.929945	0.927	0.90075
UCB-NK	0.86407	0.929945	1	0.936655	0.8901
H9-NK	0.887	0.927	0.936655	1	0.90675
NK92	0.82175	0.90075	0.8901	0.90675	1









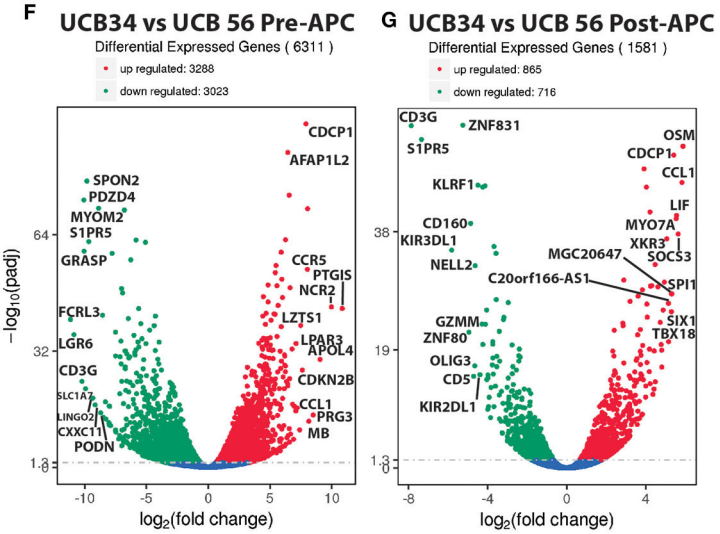


FIGURE 5 | RNA Sequencing analysis of gene expression among UCB56 NK, iPSC NK, PB NK cells, NK 92 cells, and 3 cell lines, $n = 3$ biological replicates. **(A)** Heat maps of the correlation coefficient between each NK cell population. **(B)** Plot of the number of differentially expressed genes between iPSC-NK cells and the other NK cell populations. **(C)** Cluster analysis of differentially expressed genes. Genome-wide differentially expressed genes were analyzed among each cell population.

(Continued)

FIGURE 5 | Log10(FPKM+1) value was used for clustering. **(D)** Cluster analysis of genes in the NK cell mediated cytotoxicity pathway (KEGG PATHWAY: hsa04650). Hundred twenty four genes in this pathway were analyzed. **(E)** Cluster analysis of differentially expressed genes between UCB34 and UCB56 pre- and post-expansion with antigen presenting cells (APC) as well as iPSC NK cells post-expansion. Genome-wide differentially expressed genes were analyzed among each cell population. Log10(FPKM+1) value was used for clustering. **(F)** Volcano plot of differentially expressed genes between UCB34 and UCB56 cells pre-APC expansion. The x-axis indicates the fold change in gene expression between the different samples, the y-axis indicates the statistical significance (adjusted p-values) of the differences. Significantly up and down regulated genes are highlighted in red and green, respectively. Genes that were not differentially expressed between the groups are in blue. **(G)** Volcano plot of differentially expressed genes between UCB34 and UCB56 cells post-APC expansion. The x-axis indicates the fold change in gene expression between the different samples, the y-axis indicates the statistical significance (adjusted *p*-values) of the differences. Significantly up and down regulated genes are highlighted in red and green, respectively. Genes that were not differentially expressed between the groups are in blue.

expanded UCB cells (**Figure 5E**). An analysis of differentially expressed genes demonstrates a higher number of significantly differentially expressed genes between UCB34 and UCB56 NK cells pre-APC expansion with 3,288 genes differentially up-regulated and 3,023 down-regulated (**Figure 5F**). Post-APC expansion 865 genes were differentially up-regulated significantly between the two cell populations while 716 genes were down-regulated (**Figure 5G**). Notably in the post-APC expansion UCB34 cells KIR2DL1 and KIR3DL1 were expressed at significantly lower levels compared to the UCB56 cells (**Figure 5G**).

DISCUSSION

In these studies, we demonstrate that UCB56 NK cells have consistently better cytotoxic activity compared to UCB34 NK cells derived from the same donor (sharing the same KIR and HLA genotypes). Other than expression of KIRs, CD2, and CD16, the UCB34 and UCB56 NK cell populations exhibited similar cell surface receptors. At the transcriptome level, important differences identified between the UCB56 and UCB34 NK cells include differential expression of KIR transcripts, and differential expression of genes important in differentiation. The UCB34 vs. UCB56 post-APC analysis showed markedly reduced expression of KIR2DL1 and KIR3DL1. Another notable finding was that pre-APC UCB34 and UCB56 NK cells and post-APC UCB34 and UCB56 NK cells clustered together, rather than the clustering by original tissue of derivation indicating the influence of APC expansion. Interestingly, several of the most highly differentially expressed genes in UCB34 NK vs. UCB56 NK cells post-APC expansion, such as OSM and LIF, are involved in maintenance of a undifferentiated stem cell phenotype (50, 51). The etiology of the difference in killing ability is likely multifactorial as NK cell cytotoxicity is modulated by diverse signaling pathways, where the net signaling input from both activating and inhibitory receptors determines function (52). Our derivation of UCB34 and UCB56 NK cells from individual donors controls for genetic differences and allows for the comparison of NK cell activity based on developmental origin. However, one possible confounder is that both the UCB56 and UCB34 NK cells were expanded using irradiated aAPCs which provide key activating signals including IL21 and 4-1BB (CD137) stimulation (41). In addition to the same genetic background, we found that the NK cell surface receptor profiles between the UCB34 and UCB56 NK cells are highly similar, primarily differing in CD2, CD16, and KIR expression, all markers that have been associated with NK cell differentiation (47, 53). The fact that KIR^+ UCB56 cells kill better than KIR^- UCB34 cells is similar to reports demonstrating that PB-NK cells that express more KIRs have increased cytotoxic function (54–56). The increased function of KIR^+ PB-NK cells is thought to be due to the functional maturation associated with NK cell differentiation and licensing. It is unclear if and how these processes control UCB-NK and iPSC-NK cell killing activity. UCB NK cells' cytotoxic activity is reported to be influenced by diverse stimuli including gestational age, mode of delivery, exposure to anesthetic medications, and the presence or absence of infection (57–59).

Given that the major difference we identified between the UCB cell populations was in their KIR expression, we investigated the importance of this difference in KIR expression using the 721.221 lymphoblastoid cell line system. This system allowed us to express HLA C1 (cw3) or C2 (cw4) to otherwise identical cells. Importantly, UCB56 cells from all donors expressed KIR2DL3 and KIR2DL1 while the UCB34 cells from all donors did not express these KIRs. Since the KIR ligands HLA C1 (ligand to KIR2DL3) and C2 (ligand to KIR2DL1) did not significantly alter the difference in killing activity between the UCB56 and UCB34 cells, the canonical HLA recognition role of KIRs does not explain the difference in killing activity in this system. Expression of HLA C1 and HLA C2 would have been expected to decrease the killing ability of the KIR2DL3 and KIR2DL1 expressing UCB56 cells more than it affected the KIR^- UCB34 cells if the KIR expression was driving the entire difference in cytotoxicity between the two cell types. Similar cytotoxicity results were obtained against neuroblastoma cell lines which also vary by KIR ligand HLA-C expression although HLA class I expression is generally low in these cells. Thus, in this system KIR expression is associated with enhanced functionality. The inhibitory receptors expressed by the UCB and iPSC-derived NK cells do not seem to interfere with killing of targets expressing high levels of the cognate KIR, rather these cells have increased cytotoxic activity. It is possible that feeder-based expansion drives differentiation at the same time it attenuates inhibitory signaling. Other possible influences include that the effector cells are polyclonal and not all NK cells can be inhibited. Activating KIRs, such as KIR2DS1 in the presence of Cw4, may influence these results as well. However, despite polyclonal expression we do not see an inhibitory effect in UCB56 NK lines that express the relevant KIRs at high frequencies.

NK cells develop their characteristic ability to distinguish between self and non-self through the process of NK cell education, after which they are termed licensed (licensed to

kill non-self cells). When an NK cell engages a normal "self" cell, interactions between receptors, such as KIRs, and the matching MHC class I molecule ligand inhibits NK cell lytic function (60, 61). PB-NK cells that lack this licensing step are hypofunctional as the basal level of activity in response to activating receptor signaling corresponds to the number of inhibitory receptors expressed (54, 62). A potential mechanism for this may be that NK cells that express self-specific inhibitory receptors modulate the lysosomal compartment to accumulate dense-core secretory lysosomes containing granzyme B and also uniquely compartmentalize activating and inhibitory receptors on the plasma membrane (63–65). For the donor- derived NK cells in this study, KIR^+ NK cells represent educated NK cells since donors NK cells were educated by all major KIRs (2DL3, 2DL1, and 3DL1). Our results raise the possibility that KIR-mediated education may be of relevance, although the higher degree of differentiation in the UCB56 lines, which is associated with KIR expression, must also be taken into account. Overall, our results show that increased KIR expression does not impair NK cytotoxicity against a range of target cells that express physiological levels of HLA class I. Since each NK cell population co-expresses many KIRs, it was not possible to delineate the exact role of single KIR expressing NK cells on NK cell education. However, our observation that KIR^+ NK cells displayed higher functionality is consistent with the notion that self KIR expression boosts functionality through education. As these different NK cell populations are being translated into clinical therapies, it will be important to further elucidate how KIRs contribute and affect the function and specificity of UCB and iPSC-derived NK cells. Our results suggest that KIR^+ UCB and iPSC-NK cell products are more functional, and their differentiation and expansion appear to attenuate negative signaling supporting broad implementation in an off-the-shelf setting regardless of KIR and HLA genotypes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE150363 and GSE150806.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of California, San Diego. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BG collected and interpreted data and wrote the manuscript. HZ, YW, K-JM, and DK contributed to the conception and design of the study. HZ, YW, NH, AR-C, AB, EA, and HH collected and interpreted data. HZ and YW wrote sections of the manuscript. DK and K-JM reviewed and edited the manuscript. All authors read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.561553/full#supplementary-material

Supplementary Figure 1 | Schematic of NK cell generation from CD34+ progenitors and iPSCs. **(A)** $CD56^+$ UCB NK cells (termed UCB56 NK) and $CD34^+$ UCB hematopoietic stem/progenitor cells were isolated from the same donors. The UCB34 cells were then differentiated into NK cells. Representative KIR expression for each NK cell population is indicated. **(B)** NK cells were derived from iPSCs via standard hematopoietic and NK cell differentiation protocols. iPSC-derived NK cells that were derived from different iPSC lines demonstrated significant variability in their KIR expression with distinct KIR positive and KIR negative populations.

Supplementary Figure 2 | UCB56 and UCB34 NK cell phenotypes by flow cytometry. NK cell markers by flow cytometry of Donor 1, 2, and 3 UCB34 and UCB56 NK cells (red) compared to isotype controls (blue). Representative panels are shown from $n = 3$ replicates.

Supplementary Figure 3 | UCB56 and UCB34 NK cell killing activity against neuroblastoma and myeloid K562 tumors. **(A)** Table of NK cell receptor ligand expression and HLA genotype for neuroblastoma cell lines SK-N-As, IMR32, and NBLS and chronic myeloid leukemia K562 line. **(B)** Cell death and apoptosis by caspase 3,7 activation and 7-AAD staining of SK-N-AS, IMR32, NBLS, and K562 with PBNK cells (blue), UCB56 NK cells (purple), and UCB34 NK cells (orange) after 4-h co-culture at effector:target ratios from 0.3:1 up to 5:1. Representative panels are shown from $n = 3$ replicates. All statistical analysis is of the comparisons between UCB56 and UCB34 NK cells. **(C)** Tumor cells alone (red) and tumor cell killing by PB-NK (blue), UCB56 (purple), and UCB34 NK cells (orange) measured by Incucyte live-imaging system over 24 h. Experiments were completed in triplicate.

Supplementary Figure 4 | Gene expression analysis of NK cell cytotoxicity pathway genes by qRT-PCR of UCB NK, PB NK, and iPSC NK cells. The levels of mRNA for the indicated genes were assayed by qRT-PCR. Bar graph depicts means ± SD. Comparisons by fold change between PB NK and iPSC NK cells are indicated in blue, and comparisons by fold change between UCB NK and iPSC NK cells are indicated in orange. Data are representative of two experiments.

Supplementary Table 1 | List of antibodies used in mass cytometry experiments.

Supplementary Table 2 | List of NK Cell KIR Genotypes and HLA Haplotypes. For HLA typing molecular (Mol) and serological (Sero) typing information is included.

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REVIEW
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Natural Killer Cell Integrins and Their Functions in Tissue Residency

*Michael J. Shannon and Emily M. Mace**

Department of Pediatrics, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, United States

Integrins are transmembrane receptors associated with adhesion and migration and are often highly differentially expressed receptors amongst natural killer cell subsets in microenvironments. Tissue resident natural killer cells are frequently defined by their differential integrin expression compared to other NK cell subsets, and integrins can further localize tissue resident NK cells to tissue microenvironments. As such, integrins play important roles in both the phenotypic and functional identity of NK cell subsets. Here we review the expression of integrin subtypes on NK cells and NK cell subsets with the goal of better understanding how integrin selection can dictate tissue residency and mediate function from the nanoscale to the tissue environment.

Keywords: NK cell, integrin, adhesion, cell migration, tissue residency

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Hui Peng, University of Science and Technology of China, China

****Correspondence:***
Emily M. Mace
em3375@cumc.columbia.edu

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INTRODUCTION

NK cell subsets are defined by surface receptors, especially integrins, transcription factors, and intracellular effector molecules. There is still much to learn about how tissue resident and circulating NK cells are generated and undergo specialization for functions including cytotoxicity and cytokine production. However, the classification of many tissue resident NK cell subsets by the expression of integrins, including α1 and αE integrins, when compared with non-resident cells that primarily express β2 integrins, is evidence for the relevance of integrins to environmental adaptation in addition to developmental and functional processes (**Figure 1**). To add to the complexity of the use of integrins as phenotypic parameters, multiple levels of nomenclature for integrins on immune cells exist, occasionally masking the true nature of many of the cluster of differentiation (CD) molecules that are used to define NK cell subsets as integrin subunits.

Integrins communicate signals to and from the extracellular matrix (ECM) and other cells. Integrin ligands include ECM components, including 20 different isoforms of fibronectin and multiple collagen and laminin family members, plus selectins, and cell adhesion molecules (CAMs) (1). Integrins form nanometer-scale signaling islands which are regulated by chemokine receptors and can be defined as clusters of integrins, adaptors, scaffolds, kinases, phosphatases, and actin linkers. Such signaling clusters have also been described as "nano-adhesions" in lymphocytes, which form functional units to send signals cell-wide and influence cell behavior (2). Lymphocyte nano-adhesions have the same size and many of the same molecules as nascent adhesions in non-lymphocytes (3), but have shorter lifetimes due to much faster cell migration and adhesion turnover. Each cell exists as part of a highly heterogenous 3D population of cells, all of which can influence each other to produce a cohesive, multi-faceted, system-level response. Integrin communication clusters are at the heart of complex signaling axes that include chemokine receptors and tissue architecture and mediate the homing and tissue residency of immune cells (4).

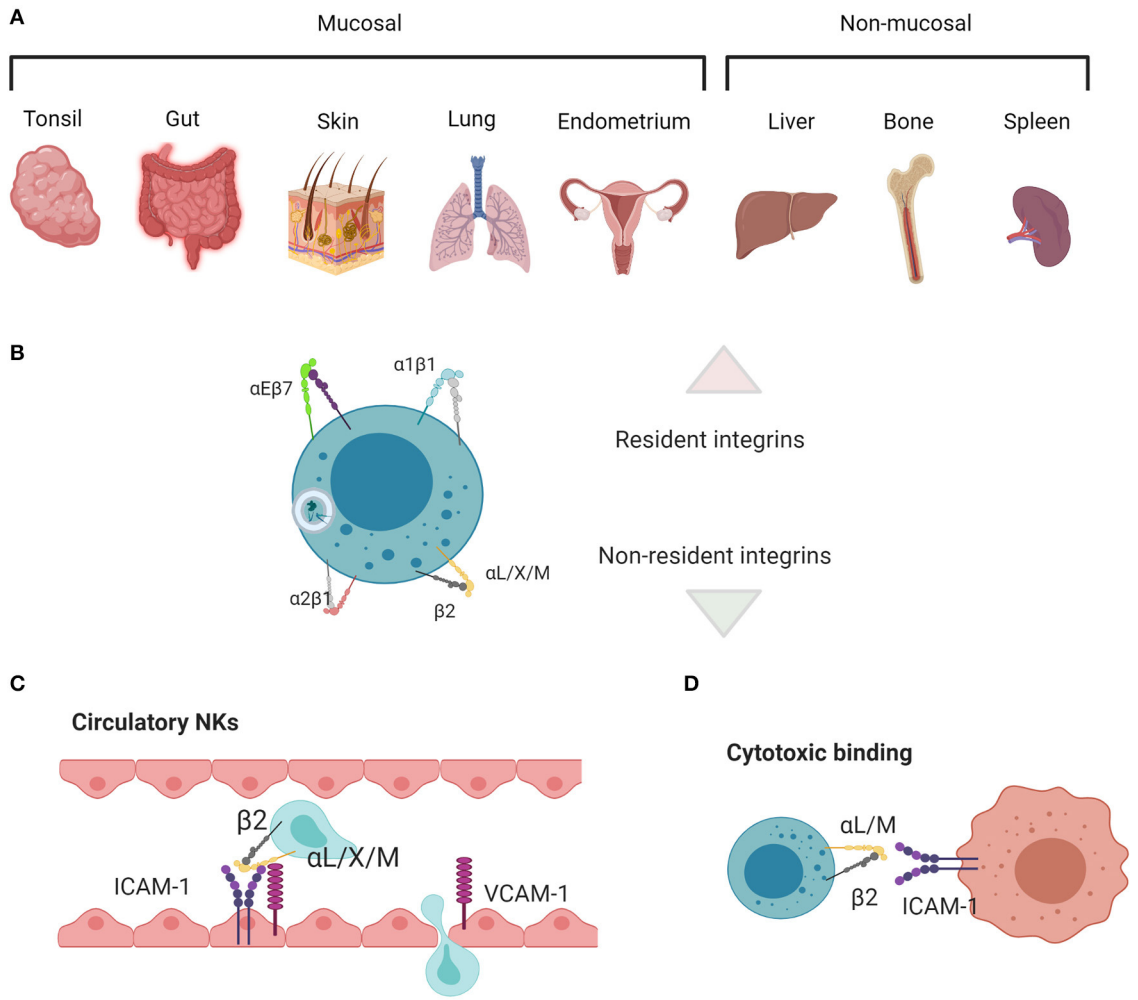

FIGURE 1 | Main tissue resident vs. non-resident integrin functions in NK cells. **(A)** Mucosal and non-mucosal tissues in which NK cell residence has been investigated. **(B)** αEβ7 and α1β1 are associated with residency, whereas α2β1 and the β2 integrins are associated with migration, adhesion, and cytotoxicity. **(C)** β2 integrins enable cells to migrate in and out of circulation and through tissues, along with **(D)** mediating adhesion and signaling during target cell lysis. Created with BioRender.com.

The use of CD nomenclature has simplified the definition of immune subsets but can also obscure the biological nature of the markers that are represented. Interpretation of genetic data related to integrins is limited by our inability to discern heterodimers. Individual β subunits form heterodimers with divergent functions depending on the α subunit that they are paired with. Antibody detection, with a few notable exceptions for those that recognize unique epitopes only present in the heterodimeric form, similarly only provides information about single subunits (5). Therefore, while ample data, particularly from flow cytometry, offers us information about the phenotype of tissue resident NK cell subsets, we still lack a comprehensive understanding of how cells become specialized to their microenvironment, what dictates their residency, and the relationship between heterodimeric integrin expression and their spatial localization and function. This knowledge gap also highlights the need for cell biological analyses that offer answers correlatively across time and length scales.

Here, we summarize integrin expression on NK cell subsets in humans and mice and ask what we can infer from the functions of integrins in relation to what is known about the tissue environments that support NK cell development and residency. By considering the single molecule functions of integrin subunits and heterodimers, we aim to integrate what is known about the likely function of integrins in different NK cell populations. Further, we discuss key outstanding questions surrounding the relative effects of inherent integrin phenotypes and plasticity in response to environmental cues. Finally, we summarize techniques which will be important for future multiscale investigations of integrins in heterogeneous tissue resident NK cell populations.

INTRODUCTION TO INTEGRIN STRUCTURE AND FUNCTION

Integrins in immunology are often described as single subunits as they are detected this way by flow cytometry, RNA-Seq and proteomic analyses. Such data are unmatched for analyzing single cells in heterogeneous populations and have driven much of our underlying knowledge of immune cell phenotypes. However, integrins are obligate heterodimers composed of an α and a β subunit, so we must start here if we are to investigate their wider function.

There are 18 known integrin α subunits and 8 β subunits, and each α subunit can differentially pair with multiple β partners to generate 24 known integrin heterodimers (6). The mix of integrins on a single cell, or group of cells, has been described as an "area code," a unique signature that directs cells to home to a particular locale and can help them perform a more generalized task, such as adhesion, migration, diapedesis, proliferation, survival, or residency (7–9). While the same integrin heterodimer can have multiple extracellular binding partners, including collagen, laminin, fibronectin, and the CAM family of adhesion receptors, these binding partners may have unique binding sites on different integrins. In addition to their diversity of ligand binding, integrins respond to intracellular and extracellular activating signals through rapid conformational change (10–12). Further, integrins are also mechanosensitive and participate in catch-bonding, meaning that force will modulate their structure, binding, and downstream signaling (13–15). Integrins also form clusters in the membrane, which change in size as cells alter their speed (T cells) (2). Therefore, both the structure and function of integrins are highly modular and adaptable, and we can consider integrin function collectively within the context of single molecule structure, bound ligand structure, and nanocluster-scale behavior. There are many open questions about how biology across these scales is linked. New quantitative microscopy techniques will be key to providing direct links between the collective behavior of a heterogeneous system of cells and nanoscale events that shape single cell responses.

Integrin Protein Structure Functionally Links With Affinity and Clustering

Integrins have been extensively structurally defined and contain an ectodomain, transmembrane domain, and cytoplasmic domain that form a shape analogous to a "head" on two "legs" (16). The ectodomain binds extracellular ligand, whereas the relatively short cytoplasmic domains bind adaptors that form signaling hubs and link integrins to the actin cytoskeleton (16). Thus, integrins mediate bidirectional signaling across the cell membrane between the actin cytoskeleton and the extracellular environment. The coupling mechanism between integrins and actin is termed the "molecular clutch" owing to the dynamism of the actin cytoskeletal network and the transient binding of integrin adhesions, which act together to translate power from the inside to the outside of a migrating cell (17–20). Integrins form clusters to effectively translate force and to allow cells to quickly change their direction or adhesive behavior. In lymphocytes, integrin clusters are very small (<100 nm) and exhibit fast turnover during migration and spreading (2, 10, 17). Nonetheless, they contain many of the same adhesion components as early or nascent adhesions in fibroblasts (3, 21–23). The status of cell-wide integrin clustering is constantly in flux, on a per adhesion basis as well as between adhesions, and likely allows for effective tuning of the behavior of a given cell within its niche. The communication and crosstalk of integrin species between nano-adhesions, and the ways in which cells translate single adhesion behavior into whole cell behavior are under active study.

The directionality and mechanism of integrin signaling across the cell membrane is often referred to as inside-out vs. outside-in signaling (24, 25). Inside-out signaling pathways are initiated by other membrane receptors, particularly chemokine receptors. They result in changes to integrin affinity, the translation of forces and signals between the integrin and the inside of the cell, and the clustering of integrin heterodimers and 3D nanoscale arrangement of focal complexes. Outside-in signaling occurs as cytoplasmic adaptors link integrins to molecules that exert force within the cell, particularly the actin cytoskeleton. In addition, outside-in signaling can lead to activation of signaling cascades including the MAPK and ERK axes (26). While less well-defined, inside-in signaling occurs via endocytosed integrins which can participate in signaling to regulate gene expression (27).

Integrins adopt three affinity states according to the "switchblade model": a low-affinity bent-closed conformation, an intermediate-affinity extended-closed conformation, and a high-affinity extended-open conformation thought only to be invoked following extracellular ligand binding (7, 28–30). αLβ2 (CD11a/CD18, LFA-1) is the best characterized integrin structurally, but electron microscopy, X-ray crystallography and NMR have confirmed similar structural changes in other integrins (31). Recent interferometric super resolution data, obtained by fluorescently tagging the membrane distal region of the α_L subunit, confirms that the αLβ2 heterodimer adopts both a bent and a stretched conformation in live cells and the length of the molecule changes by 16 nm upon binding to the ICAM-1 ligand (12). Crystallographic studies link this stretching with changes in affinity and demonstrate that the α_I domain within the α subunit of β_2 integrin heterodimers provides higher affinity ligand binding upon allosteric interaction with an internal ligand in the stretched form (31, 32). The tightening of binding to this internal ligand stabilizes the extended-open, high affinity form to facilitate strong adhesion (32). In the case of αLβ2 integrin, often used as a model for structure/function, this transition is preceded by selectin-mediated tethering, activation by endothelium-bound chemokines, then ICAM-1 binding coupled with increased tension from shear flow in the blood (33, 34).

Most cytoplasmic β integrin tails are 40–70 residues long and are largely unstructured but form helices or pack close to the membrane depending on which intermediates they bind (35). They are moderately conserved and have binding sites that can be regulated by serine/threonine or tyrosine phosphorylation, including within the highly conserved NPxY motif (36). β cytoplasmic tails mediate binding to molecules that link integrins to the actin cytoskeleton and other signaling

molecules via adaptors. α subunit cytoplasmic tails are much more structurally heterogeneous, interact with the β subunit through signaling intermediates to alter binding, and are mostly associated with specifying integrin trafficking and inhibition (35). The relationship between tertiary and quaternary protein structure caused by interactions of the β and α subunits provides specificity of binding to a given 3D motif. For example, αI and αA domains can directly bind a 6-residue collagen motif, but only form the correct structure to do this when a heterodimer is interacting with a β subunit, often in conjunction with the binding of Mn^{2+}, Mg^{2+}, or Ca^{2+} (16, 37). In this way, an area of cationic charge in the integrin head region is created that is specific for a structurally regulated grouping of amino acids. This enables a bond to form, the strength of which is subject to structural changes in the integrin that are transmitted across the cell membrane (35). The transmembrane regions form helices, and long-range interactions change their structure and non-covalent interaction with the surrounding lipid bilayer. Such structural changes occur due to alterations caused by ligand binding or mechanosensing in the ectodomain, or adaptor protein binding or mechanosensing on the cytoplasmic side (36).

Integrin-ligand bond strengthening can occur as a result of mechanical stress, inducing a transition from intermediate to high affinity conformation. This strengthening can occur in response to external forces on cells following ligand binding, such as the shear flow of the blood (38, 39) or from the pulling force of other cells during immunological synapse formation (9), as well as internal force transmission through adaptor molecules to the cytoskeleton (40, 41). Bidirectional force applied following ligand binding exposes cryptic binding sites on integrin-bound talin and relieves autoinhibition of both talin and vinculin, leading to strengthening of adhesion at focal adhesion complexes (42–44). Such strengthening manifests as a coalescence of canonical integrin adhesome proteins that regulate the lifetime and clustering of adhesions (45–48). In contrast to induction of high affinity conformation, the conformational switch from low to intermediate affinity occurs due to intracellular activation through cytoplasmic regulators in response to chemokine signaling, or in response to transient binding through other ligands such as selectins (49). Rapid adhesion strengthening and weakening through catch bond or chemokine induction helps facilitate diapedesis, where cells must strengthen adhesions to resist the shear flow of the blood prior to crawling through spaces between endothelial cells, a process which may be less dependent on integrin engagement and more dependent on cytoskeletal dynamics (50, 51). The modulation of integrin affinity and the composition of adhesion clusters are also both important for tuning the migratory capacity and dynamics of cells during inflammation. In summary, integrin affinity, clustering, trafficking and mechanosensing are regulated to give a specific response within a single cell (**Figure 2**).

Classification of NK Cell Integrins by Beta Subunits

Traditionally, integrin β subunits have been used to generalize integrin functions; however, while such groupings have utility, they are frequently a convenience rather than a biological rule. Integrins can also be defined by other structural elements, such as the presence or absence of an αI domain, which is common to some β1 and β2 containing integrins and confers structural and functional similarities (31). Here we will examine the broad groupings prior to examining more closely the functions of the specific pairings related to cell behaviors ranging from migration to residency (**Table 1**).

The β1 subunit can bind to 12 α subunits, and these heterodimers bind laminin, collagen, fibronectin, specifically LDV or RGD peptide domains, and VCAM1 (1, 90). Broadly, β1 heterodimers are often associated with tissue homing, providing a "bar code" for cells located in different kinds of tissue niches. Their functions contribute to cell adhesion, transmigration during entry or exit from tissues, and proliferation and survival (91). Specifically, α5β1 and α8β1 bind to RGD active sites in fibronectin, using a binding site at the interface between the α and β subunits. α4β1 and α9β1 bind the LDV peptide, which is structurally similar to RGD and contained within fibronectin, VCAM-1, and MAdCAM-1. Laminin and collagen binding integrins include α1β1, α2β1, α10β1, and α11β1, and their specificity is achieved through their use of the α A-domain. α3β1, α6β1, and α7β1 also bind laminin, but independently of the A-domain. Several non-β1 or β2 containing integrins also bind to RGD, LDV, laminin, and collagen (1). While β1 integrins are not leukocyte-specific, they are highly expressed on lymphocytes and play important roles in navigating tissue microenvironments. In particular, a1β1, a2β1, a4β1, and a5β1 are key components of tissue resident signatures that define residency, either by their expression or lack of expression, and are commonly found on NK cells. An overview of the main tissue resident integrins, comparing them to those found in circulatory cells or NK cells mediating cytotoxic killing, is found in **Figure 1**.

The β2 subunit forms heterodimers with 4 α subunits (αL, αM, αX, and αD), all of which bind ICAM-1 and fibrinogen and are leukocyte specific. β2 integrins have a conserved function in mediating leukocyte recruitment from circulation to the tissues [**Figure 1**; (8)]. They also help mediate target cell killing; in particular, αLβ2 initiates cell polarization through actin remodeling in the formation of an immunological synapse between NK and target cells, however αMβ2 is also found at the synapse (80–85). Nano-adhesions formed at the immune synapse (IS) in response to αLβ2 ligation are similar to those formed during NK and T cell adhesion to endothelial cells, diapedesis, or migration in tissues. The relatively small size of αLβ2 integrin signaling platforms speaks to their ability to remodel actin locally (2). Further, their catch bond function (41), in concert with other mechanosensitive molecules such as vinculin and talin (92), means that the movement of an individual cell can be fine-tuned by the spatiotemporal organization of these adhesion nodes. In the case of catch bonds, greater force applied to the cell results in a molecular response that increases the strength of binding. This is particularly important in the blood, where shear flow generated from blood pressure results in strong lymphocyte binding to endothelium, allowing the cells to subsequently crawl through to areas of infection in the tissues (93). As such, β2 integrins are important for the generalized ability of cells to

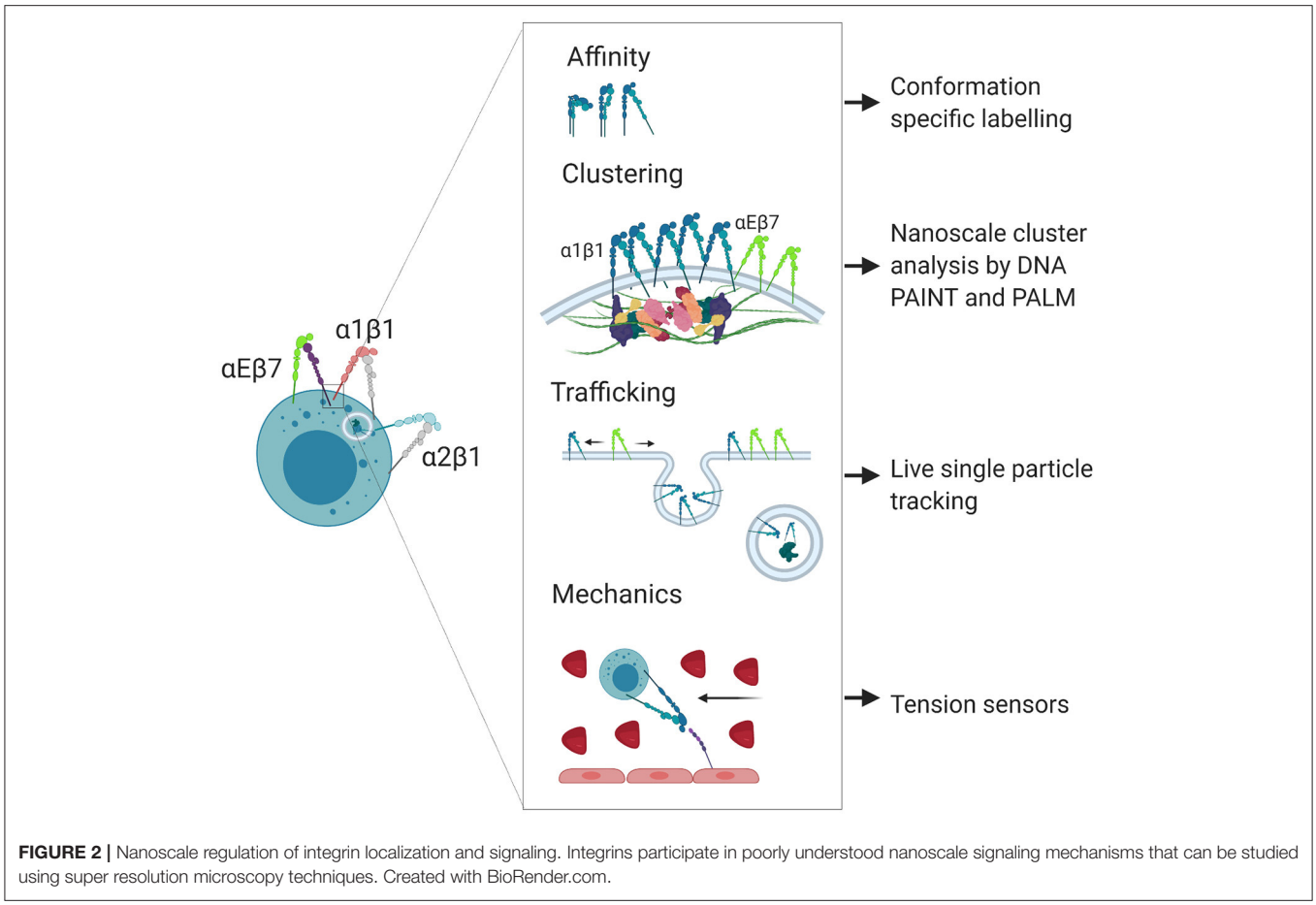

FIGURE 2 | Nanoscale regulation of integrin localization and signaling. Integrins participate in poorly understood nanoscale signaling mechanisms that can be studied using super resolution microscopy techniques. Created with BioRender.com.

migrate and communicate, and they are mostly associated with recruitment to tissues, rather than residency. While αLβ2 is most often used as a model for structural dynamics related to function (10, 12, 94–96), other integrins known to be important for NK cell locomotion and residency are also affinity regulated in this way. αDβ2 (CD11d/CD18) (6) and αMβ2 (CD11b/CD18, Mac-1) (86), both expressed by NK cells, adopt similar structural changes, which are closely regulated by the rearrangement of the MIDAS domain that is translated to I-domain movement (15, 97, 98). Integrin affinity is also regulated structurally in β1-containing heterodimers, which adopt bent-closed, extended-closed, and extended-open conformations which correspond to low, intermediate, and high binding affinity (75, 99). In some cases, the binding of integrin to ligand only occurs at a certain conformation corresponding to a given dissociation constant. α5β1 is an example of an integrin in which a single conformation can confer a low enough dissociation constant for binding, as only the extended open conformation binds to fibronectin (75).

Other β subunits further diversify the function of each heterodimer. In brief, the β4 subunit makes a heterodimer with α6, which binds laminin (1). The β5, β6, β8, and β3 subunits form heterodimers with αV, which bind the RGD motif in fibronectin (1). Finally, and of particular note in trNK cells, the β7 subunit makes a heterodimer with αE (CD103), which binds E-cadherin to form αEβ7, one of the main markers of tissue residency (100–104). β7 also makes a heterodimer with α4, which binds MAdCAM-1, VCAM1, and the fibronectin LDV peptide (1). In summary, integrins have different intracellular and extracellular ligands, and they are activated in response to a range of different signals. Diverse integrins must group together to enact whole cell change, or persistence in a given migratory state. Such specificities are intrinsically related to the tissue environment and the ligands found therein.

Diversity of Integrin Ligands Within Tissue Occupied by trNKs

If the particular collection of integrins present on a cell membrane can be considered an "area code," then it follows that the molecular composition of the environment defines the neighborhood. NK cells are located in non-lymphoid and lymphoid tissue environments, as well as in circulation in peripheral blood (105). In the blood, CAM family proteins, including VCAM, ICAM, and MadCAM, mediate leukocyte arrest and subsequent transendothelial migration between or through endothelial cells (106). During the process of extravasation, passage through the basal lamina, largely composed of collagens IV and VII and laminin, precedes entry to the underlying tissue. The non-lymphoid cells that comprise

TABLE 1 | A list of natural killer cell integrins and their function in residency and/or cell migration.

Alpha/Beta name	Alternate names	Binding partners	NK cells in tissue types	Migration types	Activity level	Residency associated signaling
β1; CD29						
α1; CD49a	VLA-1	Collagen IV (high affinity), laminin (52–54)	Liver, lung, tonsil, uterus, skin, kidney, bone, spleen (55, 56)	ECM residency. Upregulated in cNKs that become trNKs (57, 58)	trNKs with high baseline activation. No CD62L (56)	CD69 expression—S1PR antagonism (59). CXCR3/6. Receptors for retention associated CXCL16 (60–62)
β7						
αE; CD103	–	E-cadherin (63–65)	Liver, lung, tonsil, gut, skin (mucosal propensity) (66)	Epithelial residency (67)	trNKs with less cytotoxicity than α1+ αE–. Can arise in α1+ cells in response to TGF-β (68, 69)	CD69 expression—S1PR antagonism TNF-α producing. CCL5, MIP-1β, and GM-CSF—recruitment and microenvironment remodeling (59)
α4	LPAM	MadCAM-1, VCAM-1, fibronectin (LDV) (70)	Liver, lung, tonsil, gut, skin (mucosal propensity) (66)	Epithelial residency (67)	trNK or cNK	CD69 expression—S1PR antagonism (59)
β1; CD29						
α2; CD49b	VLA-2	Collagen IV, III and I (low affinity), laminin, E-cadherin (1)	Absence coupled to α1 presence (55, 56). Blood and pan-tissue	Migration from circulation to tissue if α1-(55, 56)	cNK/trNK switchable (71)	Reduced TNFα production compared to α1+ α2– (59)
α3; CD49c	VLA-3	Collagen, laminin, fibronectin (1)	Blood and ECM	Adherence (24, 48)	cNK	–
α4; CD49d	VLA-4	Collagen, laminin, VCAM-1, MAdCAM-1, Fibronectin, ADAM (1)	Blood and ECM	Adherence (72–74)	cNK	–
α5; CD49e	VLA-5	Fibronectin (RGD), ADAM (1, 75)	Blood and ECM	Adherence (72–74)	cNK	–
α6; CD49f	VLA-6	Laminin, ADAM (1)	Blood and ECM	Adherence (72–74)	cNK	–
αV; CD51		Fibronectin (RGD), vitronectin (1)	Blood and ECM	Adherence (48)	cNK	–
α10;		Laminin, collagen (1)	Blood and ECM	Adherence (48)	cNK	–
α11		Laminin, collagen (1)	Blood and ECM	Adherence (48)	cNK	–
β2; CD18						
αL	LFA-1	ICAM-1,2,3, 4 (8)	Blood, lymph nodes, migration within diverse tissues (8)	Fast cell migration (76–78)	cNKs. Diapedesis, migration in lymph nodes, synapse formation (79)	Upregulated during inflammation to speed cell migration, scanning, and enhance diapedesis and synapse formation (79)
αM	Mac-1	ICAM-1, 4. Fibrinogen (8)	Blood, lymph nodes, migration within diverse tissues (8)	Cell migration	cNKs. Diapedesis, migration in lymph nodes, synapse formation (80–86)	αM/ αX low resident cells produce IFN-γ in the lung epithelium. Coupled to survival signals through CD27 (87)
αD	—	ICAM-3, fibrinogen, fibronectin, vitronectin, VCAM-1 (40)	Blood, lymph nodes, migration within diverse tissues (8)	Cell migration	cNKs. Diapedesis, migration in lymph nodes, synapse formation (6)	αM/ αX low cells produce IFN-γ and are coupled to high αE and α1 (88)
αX	CR4	ICAM-1, 4, fibrinogen, collagen (8)	Blood, lymph nodes, migration within diverse tissues (8)	Cell migration	cNKs. Diapedesis, migration in lymph nodes, synapse formation	During inflammation, αX β2 is reduced in resident bone marrow cells coupled with an increase in α1 (70, 89)

Dark green, residency associated; Light green, non-residency.

tissue also express CAMs, glycoproteins, and other integrin ligands, whereas the ECM is composed of a huge array of RGD- and LDV-domain containing fibers, for which different integrins have different affinities. Each tissue environment is unique in their composition of these binding sites for integrins on both cells and ECM.

ECM differs vastly between and within tissues and is composed of fibrous proteins including collagens and elastin, and glycoproteins such as fibronectin, proteoglycans, and laminin (107, 108). Collagens are the most abundant macromolecule in the ECM, and there are 28 different collagen subtypes which form fibers as tight homo- or heterotrimeric helices (108). Depending on the subtypes that make up the fibers, and the three-dimensional structures that they form, different binding sites become available for integrins. In addition, collagen fibers form supramolecular complexes with laminins, which act as bridging molecules and are composed of differing α, β, and γ chains that link collagen and integrins. In this way, 16 possible Y-shaped or rod-shaped laminin heterotrimers can be formed with different binding sites for integrins, and the differential combinations of these chains allows for great complexity between and within tissues (108). Fibronectin is another tensile molecule with many binding sites that facilitate linkage to collagen, integrins, and other components of the ECM, as well as to other fibronectin molecules. Fibronectin forms a homodimer that reveals cryptic binding sites when stretched, allowing dynamic control and two-way feedback between the ECM and lymphocytes (109–114). Integrin binding, particularly to RGD sites, promotes matrix generation, and adhesions formed within cells modify the structure of fibronectin fibrils in part by providing actomyosin-driven contractile force that is required for fibrillogenesis (112, 115–117). As fibronectin networks are built around cells *in situ*, it is important to try to hypothesize how these may affect trNKs entering an ECM-rich tissue then adapting to this environment.

CAM molecules are also present or absent depending on the tissue subtype and can be up- or down-regulated during inflammation and immunological memory responses. As primary ligands of β2 integrins, CAMs dictate functional responses depending on their density. Perhaps the best characterized of these responses is the arrest of rolling leukocytes followed by the control of tissue extravasation that occurs via ICAM-1-αLβ2 interactions. The local upregulation of ICAM-1 occurs in response to inflammation, enhancing the adhesion of leukocytes to blood vessel endothelium close to sites of infection (79). In NK cells, this interaction causes the polarization of lytic granules and actin rearrangement as a precursor to cell killing, while in cytotoxic T cells, ICAM-1/αLβ2 acts as a second signal for killing after TCR engagement with MHC in addition to functioning in adhesion (80–82, 84, 85, 118–124). Chemokines alter the nanoscale spatial arrangement of integrins, such that the presence of the CCR7 ligand CCL21 makes αLβ2 adhesion clusters condense, leading to faster T cell migration (76–78). As ICAM-1 and CCL21 are highly expressed in follicular DCs in secondary lymphoid tissue, their co-expression on T cells is linked with an increased efficiency of scanning DCs for MHC molecules and speaks to the multi-modal regulation of lymphocyte migration (31). As such, ICAM-1/αLβ2 is an excellent model to illustrate how interactions between the same CAM/integrin can lead to different outcomes dependent on the microenvironment. Other integrin-CAM combinations, such as αMβ2/ICAM-1, as well as those mediated by VCAMs, MAdCAM, and ALCAMs also have diverse roles dependent on the tissue microenvironment (125).

Integrin Nanoclusters and Their Function in Lymphocyte Migration

Integrins in migrating fibroblasts group together in the lamellipodia at the leading edge, forming nascent adhesions of <100 nm in diameter (23, 126–129). Early adhesions, through the recruitment of cytoplasmic integrin adhesome proteins, coalesce into large clusters up to 1 μm in diameter at the basal membrane in the mid-body of the cell. These anchors allow cells to firmly adhere to their environment, forming large focal adhesions that are highly structured and turn over slowly (130). In contrast, lymphocytes operate a different integrin clustering program from fibroblasts owing to their need to move on a shorter time scale and quickly adapt to diverse environments. Most lymphocyte adhesion complexes are on the order of <100 nm in diameter and are only definable by super-resolution microscopy (2, 10, 17). While there are key differences between lymphocyte migration and the migration of larger, fibroblast-like cells, the model of tuning dynamic integrin-cytoskeletal linkages to promote adhesion and migration is conserved, and allows for different modes of lymphocyte migration or residency to occur in response to ligand specificity, intracellular signaling or tissue rigidity (131–134). In T cells, all αLβ2 clusters are <100 nm in diameter (2). These nanoclusters differ in their size and density depending on their location in a polarized cell membrane, namely the lamellipodia vs. the lamella, and have differential involvement of phosphorylated focal adhesion kinase (FAK) and Src family kinases. In 2D migration studies, the size of such nanoclusters, and the recruitment of active signaling intermediates, has been directly correlated with the velocity of migrating cells. Smaller, denser clusters with more phosphorylated kinases are associated with increased speed, indicating that integrins are regulated locally within signaling islands. Here, such active nano-adhesions were measured by tagging a single heterodimer in conjunction with signaling intermediates indicating active signaling (2). β1 integrins have been investigated in a similar way, albeit not in lymphocytes (135). The spatiotemporal regulation of αLβ2 integrin, and associated FAK and Src kinases works across length scales from single nanoclusters to groups of clusters that are translated into whole cell behavior providing dynamism to immune cells.

How diverse sets of integrins expressed on a single NK cell work together at this length scale is an open question. Mature NK cells co-express multiple integrins, including the β1 integrins VLA-4 and−5, and β2 integrin LFA-1 (72, 73, 136, 137). In migrating T cells, integrins are differentially localized along the front-rear axis based upon their conformation, with high affinity β1 integrins found primarily in the uropod, whereas LFA-1 is found in high-affinity conformation in the lamellipodia and mid-body focal zone, and low affinity conformation in the

uropod (138, 139). T cell integrin spatial localization is linked to differential usage, which in the case of VLA-4 and LFA-1 includes mediating up- and down-stream mechanotaxis under shear flow, respectively (139, 140). Engagement of LFA-1 or VLA-4 to ICAM-1 or VCAM-1 leads to distinct signaling pathways that help shape these specified responses (140). Thus, at least in T cells migrating under flow, differential cellular localization of integrin species direct different functions in the same context, with the passive uropod acting as a windvane in LFA-1 mediated upstream migration, and the lamellipodia passively focusing downstream migration that is mediated by VLA-4 (139, 141). While not spatially defined on the nanoscale, there are also multiple mechanisms of integrin crosstalk, including both activating and inhibitory signals, that can be passed between integrin species (142–144). It should be noted that the example of VLA-4 and LFA-1 in this case is likely distinct from chemotaxis or 3D cell migration in a tissue microenvironment, in which integrins play different roles or may even be redundant (50, 134). Regardless, this example highlights the importance of using measurements such as integrin activation, signaling, and localization in addition to cell surface expression when defining the significance of integrin expression on cellular subsets.

While spatial patterns of single integrin localization have been described in some cases, species mixing to form complex, multi-integrin subtype clusters has not been investigated on the nanoscale. Both a4β1 and LFA-1 are recruited to lipid rafts upon T cell activation yet are found in distinct membrane patches in resting cells (145). While these studies were not performed with sufficient resolution to interrogate nanoclusters, it is conceivable that mixed clusters of integrins species are formed within lipid rafts. An alternative model is that individual integrins composed of only one heterodimer subtype form discrete nanoclusters, which has been shown for the T cell receptor and its adaptor LAT (146, 147). Segregation has also been found between active ligand-bound and inactive integrin β1 in conventional focal adhesions (148). Evidence that active and inactive integrins can co-cluster, but those that are ligand-bound or -unbound remain segregated, tells a more complex story (135). Communication between clusters in the membrane might be necessary to coordinate an appropriate migratory or resident phenotype and to employ dynamism between the two. Integrins move laterally in the membrane, or rather, the cell moves over anchored integrins, during mesenchymal or crawling-type migration and during reorganization for immune synapse formation (149). This cellular movement includes the centripetal flow of cortical F-actin and myosin-based contraction, which acts to strengthen high-affinity binding of LFA-1 to ICAM-1 (40) through mechanical forces.

While nanoscale information about the colocalization of multiple integrins has not been defined in NK cells, we do have insight into differential integrin expression associated with NK subsets. As we will see, α1 and αE integrin expressing NK cells represent a resident phenotype in multiple tissues. The absence or low expression of some integrins, including αM and α2, in such cells may be as relevant as the presence of others for cell and system behavior across length scales. High content and large field of view advanced super resolution microscopy and single particle tracking will be required to definitively dissect the relationship between integrin activation, localization, clustering and mixing on NK cells that are resident vs. migratory.

Regulation of Integrins by Inside-Out Signaling From Chemokine Receptors

Chemokine receptors, the location of chemokine release or expression, and the corresponding cellular response are components of a cell's "area code" that are inseparable from integrin affinity, clustering and recycling. By having the capacity to rapidly induce integrin conformational changes and thus direct cell migration, chemokines are key regulators of the integrin-mediated response.

Chemokine receptors are grouped by their structure into four subtypes: CXCR, CCR, CX3CR, and XCR, with corresponding chemokine ligands for each (150). Functionally, chemokines can be grouped into "inflammatory/inducible," such as CCL5 (RANTES), and "homeostatic/constitutive," such as CCL19 and−21 (151). trNK cells, cNK cells, and developmental subsets of NK cells can be differentiated based on their chemokine receptor expression, which speaks to the interrelatedness of integrin and chemokine signaling in defining tissue residency and NK cell development. Key chemokines that dictate NK cell trafficking and function include CXCR1, CXCR3, CXCR4, CXCR6, CCR7, MIP-1α/β (macrophage inflammatory protein-1 alpha/beta, CCL3/4), RANTES (regulated activation, normal T cell expressed, and secreted, CCL5), and ATAC (activation-induced, T cell derived, and chemokine-related cytokine, CXCL1) (152).

Binding of chemokine to chemokine receptors results in the release of GPCR subunits into the cell that activate Rho, Rap, and Rac GTPases, which then modulate integrin affinity and clustering and actin remodeling (153). A generalizable model of the effect of how an arrest chemokine leads to rapid integrin activation includes the dissociation of Gβγ subunit, which diffuses into the cell to activate IP_3-Ca^{2+} signaling and generate rapid intracellular calcium flux. Calcium and DAG in turn activate GTPases such as Rap1A which bind via adaptors to intracellular domains of integrins and generate intermediate conformation, thus priming them for ligand binding and subsequent strengthening of adhesion (154). In this way, chemokines work in concert with selectin-mediated tethering to rapidly activate integrins and switch modes of cell migration. It is important to note that while some chemokine signaling functions are conserved, such as the capability of CXCL12 to trigger an αLβ2 high affinity state in multiple lymphocyte subsets, downstream signaling pathways can be subject to variability and can be altered in the context of malignancy (155), speaking to the mutable nature of signaling islands.

Inside-in Signaling—Integrin Trafficking and Recycling

Integrin outside-in and inside-out signaling describe a bidirectional interplay of mechanosensing, affinity and clustering by integrins and other receptors in conjunction with signaling intermediates. The process is also highly related to intracellular

integrin trafficking. Far from being a simple process of recycling, integrin trafficking reveals that integrins also signal from intracellular vesicles (156). A pool of activated integrins in vesicles may be particularly important in cells that must remain dynamic after long periods of residency within tissues to respond to a new pathogen.

A universal measure of integrin activation is the local accumulation of active kinases such as phosphorylated tyrosine 397 (Y397) on FAK, and complexes of active FAK, integrins, and talin are present in endosomes (156). Without such endosomes integrin signaling cannot proceed, as they form part of the signaling axis emanating from the adhesome and resulting in Erk/AKT signaling. β1 integrins appear to be highly important in endosomal signaling and blocking Rab21-dependent endocytosis affects cell adhesion and migration. In particular, cells in which endocytosis of active integrin vesicles is impaired undergo anoikis (156). Further, integrins maintain their intermediate- or high-affinity conformation within vesicles through interactions with talin. Such active integrins in endosomes represent those that were previously engaged with ligands. Non-engaged integrins are constitutively endocytosed and recycled but lack the activation markers in vesicles described above. Clearly, such active integrin vesicles are important for recycling to the cell membrane to form new adhesions and must be especially important for fast-moving lymphocytes to generate new adhesions (157). Along with cell migration/adhesion receptors, endocytosed active integrins may cooperate with growth factor receptors, modulating their expression or trafficking to the membrane and ultimately the fate of the cell (158). While a role for endosomal integrin signaling has not been described in NK cells, defining how the activated cytoplasmic integrin pool changes in tissue resident cells could be a fruitful area for discovery.

INTEGRINS AS MARKERS FOR RESIDENCY

Integrins and chemokine receptors are frequently used to describe differences between tissue resident subsets and circulating cells. As such, many of the key phenotypic markers that have been used with their CD nomenclature to describe unique tissue subsets are integrins. Below we will summarize these subsets with a particular focus on the integrins that define their residency and the implications of their expression.

Integrins as trNK Markers in Mice

In mice, there are multiple populations of mature NK cells that are associated with unique tissue residency and are thought to undergo some, or all, of their maturation within these tissues. These subsets include conventional NK cells (cNK), which are found in spleen, blood, and bone marrow, thymic NK cells, trNK liver and skin cells, lung NK cells, and uterine NK cells (uNK) (159). By considering the expression and function of integrins on these specialized NK cell populations, we can speculate as to their relevance in establishing and maintaining NK cell residency and mediating function in different environments (**Figure 1**).

The full spectrum of NK cell developmental subsets is found in the bone marrow. Differentiation from common lymphoid precursors occurs there, and is followed by the exit of mature NK cells to the circulation and the seeding of perfused tissue such as the spleen (160, 161). However, the presence of highly specialized tissue NK cell subsets with unique transcriptional profiles suggests that earlier progenitors may also leave the bone marrow and settle in these tissues to undergo further differentiation (162–164). The presence of circulating common ILC and NK cell progenitors in peripheral blood also supports this model (165). Alternatively, the presence of innate-like B cells, macrophages, and mast cells in tissue that are derived from embryonic pre-hematopoietic precursors suggests that a similar pathway to the development of tissue resident innate lymphoid cells may also exist (166–168).

In the bone marrow, progressive maturation of NK cells is delineated in part by the upregulation of CD11b (Mac-1, αMβ2), and subsequent downregulation of CD27 (87). αMβ2 binds ICAM-1 to facilitate cell migration through the ECM and arrest in the blood prior to diapedesis, similarly to αLβ2, and strengthens binding to target cells during cytotoxic attack (83, 169). αMβ2 binds to 30 different protein and non-protein targets that are associated with migration and cytotoxic function, rather than residency (169). While the stages of human NK cell development are less frequently classified by CD27 and CD11b expression, upregulation of αMβ2 also marks mature circulating human NK cells (87, 170).

Notably, and irrespective of their origin, trNK cell subsets have differential expression of CD49a (VLA-1, α1β1) and CD49b (VLA-2, DX5, α2β1), which may reflect how integrins either direct precursors to, or retain mature cells in, these environments (55, 56). Further investigation into the molecular composition of ECM at different sites may also provide additional insight into why the expression of certain integrins is linked to distinct tissue resident phenotypes (107). For example, liver ECM is rich in type IV collagen, a high affinity binding partner for α1β1 (VLA-1) integrin (52–54). α2β1 binds less specifically to the GFOGER motif in collagens I and IV as well as GAOGER in collagen III (171, 172). Cell tracing experiments have shown that $\alpha1^{-}\alpha2^{+}$ positive ($CD49b^{+}$) NK cells traffic to tissue sites via circulation, suggesting that their propensity for non-resident behavior may be mediated by the lower affinity of α2β1for collagen IV. The subset of liver NK cells that lacks α1 (CD49a) but expresses α2 ($DX5^{+}$ or $CD49b^{+}$) resembles splenic cNKs and are as such thought to be transient, whereas $\alpha1^{+}\alpha2^{-}$ NK cells are thought to be permanent residents of the liver (55, 56). These $\alpha1^{+}\alpha2^{-}$ liver trNK cells have low levels of αM but produce cytokines and are cytotoxic and are therefore not immature. They may represent a population that is derived after inflammation and have been linked to inflammation in the skin (55).

Liver trNK cells in mice are also functionally unique as they exhibit a higher level of baseline activation relative to splenic or liver cNK cells (56). They are larger and more granular, and express Ly49E, which mediates responses to liver-specific infections such as *T. cruzi* (173). Cytokine release is more efficient from these cells, TNFα, GM-CSF and IL-2 are all increased, and their high expression of TRAIL and FasL makes them better at

inducing target cell apoptosis by alternative killing. Together, liver trNK cells may represent a resident subtype that acts both as a recruiter of other cells and an actively cytotoxic subtype. Of note, mouse liver NK cells represent a distinct lineage of trNK cells marked by a unique transcriptional profile and integrin repertoire when compared with cNK cells (174), adding another example to a body of literature that indicates that most trNKs develop *in situ*. In addition to their high expression of α1β1, which binds collagen IV strongly, CD69 is highly expressed in murine liver trNK cells and acts as an antagonist against the S1P receptor to prevent egress (175, 176). Together, this expression pattern works to maintain the tissue residency of mature NK cells (177).

In addition to liver trNK cells, the expression of α1 and the lack of α2 can also be used to define trNK cells in the uterus, skin, and kidney (56, 178), but not in other organs, such as bone marrow, lymph nodes, lung, spinal cord, pancreas, ementum, and peritoneal cavity. It is important to note that in the liver, uterus and skin, there are clearly defined populations of trNK cells and also cNK cells (55, 56, 179). In addition, the thymus and salivary glands contain NK cells, some of which have tissue resident properties. Thymic NK cells are cytotoxic, like cNK cells, but express the IL-7 receptor α chain (CD127) and arise from thymocyte precursors (56, 180–182). Uterine NK cells are also cytotoxic, like cNKs, but may arise from a separate developmental pathway, as they appear normal in T-bet-deficient mice (56). trNKs in the liver, uterus and salivary gland express both α1 (CD49a) and α2 (CD49b), whereas adipose, kidney and small intestine lamina propria ILC1 cells express only α1 and no α2 (183). Thymic NKs and circulating cNKs express only α2 (180). αE, which heterodimerizes with β7 to form αEβ7 (CD103), is expressed highly in salivary gland trNKs (184, 185) and is also present in other subsets such as lung and liver NK cells (186).

It remains unclear whether integrin expression patterns are pre-programmed, such that cells migrate to and remain in a given microenvironment, vs. being shaped in response to their local microenvironment. However, given that NK cells change their migration characteristics and integrin expression during development, during which they are exposed to diverse microenvironments, it is likely that the answer is a mix of both of these scenarios (187). Integrin β2 deficient mouse models show us not only that NK cells lacking β2 have impaired cytotoxic function, but also that they are developmentally impaired, failing to transition to maturity in all organs and demonstrating that β2 integrin expression is necessary for the homing of precursors to niches that can subsequently support their development (188). In contrast, β1 integrin deficient murine NK cells do not have impaired maturation or function, but have inhibited proliferation, a somewhat surprising but informative observation given the specific importance of β1 integrins in cell migration and association of their expression with tissue residency (189). Together these examples suggest that tissue seeding requires transcriptionally regulated integrin expression in mice. Conversely, differences in the strength of integrin-mediated adhesion to ECM between peripheral blood and lamina propria T cells can be recapitulated by short-term incubation of peripheral blood T cells with collagen or fibronectin (190). In addition, as discussed further below, there is evidence to suggest that tissue resident signatures can be generated or strengthened *in situ*. Human transplant studies have also demonstrated that markers of tissue residency, including αE (CD103) and α1 (CD49a), are acquired over time in a stepwise fashion in recipient T cells (65, 191). As the upregulation of their expression is linked to the acquisition of a transcriptional signature associated with tissue resident T cells, it is unclear what the local signals are that drive these changes, but they likely include chemokines, cell adhesion molecules, and ECM components.

Chemokine Signaling to Integrins in Mice and Humans

Integrin expression and function are tightly linked to the expression and function of homing and chemokine receptors, which participate in inside-out signaling to regulate integrins and their downstream signaling networks. $\alpha1^{+}\alpha2^{-}$ liver trNK cells in mice highly express CXCR3 and CXCR6, both receptors for CXCL16, which is a chemokine expressed in liver sinusoids that mediates retention of NK and NKT cells in the liver (60–62). Liver trNKs also lack L-selectin (CD62L), which mediates rolling adhesion in capillaries and vessels (55). Together, these signals help generate a tissue resident phenotype by recruiting and maintaining NK cells in the liver. The specific up-regulation of molecules associated with homing and retention suggests that circulating mature NK cells can undergo phenotypic changes to become tissue resident. However, it is important to note that most literature indicates that trNKs develop through separate lineages from cNKs and establish residency phenotypes within tissues through development *in situ*.

trNK cells in the human lung are characterized by expression of CD69, a C-type lectin which antagonizes the receptor for S1P, which is a chemokine otherwise associated with egress from tissues. CD69 expression is a hallmark of tissue residency and has also been correlated with the co-expression of α1 and αE integrins, which contribute more to a distinct trNK phenotype compared to the presence of CD69 alone (59). As CD69 antagonism of S1PR prevents tissue egress, it is possible that CD69 upregulation precedes that of α1 and αE, such that circulating NK cells are trapped in the tissue and their residency is then strengthened by integrins. Further, collagen IV is rich in the basement membrane of the blood-gas barrier, so α1- expressing cells are more likely to localize here than the parenchyma. Their residency there may be reinforced by the inhibition of their egress from tissue following down-regulation of S1PR in response to CD69 ligation (59). While in many of these integrin-based mechanisms mouse and human trNKs share similarities, there are some key differences between species, and these often reside in the underlying transcriptional program. This is exemplified by Hobit, a transcriptional repressor that decreases expression of *S1PR1*, *KLF2* (which itself promotes CD69 expression), and *CCR7*, all of which promote tissue residency. In mice, Hobit directs a program of lymphocyte tissue residency that includes liver resident NK cells (192). In humans, Hobit is expressed more highly in circulating NK cells than in lymphoid tissue resident NKs (ltNKs) (70, 192, 193), but is also high in liver trNKs (194).

The high expression of Hobit in circulating CD56^{dim} human NK cells, which correlates with Hobit-regulated genes, suggests that different pathways in humans and mice can dictate tissue residency (194).

In summary, and to continue the analogy, chemokine receptors constitute an area code for a GPS guidance system, whereas integrin expression may be more like a specialized tire set. Integrins allow cells to function in different kinds of environments; when they want to move around, they change their expression or alter the spatial positions of integrins in response to chemokines.

trNK INTEGRIN EXPRESSION IN HUMANS

In humans, the enrichment of integrin ligands in given niches of organs often correlates with the expression of the integrins on immune cells, giving rise to diverse subsets of trNK cells found between organs as well as within them.

Circulating NK Cells in Peripheral Blood

Human NK cells express αLβ2 (LFA-1) and αMβ2 (Mac-1) in circulation, in addition to α4β1 (VLA-4), α5β1 (VLA-5), and α6β1 (VLA-6) (72–74). For the most part, the expression of β2 integrins confers a specialized ability of circulating NK cells to adhere to endothelial cells in the capillaries, and mediate the formation of the immune synapse. β1 integrins are associated with mediating other functions, including tissue residency. The difference between the immune phenotype of leukocyte adhesion deficiency (LAD)-I and LAD-III in humans exemplifies the differential requirements for β1 and β2 integrins. LAD-I results from β2 integrin deficiency and affects exit of precursors and mature NK cells from the bloodstream as well as immune synapse formation (195, 196). LAD-III, on the other hand, is caused by mutations in kindlin-3 or the Rap1 GEF CalDEG-GEF1, which are conserved activators of β1 and β2 integrins, and thus leads to a broader clinical phenotype including bleeding disorders (197). Some integrin functions are conserved and/or compensated for in LAD-III patients, possibly by talin enabling α4β1 to maintain sufficient adhesiveness in the absence of kindlin-3 (198).

Tissue Resident NK Cells in Humans

Tissue residency of both NK and T cells can be defined by a core transcriptional signature of genes aside from those that code for integrins. Decreased expression of *S1PR1* (S1P receptor 1) in mice (192, 199), and *SELL* (L-selectin), *RGS1* (regulator of g-protein signaling 1), and *KLF3* (Kruppel like factor 3) mark these populations in human NK and T cells (63, 200) in addition to an increased expression of CXCR6 on human NK cells (59). However, such non-integrin gene signatures also intersect with integrin profiles, as CD69$^+$ α1 (CD49a)$^+$ αE (CD103)$^+$ trNKs in the human lung have non-integrin gene signatures that are distinct from those in the bone marrow and CD8 T_{RM} cells in the lung (59). α1 (CD49a), which binds to collagen IV, and αE (CD103) which binds to E-cadherin, are both markers of tissue retention in T cells (63–65). In addition, the "area code" that can direct cell trafficking through integrin ligand expression extends not only to organs, but to specific tissue niches within those organs including the human lung (**Figure 3**).

Taking the human lung as an example, the presence of αE likely not only signifies tissue specificity, but also spatial localization within tissue. There appear to be at least three distinct populations of tissue resident NK cells in the human lung, including CD69$^+$α1$^-$αE$^-$, CD69$^+$α1$^+$αE$^-$ and CD69$^+$α1$^+$αE$^+$. These populations may reflect specific localization to specific regions in the lung. In human skin, α1 positive T cells are localized to the dermis, yet are seemingly unable to position within the epidermis. This finding has clinical implications as it affects the development of psoriasis, and it also demonstrates how sequestration of cells in tissue locales can occur based on integrin expression (67). Similarly, CD103$^+$ (αE$^+$) T cells are found within lung epithelium, suggesting that αE can be considered a marker of epithelial localization that correlates with expression of E-cadherin by epithelial cells lining the alveoli and basement membrane (201, 202). While the localization of trNK cells in the lung has not been well-defined, it is likely that the distinct populations found here are also spatially distinct, with CD103 marking a population found in the epithelium (discussed further below and summarized in **Figure 3**). These cells may undergo pre-programmed fate decisions, anchoring themselves into long term tissue residency as they undergo maturation. Alternatively, a plethora of immunological stimuli combined with the composition of the microenvironment could produce a heterogeneous population of cells which can then adapt to become resident in different locales in part through modifying integrin expression (59).

Relating Phenotypic to Functional Differences in the Lung

When we consider the differential expression of α1 and αE in lung, we can speculate as to the relative roles of these integrins on NK cells (**Figure 3**). The human lung is a mucosal environment that is frequently exposed to pathogens and where tissue resident lymphocytes are crucial for managing acute and chronic threats. Tissue resident CD8$^+$ T cells have a unique transcriptome and their frequency is correlated with lung cancer clinical outcomes (203). Unlike T cells in the lung, trNK cells have poor lytic function when challenged with tumor target cells *ex vivo*, but maintain their ability to kill virally infected cells (59). Such seemingly hyporesponsive cells also have the ability to produce inflammatory cytokines and their lytic function can be restored by stimulation with IL-15 (59). α1$^+$αE$^+$ positive cells are less cytotoxic than α1$^+$αE$^-$ cells, but both subsets produce comparable quantities of TNF (59). This suggests that αE may play a functional role in pushing cells away from a cytotoxic phenotype, potentially through its interaction with E-cadherin, although the functional role of CD103 is less well defined than that of CD49a (202, 204). The expression of α1 integrin suggests plasticity, as α1 expression can be induced by the presence of IL-15 (59, 205). Further, α1$^+$ lung trNK cells upregulate perforin, granzyme B, Ki67, and CCL5, markers of greater functional capacity, to a greater extent than cells that are α1 integrin negative in the lung (59). While blocking CD49a abrogates cell adhesion

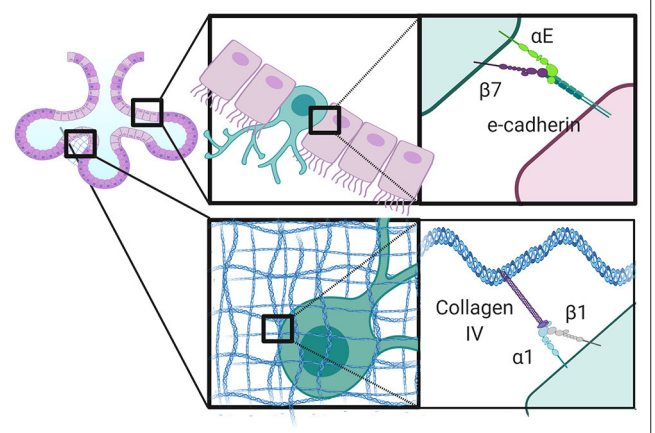

FIGURE 3 | Integrins control niche specific trNK cell localization in the lung. trNK cells expressing αEβ7 localize to alveolar epithelium binding E-Cadherin, and $\alpha1\beta1^{high}$ trNK cells preferentially localize to the collagen IV rich basement membrane. Created with BioRender.com.

and migration on collagen IV *in vitro*, cells fail to migrate on E-cadherin, and CD103 does not participate in collagen binding or cell migration (202). Together, this raises questions as to the relative contributions of α1 and αE and how they are regulated. Is α1 integrin internalized, but remaining in pools ready for quick use? Does it remain on the surface, and change its role in the presence of αE integrin as a part of inter-integrin inside-out signaling? It is important to note that trNK cells in the lung express significant amounts of CCL5, MIP-1β, and GM-CSF, which are all chemokines involved with modifying the microenvironment, shaping cell migration and recruiting other immune cells (59).

Finally, $\alpha1^+\alpha E^+$ trNK cells in the lung also express less L-selectin than α1 or αE negative cells. Expression of S1PR5, which is a receptor for S1P, a chemokine known for the induction of egress from lymphoid tissues, is also low in these cells. CD69 antagonizes S1PR1 by causing its removal from the cell membrane and its internalization, suggesting that the expression of CD69 and lack of expression of S1PR5 helps mediate retention of these cells in tissue (206). In addition, $\alpha1^+\alpha E^+$ trNK cells in the lung express more β1 integrin, which is associated with adhesion rather than migration, likely further contributing to the tissue resident phenotype (59).

Liver $\alpha1^+$ Cells Represent a Distinct Lineage of trNKs

A population of trNK cells that is partially defined by expression of the integrin CD49e ($CXCR6^+$ or $CD49e^-CD56^{bright}$) has been clearly identified in the human liver (207, 208). Unlike conventional NK cells, liver $\alpha1^+$ NK cell differentiation requires T-bet, but not EOMES (186). EOMES and T-bet, both transcription factors that can define the maturation of NK cells, are present in mature NK cells of the periphery and uterus, whereas the $EOMES^-$ population in liver appears to be rare in humans, although a minor $EOMES^-$ population expressing α1 has also been found to produce TNF during pregnancy (209, 210). This liver resident NK cell population is found in the liver tissue itself, but not in afferent or efferent hepatic blood or peripheral blood and represents an equivalent $\alpha1^+\alpha2^-$ population to that found in mice (56, 174). The presence of liver trNK cells in adult but not fetal liver suggests that these are a subset of memory NK cells that arise due to environmental stimulus, again emphasizing the potential for phenotypic switching suggested in the lung. This adds to a large body of evidence describing the development of trNK cells from precursors within tissue instead of from mature NK cells that seed tissue from the blood. Complexifying the situation, there also exists an $EOMES^{high}$ population of trNK cells detected in the liver over timepoints spanning 13 years (68). However, unlike the population described above, which seems to be a separate lineage that remains resident, the $EOMES^{high}$ population seems to be derived from cNK cells in the blood (68). It is thought that $EOMES^{low}$ cNK upregulate EOMES in response to cytokines that likely include IL-15 and TGF-β, resulting in reprogramming and the detection of many liver tissue residence molecules such as integrins α1 and αE (68, 69). These cells likely first upregulate CXCR6, which senses CXCL16, which is highly expressed in the liver especially during an inflammatory event. CXCR6 activates integrin α4β1 (VLA-4), which can enhance the recruitment of cells to the liver through binding to VCAM-1 in mice [**Figure 4**; (211)]. Upon seeding of tissue, trNK cells may upregulate EOMES and other related retention signals, including α1 and αE, to generate a tissue resident phenotype [**Figure 4**; (71)]. Therefore, the function of trNK cells, whether derived from blood or *in situ* maturation, may be to provide a version of adaptive immunity that can be shaped by integrin function in response to inflammation or infection. As with the equivalent $\alpha1^+\alpha2^-$ mouse subset, the human liver trNK subset produces IFNγ and TNF to a higher degree than conventional NK cells, and are less cytotoxic based on their inability to degranulate efficiently and their low levels of perforin (186). They are highly proliferative in *ex vivo* culture and have KIR expression that suggests clonal origins, suggesting that these are memory NK cells (186). Regardless of their origin, due to their distinct transcriptomic profiles from conventional NK cells it is likely that liver trNK cells develop or mature in the liver at least partly in response to environmental cues.

Comparing the localization of trNK cells in the liver with those in the lung, the expression of α1 means that these cells also express β1, implying that they use this receptor functionally to bind to collagen that is present at high levels in the liver parenchyma (186). In the lung, the basement membrane of the blood-gas-barrier is rich in collagen IV, which is not present in parenchyma (59). αE uses binding to E-cadherin to localize T cells to epithelial tissues in the lung (212). As such, this is another example of how the expression of integrins can result in different localization the same tissue, dependent on the binding partners that are available. This behavior is replicated in double positive α1 and αE trNK cells in the lung (59). The localization of liver $\alpha1^+\alpha E^+$ trNK cells is likely to also be biased toward E-cadherin and collagen IV rich regions, and together this highlights the specificity and degree of residency that is achieved by the plastic

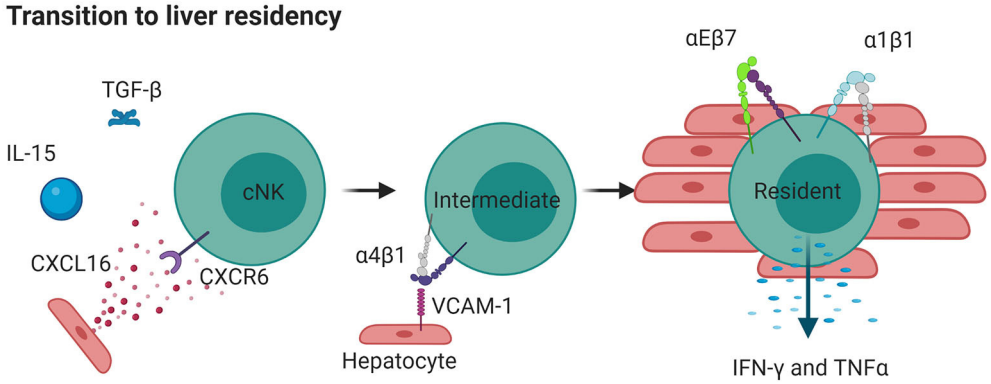

FIGURE 4 | Liver resident NK cells can originate from cNKs. IL-15 and TGFβ released during inflammation cause cNKs to upregulate CXCR6 which detects CXCL16 in the liver. This results in more α4β1, moving cNKs from blood to liver, where they upregulate αEβ7 and α1β1 and become tissue resident. Such cells are less directly cytotoxic but produce high levels of cytokines IFNγ and TNF. Created with BioRender.com.

expression of integrins together with differential expression of their binding partners in the tissue microenvironment. By tuning their killing and cytokine release abilities, which can also be correlated to their integrin expression, such cells are likely able to respond to infections common to these tissue regions.

Both α1 and αE, therefore, are associated with cell stopping and retention, rather than migration, and as such enable tissue residency. As described previously, expression of other integrins, such as αLβ2 and αMβ2, generate a more migratory phenotype within lymph nodes for scanning, or to aid in diapedesis into tissues from HEVs. Together, this points toward a shared transcriptional code for trNK cells, which can be further diversified depending on the organ and the precise tissue locale within the organ and is distinct from that of $CD8^+$ T_{RM} cells. This speaks to the combination of predetermined function and plasticity shown by NK cells that may either be programmed to become tissue resident or may respond directly to the combination of signaling molecules and other cues, such as mechanotransduction, from their environment.

Integrins and Tonsil Tissue Residency

The tonsil has been well-studied as a site of NK cell development, and in addition to other secondary lymphoid tissue represents an important site of human NK cell development and residency (162, 163, 187, 213). This is in part due to the presence of an early NK cell precursor defined by the expression of integrin β7, which is thought to seed tissue after exiting the bone marrow. This precursor can also be found in peripheral blood expressing L-selectin, suggesting that expression of L-selectin helps recruit these precursors to tissue before being subsequently down-regulated (162, 163).

Tonsil resident cells are thought to include intraepithelial ILC1 helper cells that have an integrin expression profile similar to $CD8^+$ T cells and are characterized by their expression of NKp44, αE integrin, and CXCR6 (88). While the etiology of ILCs in tonsil has since been debated (214), the tissue resident aspect of the phenotype of ieILC1s is conserved between multiple tissues. While similar in many respects, and likely belonging to the same lineage, ieILC1 cells are slightly different from trNK cells, having similar abilities to produce TNFα and IFN-γ but less cytotoxic capacity (88). Generally, cNKs do not highly express IL7Rα, and can be differentiated from ILC1s on this basis. However, some trNK cells, including those in the siLP, thymus and elsewhere, actually do express the IL7R, further complexifying their discrimination from ILCs (180, 215).

Unsurprisingly, cNK cells in the tonsil that express αE also express high levels of β7, the heterodimeric partner of αE, yet they additionally express high levels of α1 and lower levels of αM and αX (88). Like αM, αX forms a heterodimer with β2, and overlaps in many of its functions of adhesion, migration, and diapedesis in activated cells. As in the liver (216) expression of CXCR6 is high, and expression of CCR9 is low on $\alpha1^{hi}$, αE^{hi}, αM^{lo}, αX^{lo} NK cells from the tonsil (88, 216). Interestingly, NEDD9 (CasL), a protein associated with inside-out integrin signaling via its interaction with FAK, is also very highly expressed in these cells, suggesting that adhesion regulators may also mediate integrin functions associated with residency (88, 140, 217). In addition to its well-described role in liver residency, CXCR6 is associated with specificity for epithelial tissues, including the airways of the lung (61, 218, 219). Similarly, the CCR9 ligand CCL25 induces lymphocyte entry to the small intestine (220–223). α1, as previously discussed, has high affinity for collagen. Together, $\alpha1^{hi}$ αE^{hi} αM^{lo} αX^{lo} NK cells reside close to the epithelium in the tonsil and seem specialized to produce IFN-γ on demand. The specific integrin profile of tonsil resident NK cells is therefore linked both to the specific expression of chemokine receptors and adaptors.

In other secondary lymphoid tissue, CD69 and CXCR6 expression define a lymphoid tissue trNK subtype (ltNKs) that is found in isolated human secondary lymph nodes (70). In common with liver trNK cells, ltNKs downregulate S1PR1. As CD69 antagonizes S1PR1 signaling, this is a strong signal for remaining in the same locale, as S1P is high in the blood and S1P gradient disruption directs trafficking decisions (175, 176). CD62L (L-selectin) is also downregulated in secondary lymphoid tissue, leading to increased tissue retention. CCR7, again, is

strongly associated with sensing CCL21 and CCL19 in the blood and is downregulated in ltNKs (66, 70). Together, these signals act to retain NK cells in lymph node and can be tuned in response to infection. In addition, in infected lymph nodes, increased CD49b-mediated interactions with collagen confine NK cell migration to restrain them near sites of infection, and CD49b cross-linking enhances NK cell cytokine production (224, 225). CD49b (DX5) positive NK cells also home to lymph node under homeostatic conditions and migrate on collagen (224, 226). While there is debate about the relevance of integrin-mediated migration in lymph nodes (50, 134), intravital imaging clearly reveals roles for both β1 and β2 integrins in certain settings in mouse lymph node (224, 225, 227).

Endometrial trNKs Are Defined by Their Expression of α2 Integrin

Decidual and uterine NKs are important for successful gestation and are the predominant lymphoid cell type during the first trimester of pregnancy (228). In particular, dNKs are $CD56^{bright}$ and α1 integrin positive, are programmed to promote neoangiogenesis, tissue remodeling and placenta development, and may originate from cells that develop *in situ* in the uterus or traffic there from lymph node or bone marrow (229). dNK cells share the ability to secrete TNF and IFNγ with ILC1s, while also having cytolytic capabilities. In this environment, stromal release of TGFβ is a driving signal that induces αE expression, suggesting that α1 positive cells arise through development and that the expression of αE arises in response to local cytokine release to help promote residency [**Figure 5**; (209, 230)]. In mice, two subsets of dNK cells are present, both expressing EOMES and α1, but one is α2 negative and the other is α2 positive (209). A third population, negative for α1 but positive for α2 and EOMES, is also present in the uterine tissue, and represents conventional NK cells (209). As is the case in the intestine, αE expressing trNKs in human endometrium can be further dissected into two populations based on their expression of NKp44, both of which are $\alpha1^{+}\alpha2^{+/-}$ (209). Interestingly, the expression of α1 is correlated with distinct function, as $a1^{+}$ dNK have increased TNFα production relative to $a2^{+}$ cells. These phenotypes suggest that the *in situ* development of these types of cells from precursors of both peripheral and medullary origin, highlighting the plasticity of NK cells to transition into trNK cells.

Bone Marrow NK Cells and Their Tissue Resident Integrin Signature

In the adult bone marrow, HSCs maintain residency for life, but it is unknown as to whether mature NK cells found there develop *in situ* or come back and form a resident phenotype after being in circulation or developing elsewhere (164). Recently, a subset of mature trNK cells with a unique transcriptional program including integrin and chemokine receptors was defined in the human bone marrow (70). These bone marrow trNK cells have a common tissue resident phenotype marked by expression of high levels of CD69 and CXCR6 (70). However, they express more S1PR1 than lung trNKs, possibly speaking to greater plasticity of trNKs in the bone marrow. Finally, bone marrow trNK are also $EOMES^{high}Tbet^{low}$, similar to trNK cells in the liver, suggesting that these are true NK cells that are developmentally separate from ILC1s.

In addition to CXCR6, CD69 and aE, a generalizable tissue resident phenotype can be defined by the reduced expression of integrins associated with migration, diapedesis and/or scanning in lymph nodes, and synapse formation. The reduced expression of αX, α5, and β1 on human bone marrow NK cells is such an example, as αXβ2 is associated with cell migration and, in monocytes, is responsive to ligands that are upregulated during inflammation to enhance scanning, migration, and activation (70, 89). α5 couples with β1 and is also absent on human liver trNK cells (207). Distinct from the salivary glands, lung, and skin epidermis, human bone marrow trNK cells have reduced expression levels of β7, which can couple with either αE or α4 (70). The former binds cadherins, while the latter binds fibronectin or VCAM-1. In other organs, αEβ7 and α4β7 mediate cell localization close to the epithelium, the entry point for many pathogens. This difference is an example of one of the ways that trNKs in mucosal epithelial tissues such as tonsil, gut, skin, and lung, are phenotypically different from those in non-mucosal tissues including spleen, liver, and bone marrow. In terms of integrins, the main difference is that trNK cells rely on high α1 and αE expression in mucosal tissues, which appear to be reduced in non-mucosal trNK cells (66). As high α1 and αE are also accompanied by TGF-β imprinting, this points toward a propensity for some NK cells to switch to a resident phenotype upon entry into the mucosal tissue (57, 58). In the BM, it is less clear that NK cells do this, and it is possible that trNK cells here preferentially develop *in situ* through distinct developmental lineages (**Figure 6**).

αE and α1 Positive Cells Operate a First Line of Defense in the Intestine

In the fetal intestine, a population of EOMES positive, αE positive, and IL7Rα (CD127) negative trNK cells dominates and is present at a stage of development that allows them to directly and indirectly respond to viruses during a time when a newborn is vulnerable due to a developing immune system (183, 231). These innate cells provide a first line of defense while canonical adaptive immune cells are still developing. Given foreign challenges, the tissue residency of NK cells in the fetal intestine is very important and is based on their expression of integrins. It is thought that this population is replaced by an $EOMES^{+}$ T cell population later in life that similarly functions to protect this unique site (183).

In terms of integrins, trNKs in the lamina propria, the connective tissues at the base of the epithelium, are distinct from those in the epithelium. The lamina propria is an elastic, collagen III rich environment, which gets progressively denser in collagen moving away from the epithelium toward the muscle. It is an area rich in fibroblasts and adipocytes, which produce fibronectin, laminin, and collagen III (232). Within the epithelium, there are both $CD45^{+}$ $IL7R^{+}$ ($CD127^{+}$) ILCs and $CD45^{+}$ $IL7R^{-}$ NK cells. Again, trNK cells in the fetal intestine are much better at producing granzyme B and perforin than cNK cells and CD69

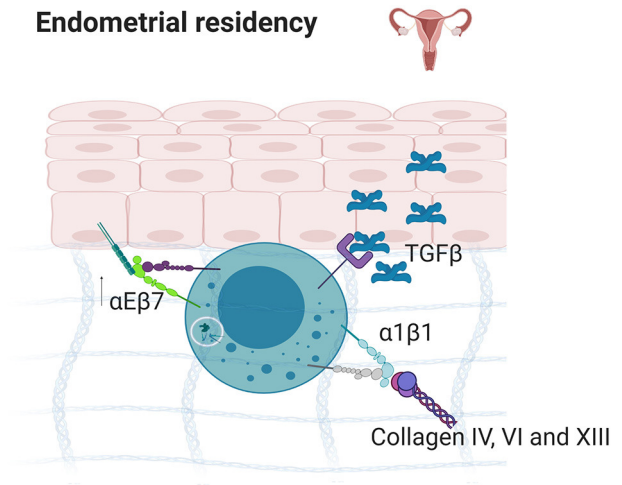

FIGURE 5 | Endometrial residency. TGFβ secretion in the endometrium results in the upregulation of α1β1 and αEβ7 which bind to E-Cadherin and collagens IV, VI, and XIII, resulting in strong tissue residency. Created with BioRender.com.

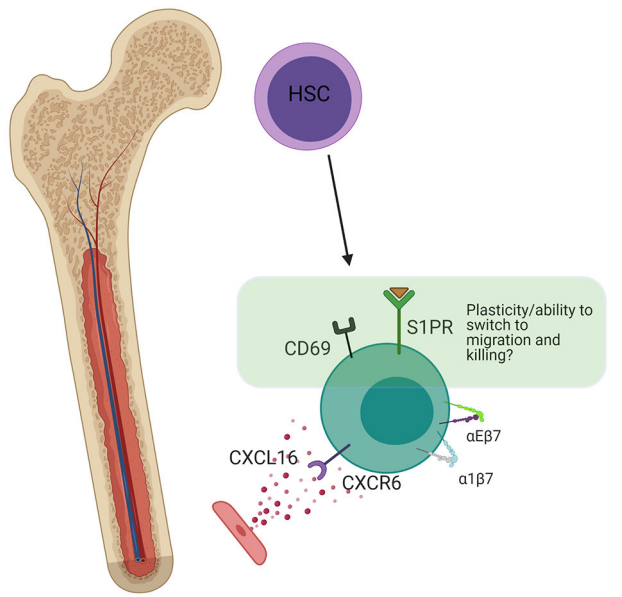

FIGURE 6 | Bone marrow residency. Bone marrow resident NK cells are thought to develop *in situ* from HSCs. The presence of a small amount of S1PR (not present in trNKs in mucosal tissues), indicates the potential for plasticity and switching into a non-resident phenotype. CD69 is still present, which antagonizes S1P signaling, but at lower levels than in the lung. CXCR6 strengthens long-term residency through upregulation of α4β7 and αEβ7. Created with BioRender.com.

positive cells (183), in the case of both epithelial and ECM resident subtypes (**Figure 7**). Neonatal trNK cells are much more potent, in these regards, relative to their adult counterparts, most of which are αE^+ and/or $\alpha 1^+$ and $CD69^+$. Notably, the cells that persist into adulthood are $NKp44^+\alpha E^+CD69^+$, whereas the $NKp44^-\alpha E^+CD69^+$ populations present in infancy die off (183). A failure of this reprogramming may relate to the development of autoimmune disorders (233–235).

THE FUTURE OF trNK STUDY: LINKING SINGLE MOLECULE AND CLUSTERING BEHAVIORS WITH SINGLE CELL AND POPULATION LEVEL BEHAVIORS

Integrins are important for tissue residency between organs and within them, and most of what we know is based on their upregulation or downregulation in terms of expression. By thinking about the upregulation of αE in mucosal trNKs, shown in T cells to be a result of TGF-β secretion, we can turn our attention to plasticity, tracking cells as they develop and move. Human organoid systems combined with DNA barcoding and/or long term microscopy imaging and quantification may now help us to understand which cNKs then go on become trNK cells, compared to which trNK cells arise directly from developmental precursors or immature NK cells. We know that the microenvironment of a given sub-tissue level environment, for example the epithelium in the alveoli at the blood-gas barrier, is distinct, and that this results in a program of distinct integrin expression. Whether trNK cells are hardwired to express this integrin program before they localize and survive in these tissues, or whether cNKs switch to this program upon entry, is an area for further study. Further, while most work so far has been focused on expression levels of integrins, their spatial localization as well as their affinity state dictates the downstream effects of their ligation. To truly understand the complex interplay of multiple integrins functioning in concert, it will be of value to carefully dissect the expression, localization and function of multiple integrins on a single-cell basis in a heterogeneous population within the microenvironment.

Cross Length-Scale Integrin Biology: Linking Nanospatial Phenomena to Heterogenous Cell and Population Behavior

The behavior of nanoscale integrin interactions is as important as their expression. Clustering is a phenomenon that has been shown conclusively for αLβ2 integrin in T cells which form very small integrin-mediated adhesions in the membrane (2, 131). The size, density, and number of molecules per nanoscale cluster is correlated with the number of phosphorylated FAK and Src family kinase molecules in individual adhesions, and the speed of the cell and its actin flow rate, despite different cells having roughly the same amount of total integrin (2). While informative, most work on the nanoscale composition of integrin adhesions has been focused on a single integrin subtype, whereas the co-clustering of multiple integrins remains relatively uninvestigated. α1 and αE are often upregulated together in trNK cells, and an open question is whether such integrins form nanoclusters in the same way as αLβ2 in T cells and whether both integrin species are found in the same cluster. If this was the case, their downstream

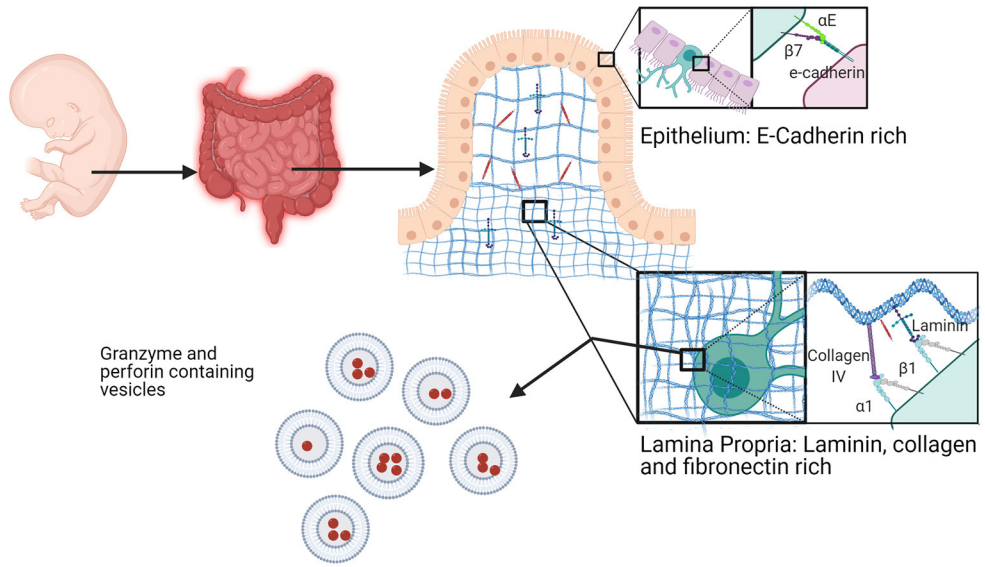

FIGURE 7 | Fetal intestine resident NK cells. NK cells in the fetal intestine exist in the epithelium and the lamina propria. In the epithelium, trNKs express high αEβ7 to bind to E-cadherin at cell junctions. trNKs upregulate α1β1 in the lamina propria, where there is high expression of fibronectin, collagen, and laminin. Fetal intestine trNK cells (of either subtype) have high levels of granzyme- and perforin-containing vesicles. Created with BioRender.com.

signaling partners might work together to produce specific cell behaviors during the switch from tissue resident to cytotoxic phenotype upon recognition of a pathogen. If α1 and αE form separate clustered islands, as seen for other molecules (236), this could indicate competition for ligands that could affect cellular responses. In the case of αEβ7, β7 also binds α4, and both are present in NK cells. Whether this results in αE outcompeting α4 for β7 binding or construction in the ER, or whether both are produced at similar amounts and are regulated spatiotemporally in the membrane is unknown. Such a phenomenon would represent an example of spatiotemporal integrin competition in response to changing conditions of the microenvironment.

trNKs exist in a primed state, where they are simultaneously long-lived and stable, but are ready to switch at a moment's notice to migratory, cytotoxic or recruitment-type behaviors. Whether the integrins that are expressed by these cells mirror this stable but also dynamic behavior is an open question, directly related to cross-length scale biology. Some integrins may act as seeds for signaling, establishing themselves in the membrane prior to recruiting other integrins. Secondary, recruited integrins might add dynamism and competition within integrin clusters that allow cells to switch their adhesive behavior, and could be addressed by using single particle tracking to watch the interaction of two integrins within the same cells. The use of a growing range of small molecule tags (237), namely frankenbodies (238), SunTag (239), and MoonTag (240), and cell permeable bright stable organic dyes (241) may allow this type of nanoscale investigation to be undertaken.

Previously, we have discussed how β1 integrin heterodimers are generally more useful for invoking residency when compared to β2 integrins which induce migratory and/or active cytotoxic phenotypes. On a single molecule level, this could be due to their reduced propensity to form catch bonds in response to mechanical stress across the molecule. This may speak to β1 integrins having different roles as single receivers, rather than as migration-inducing mechanotransducing molecules. One experimental way to address this would be to measure the tension across the integrin using new molecular tools (242) to determine how differential force affects the localization of downstream activated intermediates. Such intermediates might include phosphorylated FAK, Src kinases and phosphatases, which could be imaged by live cell microscopy in NK cells operating in different microenvironments. Such microenvironments are intricate, and new data reveals different integrin adhesome programs that arise in communication with these heterogeneous locales (243). Investigating the tension across molecules, while also measuring the behavior of cells in populations, is another way to link function to behavior using new technologies.

In migratory NK cells, as in migratory T cells, β2 integrins form adhesions which must be turned over, either removed from the membrane and degraded or recycled, very quickly due to the speed of migration of the cell over these anchor points (2). Adaptor proteins and intermediates are therefore likely to be tailored to different rates of transience in the membrane, where high transience is associated with the recruitment of Src family kinases, FAK (2) and Crk (244) in diapedesis and in response to infection. Resident phenotypes associated with high β1 incidence in the membrane may be associated with affecting the production of inhibitory molecules that further promote residence, such as CD69 that inhibits S1PR receptors to prevent egress from tissue (245). This highlights the ability of longer-lived β1 adhesions to induce different behaviors to transient β2 adhesions based on their recruited signaling intermediates. Together, linking

nanoscale/single molecule behavior of integrin αβ heterodimers in nanoclusters at the membrane and inside cells, with individual cell behavior and collective cell behavior in a heterogeneous physiological system is a wider goal of the field. New technologies such as automated super resolution microscopes that scan the plate and image cells based on their shape enable matching of nanoscale molecular organizations with cell movement and behavior (246). Machine learning increases the number of parameters for automated cell selection prior to imaging and enables the ability to segment thousands of cell behaviors post-acquisition and match them to nanoscale phenomena. These technological advances will enable us to link integrin adhesion behaviors on a molecular level to single cell behaviors and population behaviors and are primed to give us access to the molecular basis of NK cell tissue residency, migration and development.

CONCLUSION

With a greater understanding of the importance of trNKs within tissues comes a better appreciation of the cross length-scale role of integrins in their regulation. In multiple tissues, and in most cases, trNK cells arise from developmentally distinct lineages but can also derive from cNK cells that transit from the blood after inflammation in response to integrin and chemokine receptor signaling. Further, we highlight their plasticity, as trNK cells may change their integrin expression to become different upon viral infection and/or long-term residency. Integrins have multiple roles, therefore, in residency and for tuning a functional response for subsequent challenge. This makes sense as integrins are involved in adhesion, migration, and communication with both cells and ECM. Embracing heterogeneity by correlatively imaging diverse populations of NK cells in stromal or organoid based systems will help to further link the functions of nanoclusters of specific integrins that operate a tailored program within single cells to their population-level functions.

AUTHOR CONTRIBUTIONS

MS and EM contributed equally to writing and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Insights Into Human Intrahepatic NK Cell Function From Single Cell RNA Sequencing Datasets

Gráinne Jameson[1] *and Mark W. Robinson*[2]*

[1] School of Medicine, Trinity Translational Medicine Institute, Trinity College Dublin, Dublin, Ireland, [2] Department of Biology, Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Ireland

Diverse populations of natural killer (NK) cells have been identified in circulating peripheral blood and a wide variety of different tissues and organs. These tissue-resident NK cell populations are phenotypically distinct from circulating NK cells, however, functional descriptions of their roles within tissues are lacking. Recent advances in single cell RNA sequencing (scRNA-seq) have enabled detailed transcriptional profiling of tissues at the level of single cells and provide the opportunity to explore NK cell diversity within tissues. This review explores potential novel functions of human liver-resident (lr)NK cells identified in human liver scRNA-seq studies. By comparing these datasets we identified up-regulated and down-regulated genes associated with lrNK cells clusters. These genes encode a number of activating and inhibiting receptors, as well as signal transduction molecules, which highlight potential unique pathways that lrNK cells utilize to respond to stimuli within the human liver. This unique receptor repertoire of lrNK cells may confer the ability to regulate a number of immune cell populations, such as circulating monocytes and T cells, while avoiding activation by liver hepatocytes and Kupffer cells. Validating the expression of these receptors on lrNK cells and the proposed cellular interactions within the human liver will expand our understanding of the liver-specific homeostatic roles of this tissue-resident immune cell population.

Keywords: liver, RNA-seq, liver-resident, intrahepatic, NK cell

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***Correspondence:**
Mark W. Robinson
mark.robinson@mu.ie

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INTRODUCTION

Natural Killer Cells: From Blood to Tissues

The phenotype and function of natural killer (NK) cells have been extensively studied in both mice and humans, predominantly utilizing NK cells isolated from circulating peripheral blood (PB). Circulating NK cells account for 5–15% of the total lymphoid population in PB. In humans two functionally distinct subsets of NK cells are recognized, based on their expression of CD56 and CD16: $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{lo/-}$ (1). The $CD56^{dim}$ NK cells represent the majority of circulating NK cells (∼90%) and are more cytotoxic than $CD56^{bright}$ NK cells (2).

Circulating NK cells are equipped to recognize and kill both tumor and virally infected cells as well as possessing the ability to regulate other immune cells (3, 4). To carry out their effector functions NK cells have an extensive repertoire of stimulatory, costimulatory and inhibitory receptors but, unlike T and B cells, the genes encoding these receptors do not undergo somatic gene recombination. These NK cell receptors include the killer immunoglobulin-like receptor (KIR) family, the CD94(KLRD1)/NKG2 family of C-type lectins, the natural cytotoxicity receptors (NCR)

and as well as a variety other activating and inhibitory surface receptors, as reviewed previously (5–7). It is the balance of expression of these receptors that is necessary to discriminate between healthy host cells and cells in distress, as well as self and non-self-cells. This balance ensures NK cells remain tolerant of healthy tissue whilst effectively clearing any potential threats, such as viral infection or cancerous cells.

In addition to these circulating NK cell populations it is evident that a variety of tissue-resident NK cell subsets exist (8). Tissue-resident NK cells are enriched in lymphoid tissues, the uterus and the liver, as well as a number of other tissues (9–12). Tissue-resident NK cell populations are predominantly $CD56^{bright}$ in humans and they appear to be phenotypically and functionally distinct from their circulating counterparts (8). In the human liver NK cells constitute up to half of total lymphocytes. The majority of intrahepatic NK cells possess a $CD56^{bright}$ phenotype, however their functional roles within the liver remains unclear (8, 13–16).

THE LIVER AS AN IMMUNOLOGICAL ORGAN

The liver has vital metabolic functions, detoxification functions, and immunological functions (17, 18). It is home to a diverse repertoire of resident immune cells including Kupffer cells (KC), NK cells and other innate and adaptive immune cells which are thought to contribute to maintain hepatic tolerance (19–21). The liver receives up to 80% of its blood supply from the gut via the hepatic portal vein, and this blood contains a high concentration of foreign molecules derived from our diet and gut microbiome. Resident immune cells must remain tolerant to this continuous exposure to harmless dietary and commensal bacterial products, while simultaneously being ready to clear any pathogens, malignant cells or toxic products that it may encounter, ensuring organ homeostasis (18).

Intrahepatic NK Cells

Intrahepatic NK cells were first described in rat livers as "pit cells" in the 1970s after their cytoplasmic granules which resembled grape pits (22). For many years it was known that NK cells were enriched in the liver, however the functional significance of this was unknown (14, 23). Murine studies investigating memory-like NK cell responses identified a population of intrahepatic NK cells in murine livers defined as $DX5^{-}CD49a^{+}CXCR6^{+}TRAIL^{+}$ (24–28). In 2015 an equivalent human intrahepatic NK cell population was described within hepatic sinusoids defined by the expression of CD49a (29). These $CD49a^{+}$ intrahepatic NK cells display enhanced pro-inflammatory cytokine responses and memory-like recall responses in *in vivo* murine and *ex vivo* human studies (25, 29, 30).

In human liver this $CD49a^{+}$ intrahepatic NK cell population is highly variable between donors and only represents a minority of the total intrahepatic NK cell population. The majority of human intrahepatic NK cells consist of a $CD56^{bright}$ liver-resident (lr)NK cell population, and are defined phenotypically as $CD56^{bright}CXCR6^{+}CD69^{+}Eomes^{hi}Tbet^{lo}CD49a^{-}CD49e^{-}$ (11, 13, 16, 29, 31–33). Due to the species specific differences between mice and humans this review will focus only on human studies, with the term "lrNK" being used to specifically refer to the human $CD56^{bright}CXCR6^{+}CD69^{+}Eomes^{hi}Tbet^{lo}CD49a^{-}CD49e^{-}$ NK cell population.

Using these defined phenotypical markers the effector function of human lrNK cells have been explored in a number of studies (summarized in **Table 1**). These studies have identified equivalent or reduced cytokine responses and increased degranulation compared PB NK cells and intrahepatic $CD56^{dim}$ populations (**Table 1**). However, to date these findings are limited to functional assays assessing a limited range of markers, upon stimulation with cytokines or HLA class I deficient immortalized cell lines. In this context, data generated from single cell RNA sequencing (scRNA-seq) studies can provide novel insights into potential roles for human lrNK cells beyond IFN-γ and TNF-α production and cytotoxic responses to cytokine activation or HLA-deficient tumor cells.

SINGLE CELL RNA SEQUENCING ANALYSIS OF INTRAHEPATIC IMMUNE CELL POPULATIONS

Recent advancements in scRNA-seq have provided us with a detailed view of the cellular components that make up the human liver (17, 32, 36–39). A number of studies utilizing scRNA-seq technologies have profiled the human liver (summarized in **Table 2**) (36–40). These studies have provided a global overview of the unique liver-specific adaptations of both parenchymal and non-parenchymal cells, including tissue-resident immune cells. This data represents an important resource for the international research community.

These scRNA-seq datasets have sampled all tissue-resident immune cell populations (limited only by the number of single cells sequenced in each dataset). However, subsequent validation experiments and functional work in these studies have, out of necessity, focused on only a few selected cell populations present in the human liver. One population currently lacking a detailed interpretation of the transcription profiles generated in these datasets is lrNK cells.

In a number of published scRNA-seq studies of the human liver NK cell clusters have been identified although the composition of these NK cell clusters varies between studies (**Table 2**). The requirement to define clusters based on genes characteristic of specific cell types represents a limitation of scRNA-seq studies. While phenotypic markers have been well defined, the transcriptional profiles that distinguish between immune cell subpopulations, in particular subpopulations of NK cells, lack extended validation. This issue is compounded by the fact that the optimal number of clusters differs depending on the specific research question. The datasets generated by MacParland et al. (37), Aizarani et al. (38), and Zhang et al. (40), all identified a single cluster of NK cells (**Table 2**). It is likely this single NK cell cluster contains both lrNK cells and circulating NK cells. As such, further analysis of NK cell subpopulations is not possible without reanalysing the raw sequencing datasets in order to generate additional NK cell clusters.

TABLE 1 | Overview of studies that have investigated human lrNK cell function.

References	Intrahepatic NK cell subset (A)	Comparison cell subset (B)	Functional difference (A) vs. (B)	Stimulation
Harmon et al. (16)	Hepatic $CD56^{bright}$	PB $CD56^{bright}$	Enhanced CD107α and reduced IFN-γ expression	MHC Class I deficient K562 target cells effector:target 5:1 for 4 h either with or without overnight rhIL-2 priming
Hudspeth et al. (13)	Hepatic $CD56^{bright}$	PB $CD56^{dim}$	Similar IFN-γresponses	20 ng/ml rhIL-12 and 200 U/ml rhIL-2 for 18 h
Stegmann et al. (11)	Hepatic $CXCR6^{+}$	Hepatic $CXCR6^{-}$	Lower IFN-γresponses	IL-12/IL-18 (5 ng/ml, 50 ng/ml) for 4 h
Aw Yeang et al. (33)	Hepatic $CD49e^{-}$	Hepatic $CD49e^{+}$	Similar IFN-γ and TNF-αresponses	PMA (20 ng/ml) and ionomycin (1 mg/ml) for 6 h
Sun et al. (34)	Hepatic $CD160^{+}$	Hepatic $CD160^{-}$	Higher basal IFN-γ	Unstimulated
Lunemann et al. (35)	Hepatic $CXCR6^{+}CD56^{bright}$	Hepatic $CXCR6^{-}CD56^{bright}$	Reduced IFN-γ and TNF-α	0.221 cells at an effector:target cell ratio of 5:1 for a total of 6 h
Zhao et al. (36)	Hepatic $CXCR6^{+}CD16^{-}$	PB and hepatic $CXCR6^{-}CD16^{+}$	More CD107α vs. both PB and hepatic $CXCR6^{-}CD16^{+}$ More IFN-γ vs. hepatic $CXCR6^{-}CD16^{+}$	IL-12/IL-18 (50 ng/ml) for 6 h

In contrast both the study by Zhao et al. (36) and the study by Ramachandran et al. (39) identified multiple NK cell clusters (**Table 2**). The study by Zhao and colleagues identified differentially expressed genes comparing between $CXCR6^{+}$ lrNK cells and $CX3CR1^{+}$ circulating (c)NK cells (36). The study by Ramachandran and colleagues identified two lrNK cell clusters and one cNK cell cluster, and included lists of differentially expressed genes comparing each individual cluster to all others (39). This study includes liver samples from both healthy controls and patients with liver cirrhosis, and one of the lrNK cell clusters is largely absent in cirrhotic patients highlighting the possibility that NK cell subpopulations may be altered in the diseased liver (39).

To explore the transcriptional profile unique to lrNK cells our review focuses on genes identified in the Ramachandran et al. and Zhao et al. datasets (36, 39). Comparing the lrNK cell and cNK cell clusters between these 2 datasets identifies 180 up-regulated genes associated with lrNK cells, 146 up-regulated genes associated with cNK cells and 19 up-regulated genes shared between lrNK and cNK clusters (**Figure 1** and **Supplementary Data**). Genes up-regulated in lrNK cell clusters include *EOMES* and *CXCR6*, which have been validated at a protein level as phenotypic markers of lrNK cells (11, 13, 16). Genes up-regulated in cNK cell clusters include *ITGAM, S1PR1,* and *SELL*, which are markers of immune cell migration that are down-regulated at a protein level in tissue-resident immune cell populations (31, 33). A variety of activating and inhibitory receptors, downstream signaling molecules, and effector molecules are differentially expressed between lrNK cells and cNK cells (**Figure 1** and **Supplementary Data**). These differentially regulated genes provide opportunities to identify novel markers of lrNK cells as well as providing intriguing insights into potential functions of lrNK cells within the human liver, which are distinct from cNK cells and extend beyond the recognition of virally infected cells or tumors.

Activating the lrNK Cell Immune Response

A number of genes involved in the activation of NK cells are differentially regulated in lrNK and cNK cells in these scRNA-seq datasets (**Figure 1** and **Supplementary Data**). The lrNK cell clusters up-regulate activating receptor genes *CD160*, *CD27*, *CD7*, *IL2RB*, *TMIGD2,* and *TNFSF14* as well as *SH2D1A*, which is involved in signal transduction. In contrast the cNK cell clusters up-regulate activating receptor genes such as *CD226*, *FCGR3A*, *IL12RB1*, and *NCR3*.

A number of these genes are involved in NK cell interactions with hepatocytes and other immune cell populations. *IL2RB* encodes the interleukin-2 receptor β-chain (IL-2Rβ; CD122) and is a major marker of NK-committed cells or NK-progenitors, which allows the cells to respond to IL-15 (41). IL-2Rα forms part of the IL-2 as well as the IL-15 receptor (in complex with IL-15Rα) (42). IL-15 is important for NK cell survival, activation and function (43, 44), is strongly expressed in the liver (45–47), and hepatocyte- and KC-derived IL-15/IL-15Rα directly regulates the homeostasis of liver NK cells via trans-presentation (48). Conversely *IL12RB,* which encodes IL-12Rβ, is upregulated on cNK cells and induces IFN-γ production when stimulated by IL-12, highlighting that cNK and lrNK cells are regulated by different cytokines in the liver microenvironment (49).

$CD27^{+}$ NK cells are rare in the PB yet are enriched in tissues and associated with lower cytotoxic abilities with reduced granzyme B and perforin in comparison to their $CD27^{-}$ counterparts (50). Interestingly, upon binding of its ligand CD70, CD27 is downregulated in an IL-15R-dependent manner suggesting that these lrNK cell's cytotoxicity is tightly controlled

TABLE 2 | Summary of human liver scRNA-seq studies and the NK cell clusters identified.

References	Year	Journal	Sample type	Technology platform	Cells sequenced	GEO dataset ID	NK cell clusters	Descriptor of gene sets available
Zhao et al. (36)	2020	Cell Discovery	Liver perfusate obtained during orthotopic liver transplantation (*n* = 3); Fresh	Library = 10X Genomics, Sequenced = Illumina Hiseq XTEN platform	Magnetically sorted $CD45^+$ cells	GSE125188	Clusters 12 and 13; Figure 2, Supplementary Table 4	DEG comparing CXCR6+ NK vs. CX3CR1+ NK cell clusters (up-regulated genes in each cluster)
Zhang et al. (40)	2020	Journal of Hepatology	Treatment naïve-ICC tissues and paired adjacent normal samples	Library = 10X Genomics, Sequenced = Illumina Xten or NovaSeq 6000 system	Viable single cell suspension post tissue digestion	GSE138709, GSE142784	NK cell; Supplementary Table 2	DEG comparing NK cell cluster vs. all other clusters (no information on up- or down-regulation)
Aizarani et al. (38)	2019	Nature	Healthy liver tissue obtained during resections for CRC metastasis or CC (*n* = 9); Cryopreserved and fresh	mCEL-Seq2	PHH and NPCs or sorted directly after tissue digestion if HCC sample.	GSE124395	Cluster 5; Supplementary Table 1	DEG comparing NK cell cluster vs. all other clusters (up- and down-regulated genes)
Ramachandran et al. (39)	2019	Nature	Healthy liver tissue obtained during resections for CRC metastasis (*n* = 5) Cirrhotic tissue obtained during orthotopic liver transplantation (*n* = 5); Fresh	Library = 10X Genomics/Sequenced= Illumina HiSeq 4000	Viable $CD45^+$ leucocytes vs. $CD45^-$ non-parenchymal cells were sorted via FACS	GSE136103	Clusters NK cell (1), NK cell (2), and cNK cell; Extended Data Figure 3E, Supplementary Table 6	DEG comparing each NK cell cluster vs. all other clusters (up-regulated genes in each cluster)
MacParland et al. (37)	2018	Nature Communictions	Healthy liver tissue obtained during orthotopic liver transplantation (*n* = 5); Fresh	Library = 10X Genomics/Sequenced= Illumina HiSeq 2500	Total liver homogenate	GSE115469	Cluster 8; Supplementary Data 1	DEG comparing NK cell cluster vs. all other clusters (up- and down-regulated genes)

CRC, Colorectal cancer; CC, cholangiocarcinoma; PHH, primary human hepatocytes; NPCs, non-parenchymal cells; FACS, fluorescence-activated cell sorting; DEG, differentially-expressed genes.

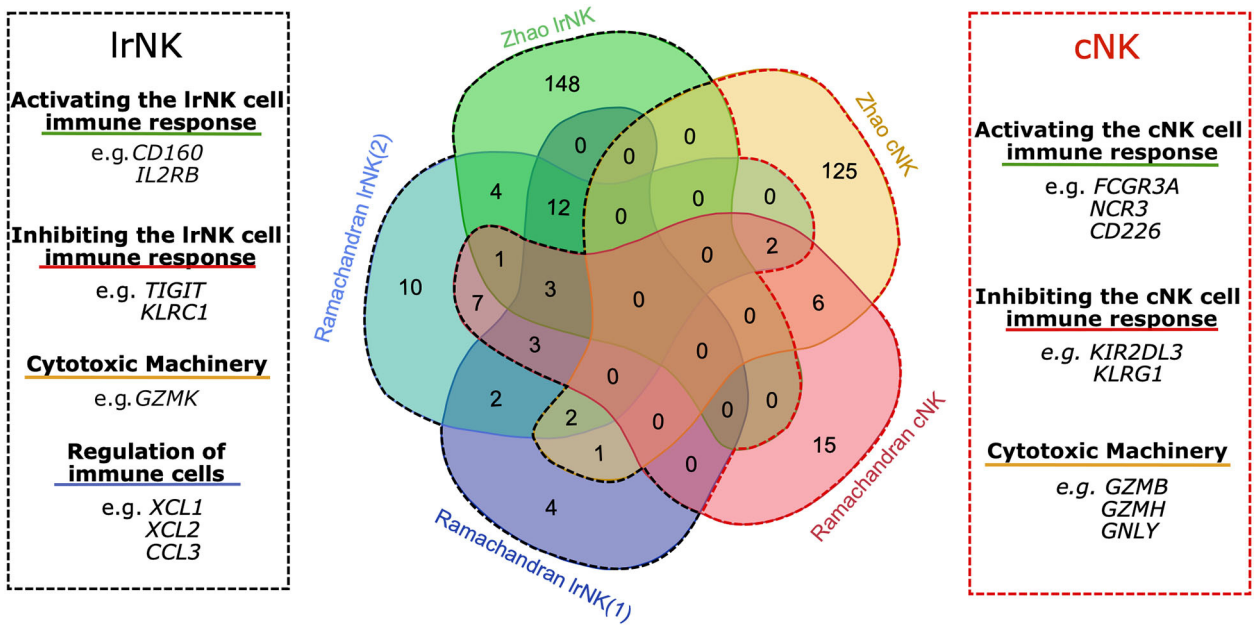

FIGURE 1 | Overlap of up-regulated genes in lrNK cell populations from human liver scRNA-seq datasets. Venn diagram illustrating the overlap of DEG within each NK cell cluster of both the Zhao et al. (36) and Ramachandran et al. (39) datasets. The black dashed line and the red dashed line indicate genes up-regulated in lrNK cell clusters and cNK clusters respectively.

by their expression of IL-15R and CD27 and the local availability of IL-15 and CD70 (51). In the steady state, CD70 levels are quite low, with tightly regulated expression observed upon activation on T cells, B cells and subsets of DCs (52).

Both *CD160* and *TNFSF14* (also known as *LIGHT*) encode proteins capable of binding to herpes virus entry mediator (HVEM; also known as TNFRSF14) (53, 54). HVEM is widely expressed by monocytes, dendritic cells (DC), neutrophils, NK cells, T- and B-cells and downregulated in activated T cells (55, 56). CD160 also binds weakly to classical MHC-I such as HLA-C (57, 58) and the non-classical MHC-I soluble HLA-G (59), while LIGHT also binds to lymphotoxin-receptor (LT-R, also known as TNFRSF3) (60).

Engagement of CD160 or LIGHT on PB CD56^{dim} NK cells activates their cytotoxic effector function enhancing NK-mediated lysis of target cells (54, 58, 61), indicating the possibility that NK cells directly regulate HVEM-expressing immune cells in the liver. Both KCs and hepatocytes express LT-R within the liver. The engagement of LIGHT and LT-R on hepatocytes has also been shown to regulate hepatic lipase expression (62) as well as directly regulating hepatocyte proliferation and liver regeneration in response to partial hepatectomy (63–65).

CD7 encodes an early differentiation marker on common lymphoid progenitors and crosslinking of CD7 induces NK cell activation, proliferation, cytokine production and cytotoxicity (66, 67). Ligands for CD7 include SECTM1 (68) (secreted and transmembrane protein 1) and LGALS1 (galectin-1) (69). SECTM1 is highly expressed by epithelial cells and acts as a chemoattractant for CD7 expressing cells (70, 71). Galectin-1 is also expressed by epithelial cells as well as T regulatory cells. Galectin-1 is capable of suppressing inflammatory immune responses and is essential for regulating inflammation and hepatocyte proliferation during liver regeneration (72).

SH2D1A encodes the signaling lymphocyte activation molecule (SLAM)-associated protein SAP. This adaptor protein mediates downstream signaling from SLAM family receptors on NK cells leading to stable conjugate formation with target cells via LFA-1 (73) and target cell-directed cytotoxic granule polarization and exocytosis (74). SLAM family receptors are immunoglobulins expressed on a variety of immune cell populations that are activated via homophilic binding (except for SLAMF4/2B4 which binds SLAMF2/CD48) (75).

In addition to these up-regulated genes, a number of activating receptors are expressed by cNK cell but not lrNK cells. cNK cells upregulate *FCGR3A* which encodes the CD16 protein, a key marker of CD56^{dim} cNK cells, which is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC). *NCR3* encodes NKp30 and is upregulated on cNK cells equipping them with anti-tumor specific cytotoxic abilities (76). Likewise the *CD226* gene is upregulated on cNK cells and encodes the activating receptor DNAM-I. Interestingly this receptor shares ligands, which include polio virus receptor (PVR/CD155) and Nectin-2 (PVRL2/ CD112), with the inhibitory molecule T cell Ig and ITIM domain (TIGIT) that is upregulated in lrNK cells and is discussed in detail in the next section (77). This differential expression emphasizes how certain stimuli can regulate cNK and lrNK cell populations in different directions.

These differentially expressed activating receptor genes highlight the importance of hepatocytes in regulating lrNK cell survival and imply an important role for lrNK cells in regulating

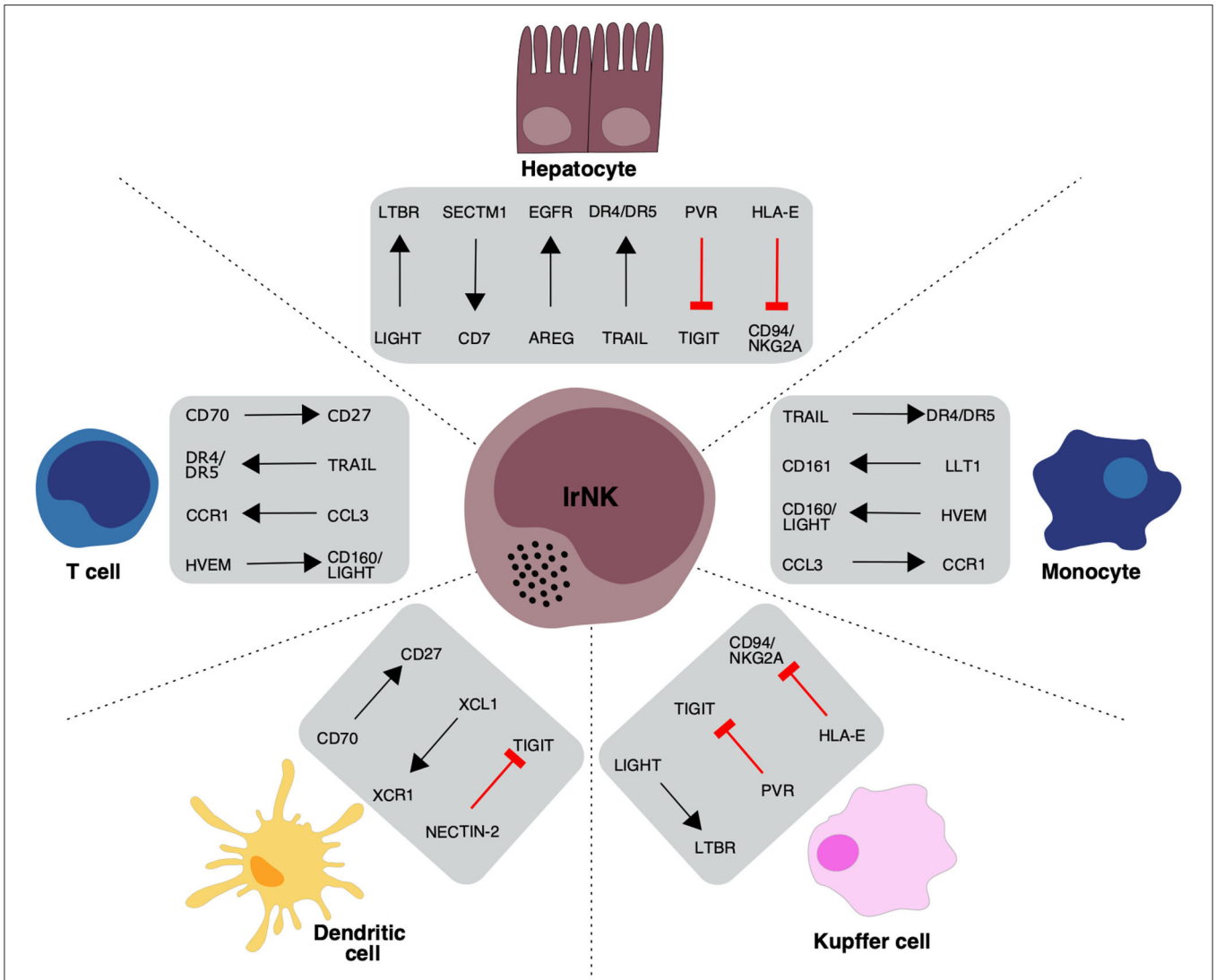

FIGURE 2 | Overview of proposed receptor-ligand interactions between IrNK cells and haemopoietic and non-haemopoietic cell populations in the liver. Black arrows indicate the direction of a stimulatory ligand-receptor interaction and red lines indicate an inhibitory ligand-receptor interaction between interacting cell types.

infiltrating immune cell populations within the human liver (**Figure 2**).

Inhibiting the IrNK Cell Immune Response

In addition to activating receptors, a number of inhibitory receptors are differentially regulated in IrNK and cNK cells in these scRNA-seq datasets (**Figure 1** and **Supplementary Data**). The IrNK cell clusters up-regulate genes including *KLRB1*, *KLRC1*, and *TIGIT*. Conversely, the cNK cell clusters up-regulate *CLEC2D*, *KIR2DL3*, *KIR3DL2*, and *KLRG1*.

TIGIT is an inhibitory receptor expressed by T and NK cells and up-regulated upon activation (78, 79). As mentioned previously TIGIT shares its ligands PVR and Nectin-2 with the activating receptor DNAM-I that is up-regulated in cNK cells (80). TIGIT binds with high affinity to PVR and with lower affinity to Nectin-2, with both interactions resulting in inhibition of NK cell cytotoxicity and IFN-γ production (80–82). Hepatocytes and KCs in the liver express PVR equipping them with the ability to directly inhibit NK cell function (83). Hepatocyte PVR levels are increased during liver regeneration, which is suggested to be a mechanism by which hepatocytes safeguard themselves from TIGIT$^+$ NK cell killing (83, 84). DCs express both PVR and Nectin-2, and engagement of PVR with a TIGIT-Fc fusion protein increased DC IL-10 production, which may further suppress NK cell function in the liver (79). Using ligand-receptor pair databases Zhang and colleagues predicted that LAMP3$^+$ DCs in the liver interact with conventional circulating NK cells via the activating Nectin2-DNAM-I axis and with IrNK cells via inhibitory Nectin-2-TIGIT interactions suggesting that liver DCs may be regulating different NK subsets in opposing directions (85, 86).

KLRD1 (CD94) is up-regulated in both cNK and lrNK cells and can combine with KLRC1 (NKG2A) which is specifically up-regulated in lrNK cells. This heterodimeric complex is a C-type lectin receptor and interacts with the non-classical HLA class I molecule HLA-E on target cells resulting in inhibition of NK cell function. High protein levels of NKG2A have been confirmed on CD49e$^-$ intrahepatic NK cells (29, 35), and NKG2A can be induced by the immunosuppressive cytokines TGF-β and IL-10 which are present at high levels in the liver (87, 88). HLA-E is expressed in the liver by both KCs and hepatocytes providing another molecular pathway through which hepatocytes, and KCs, can inhibit lrNK cell activation. In circulating PB NKG2A$^+$ NK cells that lack KIR have been shown to kill autologous immature DCs that have reduced HLA-E expression *in vitro* (89), and it is possible lrNK cells are likewise capable of effectively targeting immature DCs that express low levels of HLA-E.

An up-regulation of the *KLRB1* gene in lrNK cells provides a further mechanism that may lead to inhibition of lrNK cell function. Engagement of KLRB1 (CD161) by its ligand lectin-like transcript 1 (LLT1 also known as CLEC2D) inhibits NK cell cytotoxicity and cytokine production (90, 91). LLT1 is expressed by B cells and monocytes and the level of LLT1 decreases on monocytes upon activation (92) but it also increased in cNK cell clusters in these scRNA-seq datasets suggesting that cNK cells may regulate lrNK cell function. While hepatocytes in healthy livers express relatively low levels of LLT1, it is increased in the context of liver cirrhosis and this results in reduced NK cell function (93).

In contrast to lrNK cells, cNK cells express a distinct profile of inhibitory receptors. The scRNA-seq datasets reveal an up-regulation of inhibitory KIRs in cNK cells (*KIR3DL2*, *KIR2DL3*). This low expression or absence of KIRs on lrNK cells has been validated at the protein level (33). *KLRG1* is also up-regulated in cNK cells. *KLRG1* encodes an inhibitory C-type lectin receptor that recognizes E-cadherin and N-cadherin and inhibits NK cell activation (94).

These differentially expressed inhibitory receptor genes highlight a number of key regulatory mechanisms by which liver hepatocytes and intrahepatic KCs restrain lrNK cell activity and maintain overall organ homeostasis (**Figure 2**).

Cytotoxic Machinery

Upon activation by a target cell, NK cells can release cytotoxic granules and up-regulate death-inducing ligands, each of which result in target cell apoptosis. These scRNA-seq datasets indicate an up-regulation of *GZMK* and *TNFSF10* in lrNK cells, and an up-regulation of *GNLY*, *GZMB,* and *GZMH* in cNK cells (**Figure 1** and **Supplementary Data**).

TNFSF10 encodes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a transmembrane protein that mediates a death-receptor mediated pathway of cytotoxicity. It has been described as important for both NK cell anti-viral and anti-tumor activity in the liver as it binds to TRAIL death receptors (DR) including DR4 and DR5 on target cells to induce target cell-apoptosis (95–97). Decoy receptors (DcR) also exist that do not induce apoptosis and include DcR1 and DcR2. DR4 and DR5 are expressed in by hepatocytes (98), monocytes, monocyte derived macrophages and activated T cells (99).

Granule-mediated cytotoxicity involves the targeted release of secretory granules containing granzymes (e.g., granzyme-A, -B, -H, -K, and -M), together with perforin and granulysin (100). LrNK cells have elevated transcription of *GZMK* which encodes granzyme-K. Granzyme-K has trypsin-like activity and can induce a rapid cell death independently of caspases (100). In contrast cNK cells have elevated expression of *GZMB* encoding for granzyme-B and *GZMH* encoding for granzyme-H. Granzyme-B mediates apoptosis of target cells using caspase-dependent mechanisms (101–103), while granzyme-H can induce cell death independently of caspases (104). Granulysin is a saposin-like protein that has pore forming abilities as well as anti-tumor and anti-microbe cytotoxic activity and is selectively up-regulated in cNK cells (105–107).

The elevated expression of *GZMK* and *TNFSF10* indicates lrNK cells can utilize both death-receptor and granule-mediated cytotoxic pathways to kill target cells, however, they use distinct mediators in comparison to cNK cells where granzyme-B and granulysin are key cytotoxic mediators.

Regulation of Parenchymal and Non-Parenchymal Cells in the Liver

In addition to mediating cytotoxic responses, NK cells are also capable of producing a wide variety of immunoregulatory molecules, most notably IFN-γ. Within the scRNA-seq datasets lrNK cells upregulate a variety of immunoregulatory genes including *XCL1*, *XCL2*, *CCL3*, and *AREG* which may regulate both parenchymal and non-parenchymal cells in the liver and shape the hepatic immune response (**Figure 2**).

A number of chemokines were identified in lrNK clusters highlighting potential roles in regulating the migration of other immune cell subsets in the liver. XCL1 and XCL2 bind to the XC chemokine receptor 1 (XCR1), which is selectively expressed by cross-presenting CD141$^+$ DC's in circulating PB, commonly referred to as cDC1 (108). These cDC1s are functionally equivalent to CD8$^+$ DC in the mouse, a subset of DC that endocytose stressed cells and dying cells (109, 110) and preferentially present this processed antigen to CD8$^+$ T cells (111, 112). This XCL1-XCR1 axis may provide lrNK cells the ability to regulate DCs within the human liver (38, 39, 113, 114).

The transcripts for *CCL3*, up-regulated in lrNK cells, and *CCL4*, up-regulated in both cNK and lrNK cells, encode CCL3 (also known as MIP-1α) and CCL4 (also known as MIP-1β). Both of these chemokines signal via the CCR1 and CCR5 receptors (115) which are expressed by several cell types including monocytes, NK cells and activated CD4$^+$ and CD8$^+$ T cells (116, 117). Expression of CCL3 and CCL4 allows lrNK cells to directly regulate migration of myeloid cell populations and T cells into the liver. HSCs also express CCR5 and CCL3-engagement induced their proliferation *in vitro* and implicating the CCL3-CCR5 axis in the progression of liver fibrosis (118, 119).

The liver has a remarkable ability to regenerate itself via hepatocyte proliferation. One of the key pathways involved in regulating hepatocyte proliferation is the epidermal growth

factor (EGF) receptor (EGFR). A number of EGFR ligands are known, one of which is amphiregulin (AREG) (120–122), which is up-regulated at a transcriptional level in lrNK cells. Interestingly, *AREG* was only identified in lrNK cell cluster from the Ramachandran et al. dataset. This cluster included lrNK cells from both healthy and cirrhotic liver tissues, suggesting this gene may be up-regulated in lrNK cells in the context of liver disease. AREG is a membrane-bound precursor protein that can be processed and secreted to act in a paracrine or autocrine fashion and is involved in in multiple processes including proliferation, apoptosis and migration of both epithelial and immune cells (123). EGFR is expressed by hepatocytes and HSCs in the liver and the AREG-EGFR axis is known to specifically modulate the hepatic acute phase reaction in the liver regeneration process and is essential for normal hepatocellular survival and proliferation (124, 125). Overall, immune cell derived AREG is suggested to be associated with type 2 immune-mediated resistance and tissue protective mechanisms (123, 126, 127).

CONCLUSION

The up-regulation of specific signaling molecules and receptors on lrNK cells and their enrichment in the liver suggest an important liver-specific role for these cells. Attempts to understand these NK cell functions have been limited by the use of functional assays that assess a limited range of markers. Here we show that scRNA-seq datasets can provide novel insights into possible functional roles of human lrNK cells (**Figure 2**) that may support the development of novel hypotheses to be explored in future functional investigations, once expression at the protein level is validated.

The increased expression of cytotoxic machinery components, including perforin and granzyme-K, equip these lrNK cells with the ability to mediate cytotoxic responses. The activation of this cytotoxicity is tightly controlled by the careful balance of activating and inhibitory receptors, of which lrNK cells express a distinct repertoire. This distinct receptor repertoire may confer the ability to regulate a number of infiltrating immune cell populations within the liver. At the same time this distinct receptor repertoire ensures hepatocytes and KCs can restrain lrNK cell activity and promote organ homeostasis.

Detailed investigations into specific intrahepatic cells that express these receptor ligands and the conditions under which they are induced will aid in our understanding of the liver-specific role of lrNK cells. Validation of potential liver-specific functions of lrNK cells requires a comprehensive analysis of human liver parenchymal and non-parenchymal cell populations, together with the development of novel functional assays. It is evident that a wide diversity of resident intrahepatic NK cell populations exist in the human liver and a detailed understanding of their roles in liver homeostasis and liver disease is essential for our understanding of tissue-resident immune responses within the liver.

AUTHOR CONTRIBUTIONS

GJ and MR contributed equally to the conception and design of the article, interpreting the relevant literature, and drafting and reviewing the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.649311/full#supplementary-material

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REVIEW
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How Do Uterine Natural Killer and Innate Lymphoid Cells Contribute to Successful Pregnancy?

*Oisín Huhn[1,2], Xiaohui Zhao[2], Laura Esposito[2,3], Ashley Moffett[2,3], Francesco Colucci[1,2] and Andrew M. Sharkey[2,3]**

[1] Department of Obstetrics and Gynaecology, National Institute for Health Research Cambridge, Biomedical Research Centre, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom, [2] Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience University of Cambridge, Cambridge, United Kingdom, [3] Department of Pathology, University of Cambridge, Cambridge, United Kingdom

Innate lymphoid cells (ILCs) are the most abundant immune cells in the uterine mucosa both before and during pregnancy. Circumstantial evidence suggests they play important roles in regulating placental development but exactly how they contribute to the successful outcome of pregnancy is still unclear. Uterine ILCs (uILCs) include subsets of tissue-resident natural killer (NK) cells and ILCs, and until recently the phenotype and functions of uILCs were poorly defined. Determining the specific roles of each subset is intrinsically challenging because of the rapidly changing nature of the tissue both during the menstrual cycle and pregnancy. Single-cell RNA sequencing (scRNAseq) and high dimensional flow and mass cytometry approaches have recently been used to analyse uILC populations in the uterus in both humans and mice. This detailed characterisation has significantly changed our understanding of the heterogeneity within the uILC compartment. It will also enable key clinical questions to be addressed including whether specific uILC subsets are altered in infertility, miscarriage and pregnancy disorders such as foetal growth restriction and pre-eclampsia. Here, we summarise recent advances in our understanding of the phenotypic and functional diversity of uILCs in non-pregnant endometrium and first trimester decidua, and review how these cells may contribute to successful placental development.

Keywords: uterine natural killer cell, innate lymphoid cell, pregnancy, tissue resident natural killer cell, placenta, decidua, endometrium

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***Correspondence:**
Andrew M. Sharkey
as168@cam.ac.uk
orcid.org/0000-0002-5072-7748

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INTRODUCTION

The endometrial lining of the uterus is a highly unusual mucosal surface. It is a dynamic tissue that, in response to steroid hormones from the ovary, undergoes shedding, repair, extensive growth and remodelling up to 400 times between menarche and menopause (**Figure 1A**). If pregnancy occurs, the endometrium transforms into decidua to support implantation of the semi-allogeneic blastocyst

Abbreviations: Uterine NK (uNK) and uILC collectively refer to either NK or ILC cells respectively in both non-pregnant (endometrium) or pregnant (decidua) uterine tissue.

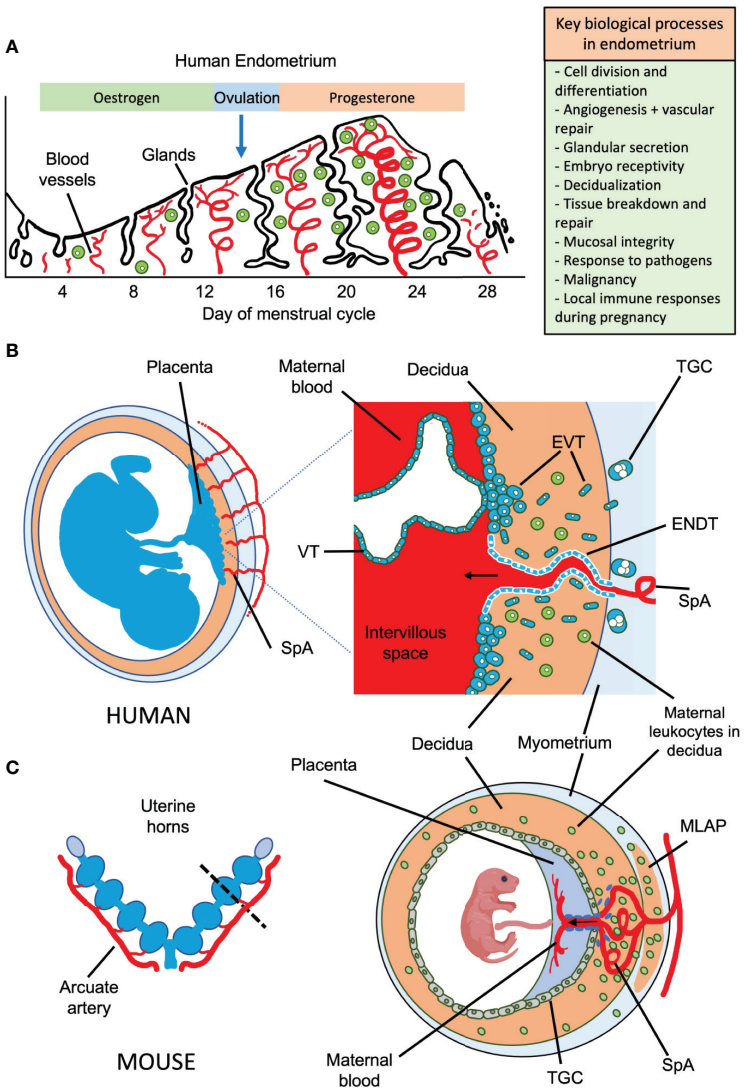


FIGURE 1 | Possible roles of uterine NK cells and ILCs in human endometrium and placental development in humans and mice. **(A)** Important biological processes in endometrium during the human menstrual cycle are shown, which may be influenced by local immune cells. Following menstruation, the endometrium undergoes repair and proliferation under the influence of oestrogen from the ovary and blood supply is re-established involving both spiral arterioles and new capillaries. Following ovulation, progesterone action results in decidualization of the endometrial stroma and a rapid increase in the number of uNK cells, which comprise up to 70% of the leukocytes (shown in green) towards the end of the cycle. Dynamics of other ILC populations in human endometrium through the cycle are less well understood. Murine endometrium cycles by exposure to oestrogen then progesterone but the estrous cycle only lasts ~5 days and there is no menstruation. **(B)** The human maternal-fetal interface. Spiral arterioles (SpA) branch from the radial arteries and penetrate the decidua to supply the placenta. Right hand panel shows placental villi bathed in maternal blood which enters the intervillous space. Placental villi are covered by syncytiotrophoblast, beneath which is the villous cytotrophoblast layer (VT). Anchoring villi attach to decidua *via* columns of extravillous trophoblast (EVT) which differentiates from VT. Some EVT migrates down spiral arteries as endovascular trophoblast (ENDT) while other EVT cells migrate into the decidua as far as the myometrium where they transform into trophoblast giant cells (TGC). Interstitial EVT home to maternal spiral arteries and participate in their remodelling to provide a low pressure, high capacity blood supply to the placenta. During the first half of pregnancy, when spiral artery remodelling occurs, trophoblast invasion in humans is much deeper and more extensive than in mice. EVT invading into the decidua encounter maternal leukocytes (shown in green). In humans in the first trimester, these comprise: ~70% NK and ILCs, 20% macrophages and ~10% T-cells, which include CD4 and CD8 cells. Figure **(B)** adapted from Moffett and Colucci (1). **(C)** Schematic view of pregnant mouse uterus showing multiple implantation sites. Radial arteries branch from the arcuate artery to supply each developing fetus. The right hand panel shows a uterine cross section cut as indicated by the dotted line, with the arrangement of the placenta and decidua at gestation day (gd) ~12.5. The radial arteries penetrate through the myometrium, traversing a specialized structure that develops around mid-gestation, known as the mesometrial lymphoid aggregate of pregnancy (MLAP). The MLAP is not present in humans. Spiral arteries (SpA) branch from the radial artery into the decidua, rich in uterine NK cells but then merge at the interface between the placenta and the decidua to form a large blood canal that supplies the labyrinthine layer of the placenta where gaseous exchange takes place between maternal and fetal blood. This blood canal is lined with specialized fetal trophoblast cells (shown coloured purple). The boundary between the placenta and decidua is delineated by trophoblast giant cells (TGC), which show minimal invasion into the decidua - those few that do invade are largely perivascular. From gd12.5, glycogen rich trophoblast cells invade more extensively into the decidua (later than timepoint shown here), but spiral artery remodelling is largely complete by gd12.5. Panel **C** based on data from Adamson et al. (2).

and subsequent placental development (3). During the first trimester of pregnancy, decidual glandular secretions nourish the developing embryo. Extravillous trophoblast cells (EVT), derived from the blastocyst, invade into the decidua and remodel maternal spiral arteries to become high conductance vessels. These ensure a sufficient blood supply to the developing foetus from ~10 weeks gestation until term (4). This arterial transformation by trophoblast must be carefully regulated to avoid both extremes of insufficient or excessive invasion. Reduced invasion alters resource allocation between mother and foetus and is associated with common diseases of pregnancy including foetal growth restriction (FGR), pre-eclampsia, and miscarriage (5). Excessive invasion by trophoblast can also lead to pregnancy complications, for example when the placenta attaches over caesarean scars or in ectopic pregnancies. Thus, proper development of the maternal-foetal interface is a tightly controlled balancing act. The uterine immune system is involved in regulating this process.

Uterine natural killer cells (uNK) are the most abundant maternal immune cell in secretory phase endometrium and, if pregnancy ensues, constitute up to 70% of leukocytes in first trimester decidua. Numbers then decline substantially towards term (6). Non-NK ILCs are also present in the uterus, albeit in lower numbers, throughout the reproductive cycle. These include ILC1s, ILC3s and LTi-like cells, while the status of uILC2s is unclear. A unique feature of uILCs is that during pregnancy they can encounter trophoblast from a genetically different individual- the foetus. Therefore, uILC responses are regulated by interactions with maternal stromal cells, leukocytes in the decidua and with ligands expressed on EVT. Growing genetic and functional evidence suggests that interaction of uNK and other uILC subsets with EVT regulates the depth of foetal trophoblast invasion (1, 7). In addition, uILCs likely play important roles in homeostasis and tissue remodelling as well as in more canonical immune functions such as controlling pathogens and malignancies. Indeed, uNK can kill CMV-infected decidual stromal cells and are required for effective responses to *Chlamydia trachomatis* (8, 9). Thus, uILCs likely fulfil multiple functions depending on the context and their location (10). Moreover, these are likely to differ between species which have developed different mechanisms to sustain pregnancies. The maternal-foetal interface in human and mouse, and the potential roles uILCs play are summarised in **Figures 1B, C**. In both species, foetally-derived trophoblast cells invade the uterine mucosa during development of the placenta, although the extent of invasion is much greater in humans. This brings them into direct contact with decidual leukocytes, leading to the suggestion that uNK and uILCs regulate key processes during pregnancy.

The ability to perform a wide range of effector functions may be facilitated by heterogeneity within the uILC compartment. The most studied uILC are uNK, originally defined as CD3- CD56+ lymphocytes. There are several recent reviews of uterine ILCs (11–14). The diversity of uNK was apparent from the first phenotypic characterisations (15–17) and has become increasingly evident as high dimensional single cell technologies have been applied to the endometrium and decidua. Here, we focus on how these new approaches have expanded our understanding of the diversity of the uNK niche within ILCs and how these new uNK subsets may regulate normal placental development. We place recent findings in the wider context of ILC biology and discuss several outstanding questions that should guide future studies.

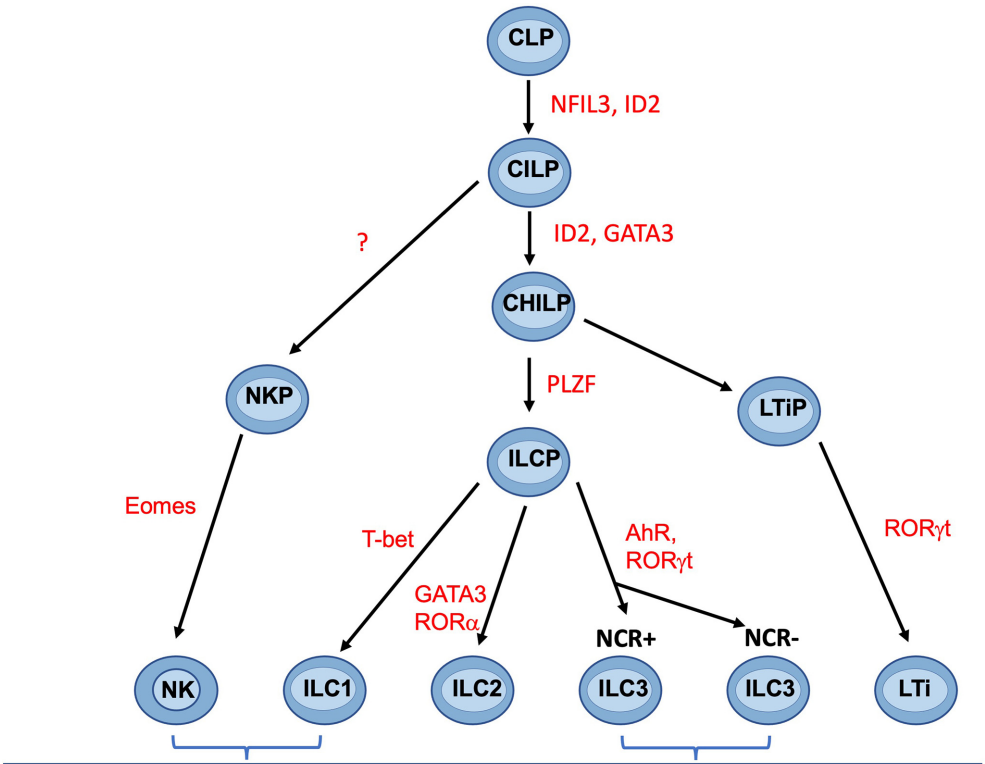
NK AND THE ILC FAMILY OF LYMPHOCYTES

NK cells are members of the ILC family which also contains ILC1, ILC2, ILC3 and lymphoid tissue inducer cells (LTi) (18). Lineage studies show that, in both mice and humans, NK cells and ILCs develop through a common lymphoid progenitor (CLP) but NK and ILCs then diverge (**Figure 2**). The latter proceed through a common helper-like ILC precursor (ChILP) dependent on the transcription factor PLZF (20). Subsequent development of the ILC1, ILC2 and ILC3 subtypes depends on differential expression of transcription factors T-bet, GATA-3 and RORγt respectively (21). In contrast, NK cell development from CLPs proceeds *via* an NK precursor and depends upon Eomes in combination with T-bet (20, 22–25). Several precursors have been identified that can develop into tissue ILC subsets. Identification of specific intermediates in human and murine ILC development is still underway and the mechanisms controlling ILC development may differ from the proposed scheme in certain tissues (26). Tissue ILCs also exhibit considerable plasticity within tissues, so the pathways leading to NK and other ILCs appear to vary in different organs (19). Tissue residency is a hallmark of ILCs, which like NK cells, lack antigen-specific receptors generated by somatic recombination. Although parabiosis experiments and constitutive cell tagging approaches in mice show that, under physiological conditions, ILCs and some NK subsets in non-lymphoid organs are largely tissue resident and maintained by self-renewal (27, 28), recent experiments suggest that ILC circulation is more extensive than previously thought (29). ILC3s can migrate from the intestinal mucosa to local lymph nodes and low numbers of ILCs circulate in the blood (30). Thus, the extent to which individual ILC and NK subsets can exchange with the circulation in different organs including the uterus is still unclear.

ILCs orchestrate context-specific immune responses and fulfil non-immunological roles including tissue remodelling and metabolic homeostasis (21, 31). Whilst the main ILC subtypes have common features across different organs, they acquire distinctive characteristics in response to local cytokines and the tissue microenvironment. Growing evidence for plasticity between some ILC subsets is also emerging, emphasising the need for detailed phenotypic and functional characterisation within each tissue (23, 32, 33). This variation has hampered efforts to define the identity and function of uterine ILCs.

UTERINE ILCs

Although all five main ILC subsets have been identified within the human uterus, the reports are somewhat conflicting and their



Stimulated by	IL-12 IL-15 IL-18		IL-25 IL-33 TSLP	IL-23 IL-1β	IL-23 IL-1β
Cytokines secreted	IFNγ TNFα GM-CSF		IL-4, IL-5 IL-9, IL-13 Areg	IL-22, IL-17 GM-CSF	IL-22, IL-17 LT, TNFα
Selected functions	Viral and bacterial infections Tumour surveillance		Extracellular parasites Airway inflammation	Gut inflammation Mucosal integrity	Formation of secondary lymphoid tissues
Typical phenotypes (human)	NK CD127- CD94+/- CD56+ CD16+/-	ILC1 CD127+ CD161+ CD94-	ILC2 CD127+, CRTH2+ CD117- GATA3^{hi}	ILC3 NCR+ CD127+, CRTH2- CD117+ NKp44+	ILC3 NCR- CD127+, CRTH2- CD117+ NKp44-	LTi CD127+, CRTH2- CD117+ NKp44-, CCR6+

FIGURE 2 | Development and functions of NK cells and ILCs Simplified overview of key stages of ILC development. Scheme shown is largely based on murine data. Table shows typical cytokines that stimulate the main ILC subsets, together with their principal effector molecules and known immune functions. Phenotypic markers commonly used to identify human subsets are shown and assumes prior gating on lin-CD45+ cells. CLP common lymphoid progenitor, CILP, common innate lymphoid progenitor; CHILP, common helper ILP; ILCP, innate lymphoid cell precursor; NKP, NK progenitor; LTiP, lymphoid tissue inducer precursor; LTi, lymphoid tissue inducer; NFIL3, Nuclear factor, interleukin-3 regulated; ID2, inhibitor of DNA binding 2; GATA3, GATA binding protein 3; PLZF, promyelocytic leukemia zinc finger; T-bet, T-box transcription factor 21; Eomes, eomesodermin; AhR, aryl hydrocarbon receptor; ROR, Retinoic acid–related orphan receptor; TSLP, thymic stroma lymphopoietin; Areg, amphiregulin; LT, lymphotoxin; IFNg, interferon-gamma; IL, interleukin; NCR, natural cytotoxicity receptor; NK, natural killer. Figure and phenotypic markers adapted from Vivier et al. (18) and Guia and Mancinelli (19).

exact phenotypic profiles and functional roles remain undefined (34–37). This is partly because studies of the functional properties of uterine ILCs and NK cells were based on phenotypic markers derived from other tissues. This can be problematic because of the phenotypic variability in different locations. Enzymatic disaggregation of tissue can also result in loss of key markers such as CD56 and NKp44; this is reduced by use of mechanical dissociation of tissue (35).

Uterine NK Cells

NK cells were identified phenotypically as CD3−CD56+ granulated lymphocytes in blood (38). Large granular lymphocytes had been

described early in the 20th century in the uterine mucosa, but it was only with the advent of immunohistochemistry that these were shown to belong to the NK lineage (15–17). Studies over decades have relied on gating on CD3−CD56+ to capture NK cells, but this is now known to capture a mixture of both NK and non-NK ILCs in various tissues including the uterus (33, 37, 39, 40).

Endometrial NK Cells: NK cells in proliferative phase endometrium (eNK) comprise ~20% of total leukocytes. After ovulation, eNK then proliferate vigorously during the secretory phase which continues through early pregnancy (17, 41). This is driven by IL-15 secreted by stromal cells in response to progesterone (42). eNK are $CD56^{bright}$, express markers of tissue residency (CD49a, CD69 and CD9), and many canonical NK cell markers (NKG2A, NKG2D and NKp46) but lack CD16 and CD57. The family of killer cell immunoglobulin-like receptors (KIR) are also important in regulating NK activation. KIR expression on eNK differs compared to matched pbNK as well as to dNK and is stable over multiple menstrual cycles (43, 44).

Compared with decidual NK cells (dNK), there are very few functional studies using eNK. They show cytotoxicity against K562 targets and low levels of spontaneous cytokine secretion, which is increased upon activation with IL-15 (41, 45, 46). Their rapid increase after ovulation has led to suggestions that eNK may be important in implantation. Accurate counting is difficult because of their uneven distribution and very rapid changes in number through the cycle, but numerous studies have failed to establish any consistent alterations in numbers of eNK and implantation failure or recurrent miscarriage (47). Nor is it clear that the wide variation in eNK numbers observed between these patients is functionally significant since it is also seen in women with normal fertility (48).

First Trimester Decidual NK Cells: In comparison to eNK, first trimester dNK have been far more extensively studied. Phenotypically they share many similarities with eNK including expression of tissue residency markers, lack of CD16 and CD57, and increased proportion of cells that are KIR+ and NKG2A+ (43, 49–52). Comparison of eNK from the secretory phase with first trimester dNK, identified over 150 transcripts that differed >3 fold, highlighting significant changes that arise after the onset of pregnancy (53). As well as altered transcript levels, there are changes in RNA splicing, resulting in expression of inhibitory rather than activating isoforms of individual natural cytotoxicity receptors (NCR). Ligation of NKp30 or NKp44 therefore induces inhibitory responses in dNK but activation of pbNK (54). This may contribute to the low cytotoxicity displayed by dNK towards HLA class I null cell lines and to trophoblast (55). NK cells can mediate allo-recognition and dNK interact with EVT as the latter invade into the decidua. Because some of these receptors differ between mouse and human, the ligand/receptor interactions between dNK and foetal EVT are distinct in the two species, requiring caution in extrapolating results between the two (**Figure 3**).

Term Decidual NK Cells: As gestation proceeds, the proportion of dNK decreases and by term lin−CD56+ cells represent ~20% of CD45+ cells in decidua parietalis (60). They retain expression of CD9, lack CD16 and CD57, and display decreased expression of KIR, LILRB1 and 9kDa granulysin. There are also functional differences compared with their first trimester counterparts; term dNK degranulate more readily in response to PMA/ionomycin stimulation or K562 but show decreased cytotoxicity to HCMV-infected stromal cells. Thus, uNK are a dynamic population which vary in both function and phenotype depending on the stage of the reproductive cycle and location within the tissue.

ILC1s

ILC1s, originally identified in the tonsils and gut mucosa, are Tbet+ cells that produce IFN-γ in response to IL-12 stimulation (61). Two human ILC1 subsets have been described: "classical" ILC1s (CD56−CD94−CD127+CD117−NKp44−) and intra-epithelial ILC1s (ieILC1s) (CD56+ CD103+) (62). The ILC1 cells identified in the decidua, resemble ieILC1s described in the gut, which express CD56, perforin and granzymes and are cytotoxic. ieILC1s express a unique integrin profile including CD103 and are found enriched in epithelial regions (62). ILC1s appear phenotypically heterogeneous and even display different transcription factor requirements for their development in different tissues so do not readily conform to the scheme outlined in **Figure 2** (63). In secretory phase endometrium no 'classical CD127+ ILC1' cells were detected (35) but both ILC1 and ieILC1 subsets are reported in decidua (36, 37). Indeed, NKp44+CD103+ cells were identified as the major source of IFN-γ within the decidual Lin−CD56+ compartment (64). Although CD56− ILC1s, 'classical ILC1s', are reported in term decidua, comprising ~3% of the Lin−CD127+ compartment, their existence is controversial (64). The gating strategy used to identify ILC1s can capture a mixed population of other leukocytes and hematopoietic stem cells in some tissues (33). Current evidence therefore supports the presence of uterine ieILC1s but the presence of classical ILC1s needs confirming.

ILC2s

Mixed reports also exist for the presence of uterine ILC2s, typically identified as Lin−CD127+CRTH2+ cells. A preliminary report showed ILC2s in small numbers in human non-pregnant endometrium and decidua and suggested they can promote foetal growth in mice (65). In human term decidua, ILC2s (Lin− CD56− CD127+ GATA-3+) were the most frequent CD56− CD127+ ILC, with increased ILC2 in decidua basalis of women with preterm labour (64). Other studies did not detect CRTH2 expression in endometrium or decidua (35, 36). These discrepancies may be explained by the gate used to identify ILCs. As found for ILC1s in the uterus, when gating on CD127+ cells, no ILC2s are detected in early decidua but CD127low/neg are fairly abundant (65). Whether cells corresponding to ILC2s exist in the uterus at all stages of the reproductive cycle is therefore controversial.

ILC3s

ILC3s and Lymphoid Tissue-inducer-like cells (LTi-like) are defined by their expression of RORγt (66). LTi-like ILCs are phenotypically similar to LTi cells, which promote lymph node formation in the developing foetus. ILC3s can be further

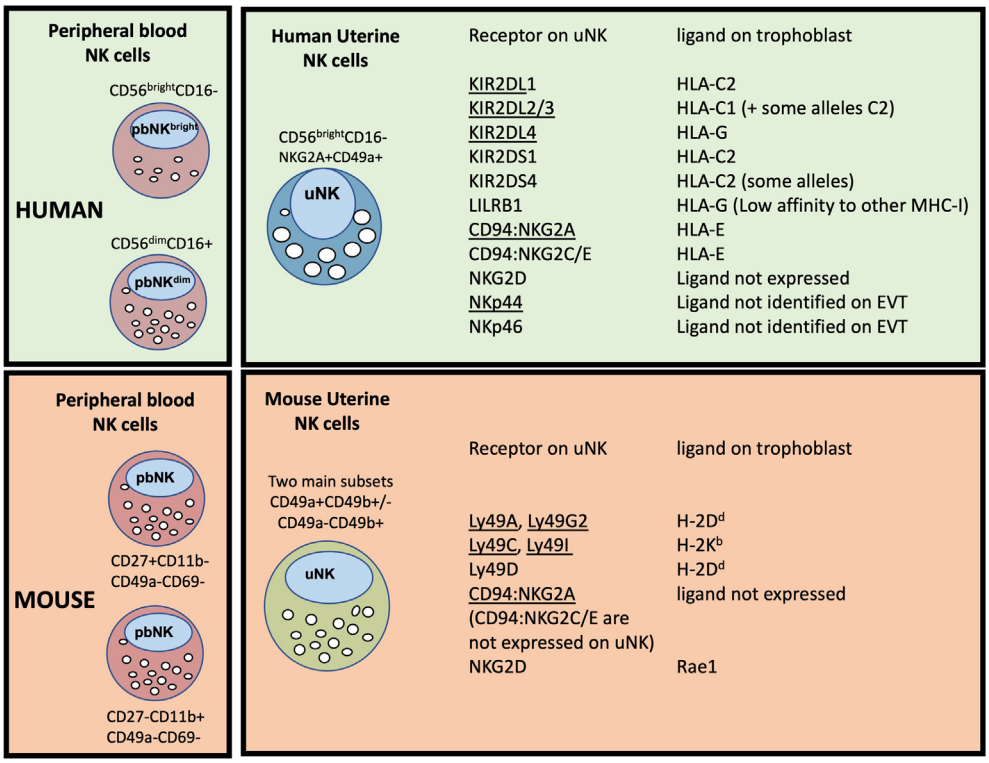


FIGURE 3 | Major peripheral blood (pbNK) and uterine NK cell (uNK) subsets in human and mouse, with possible trophoblast MHC ligands for selected uNK receptors. The two main human pbNK subsets are Lin-CD56bright (~10% of total NK) and CD56dim (~90%); in mice the equivalent NK subsets are lin-CD27+ CD11b+ and CD27-CD11b+ (56). In the human uterus, uNK cells are defined as lin-CD56+CD49a+ (or CD56+CD9+, since both CD49a and CD9 are markers of tissue residency in the uterus). Mouse uterine NK cells are typically gated as lin-NK1.1+NKp46+ and comprise two main subsets, distinguished on the basis of CD49a and CD49b expression (CD49b is often referred to as DX5). Recent studies have shown that uNK in both species exhibit further heterogeneity (described in more detail later). The right hand panel shows selected receptors expressed on uNK cells and whether the corresponding MHC ligands are expressed on human or murine trophoblast cells. Receptors that normally inhibit NK activity are underlined. Note that LILRB1 which is normally considered an inhibitory receptor, has been reported to function as an activating receptor in uNK cells by some authors (57, 58); conversely NKp44 may function as an inhibitory receptor in uNK (54), so is shown underlined. C2+HLA-C, indicates an HLA-C allele carrying a C2 epitope. Full details and original references describing expression of each ligand on trophoblast are available in Gaynor and Colucci (59).

subdivided based on NKp44 expression into NCR+ ILC3s and NCR− ILC3s; the latter are difficult to distinguish from LTi-like ILCs and have typically been treated as one population. ILC3s are described in both human and murine uteri (34, 35, 37). In human endometrium, the majority are RORγt+NKp44+ (~3% of leukocytes) corresponding to NCR+ILC3s (35, 37). Within the decidua, NCR+ILC3s produce IL-22 whilst NCR− ILC3s/LTis produce TNF upon activation (36). Both ILC3 and LTi subsets also induce the upregulation of adhesion molecules ICAM-1 and VCAM-1 on decidual stromal cells, suggesting they may be involved in recruitment of other leukocytes or can perform LTi-like functions (13, 36). Increased ILC3 proportions have also been detected in decidua parietalis of women with preterm labour (64).

These studies suggest that ILCs are represented across all stages of the human reproductive cycle and are dominated by uNK cells. However, the gating strategies needed to distinguish these subsets from one another in the uterine mucosa are not yet clear. For example, new findings that human dNK are CD56+CD94+ but heterogeneous for CD103 expression means it is problematic to identify ieILC1s, which in other tissues are typically gated as CD56+CD94+/−CD103+ (33, 37, 39, 40). For the same reason the presence of uterine ILC2s and classical ILC1s is not clearly established.

Origin of Uterine ILCs

The dynamic changes in numbers of uNK and other uILCs in the endometrium and decidua has led to interest in where they originate. uNK cells have been the focus of most of these studies. Since NK cells originate from bone marrow derived precursors, work has centred on whether uNK cells derive from circulating CD34+ hematopoietic progenitor cells (HPCs) or NK lineage-committed precursors that migrate to the uterine mucosa. Others have examined whether mature blood NK cells can acquire uNK characteristics in the uterine microenvironment. Following bone marrow transfer, donor-derived leukocytes, including NK cells, are detected in endometrium and decidua in both humans and mice, suggesting that *in vivo* these can arise from transferred HPCs (67, 68). Human stage 3 NK-committed precursors can be

detected in blood and can differentiate into more mature NK cells in the presence of IL-15, which is expressed in the endometrium and decidua (34). However CD34+ progenitors are also detectable in both human endometrium and decidua and can rapidly differentiate into NK cells *in vitro* (69). This suggests homing of NK precursors derived from bone marrow or differentiation of stem cells resident in the tissue may both contribute to uNK populations. The latter is supported by the finding that CD56+ NK cells develop in human endometrial tissue xenografted into immunodeficient mice, in response to steroid hormones (70). Mature human NK cells circulating in blood also acquire characteristics of uNK cells, after treatment with cytokines including TGF-β, suggesting that recruitment of pbNK can contribute to uNK populations (71). The idea that both *in situ* differentiation from tissue resident precursors and recruitment from cells circulating in blood contribute to uNK populations is supported by elegant parabiosis studies in mice. These show both mechanisms contribute to increases in uNK at different times: tissue resident cells proliferate during early pregnancy in response to decidualization, while a second wave of recruitment of circulating NK cells augments uNK cell numbers in the second half of gestation (72, 73). Recent data from HLA mismatched uterus transplant recipients in humans suggests that uNK can indeed be replenished from the circulation (74). This approach could be applied to establish whether other uILC subsets can also originate from the periphery and whether peripheral or tissue resident contributions vary during pregnancy, as seen in the mouse.

HIGH RESOLUTION ANALYSIS OF DECIDUAL NK AND ILC POPULATIONS

It is now apparent that gating strategies used to define specific ILCs in one tissue may not readily translate to another because key markers can differ. Recently single cell RNA sequencing (scRNAseq) and high dimensional mass cytometry (CyTOF) have allowed ILCs and NK subsets to be reliably identified in different tissues despite this variation in phenotype and functional responses (33, 56). These techniques, as well as high parameter flow cytometry, have recently been applied to first trimester decidua to provide a more accurate characterization of the phenotype and functions of ILC subsets in the uterus (39, 40, 75–77).

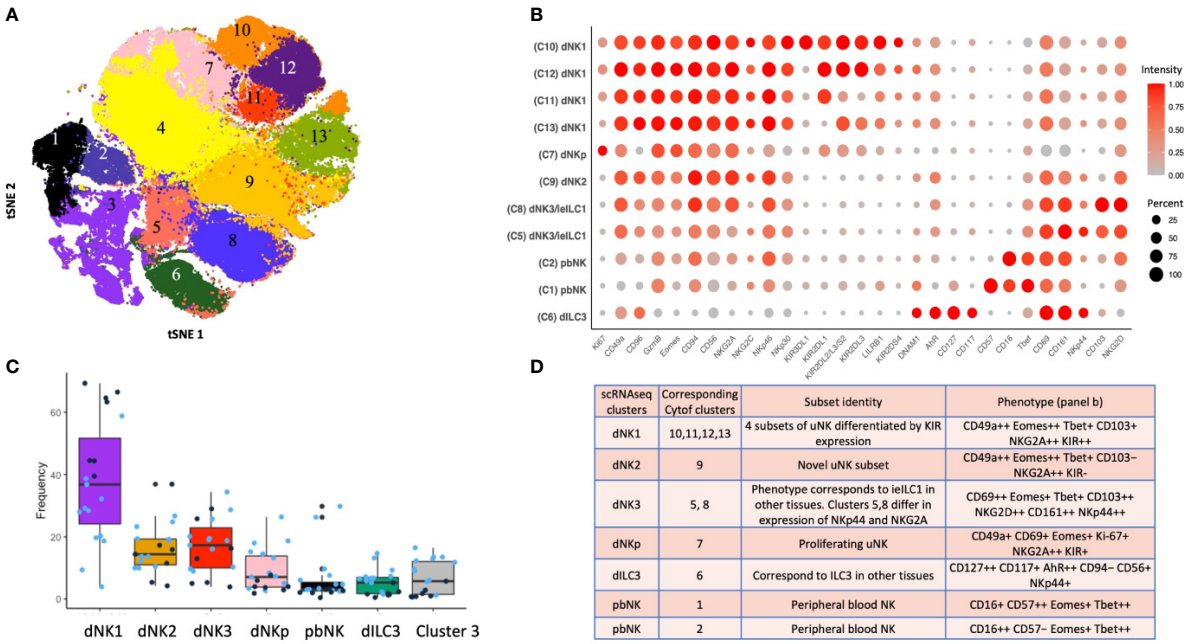
Analysis by scRNAseq provided the first description of three distinct decidual NK populations, termed dNK1-3, as well as a proliferating NK subset (dNKp) and ILC3s (40, 77). dNK1 are characterised by expression of KIR, LILRB1, CD39 and increased granzymes. dNK2 express high levels of ITGB2 and anti-inflammatory ANXA1. Based on their expression signature, dNK3 resemble intra-epithelial ILC1s. No clusters corresponding to ILC2s or classical ILC1s were identified. This analysis also identified significant heterogeneity within other immune, stromal and epithelial cells and allowed the first systematic analysis of potential ligand/receptor interactions between uILCs and other decidual and placental cell types (40, 76, 78). scRNAseq analysis of luteal phase endometrium also identified several NK subsets suggesting that heterogeneity in uNK arises before pregnancy (79).

A complementary analysis of lin−CD45+ decidual cells by CyTOF using a panel of 41 antibodies identified 13 separate clusters of which 11 can be phenotypically assigned to NK or ILC subsets (**Figures 4A, B**). This confirms the presence of multiple dNK subsets at the protein level (39). Clusters 10-13 (c10-13) correspond to dNK1 in the scRNAseq analysis but are further separated by differential KIR expression. Based on a similar receptor staining profile, including high expression of NKG2A and low expression of KIR and LILRB1, c9 corresponds to dNK2, and c5 and c8, which differ based on NKG2A and NKp44 expression, represent dNK3/ieILC1. Two small CD16+ pbNK-like clusters (c1-2) are probably maternal blood contaminants as immunohistochemistry has revealed few CD56+CD16+ NK cells within the decidua itself (17). dNK1 are the most abundant dILC followed by dNK2 and dNK3/ieILC1, although proportions vary between donors and can also be affected by cryopreservation (**Figure 4C**). These ILC subsets and their phenotypes are summarised in **Figure 4D**. Stimulation of ILCs by 'missing self' (K562) or PMA/ionomycin revealed that these dNK subsets significantly differ in their responsiveness, with dNK2 and dNK3/ieILC1 more responsive than dNK1. These are non-physiological stimulations and how each subset responds in more relevant assays such as co-cultures with trophoblast needs exploring.

In the light of these new findings a re-evaluation of previous studies using pre-determined gating strategies to identify dNK subsets is possible. Three populations were found using NKp44 and CD103: NKp44+CD103+, NKp44−CD103+ and NKp44−CD103− (37). Although NKp44+CD103+ are enriched for dNK3/ieILC1, the remaining subsets do not directly correlate with those defined by CyTOF. pbNK cells can display memory-like properties with expansion of specific NK subsets in CMV infection (80). Expansions of uNK subsets have been described in both mice (81) and humans (44, 57, 82). A population of NKG2C^{high} LILRB1+, termed pregnancy-trained decidual NK (PTdNK) is increased in secondary and subsequent pregnancies. Phenotypically, these most closely resemble dNK1 and probably represent an expanded dNK1 subpopulation. PTdNK have epigenetic and transcriptomic profiles that favour IFN-γ and VEGF-A production although the functional studies were performed with dNK activated after IL-15 priming (57). IFN-γ and VEGF-A are not normally produced by freshly isolated dNK1 unless they are strongly activated after isolation, so their role *in vivo* is unclear (83, 84). First pregnancies show a higher risk of low birth weight and pre-eclampsia, which may be due to better extra-villous trophoblast invasion and arterial transformation in subsequent pregnancies (85–87). Further work is required to define the PTdNK subset more accurately to ascertain if they contribute to this effect.

How Do Decidual ILC Populations Compare With Other Tissues?

ILC subset composition varies between tissues and pathological states but in first trimester decidua it differs from other normal mucosal tissues (**Figures 4C**, **5**) (33, 39). Some caution is needed with this comparison as the gating strategies used in the two studies are not directly comparable. The lung contains relatively fewer ieILC1 cells whilst colon, adenoid and tonsil all possess larger proportions of ILC3s than decidua. Intriguingly, in this



scRNAseq clusters	Corresponding Cytof clusters	Subset identity	Phenotype (panel b)
dNK1	10,11,12,13	4 subsets of uNK differentiated by KIR expression	CD49a++ Eomes++ Tbet+ CD103+ NKG2A++ KIR++
dNK2	9	Novel uNK subset	CD49a++ Eomes++ Tbet+ CD103− NKG2A++ KIR-
dNK3	5, 8	Phenotype corresponds to ieILC1 in other tissues. Clusters 5,8 differ in expression of NKp44 and NKG2A	CD69++ Eomes+ Tbet+ CD103++ NKG2D++ CD161++ NKp44++
dNKp	7	Proliferating uNK	CD49a+ CD69+ Eomes+ Ki-67+ NKG2A++ KIR+
dILC3	6	Correspond to ILC3 in other tissues	CD127++ CD117+ AhR++ CD94− CD56+ NKp44+
pbNK	1	Peripheral blood NK	CD16+ CD57++ Eomes+ Tbet++
pbNK	2	Peripheral blood NK	CD16++ CD57− Eomes+ Tbet++

FIGURE 4 | ILC subsets identified by mass cytometry of first trimester decidual cells. **(A)** tSNE landscape of CD45+ CD3- CD19- CD14- HLA-DR- (Lin-) cryopreserved decidual cells stained by mass cytometry. tSNE is coloured by clusters identified by DensVM clustering. 11 of these clusters can be assigned to NK or ILC subsets. Cluster 3 is heterogeneous and cluster 4 appears to contain cells damaged by cryopreservation so these were excluded from subsequent analysis. **(B)** Phenotypic characterisation of selected clusters. Size of the circle is representative of the proportion of the cluster positive for that marker. Circles are coloured by intensity of staining for that marker. Intensities have been scaled by marker (ie within each column). Red corresponds to higher expression and grey to lower expression. Percentage of cells in the cluster staining for each marker is indicated by size of the circles, scaled as shown on the right of the figure. **(C)** Box plots show the proportions of designated clusters within the total CD45+ Lin- decidual compartment. Blue dots = cryopreserved samples, Black dots = fresh samples. [**Figure 4A** adapted from Huhn et al. (39), where details of the antibody panel are described]. **(D)** Comparison of decidual NK and ILC clusters identified by single cell RNA sequencing (scRNAseq) (40) and by mass cytometry (Cytof) (39). Identity of each cluster is based on their profiles of RNA or protein marker expression respectively. Cytof clusters 10-13, correspond to scRNAseq cluster dNK1 and are distinguished by their KIR expression. Cytof clusters 5,8 are distinguished by NKp44/NKG2A expression and correspond to scRNAseq cluster dNK3, representing ieILC. Cytof clusters 3 and 4 identified in panel A appear heterogeneous and don't match any scRNAseq clusters; identities are not established. ++ is > 75% positive staining of cells with antibody in Cytof; + is 25% to 75%; – is <25%.

analysis the decidual ILC composition more closely resembles that seen in colorectal tumours with NK and ieILC1-like cells predominant and few ILC3s (**Figure 5**). This relative lack of ILC3s in decidua is consistent with a recent analysis of normal mucosal tissues, which also demonstrated significant variability in ILC composition at different sites within the same tissue (63). dNK1 are unlike tissue resident NK (trNK) present elsewhere; their expression of high levels of both KIR and NKG2A is unique (33, 88). For example, liver resident NK (lrNK) subsets are defined by CD49a and CXCR6. However, CD49a+ lrNK are KIR+ NKG2A– and CXCR6+ lrNK are KIR– NKG2A+ (89–91). Similarly, dNK2 may resemble CXCR6+ lrNK with respect to KIR and NKG2A expression but differ in terms of CD49a expression. In contrast, dNK3/ieILC1 and dILC3 more closely resemble their counterparts in other tissues. The presence of multiple uNK subsets is suggestive of subset-specific functions.

Microheterogeneity Within uNK Subsets

Our mass cytometry analysis has revealed further phenotypically discrete populations within these novel uNK subsets. Many NK receptors are expressed stochastically, further diversified by environmental factors including NK cell education, epigenetics and viral infections as well as genetic differences between individuals (92, 93). Using Boolean analysis of receptor combinations, pbNK exhibit a large level of receptor diversity within the main $CD56^{bright}$ and $CD56^{dim}$ fractions (94). A similar analysis of CD45+ Lin– CD56+ cells using our CyTOF data reveals comparable diversity within the decidual NK subsets (**Figure 6**). 25 additional markers were included so 2^{25} phenotypic combinations are theoretically possible. For pbNK, up to 6173 phenotypes were identified for a single donor using 28 markers, with the most frequent phenotype present representing ~0.6-4.6% of total pbNK from six donors. Subsets comprising the most frequent 20 phenotypes accounted for between 6.1-25.9% in each donor's pbNK (**Table 1**). For dNK, up to 4745 phenotypes were detected for a single donor. In contrast to pbNK, the top 20 phenotypes represented a larger proportion of the total NK compartment, ranging from 25.2-44.3% between donors. Cells expressing KIR (mainly corresponding to dNK1) occur more frequently in the most common dNK phenotypes in comparison to pbNK. While overall phenotypic diversity appears similar in

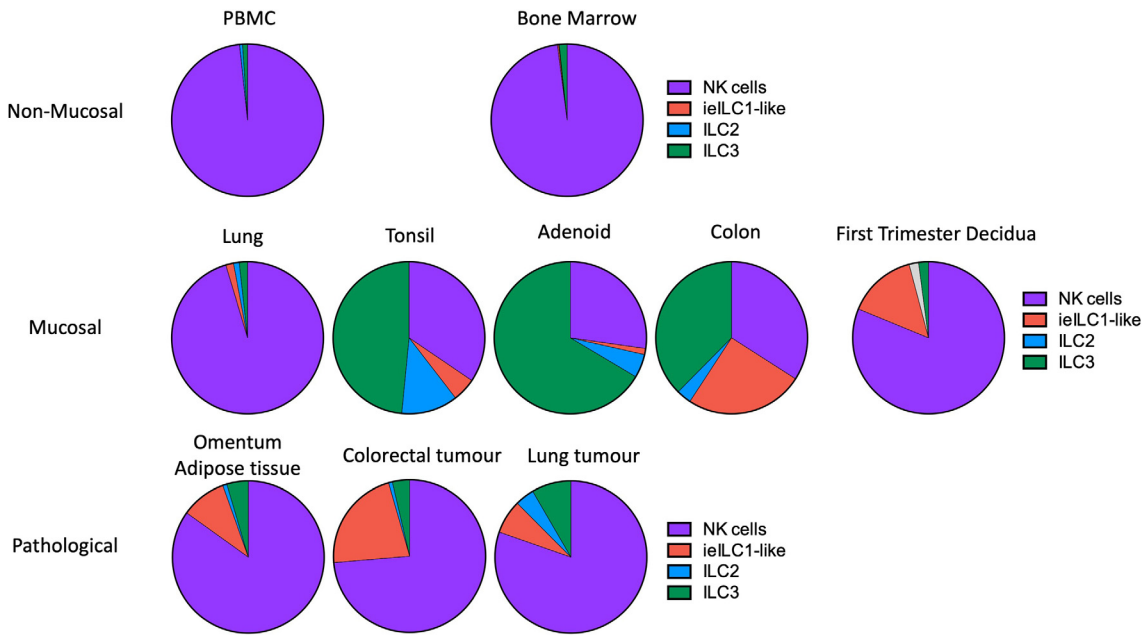


FIGURE 5 | Frequencies of NK and ILC populations in decidua compared with other human tissues. Pie charts showing the frequencies of NK and ILC subsets in decidua (N = 7) compared with normal and pathological human tissues determined by mass cytometry. All charts, with the exception of first trimester decidua come from Simoni et al. (n = 4-9 separate donors for each tissue type) (33). For first trimester decidua, Live CD45+CD3-CD19-CD14-HLA-DR- cells were gated on as the parent population [data from Huhn et al. (39)]. Specified subsets were then identified on a tSNE landscape based on phenotypic profiles and their proportion within this population calculated. Unassigned cells (grey in decidua) could not be confidently labelled as a particular subset based on the markers included in the CyTOF panel. For all other tissues, samples were first depleted of T and B cells and then gated on Live CD45+ FcεR1α– CD14– CD19– CD123–CD34-CD5- cells. Gating strategies to identify each subset were then as follows: NK cells (purple) = CD94+/-CD127+/-CD56+CD103-; ieILC1-like (red) = CD94+/-CD127+/-CD56+CD103+; ILC2 (blue) = CD94-CD127+CRTH2+; ILC3 =CD94-CD127+CRTH2-. [Figure adapted from Simoni et al. and Huhn et al. (33, 39)].

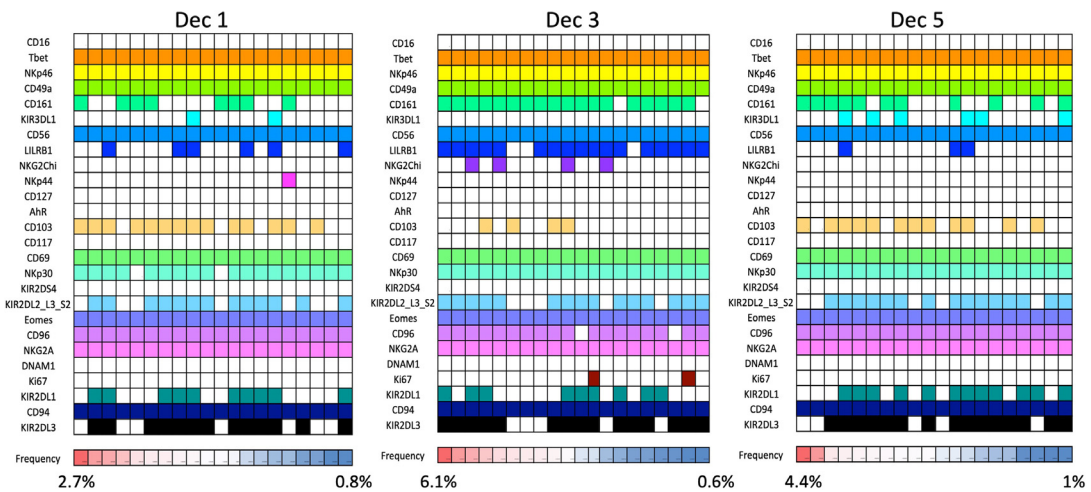


FIGURE 6 | dNK receptor repertoire diversity Frequencies of the most common NK cell phenotypes were determined by mass spectrometry in freshly isolated CD45+Lin–CD56+ decidual NK cells for three selected individuals based on combinations of 26 individual phenotypic markers expressed on each cell. The twenty most frequent phenotypes detected are shown for each donor Dec1,3,5. Each column represents a phenotype shared by a number of cells; boxes are coloured if the marker is expressed in that phenotype. Frequencies of each phenotype as a percentage of total CD45+Lin–CD56+ are displayed as a heat map at the bottom of the figure. Based on analysis of data from Huhn et al. (39).

TABLE 1 | Phenotypic diversity of CD45+Lin–CD56+ cells in human blood and decidua based on receptor profiles of individual cells analysed by mass cytometry.

Donor	Tissue	Input number of cells	Number of phenotypes	Frequency of most frequent NK phenotype[1](%)	Total frequency captured by 20 most frequent phenotypes[1](%)
Pb1	Blood	25683	6173	1.9	18.6
Pb2	Blood	9736	4509	1.9	13
Pb3	Blood	7892	3563	2.2	14.5
Pb4	Blood	10787	3336	4.6	25.9
Pb5	Blood	4164	2882	0.6	6.1
Pb6	Blood	14483	4673	2.2	19.6
Dec 1	Decidua	24031	3792	2.7	25.2
Dec 2	Decidua	24740	4428	3.8	32.5
Dec 3	Decidua	32197	4745	6.1	36.8
Dec 4	Decidua	24670	3206	5.6	36.2
Dec 5	Decidua	32507	3658	16	44.3
Dec 6	Decidua	32935	3261	4.4	31.4

[1]*Frequencies expressed as % of total CD45+Lin–CD56+ cells in blood or decidua. Blood and decidual samples not from matched donors. Data derived from Huhn et al. (39).*

pbNK and dNK, the decidual NK cell repertoire is dominated by a more limited number of highly represented phenotypes, possibly selectively favoured during NK proliferation and maturation. Some may represent developmental intermediates while others include effectors that drive important physiological processes during pregnancy. The immediate challenge is to determine how the phenotypic diversity of uNK and ILC populations within and between women influences pregnancy outcomes.

WHAT CAN WE LEARN ABOUT THE FUNCTION OF UTERINE ILCs FROM MOUSE MODELS?

Establishing the function of human NK cells and ILCs is challenging because of the rapid changes in cell types and ongoing uncertainty about which ILCs are present at different stages of pregnancy. Implantation and early placental development in the mouse do share some features with humans, including decidualisation of stromal cells of the uterine mucosa, and invasion of foetal trophoblast cells into the decidua (**Figure 1** and **Table 2**). Although trophoblast invasion and spiral artery remodelling is necessary in both species to increase the blood supply to the developing placenta, this invasion is far more extensive in humans. Human trophoblast invades both down the blood vessels (endovascular) and through the decidua (interstitial) extending as far as the inner myometrium (**Figure 1B**). Trophoblast cells mediate the classical fibrinoid necrosis of the arterial media that transforms these vessels (95, 96). In mice, trophoblast invasion is minimal and limited to the junctional zone of the placenta during the first 12 days of gestation. Instead, spiral artery modification is associated with NK cells which move into the arterial wall (59, 97, 98).

TABLE 2 | Key Features of human and murine pregnancy models.

Description	Human	Mouse
Duration	40 weeks	~19 days
Implantation sites	Typically one	Multiple implantation sites in each uterine horn
Decidualisation of endometrial stromal cells	Begins in non-pregnant endometrium. Progesterone dependent	Triggered by embryo at site of attachment. Also progesterone dependent
Uterine NK cell numbers	Proliferate in endometrium before pregnancy Increase rapidly in first trimester then decline	Few in endometrium Rapid increase in trNK after implantation starting ~ gestation day (gd) 4.5 Peak at mid-pregnancy ~gd 10 then decline
Trophoblast invasion	Extensive: through decidua and even into myometrium by: Interstitial trophoblast, Endovascular trophoblast, Placental bed giant cells	Limited: to junctional zone until gd12. Limited invasion of decidua thereafter by: Trophoblast giant cells and glycogen cells
Vascular remodelling	Associated with endovascular trophoblast in lumen. Interstitial trophoblast in muscle wall. Some evidence for NK involvement before trophoblast invasion and away from placental bed	Associated with NK cell intravasation into smooth muscle of vessel Mediated in part by IFNγ from NK cells Endovascular Trophoblast limited to central canal in placenta
Localisation of NK cells	Isolated cells and in aggregates In pregnancy, is limited to decidua, Not in myometrium	NK cells in decidua and in the MLAp, embedded in the myometrium

(MLAp is the mesometrial lymphoid aggregate of pregnancy, not seen in humans).

Nonetheless, invading trophoblast cells do encounter maternal NK and ILCs in the murine decidua later in pregnancy after spiral artery remodelling is completed.

There are also important differences in timing as well as localisation of ILC populations. Decidualization is always characterized by extensive accumulation of NK cells in both species, but in humans this process begins in the endometrium, so large numbers of proliferating NK cells are present even before pregnancy begins. In the mouse, NK numbers do not increase until decidualization is triggered by implantation of the embryo. In both species NK cells increase rapidly during the first trimester but decline by term (6, 11). In mice, at mid-gestation, ILCs are located in the mesometrial lymphoid aggregate of pregnancy (MLAp) which develops in the myometrium (11). No such structure exists in humans and the function of the MLAp is unknown. It may participate in the regulation of blood flow since the uterine artery that supplies each implantation site coils through it (99). In addition, NK-derived IFNγ is a key cytokine in murine pregnancies but it is not a major cytokine produced by the main dNK1 subset in humans. Despite these apparent differences in anatomy and cellular dynamics, human and murine pregnancy share key features. These include trophoblast invasion into the decidua and the involvement of both foetal trophoblast and maternal uNK cells in spiral artery remodelling to ensure sufficient blood reaches the haemochorial placenta. This suggests mice can provide a useful model to study the role of NK cells and ILCs in placental development.

NK cells and ILC1s are the most abundant leukocytes in the pregnant mouse uterus (~30% of total leukocytes at mid-gestation), significantly lower than in human first trimester decidua (35). There are three subsets: tissue resident NK (trNK), CD49a+Eomes+, conventional NK (cNK), CD49a−Eomes+, and ILC1s, CD49a+ Eomes− (**Figure 7A**). These all change rapidly in number and location throughout the reproductive cycle (**Figure 7B**). Prior to puberty, mouse endometrium contains predominantly ILC1s. These decline after puberty while cNK and trNK increase (81). ILC2s and ILC3s are also present in low numbers prior to pregnancy, the latter comprising both LTi-like and NCR+ ILC3s (35, 100). ILCs are distributed throughout the tissue in a subset-specific manner. At gestational day 11.5 of pregnancy (gd 11.5), the decidua basalis and MLAp are composed of mainly cNK or trNK respectively. ILC2 and ILC3s are absent from the decidua (35, 36). As outlined when discussing the origins of uNK and ILCs, different rates of subset proliferation, influx from blood or conversion between ILC subsets within the uterus may all contribute to these rapid changes in cellular composition.

Relating human decidual ILC subsets to their murine counterparts is difficult. dNK1 may be analogous to trNK that express high levels of Ly49, Eomes and Ki-67 (28, 81). Murine cNK resemble circulating NK in humans but cNK are present in large numbers in murine decidua whilst CD16+ pbNK are rarely seen in human decidual tissue (17, 35, 73). Murine uILC1 expand in secondary pregnancies and express CXCR6, a hallmark of antigen-specific NK memory cells (101). Thus, they may be the functional counterparts to human PTdNK 'memory' cells but differ in their low Ly49 expression (57, 81). How human dNK2 and dNK3/ieILC1 relate to murine decidual ILCs is also unclear. Murine ieILC1 in the gut are Nfil3-dependent NKp46+ NK1.1+ CD160+ (62). However, *Nfil3*$^{-/-}$ mice contain both uterine trNK and ILC1 but lack cNK cells (100). The difficulties in directly correlating murine and human dILC subsets may reflect the anatomical differences between human and mouse placentation as well as the menstrual versus oestrus cycles in which ILCs and uNK develop preceding pregnancy. Nevertheless, further characterisation may reveal functional homologies between phenotypically distinct uterine ILC subsets between the species.

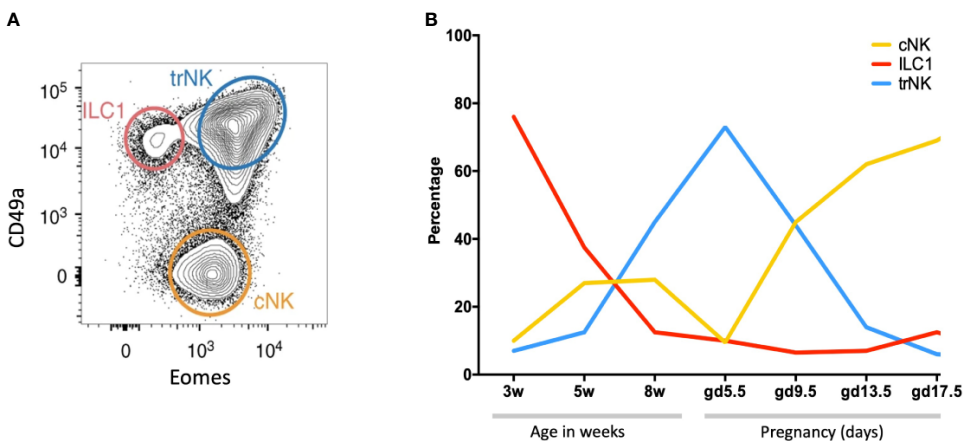


FIGURE 7 | Dynamic changes of NK and ILC1s in the mouse uterus before and after pregnancy **(A)** Gating strategy to identify uterine NK and ILC1 subsets. Cells isolated from whole uterus were analysed together. Representative 2D FACS plot is from gestational day 9.5 (gd 9.5) and gated on Live CD45+CD3−CD19− CD11blow/- NK1.1+NKp46+ cells. ILC1s (red line) are CD49a+ Eomes−, tissue resident NK (trNK, blue) are CD49a+Eomes+, conventional NK (cNK, gold) are CD49a− Eomes+. **(B)** Proportions of indicated uterine subsets at different stages of the reproductive cycle as a percentage of the total Live CD45+CD3−CD19− CD11blow/- NK1.1+NKp46+ cells. w = weeks of age, gd = gestational day [Figure adapted from Filipovic et al. (81)].

FUNCTIONS OF UTERINE ILC POPULATIONS DURING PREGNANCY

Evidence highlighting the importance of uNK cells in supporting successful pregnancies has come from both humans and mice. Loss of functional NK cells and/or ILCs in a variety of mouse models is associated with decidual abnormalities and reduced vascular remodelling (102) and foetal growth restriction (98, 100, 103, 104). For example, pregnancies from an NK- and T cell deficient mouse model, *tgϵ26*, exhibit smaller litters and lower birth weights. Normal birth phenotypes could be restored by bone marrow grafts from SCID mice that lack B and T cells but possess NK cells (105). These changes are in part due to loss of IFNγ secretion by NK cells, since they are rescued by systemic IFNγ administration and similar effects are seen in mice lacking IFNγ or its receptor (102, 103). Even deletion of single receptors on maternal NK cells can result in abnormal foetal development. Mice lacking Nkg2a, Ncr1 or AhR show defective NK cell maturation, abnormal spiral artery remodelling and lower foetal weights (106, 107). Depletion of NK cells in pregnant rats also causes abnormal arterial remodelling and hypoxia, accompanied by differentiation of more invasive trophoblast resulting in abnormal placental development (108). The roles of other ILCs in pregnancy are much less well understood. Mice lacking the transcription factor Nfil3/E4bp4 show greatly reduced numbers of cNK and ILC2s in the uterus but retain trNK, ILC1 and ILC3s. This is associated with placental abnormalities and reduced foetal growth indicating that the altered ILC repertoire affects the outcome of pregnancy (100). The foetal growth restriction in *Nfil3*-/- mice is reversed by infusion of pleiotrophin, supporting the idea that NK or ILC subsets produce factors in addition to IFNγ that promote placental development, but the detailed mechanisms responsible are unclear (104).

Regulation of Trophoblast Migration

A unique feature of pregnancy is that dILCs interact with EVT from the foetus as well as with maternal cells in the decidua. Following implantation, in both humans and mice, foetally-derived trophoblast migrate into the decidua where they encounter maternal immune cells. These foetal cells express an unusual repertoire of MHC class I antigens: human EVT is negative for HLA-A and -B but expresses polymorphic HLA-C and oligomorphic HLA-E and HLA-G molecules (109). Trophoblast are always MHC class II negative. Murine trophoblast from C57BL/6 mice express the MHC class I antigen H-2K^b but very low levels of H-2D^b and Qa-1b. Many of the receptors on uNK that recognise these polymorphic MHC molecules are also highly variable and include members of the killer cell immunoglobulin-like receptors (KIRs) in humans or the Ly49 family in mice (**Figure 3**). The dNK1 subset in particular expresses relevant receptors for trophoblast HLA molecules, including NKG2A, LILRB1 and members of the KIR family (**Figures 3**, **4B**). Their activation status can therefore be influenced by both foetal and maternal ligands. Maternal KIR recognition of HLA-C molecules on EVT is of particular interest because both KIR and HLA-C genes are highly polymorphic so each pregnancy is characterized by different combinations of these genes, resulting in variable activation or inhibition of dNK. Genetic studies of large pregnancy cohorts suggest that women with a KIR AA genotype are at increased risk of pregnancy disorders when the foetus has an HLA-C allele carrying a C2 epitope (C2+HLA-C). This combination results in strong inhibitory signals to uNK *via* maternal inhibitory KIR2DL1 binding to foetal C2+HLA-C on EVT and is associated with increased risk of pre-eclampsia, foetal growth restriction and miscarriage, although not all studies concur (110–113). In contrast, combinations of maternal KIR and foetal HLA-C which favour activation through KIR2DS1 or KIR2DS5 are associated with protection from pre-eclampsia and increased birth weight (110, 112, 114–116).

These are all disorders of pregnancy in which EVT migration and vascular remodelling is reduced (5). More recently the presence of the strongly inhibitory allele *KIR2DL1*003* in mothers, combined with foetal C2+HLA-C ligand has been shown to further increase risk in a dose dependent manner (117). Thus, binding of a specific inhibitory receptor expressed on uNK with the corresponding ligand on EVT can affect pregnancy. These effects are likely to be mediated by dNK1 since they are the main KIR-expressing cells (**Figure 4B**). This idea is supported in a mouse model in which the addition of a single additional MHC molecule (H-2D^d) to the foetal genome results in abnormal uterine artery remodelling and reduced foetal growth. This suggests that maternal recognition of a single paternally-derived H-2D^d molecule influences pregnancy outcome (98). Whether the mechanism involves direct recognition of paternal H2-D^d expressed on trophoblast by inhibitory Ly49 receptors on maternal dNK cells, as seems to occur for human dNK1 and C2+HLA-C on EVT, is not yet certain. In humans, the role of other dILC subsets is less clear. dNK2 and dNK3/ieILC1 express receptors for HLA-E and HLA-G and other ligands on trophoblast, but these are not significantly polymorphic, so will not vary between pregnancies (39, 40). dNK2 and dNK3 respond vigorously to activation, but how they respond to EVT *in vivo* and how the resulting cytokine responses contribute to pregnancy success, is not known. Patients with SCID due to mutations in *IL2RG* or *JAK3*, have very low levels of ILC and NK cells in blood, gut and skin after hematopoietic stem cell transplantation (HSCT). Despite this, two women had clinically normal pregnancies after HSCT and healthy babies of average weight (30). While this study did not investigate uterine ILCs in these women, the results suggest NK cells and other ILCs are not essential for reproduction under normal conditions, but can influence pregnancy outcome by fine-tuning placental development and hence foetal growth. This is likely achieved by fulfilling multiple functions including regulating trophoblast invasion, and remodelling of the vasculature and decidual tissue.

What are the critical responses triggered by KIR in dNK that might affect placental development and pregnancy outcome? dNK produce a wide array of chemokines and cytokines upon stimulation with PMA plus ionomycin, or by ligation of activating receptors (37, 52, 118, 119). Ligation of activating

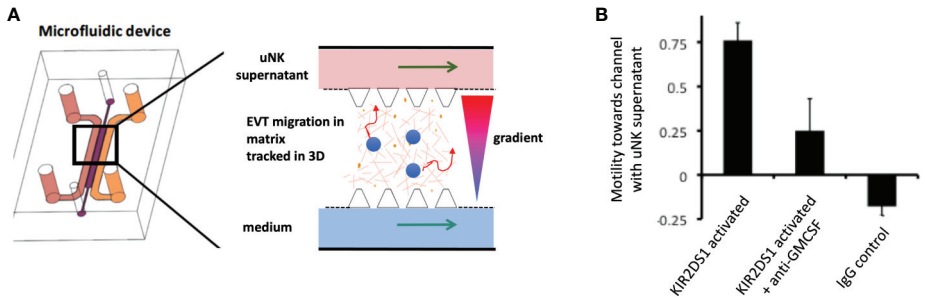


FIGURE 8 | Ligation of activating KIR on uNK affects human trophoblast migration *in vitro*. **(A)** Cartoon showing a cross section of a microfluidics device to study effects of uNK supernatants on migration of extravillous trophoblast (EVT) . Primary human EVT isolated from first trimester placentas are stained with tracker dye (blue) and embedded in Matrigel in the central channel. Cells are tracked in real time as they move between two side channels. These side channels have medium constantly flowing through them and can be supplemented with other factors or supernatants conditioned by uNK cells to create a gradient. **(B)** Plot showing motility of EVT towards or away from a microchannel containing uNK-conditioned medium. Freshly isolated uNK were purified from decidua using microbeads and KIR2DS1 was activated by EB6 antibody cross-linking for 12 hours. Stimulation conditions are shown on x-axis. On average, EVT display increased motility towards a channel containing supernatant from uNK stimulated by KIR2DS1 cross-linking. This is reduced by the addition of neutralising anti-GM-CSF in the supernatant. Control is ligation with isotype matched irrelevant IgG. Motility is calculated by subtracting the number of cells migrating upstream towards the uNK supernatant channel by the number of cells migrating away and dividing this by the total number of cells, [Figure adapted from Abbas et al. (120)].

KIR to simulate dNK/EVT interactions in the decidua stimulates secretion of GM-CSF and CXCL10, which have been shown to increase EVT migration (52, 115, 119). In a 3D microfluidics model, primary trophoblast migrate towards a microchannel containing uNK-conditioned medium following activation of KIR2DS1 (**Figure 8**) (120). uNK have also been shown to secrete TGF-β which reduces EVT migration and this effect changes with gestational age, so uNK responses can potentially enhance or decrease EVT migration (121). Whilst no migration model can replicate the complex tissue environment of the decidua, these genetic and functional studies suggest ligation of activating KIR on dNK can modulate EVT behaviour and thus affect blood flow to the placenta.

Vascular Remodelling in Decidua and Non-Pregnant Endometrium

The role of uNK-derived IFNγ in vascular remodelling in murine decidua is well established (102). Impaired vascular remodelling in the decidua of mice lacking NK cells or specific NK receptors, early in gestation shows that uNK cells may directly participate in this process prior to the arrival of trophoblast (122). This may be a direct effect on the arteries as they infiltrate the media, unlike in humans where uNK are seen around the arteries but not in their wall. Murine uNK also secrete VEGF-C and mice lacking the corresponding receptor VEGFR3 on endothelial cells show reduced vessel remodelling and foetal growth restriction. This shows soluble factors secreted by uNK in mice can target endothelium lining the arteries, as well as the surrounding smooth muscle (123). There is indirect evidence that uNK may also influence spiral arteries around which they are seen to cluster in human endometrium and decidua (95, 124). dNK produce MMP-9 which breaks down extracellular matrix (ECM) of the vascular smooth muscle wall and angiogenic factors such as VEGF-C and angiopoietins-1 and -2 (119, 125–127). Although these could disrupt the vascular smooth muscle wall, as their effect is partially abrogated by Ang-2 inhibition using *in vitro* models, little is known about the mechanisms by which human uNK or EVT actually influence vascular remodelling *in vivo* (121, 128, 129). Evidence that NK cells can play a role in regulating the vascular remodelling of the human endometrium prior to pregnancy is more compelling. Clusters of eNK around spiral arteries express angiogenic factors including VEGF-C and PLGF which can regulate endothelial cell function (125). Administration of a progesterone modulator (Asoprisnil) to women results in absence of eNK, altered arterial morphology and no menstrual bleeding (42). Asoprisnil blocks IL-15 secretion by stromal cells in response to progesterone. Whether the outcomes are due to the reduced uNK or other uILCs or a consequence of blocking other actions of progesterone is unclear. The role of uNK in modifying the arterial media needs exploring further as the signals triggering menstruation and decidual breakdown in miscarriage are still essentially unknown (129). One suggestion is that eNK use the activating receptor NKG2D to kill senescent stromal cells emerging during the secretory phase (130). NK cells kill senescent cells in tumours and in mouse models of liver fibrosis, but whether this is important for homeostasis in cycling human endometrium *in vivo* needs verification (131, 132).

Regulation of Local Immune Responses in the Decidua

It is clear that dNK can recognise both maternal and paternal HLA-C expressed by invading EVT, but allogeneic trophoblast are also potential targets for maternal T cells. dNKs have been shown to secrete cytokines and chemokines which have immunoregulatory functions, leading to suggestions they contribute to foetal-specific T cell tolerance in the decidua *via* a plethora of mechanisms (133, 134). For example, dNK activated *in vitro* to secrete IFNγ, drives IDO production by decidual CD14+ cells and induces expansion of regulatory T cells

(Treg) (135). A caveat is that many functional *in vitro* studies use dNK stimulated by unphysiological levels of IL-15 or other factors and their relevance is questionable. Freshly isolated dNK typically produce low levels of IFNγ and other cytokines, unless strongly activated after isolation (83, 126, 136), and decidual stromal cells have also been reported to secrete IFNγ (137). New approaches such as scRNAseq and spatial transcriptomics are required to investigate how uNK influence responses of other immune cells within the decidua *in vivo*.

Recently, the ectonucleotidase, CD39, was found to be expressed by dNK1; this could combine with CD73 on EVT to convert ATP to adenosine, which is associated with local immune suppression (40, 74). Trophoblast-specific T cells are likely to be HLA-C restricted, since this is the only polymorphic HLA-molecule on trophoblast. In term decidua, Tregs are induced following expansion of T cells in the decidua of HLA-C mismatched pregnancies (138). Many potential mechanisms favouring T cell tolerance in the decidua have been demonstrated *in vitro*, but whether effector T cells ever cause foetal loss in humans, and if dysfunction of dNK or other dILCs contribute to this, is not yet established (1, 133, 139).

Despite the immunomodulatory environment in the decidua, maternal immune cells including ILCs must be able to respond to pathogens. For example, uILC3s produce IL-22 which maintains mucosal integrity, and has been shown to maintain pregnancies in a mouse model of infection-induced pregnancy loss (140). dNK can also counteract infections directly; they inhibit infection of human decidual macrophages by HIV *in vitro* and kill decidual stromal cells infected with HCMV (141). dNK expressing an activating KIR were more cytotoxic against infected decidual stromal cells than dNK lacking that activating receptor. This effect was enhanced when the stromal cells also expressed the cognate ligand for the activating KIR (8, 83). dNK have also been shown to suppress infection by *Listeria monocytogenes* of primary trophoblast cells and decidual macrophages without killing the infected cells (142). Exactly which dNK or dILC subsets are responsible for these antipathogen effects is not established. Thus, uILCs contribute through multiple redundant mechanisms to enhance the resilience of pregnancy to various challenges including infection of the uterine mucosa, while accommodating the invasion of semiallogenic trophoblast.

SUMMARY AND FUTURE QUESTIONS

Uterine ILCs have proved challenging to study because, although they share characteristics with ILCs in other tissues, they have distinctive phenotypes and functions. The factors responsible for this in the non-pregnant endometrium and the decidual microenvironment remain poorly understood. Through the application of scRNAseq combined with mass and flow cytometry we now have a better understanding of which ILCs are present before and during pregnancy (**Figures 4C**, **5** and **Table 3**). In first trimester decidua the predominant ILCs are dNK1, approximately two fold higher than either dNK2 or dNK3/ieILC1s, and the overall composition most resembles mucosal tumours. Our current understanding of uterine ILCs and their responsiveness is summarised in **Figure 9**. Many experiments

TABLE 3 | Summary of NK and ILC populations in human first trimester decidua.

Cell Type	Subset	Phenotype	Frequency[1]	Comment	Key References
NK and ILC1s	dNK1	CD49a+ KIR^{hi} $NKG2A^{hi}$ $LILRB1^{hi}$ $CD39^{hi}$	~55%	Receptor profile suggests direct interactions with EVT	(39, 40, 74)
	PTdNK	$NKG2C^{hi}$ $LILRB1^{hi}$	Not detected	CD56+CD3-$NKG2C^{hi}$ primiparous ~3% multiparous ~17% May be expansions of specific dNK1 subsets?	(57)
	dNK2	CD49a+ KIR^{lo} $NKG2A^{hi}$ $CD103^{lo}$	~15%	Distinct receptor profile from dNK1, but do have receptors for ligands on EVT	(39, 40)
	dNK3/ieILC1	CD49a+ $CD69^{hi}$ $CD103^{hi}$ $NKG2D^{hi}$ $CD161^{hi}$ KIR^{lo} NKG2A +/- NKp44+/–	~15%	dNK3 resemble ieILC1s as identified in many tissues.	(33, 36, 37, 39, 40, 62, 63)
	dNKp	$CD69^{lo}$ Ki-67^{hi} $NKG2A^{hi}$ KIR+/–	~5%	KIR profile suggests these are mainly proliferating dNK1	(39, 40, 50)
	pbNK	$CD49a^{lo}$ CD16+/– CD57+/–	<5%	Contaminants from blood. CD56+ CD16+ cells are rare in decidua by immunohistochemistry	(17, 39, 40)
	Classical ILC1s	CD56– CD117– CD127+	Not detected	Identified at very low frequencies?	(36)
ILC2	ILC2	Disputed	Not detected	Expression of CRTH2 not detected in decidua. Lin-CD56-GATA3+ reported in term decidua	(35, 36, 39, 40, 64)
ILC3	NCR+	$CD127^{hi}$ $CD117^{hi}$ CD94– $NKp44^{hi}$	~2%		(34–37, 39, 40)
	NCR-/LTi-like	$CD127^{hi}$ $CD117^{hi}$ CD94– $NKp44^{lo}$	~2%	NCR-ILC3s are phenotypically very similar to LTIs	

Frequencies (%) as detected by Huhn et al. (39), using CD45+HLADR–CD14–CD19–CD3– (Lin–) cells as the parent population. Samples are fresh (not cryopreserved).

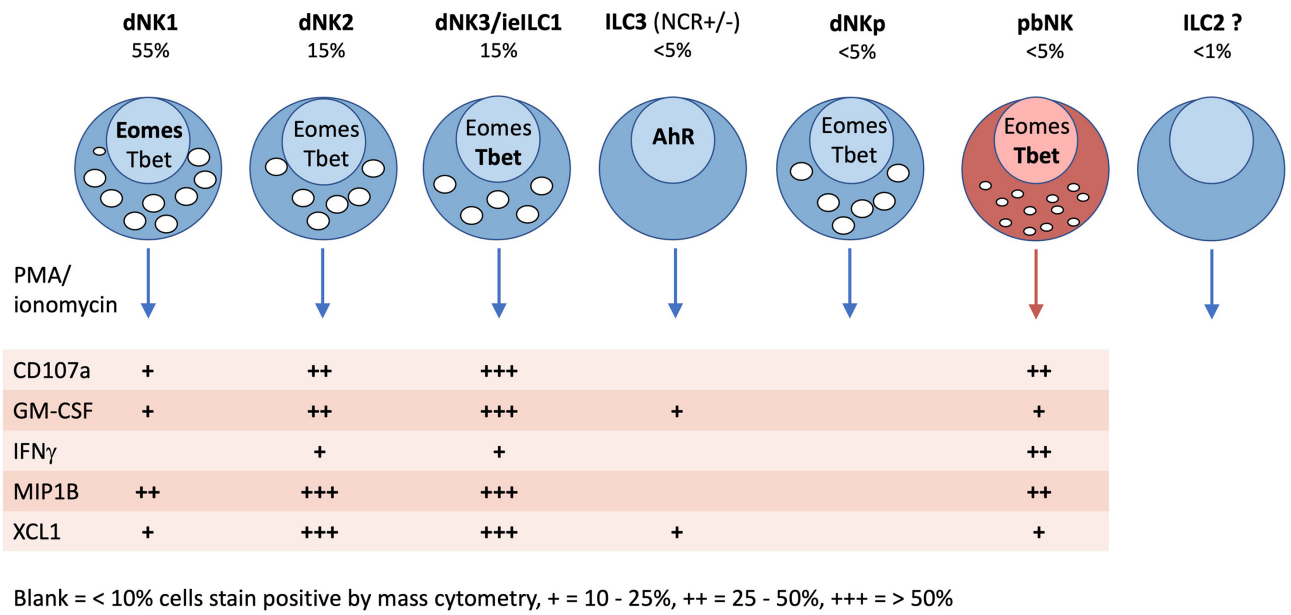


FIGURE 9 | Characteristics of innate lymphoid cells identified in the human uterus by scRNAseq and mass cytometry. Typical frequencies of each ILC type are given as a percentage of total decidual CD45+HLADR –CD14–CD19–CD3– (Lin–) gate in freshly isolated cells (not cryopreserved). Frequencies in endometrium will be different. Transcription factor (TF) expression and cytokine responses following PMA/ionomycin stimulation are based on mass cytometry data of Huhn et al. (39). TFs and cytokines listed in bold, indicate stronger staining. For functional responses, blank = 0-10%, + = 10-25%, ++ = 25-50%, +++ = 50+% staining by mass cytometry.

investigating uNK functions were carried out prior to the discovery of other uILCs, which would have contaminated these preparations. Some of these questions will need to be revisited to establish the functions of different uILC subsets at each stage of the reproductive cycle. A key new finding has been the identification of three distinct decidual NK subsets, dNK1-3. Much of the existing dNK literature focuses on KIR-expressing dNK which are largely dNK1; the properties and functions of the dNK2 and dNK3 subsets remain to be determined, with dNK3 resembling ieILCs in other tissues. These findings raise a number of questions for future basic and clinically applied research regarding uILCs and uNK cells in particular:

1. **What NK and ILC subsets are present and how can they be identified?**
   Identification of clusters in high dimensional datasets often relies on 'emergent' properties where subtle differences across many markers in combination distinguish a subset (143). For human uILCs there is no consensus on simple 2D gating strategies to identify subsets across all the stages of the reproductive cycle. Further work is required to reliably identify and analyse the phenotype and functions of uILC subsets in endometrium and throughout pregnancy.
2. **Where are the NK and ILC subsets located and how does this change?**
   Recent mouse data suggests that the composition of mouse uILCs changes throughout reproductive life and that some uILCs are concentrated at discrete locations. Understanding where human and mouse uILC and uNK subsets localise in the endometrium and during pregnancy will be essential to understanding their functions.
3. **What are the origins of NK and ILCs in the uterus?**
   ILC development in different tissues appears to have divergent pathways as demonstrated by the effects of Nfil3 deficiency on trNK and cNK subsets in the mouse uterus. Which subsets develop *in situ* and which arise from circulating precursors in the human uterus can be addressed by analysing uILCs from patients with bone marrow or uterine transplants with HLA-mismatched donors (74, 144). ILCs also demonstrate considerable plasticity and the relationships between uNK subsets and other uILCs is unclear, although recent evidence suggests that dNK2 can convert to cells resembling dNK1 *in vitro* (76).
4. **What roles do NK and ILC play in repair and homeostasis of the uterine mucosa?**
   While dNK1 appear to be specialised for interactions with EVT through KIR, the roles played by other uNK and uILC subsets is poorly understood in physiological or pathological situations. It will be important to understand how they contribute to mucosal integrity, metabolism, tissue remodelling and antipathogen responses in endometrium and decidua.
5. **Is the huge diversity of phenotypes within known subsets biologically meaningful?**
   In addition to subset discovery, mass cytometry has revealed a previously unappreciated diversity even within well characterised subsets. Lin−CD56+ uNKs exhibit comparable diversity to that reported in pbNK with thousands of related phenotypes defined by stochastic NK

receptor expression (**Figure 6**). Determining if this population level variation is biologically meaningful and whether it affects functions such as embryonic implantation and pregnancy outcome is a future challenge.

6. **Do uNK have memory?**

uILC subsets with a specific phenotype may expand in response to pregnancy or pathogens as previously observed in pbNK (44, 57, 81), where some NKG2C+ pbNK expand in response to CMV infection. How these adaptive NK responses influence pregnancy outcome or pathology will be an important future research question.

In summary, current evidence suggests that uNK and uILCs are important in supporting aspects of normal placental development in both humans and mice, but the effects of NK or ILC deficiency are nuanced. Loss of functional uNK or uILCs is associated with increased incidence of foetal growth restriction, pre-eclampsia or premature labour in murine models. In humans, these can have life-long consequences including increased cardiovascular and neurological disease (145). Genetic studies of large human cohorts support the idea that variants known to alter NK responsiveness are associated with increased risks of pregnancy disorders (110–112, 116). Understanding how uNK and other uILCs contribute to disorders of implantation and pregnancy will be a major challenge for future studies. Knowledge of subset functions will allow accurate assessment of changes in clinical pathology and infertility and may pave the way for targeted immunotherapies.

AUTHOR CONTRIBUTIONS

Conceptualisation: OH, FC, LE, AM, and AS. OH and XZ undertook additional analysis and interpretation of published data. Writing: AS, OH, AM, and FC drafted the manuscript and LE and XZ revised and amended it. Visualisation: AS, OH and XZ developed original figures. All authors contributed to the article and approved the submitted version.

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