NATURAL KILLER CELLS AND MICROBES: BEYOND THE LICENSE TO KILL

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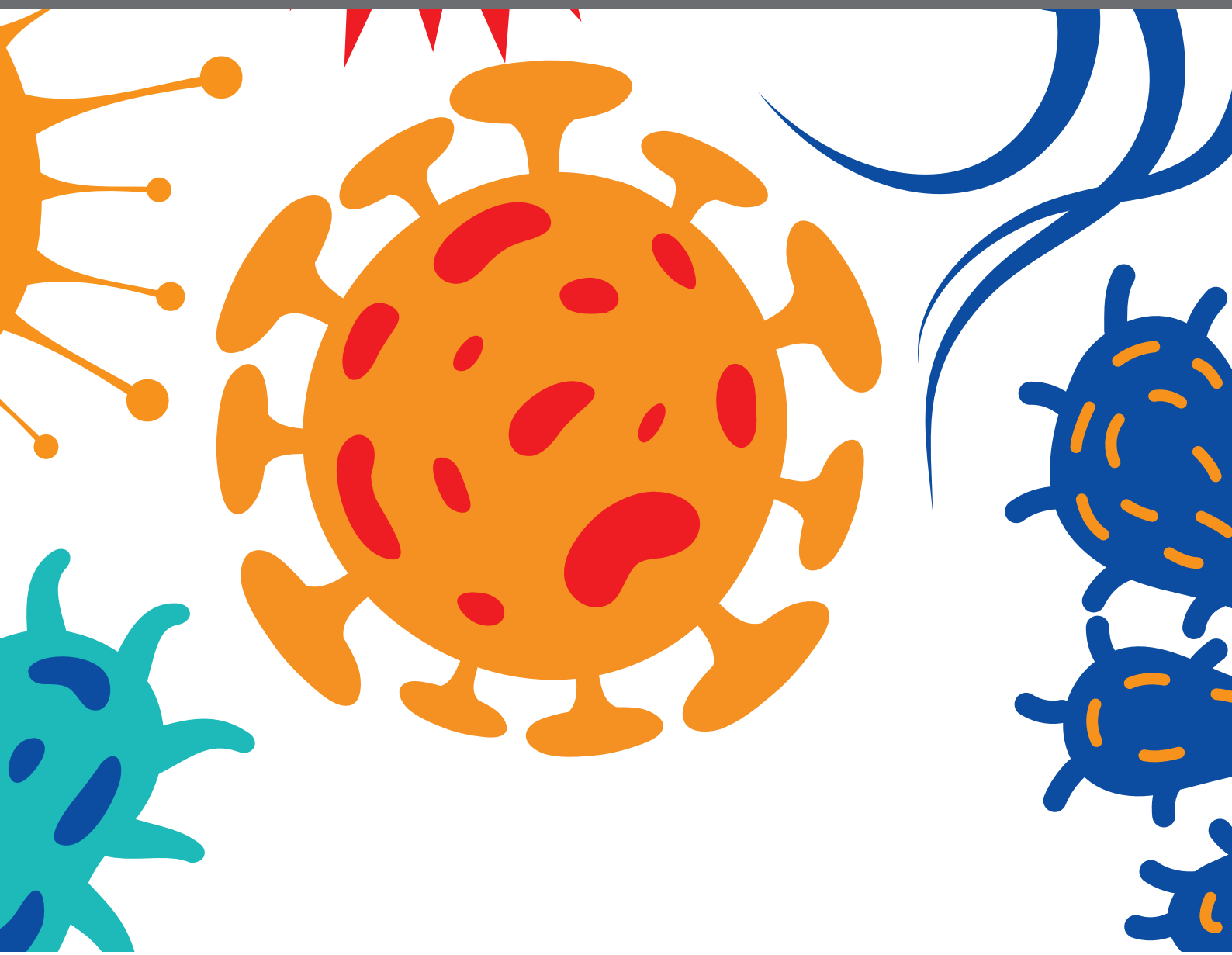

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NATURAL KILLER CELLS AND MICROBES: BEYOND THE LICENSE TO KILL

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BRIEF RESEARCH REPORT
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HLA Upregulation During Dengue Virus Infection Suppresses the Natural Killer Cell Response

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Dengue virus (DENV) is the most prevalent mosquito-borne virus in the world and a major cause of morbidity in the tropics and subtropics. Upregulation of HLA class I molecules has long been considered a feature of DENV infection, yet this has not been evaluated in the setting of natural infection. Natural killer (NK) cells, an innate immune cell subset critical for mounting an early response to viral infection, are inhibited by self HLA class I, suggesting that upregulation of HLA class I during DENV infection could dampen the NK cell response. Here we addressed whether upregulation of HLA class I molecules occurs during *in vivo* DENV infection and, if so, whether this suppresses the NK cell response. We found that HLA class I expression was indeed upregulated during acute DENV infection across multiple cell lineages *in vivo*. To better understand the role of HLA class I upregulation, we infected primary human monocytes, a major target of DENV infection, *in vitro*. Upregulation of total HLA class I is dependent on active viral replication and is mediated in part by cytokines and other soluble factors induced by infection, while upregulation of HLA-E occurs in the presence of replication-incompetent virus. Importantly, blocking DENV-infected monocytes with a pan-HLA class I Fab nearly doubles the frequency of degranulating NK cells, while blocking HLA-E does not significantly improve the NK cell response. These findings demonstrate that upregulation of HLA class I during DENV infection suppresses the NK cell response, potentially contributing to disease pathogenesis.

Keywords: dengue virus, anti-viral response, human leukocyte antigen class I, HLA-E, natural killer cells, monocytes

INTRODUCTION

Dengue virus (DENV) is a positive-strand RNA virus of which there are four serotypes (DENV-1 to DENV-4). The virus is transmitted between humans by its vector, *Aedes* mosquitoes. Each year, an estimated 390 million people are infected with DENV (Bhatt et al., 2013). While most DENV infections are not life-threatening, severe infections can result in hemorrhage, plasma leakage, shock, organ failure, and death (Kyle and Harris, 2008).

The incidence of dengue is rapidly rising (World Health Organization, 2012), increasing the need for a better understanding of how the human immune system responds to DENV infection. There is significant interest in elucidating the role of natural killer (NK) cells during DENV infection. NK cells are innate lymphoid cells that play a key role during the early stages of viral infection. Previous studies have shown that NK cells are activated *in vivo* during DENV infection (Azeredo, 2006; Petitdemange et al., 2016) and that activated NK cells may be an indicator of a positive prognosis (Azeredo, 2006). NK cell activation in response to virally infected cells is dependent on the balance of activating and inhibitory signals from numerous germline-encoded receptors. One such activating receptor, FcRγIIIa (CD16a), mediates antibody-dependent cell cytotoxicity (ADCC), a key bridge between the adaptive and innate immune systems in which antibodies bound to infected cells target them for NK cell killing (Laoprasopwattana et al., 2007; Sun et al., 2017, 2019). NK cells can also kill DENV-infected cells in the absence of ADCC (Costa et al., 2017). Several NK cell receptors, namely DNAM-1, NKG2D, and NKp44 have been implicated in this direct recognition of DENV-infected cells (Beltrán and López-Vergès, 2014; Petitdemange et al., 2014; Costa et al., 2017; Mathew, 2018). However, DENV may also evade the NK cell response, most notably through upregulation of HLA class I (Lobigs et al., 1996; Momburg et al., 2001; Hershkovitz et al., 2008; Glasner et al., 2017; Drews et al., 2018).

HLA class I molecules can bind inhibitory NK cell receptors, mitigating NK cell effector functions against healthy cells. The classical HLA-A, -B, and -C molecules do this by binding to various inhibitory killer-cell immunoglobulin-like receptors (KIRs). The non-classical HLA-E, which presents peptides derived from leader sequences of other HLA molecules, does this by binding to the inhibitory heterodimer CD94/NKG2A (Braud et al., 1998). Viruses can evade NK cell recognition by taking advantage of these inhibitory interactions. *In vitro* studies have shown flaviviruses, including DENV, upregulate total HLA class I as well as HLA-E, leading to inhibition of NK cell activation (Lobigs et al., 1996; Momburg et al., 2001; Hershkovitz et al., 2008; Glasner et al., 2017; Drews et al., 2018). Immune cells, particularly monocytes, are the main targets of DENV infection *in vivo* (Durbin et al., 2008). However, previous studies investigating DENV-mediated HLA class I upregulation and its effect on NK cell activation have used mouse and human cell lines derived from non-immune cells or differentiated primary immune cells (Lobigs et al., 1996; Libraty et al., 2001; Momburg et al., 2001; Cheng et al., 2004; Hershkovitz et al., 2008; Nightingale et al., 2008; Shwetank et al., 2013; Glasner et al., 2017; Drews et al., 2018). This has left a critical gap in our understanding of how undifferentiated primary human immune cell expression of HLA class I is affected by DENV infection, and whether any such changes impact NK cell responses to DENV.

We aimed to determine whether upregulation of class I HLAs, including HLA-E, occurs during *in vivo* DENV infection and, if so, whether this serves to suppress the NK cell response. To address this question, we analyzed peripheral blood mononuclear cell (PBMC) samples from a Panamanian cohort of adult dengue patients and healthy controls for expression of total HLA class I and HLA-E. We then used *in vitro* DENV-infected primary monocytes to determine mediators of HLA class I upregulation. Finally, we co-cultured primary NK cells with autologous, DENV-infected monocytes in the presence of HLA class I blocking Fabs to determine the impact of HLA class I expression on the NK cell response.

MATERIALS AND METHODS

DENV Patients and Ethical Statement

Adult DENV patients with <5 days of symptoms consistent with acute DENV infection (fever over 38°C, severe headache, retro-orbital pain, intense myalgia, arthralgia, exanthema, conjunctivitis, diarrhea, chills, nausea, vomiting, abdominal pain, petechiae, and/or bleeding) were recruited at public health institutions (hospitals belonging to the Ministry of Health, the Social Security System in Panama City, Republic of Panama, and suburban areas). Healthy Panamanian control donors volunteered at Gorgas Memorial Institute of Health Studies. All dengue cases were confirmed by qRT-PCR, NS1 antigen, DENV-specific IgM, and IgG serological testing. The study protocol was approved by the IRB of Hospital del Niño (CBIHN-M-0634), then confirmed by the committees of ICGES, CSS, Santo Tomas Hospital, and Stanford University. Anonymous healthy adult PBMC samples for *in vitro* studies were collected from leukoreduction system chambers purchased from the Stanford Blood Center.

PBMC Sample Processing, Storage, and Thawing

PBMCs were isolated using gradient centrifugation separation by Ficoll-Paque, suspended in freezing media (90% FBS, 10% DMSO), stored at −80°C for 24–72 h, then transferred to liquid nitrogen. PBMCs were thawed, added to complete media (RPMI-1640, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin), centrifuged, and counted.

Mass Cytometry Staining, Data Acquisition, and Analysis

Antibodies for mass cytometry were conjugated using Maxpar® X8 Antibody Labeling Kits (Fluidigm). PBMCs were stained with 25 μM cisplatin (Enzo Life Sciences), live cell palladium barcoded for 30 min at 4°C (Mei et al., 2015), pooled, and stained with surface antibodies for 30 min at 4°C. Cells were fixed with 2% paraformaldehyde in PBS and permeabilized (eBioscience Permeabilization Buffer) prior to intracellular staining for 45 min at 4°C. Finally, the cells were incubated at 4°C in iridium-191/193 intercalator (DVS Sciences) for up to a week, washed once with CyPBS (10X Rockland PBS diluted to 1X in MilliQ water), washed three times with MilliQ water, and diluted with EQ Four Element Calibration Beads before being run on a Helios mass cytometer (Fluidigm). Raw FCS files were normalized using the Normalizer multivariate curve resolution. Normalized files were then de-barcoded using the ParkerICI Premessa de-barcoder. FlowJo® 10.2 was used to gate on live cells. viSNE analysis was performed in Cytobank.

Human sHLA-E ELISA Testing

Human sera from 6 DENV confirmed patients and 31 healthy donors were diluted 1:10 and assayed with the Human MHCE/HLA-E ELISA Kit (Biomatik) per manufacturer's instructions. Optical densities were used to calculate concentration (ng/mL) with a 4 parametric logistic regression analysis using GraphPad Prism 7.

Monocyte and NK Cell Preparation

Monocytes were isolated from PBMCs by negative selection using a human Pan Monocyte Isolation Kit (Miltenyi). Autologous NK cells were isolated by negative selection using a human NK Cell Isolation Kit (Miltenyi) and cultured in complete RPMI-1640 media with 300 IU/mL of IL-2 (R&D Systems) for 22 h.

DENV Infection of Primary Monocytes and Analysis of HLA Expression

Aedes albopictus C6/36 cells were infected with DENV-2 laboratory strain 429557 (NR-12216). Supernatants were harvested on day 7 or 8 post-infection, filtered, and ultracentrifuged on a D-sorbitol cushion at 59,439 RCF at 4°C for 3 h. Virus was titrated using a Vero cell focus-forming assay (Bayless et al., 2016). Concentrated virus was stored at −80°C. Virus was UV-inactivated at 500 μJ × 100 on ice in flat-bottom 96-well plates using a Stratagene UV Stratalinker. Virus inactivation was verified by focus-forming assays. All experiments were repeated with multiple virus batches. Monocytes were mock-infected, exposed to UV-inactivated DENV-2, or infected with active DENV-2 at a multiplicity of infection (MOI) of 2 for 2 h in infection media (RPMI-1640 media with 2% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 20 mM HEPES). After 2 h, cells were washed, resuspended in 24 h infection media (infection media without HEPES), and incubated at 37°C, 5% CO_2 for 22 or 46 h. Cells were stained with FITC-conjugated anti-CD3 (UCHT1, BioLegend), FITC-conjugated anti-CD7 (CD7-6B7, BioLegend), PE-Cy7-conjugated anti-HLA-E (3D12, BioLegend), PE-conjugated anti-pan HLA class I (W6/32, BioLegend), flavivirus group antigen (4G2, Novus Biologicals) conjugated to Alexa Fluor™ 647 using Alexa Fluor 647 Antibody Labeling Kit (Life Technologies), and LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (Life Technologies) before analysis on a MACSQuant Analyzer and FlowJo® 10.2.

Supernatant Swap Assay

Conditioned supernatants from aforementioned DENV-infected primary monocyte cultures were UV-inactivated as previously described. They were then used to culture primary monocytes from the same donors from which the supernatants were collected. After a 24 h incubation, monocytes were stained with APC-conjugated anti-CD3, APC-conjugated anti-CD7, PE-conjugated anti-pan HLA class I, and Zombie Aqua Fixable Viability dye (BioLegend), then analyzed using a Cytek™ Aurora analyzer and FlowJo® 10.2.

Quantification of Cytokine Production by Luminex

The concentrations of cytokines in conditioned supernatants from the aforementioned DENV-infected primary monocyte cultures were assessed in duplicate using a multiplex cytokine assay by Luminex per the manufacturer's instructions.

NK Cell Degranulation Assay

Monocytes were infected with DENV-2 at an MOI of 2 and incubated for 24 h. After incubation, monocytes were left unblocked, blocked with an anti-pan human HLA class I Fab (generated from DX17, BD Bioscience), with an anti-human HLA-E Fab (generated from 3D12, BioLegend), or with isotype-matched control mouse IgG1 Fab (generated from MG1-45, BioLegend) all at 7.3 μg/mL for 30 min before adding autologous, IL-2-activated NK cells at a 1:5 effector to target (E:T) ratio. Fabs were produced using mouse IgG1 Fab F(ab)2 Kits (Thermo Scientific) and verified by gel electrophoresis and Coomassie Blue staining. During the 4 h co-culture, cells were incubated with brefeldin A (eBioscience), monensin (eBioscience), and APC-H7-conjugated anti-CD107a (H4A3, BD Bioscience) per manufacturer's instructions. Cells were stained with PerCP-Cy5.5-conjugated anti-CD3, FITC-conjugated anti-CD7, Alexa Fluor 700-conjugated anti-CD16 (3G8, BioLegend), PE-Cy7-conjugated anti-CD56 (HCD56, BioLegend), and Zombie Aqua Fixable Viability dye, then analyzed using a Cytek™ Aurora analyzer and FlowJo® 10.2.

Statistical Analysis

A Friedman test, followed by Dunn's multiple comparisons test was used to determine significant differences between paired data with three conditions. A paired Wilcoxon signed-rank test was used to determine significant differences between DENV– and DENV+ cells. A Friedman test with FDR correction followed by a one-tailed Wilcoxon matched-pairs signed-rank test with a holm correction was used to analyze the Luminex data. Fab blocking data was analyzed using a Friedman test followed by paired Wilcoxon signed-rank tests. All statistical analysis was done using GraphPad Prism 8, R version 3.4.2, R version 3.6.0, and the compare_means function in the open source ggpubr R package.

RESULTS

We evaluated HLA class I expression on PBMCs from a Panamanian cohort of 8 qRT-PCR confirmed, DENV-2-infected adults within 5 days of symptom onset and 31 healthy Panamanian adult controls (**Supplementary Table 1**). The expression patterns of HLA class I across cell subsets were visualized with viSNE. This algorithm separated immune cell subsets into clusters based on expression of key lineage markers; manual gating confirmed cluster identity (**Figure 1A** and **Supplementary Figure 1A**). Analysis based on protein expression revealed marked upregulation of total HLA class I and HLA-E in DENV-infected adults compared to healthy controls across multiple immune cell subsets (**Figure 1B** and **Supplementary Figure 1B**). As HLA-E can also be shed as

soluble HLA-E (sHLA-E), which has been implicated as a potential viral mechanism of NK cell evasion (Shwetank et al., 2013, 2014), we performed an sHLA-E ELISA. There was no significant difference in the concentration of sHLA-E between the DENV-infected adults and healthy controls (**Supplementary Figure 2**). Together, these findings indicate that upregulation of HLA class I occurs on the cell surface of multiple immune cell subsets during acute *in vivo* DENV infection.

Interestingly, viSNE visualization revealed that upregulation of total HLA class I and HLA-E was not uniform across all monocytes. Instead, there were clear HLA class I^{high} and HLA-E^{high} expressing monocytes. We gated on these cells (**Figure 1C** and **Supplementary Figure 3A**) and used an unbiased generalized linear mixed model (GLMM) (Seiler et al., 2019) to identify associated markers. The GLMM identified 10 markers (CD14, HLA-DR, HLA-E, LFA-3, CCR2, CD95, CD48, ICAM-1, CD163, and Nectin-1) whose expression was associated with HLA class I^{high} monocytes (**Figure 1D**) and 5 markers (HLA class I, CD11b, ULBP-1,2,5,6, CD163, and MICA/B) whose expression was associated with HLA-E^{high} monocytes (**Supplementary Figure 3B**). We then verified these markers by comparing the expression level of each marker in HLA^{high} expressing monocytes to the expression level in HLA^{low} expressing monocytes from DENV-infected adults (**Supplementary Figures 3C, 4A**). Finally, we gated down to monocytes expressing all 10 or all 5 markers in DENV-infected adults (**Supplementary Figures 3D, 4B**), and found that the 10 marker subset and the 5 marker subset expressed HLA class I and HLA-E, respectively, at significantly higher levels than total monocytes from the same donor (**Figures 1E,F** and **Supplementary Figures 3E,F**) verifying that combinatorial gating using the aforementioned markers accurately identifies HLA class I^{high} and HLA-E^{high} monocytes. Using the same gating scheme, we gated on the 10 and 5 marker monocyte subsets in healthy controls and found that HLA class I expression by the 10 marker subset and HLA-E expression by the 5 marker subset were both 1.6-fold higher in DENV-infected adults compared to healthy controls (**Supplementary Figures 3G, 4C**). These results indicate that upregulation of HLA class I and HLA-E by monocytes during *in vivo* DENV infection only occurs on specific monocyte subsets.

To better understand the effects of HLA class I upregulation, we modeled DENV infection *in vitro* using primary monocytes isolated from healthy donors. Further, to understand how viral replication vs. the presence of viral proteins alters HLA class I expression, we compared the effects of "active," replication-competent DENV, with that of UV-inactivated virus incapable of viral replication. At 24 h post-infection (hpi), HLA class I expression did not significantly differ between active DENV, UV-inactivated DENV, or mock-infected conditions (**Supplementary Figures 5A,B**). By 48 hpi, HLA class I expression was 2.1-fold higher in monocytes infected with active DENV compared to mock (**Figures 2A,B**). Interestingly, in the active virus cultures, the uninfected bystander monocytes had a modest 1.1- and 1.2-fold higher HLA class I expression at 24 (**Supplementary Figures 5C,D**) and 48 (**Figures 2C,D**) hpi, respectively, than infected monocytes in the same culture. This suggests HLA class I upregulation is primarily restricted to infected cells with a modest impact on uninfected cells, likely due to changes in the cytokine milieu. Alternatively, bystander cells in our assay may have been infected at a level below our limit of detection.

Expression of HLA-E was also altered during DENV infection. At 24 and 48 hpi, exposure to UV-inactivated DENV or infection with active DENV resulted in a majority of monocytes becoming HLA-E^{high} (**Supplementary Figure 5E** and **Figure 2E**). Similarly, at 48 hpi, the percentage of HLA-E^{high} monocytes in the UVed and active DENV cultures was 2.8- and 3-fold higher, respectively, compared to mock-infected monocytes (**Figure 2F**). The increase of HLA-E^{high} monocytes was 3-fold for both virus conditions compared to mock at 24 hpi (**Supplementary Figure 5F**). At both time points, bystander monocytes in the active DENV cultures had a bimodal distribution of HLA-E^{low} and HLA-E^{high} cells, while infected cells were all HLA-E^{high} (**Figure 2G** and **Supplementary Figure 5G**). Infected monocytes displayed a 1.3-fold increase in the percentage of HLA-E^{high} monocytes at 24 hpi compared to bystander monocytes (**Supplementary Figure 5H**), but at 48 hpi there was no longer a significant difference (**Figure 2H**). These results suggest that the response to viral proteins, rather than viral replication, is the main driver of HLA-E upregulation.

Considering that bystander monocytes expressed higher levels of HLA class I than DENV-infected monocytes, we wanted to test whether secreted factors, such as cytokines, viral proteins, and other molecules produced during active DENV infection were sufficient to upregulate HLA class I. To this end, we UV-treated conditioned supernatants from previous 48 h cultures of primary monocytes that were mock-infected, exposed to UV-inactivated DENV, or infected with active DENV. We then isolated uninfected monocytes from the same donors from which the supernatants were collected and cultured them in the conditioned supernatants for 24 h. Supernatants collected from active, DENV-infected cultures led to a significant, if modest, 1.1-fold increase in HLA class I expression compared to monocytes cultured in supernatants collected from the mock-infected and UV-inactivated DENV cultures (**Figures 3A,B**). This increase in HLA class I expression shows that soluble factors secreted during active DENV infection could be contributing to HLA class I upregulation. In order to investigate the potential role of cytokines in mediating this increase in HLA class I expression, we used Luminex to determine the concentration of cytokines present in the conditioned supernatants. We found that the concentrations of IFN-α, IFN-β, and TNF-α were 759.2-, 26-, and 271.7-fold higher, respectively, in the active condition compared to mock and 59.9-, 12.3-, and 2.8-fold higher, respectively, in the active condition compared to the UVed condition (**Figure 3C**). These findings suggest that upregulation of HLA class I during active DENV infection is largely driven by viral replication, and is likely mediated to some extent by cytokines. It is important to note that viral RNA, proteins, and particles present in the supernatant of

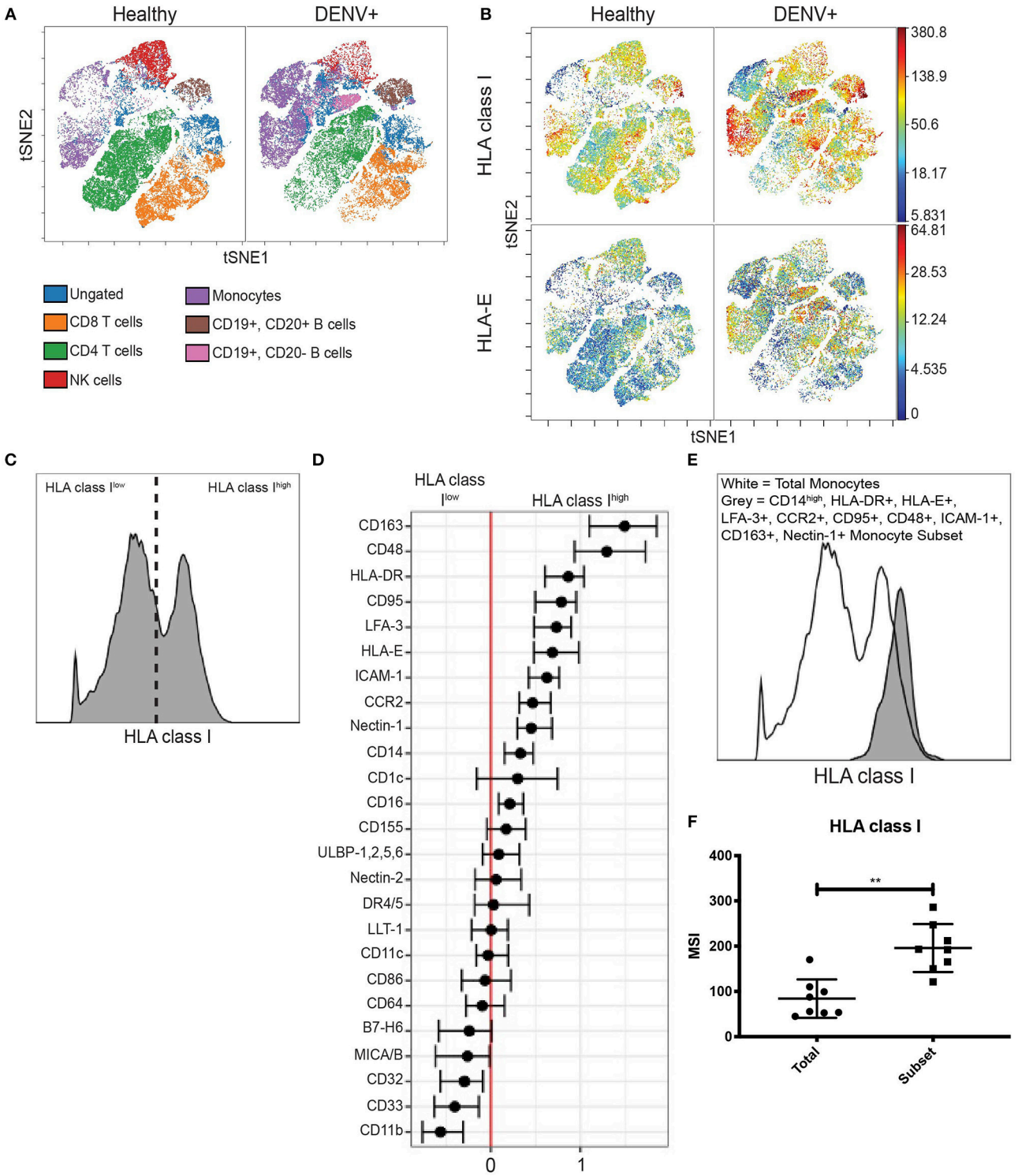

FIGURE 1 | HLA class I upregulation occurs during *in vivo* DENV infection. **(A)** Visualization of immune cell subsets in PBMCs from acute Panamanian DENV patients and healthy Panamanian controls using viSNE. The plots represent pooled data from $n = 8$ DENV patients and $n = 31$ controls. To assure equal donor representation, 4,375 events were used from each DENV patient and 1,129 events were used from each healthy control, resulting in 34,999 pooled events to generate both the DENV+ and healthy control viSNEs. Color key demonstrates major cell populations as determined by gating overlaid upon the viSNE visualization, demonstrating clusters of major cell subsets. **(B)** viSNE visualization of total HLA class I and HLA-E expression in whole PBMCs from DENV patients and healthy controls, generated as in **(A)**. **(C)** Representative histogram from a DENV patient illustrating HLA class I^{high} and HLA class I^{low} expressing monocytes gated on for generalized linear

(Continued)

FIGURE 1 | mixed model (GLMM) analysis. **(D)** GLMM analysis of markers associated with HLA class I^{high} and HLA class I^{low} expressing monocytes. **(E)** Representative histogram from a DENV patient showing increased HLA class I expression by the monocyte subset gated on using the 10 markers identified in **(D)** (CD163, CD48, HLA-DR, CD95, LFA-3, HLA-E, ICAM-1, CCR2, Nectin-1, and CD14) compared to total monocytes from the same donor. **(F)** Summary data from all eight DENV patients. Wilcoxon signed-rank test $^{**}P < 0.01$.

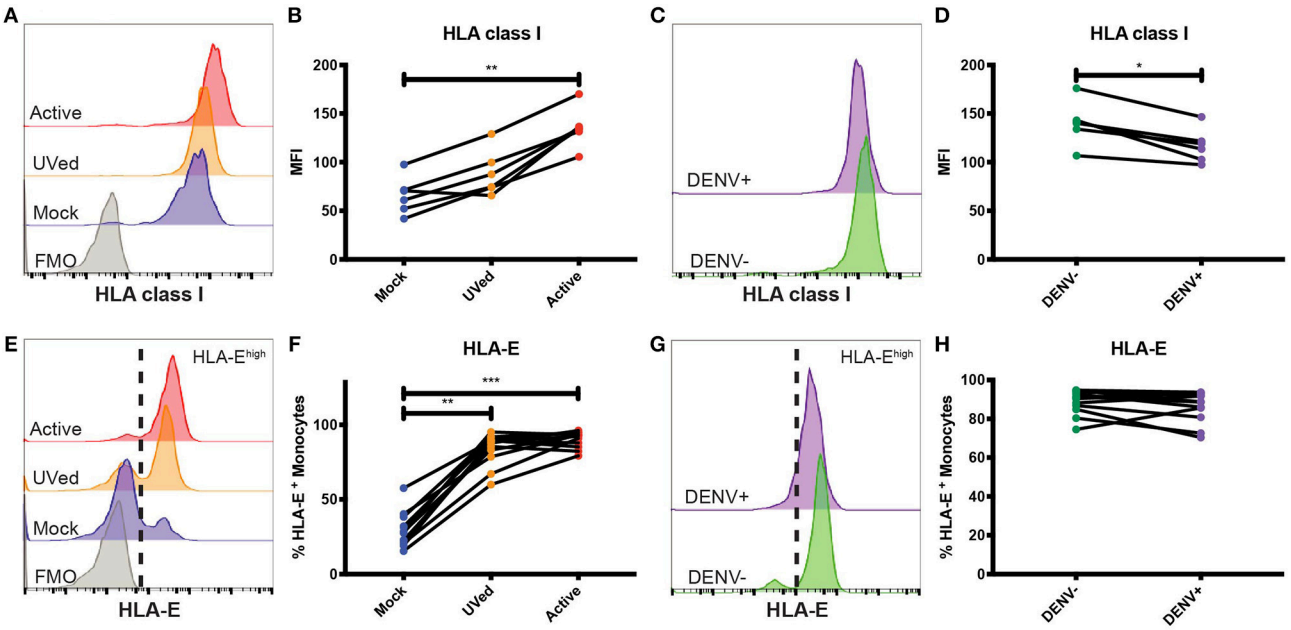

FIGURE 2 | Primary monocytes upregulate HLA class I and HLA-E during *in vitro* DENV infection. Primary monocytes isolated from whole PBMCs from healthy blood bank donors were mock-infected (blue), exposed to UV-inactivated DENV (orange), or infected with active DENV (red) for 48 h. Representative histograms of HLA class I **(A)** and HLA-E **(E)** expression in total monocytes cultured in the respective conditions. Fluorescence minus one (FMO) shown in gray. HLA class I MFI in total monocytes **(B)** as well as bystander (DENV–) and infected (DENV+) monocytes **(D)**. Representative histograms of HLA class I **(C)** and HLA-E **(G)** expression in bystander monocytes (DENV–, green) and infected monocytes (DENV+, purple). Percentage of HLA-E^{high} total monocytes **(F)** as well as bystander and infected monocytes **(H)**. Two independent experiments measuring HLA class I were performed with 6 donors. The average for each donor is represented in the graphs. $n = 12$ for HLA-E experiments. Friedman test followed by Dunn's multiple comparisons test was used to analyze total monocytes. Wilcoxon signed-rank test was used to analyze DENV– vs. DENV+ monocytes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

active infection cultures could also contribute to HLA class I upregulation.

Given that NK cell activation is dampened by the expression of self-HLA class I on potential target cells, we investigated the impact of DENV-mediated HLA class I upregulation on NK cell degranulation in response to DENV-infected cells. We co-cultured DENV-infected primary monocytes with autologous primary NK cells in the presence of an isotype-matched control Fab, an anti-pan HLA class I blocking Fab, or an anti-HLA-E blocking Fab for 4 h and measured the percentage of CD107a+ NK cells as a marker of degranulation and killing activity (**Figures 4A,B**). Fabs were used instead of whole IgG to avoid killing via ADCC following binding of the anti-HLA antibodies to the target cells. Blocking HLA class I on mock-infected monocytes led to a 9.7% frequency of CD107a+ NK cells, a 4.5% increase from 5.2% in the unblocked, mock-infected monocyte condition. Because NK cells can become activated when they are unable to bind self-HLA class I, this result demonstrates that the Fabs were effectively blocking their targeted proteins. Blocking HLA class I on DENV-infected monocytes resulted in nearly double the frequency of CD107a+ NK cells compared to DENV-infected monocytes blocked with the isotype-matched control Fab, increasing from 9.9 to 18.8%. Additionally, HLA class I-blocking of DENV-infected monocytes resulted in a 6.7% increase in CD107a+ NK cells compared to blocking HLA-E. Blocking HLA class I on DENV-infected monocytes also nearly doubled the frequency of CD107a+ NK cells compared to blocking HLA class I on mock-infected cells, increasing from 9.7 to 18.8% ($P = 0.0312$). For all of the blocking conditions, except HLA-E, the DENV co-cultures had a statistically significant increase in CD107a+ NK cells compared to the mock-infected co-cultures. Thus, these data demonstrate that HLA class I upregulation can dampen the NK cell response to DENV-infected cells.

DISCUSSION

Roughly one-third of the world's population is at risk of acquiring DENV, making it critically important that we elucidate immune factors that contribute both to disease protection and

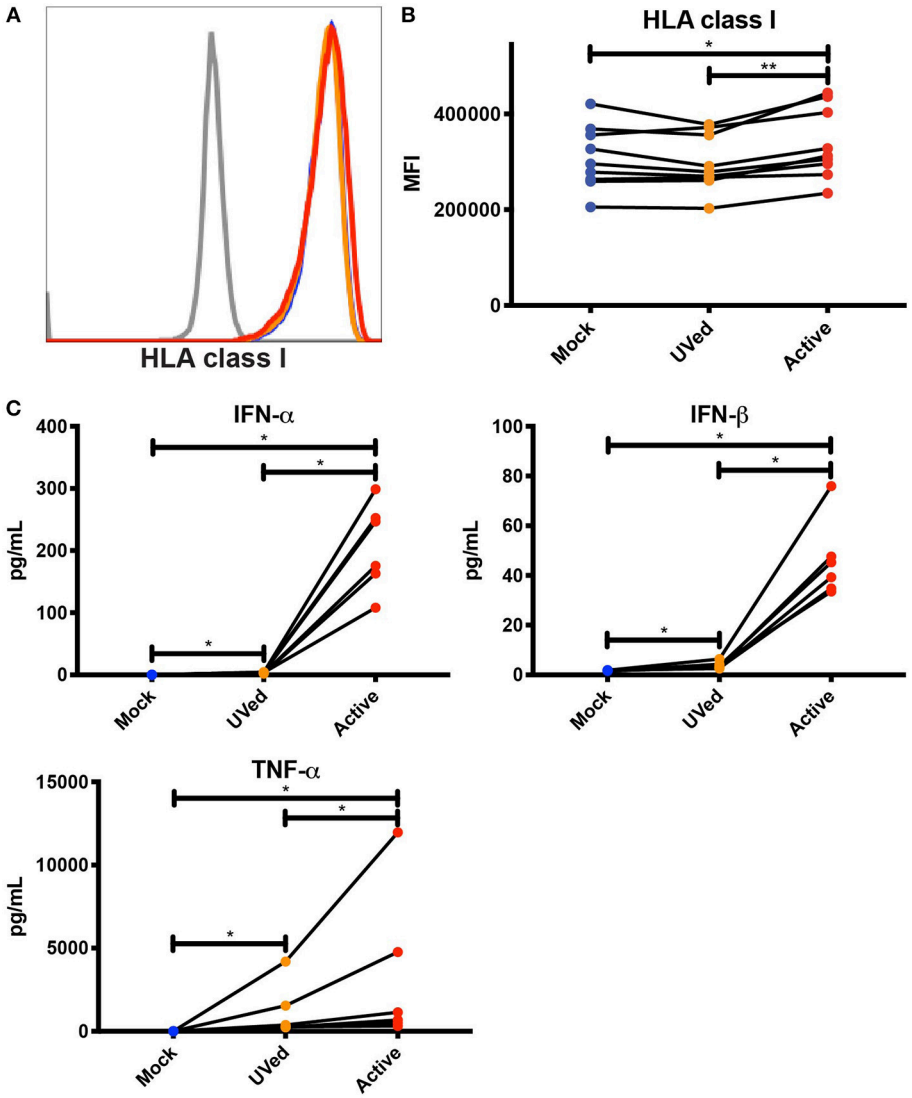

FIGURE 3 | Soluble factors secreted during active DENV infection upregulate HLA class I expression. Conditioned supernatants from experiments shown in **Figure 2** were UV-treated and used to culture primary monocytes from healthy blood bank donors ($n = 9$). After a 24 h incubation, expression of total HLA class I was analyzed by flow cytometry. Histograms from a single representative donor **(A)** as well as summary data from all 9 donors **(B)** are shown. Friedman test followed by Dunn's multiple comparisons test, $^{*}P < 0.05$, $^{**}P < 0.01$. **(C)** Cytokine concentrations in conditioned supernatants from experiments shown in **Figure 2** were analyzed by Luminex. Values shown are the average of two reads for each sample ($n = 6$). Friedman test with FDR correction followed by a one-tailed Wilcoxon matched-pairs signed-rank test with holm correction, $^{*}P < 0.05$.

pathogenesis. Mechanisms by which DENV evades the innate immune response by inhibiting the production and signaling of type I IFNs, as well as other aspects of the cellular antiviral response, have been well-reported (Morrison et al., 2012; Green et al., 2014). Similarly, pathways that might promote DENV escape from NK cell recognition have been proposed, including a potential role for the upregulation of HLA class I molecules by DENV-infected cells to inhibit the NK cell response by binding inhibitory KIRs or CD94/NKG2A (Beltrán and López-Vergès, 2014; Petitdemange et al., 2014; Mathew, 2018). However, these data have primarily arisen from *in vitro* infection of mouse or human cell lines, rather than more physiologic systems such as natural infection or infection of undifferentiated primary human immune cells. Here, we show natural DENV infection leads to increased expression of HLA class I and HLA-E in adult patients. We also found that soluble factors produced during active infection contributed to HLA class I upregulation. Finally, blocking HLA class I on DENV-infected monocytes enhanced the ability of NK cells to degranulate in response to DENV-infected cells. Together, these findings show HLA class I upregulation during active DENV infection suppresses NK cell degranulation.

HLA class I upregulation during flavivirus infection has been previously described and attributed to various mechanisms such as NFκB activation (Kesson and King, 2001), increased transport

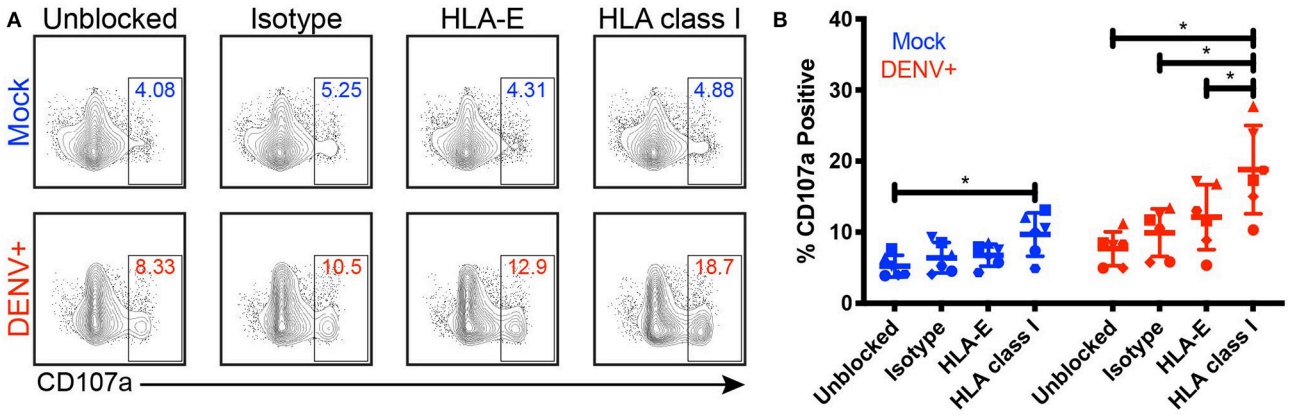

FIGURE 4 | Blocking HLA class I improves NK cell responses to DENV-infected cells. Primary NK cells and monocytes were isolated from whole PBMCs from healthy blood bank donors ($n = 6$). NK cells were activated for 22 h with IL-2. Monocytes were mock-infected (blue) or infected with active DENV (red) at an MOI of 2 for 24 h. Prior to co-culture with autologous NK cells, monocytes were blocked for 30 min with an isotype-matched control Fab, an anti-HLA-E blocking Fab, or an anti-pan HLA class I blocking Fab. Monocytes and NK cells were co-cultured for 4 h before NK cell expression of CD107a was evaluated by flow cytometry. Flow cytometry plots from a single representative donor **(A)** as well as summary data from all 6 donors **(B)** are shown. Friedman test followed by paired Wilcoxon signed-rank tests, $^*P < 0.05$.

of peptides into the endoplasmic reticulum for HLA loading (Momburg et al., 2001), and the presence of IFN-β (Glasner et al., 2017). We show replication-competent virus was required for significant HLA class I upregulation, but that HLA class I expression was highest in uninfected bystander monocytes. Further, supernatants collected from active DENV cultures were able to upregulate HLA class I on uninfected monocytes and contained higher concentrations of IFN-α, IFN-β, and TNF-α compared to supernatants from mock-infected or UV-inactivated DENV cultures indicating HLA class I upregulation is mediated at least in part by soluble factors. These findings pose a potential mechanism for HLA class I upregulation in which soluble factors secreted by DENV-infected cells induce increased HLA class I expression on all cells in an effort to promote cytotoxic T lymphocyte responses. However, the DENV-infected cells express lower levels than the bystander cells because DENV may encode proteins that interfere with processes driving HLA class I upregulation to escape the T cell response (Ye et al., 2013; Green et al., 2014; Guzman and Harris, 2015; Glasner et al., 2017).

Interestingly, upregulation of HLA-E likely involves different mechanisms than upregulation of other HLA class I molecules. We observed a significant increase in HLA-E expression in response to active DENV as well as UV-inactivated DENV. This suggests that direct sensing of viral products by innate immune receptors and the resulting cytokines, rather than pathways induced during viral replication, may be the primary contributors to HLA-E upregulation. However, DENV-infected monocytes expressed the highest levels of HLA-E, implying DENV itself is also mediating HLA-E upregulation. Intriguingly, cytomegalovirus proteins can modulate the surface expression of HLA-E by encoding HLA leader sequence mimics with reduced binding affinity to the CD94/NKG2 receptors (Heatley et al., 2013). Similarly, human immunodeficiency virus-1 has been found to upregulate HLA-E expression, resulting in the presentation of a capsid peptide which prevents HLA-E engagement with CD94/NKG2A (Nattermann et al., 2005; Davis et al., 2016). No such mechanisms for modulating HLA-E expression and its affinity for the CD94/NKG2 receptors have been reported for DENV. The NetMHCpan 4.0 server predicts several DENV-2 peptides with strong and weak binding to HLA-E*01:01, making it possible that DENV peptides modulate NKG2A/C binding. Together, these results suggest HLA-E upregulation is mediated by both virus-dependent and virus-independent mechanisms, and could influence NK cell recognition through NKG2A/C.

Here we extend prior studies demonstrating that HLA class I upregulation during flavivirus infection in cell lines can inhibit NK cell activation (Lobigs et al., 1996; Momburg et al., 2001; Hershkovitz et al., 2008; Glasner et al., 2017; Drews et al., 2018). For the first time, we used patient samples and more physiologically relevant *in vitro* infection and co-culture systems with undifferentiated primary human immune cells. By directly blocking NK cell binding to HLA class I using Fabs, we found HLA class I expression on DENV-infected cells significantly dampens NK cell degranulation. This provides the first direct evidence that upregulation of HLA class I is responsible for inhibition of NK cell responses to flavivirus-infected cells. We did observe some non-specific increase in NK cell degranulation in the presence of the isotype-matched control antibody, but this effect was dwarfed by the increase in NK cell degranulation in response to DENV-infected cells in the presence of HLA class I blocking Fabs. These findings indicate that HLA class I expression dampens the magnitude of the NK cell response.

Previous studies using cytokine-activated endothelial cells showed that blocking surface HLA-E and sHLA-E increased NK cell killing, illustrating the importance of HLA-E expression as a potential NK escape mechanism in some vascular diseases (Coupel et al., 2007). However, similar to Drews et al. we did not

observe a significant modulating effect of HLA-E expression on NK cell activity against DENV-infected cells (Drews et al., 2018). HLA-E binds to both inhibitory NK cell receptor CD94/NKG2A and activating receptor CD94/NKG2C (Braud et al., 1998; Valés-Gómez et al., 1999; Kaiser et al., 2005). Notably, HLA-E binds to CD94/NKG2A with higher affinity (Valés-Gómez et al., 1999; Kaiser et al., 2005). The fact that NK cell binding to HLA-E can result in both activating and inhibitory signaling with a dominant advantage toward inhibitory signaling could explain our results and those of Drews et al. HLA-E's greater affinity toward CD94/NKG2A also suggests that increased HLA-E expression on DENV-infected cells might be part of the viral escape strategy. Specifically blocking NKG2A or NKG2C in NK cell-infected cell co-cultures could identify the role of inhibitory signaling through NKG2A vs. activating signaling through NKG2C in DENV recognition.

In contrast to Drews et al. and Shwetank et al. who observed an increase in sHLA-E in the supernatants of DENV-infected HMEC-1 cells and Japanese encephalitis virus-infected human brain microvascular endothelial cells, respectively, we saw no significant increase in sHLA-E in the serum of DENV patients compared to healthy controls (Shwetank et al., 2013; Drews et al., 2018). This suggests that sHLA-E shedding is not increased at the systemic level during *in vivo* DENV infection and is consequently unlikely to contribute strongly to suppression of the NK cell response.

This study has limitations, the most significant of which is the small sample size of DENV-infected adults in our cohort. Despite the modest numbers, the conclusions we drew from these *in vivo* data were clear and supported by our *in vitro* experiments using primary human immune cells. The second limitation is that we were unable to clearly determine the role of HLA-E on the NK cell response to DENV infection given its binding to both inhibitory CD94/NKG2A and activating CD94/NKG2C receptors.

To our knowledge, ours is the first study showing upregulation of HLA class I molecules in acute dengue patient samples, suggesting different drivers of HLA-E upregulation vs. upregulation of other HLA class I proteins during DENV infection, and showing enhanced primary NK cell degranulation upon blocking HLA class I on primary DENV-infected monocytes. Our *in vivo* HLA class I expression data need to be confirmed with additional DENV cohorts at different time points of the disease, spanning all serotypes and degrees of disease severity, and including pediatric patients. This will be vital to determining what temporal, viral, and age-related factors affect HLA class I upregulation, as well as whether there is a correlation between disease severity and HLA class I expression. Future experiments are also required to determine what factors and pathways mediate HLA class I upregulation in monocytes and how HLA class I expression is modulated in other immune cell subsets. Overall, this study furthers our understanding of the impacts of DENV infection on innate immune cells and their intercellular interactions.

DATA AVAILABILITY

Requests to access the dataset should be directed to Catherine Blish at cblish@stanford.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB of Hospital del Niño (CBIHN-M-0634), then confirmed by the committees of ICGES, CSS, Santo Tomas Hospital, and Stanford University. Written informed consent to participate in this study was provided by the participants or participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JM, DB, CB, and SL-V contributed to the conceptualization, formal analysis, investigation, data curation, and the preparation of the writing of the original draft. JM, DB, AP, LS, AA, and RV contributed to the methodology. DB, EH, LL, CB, and SL-V contributed to the resources. EH and LL contributed to the reviewing and editing of the manuscript. CB and SL-V contributed to the supervision and project administration. DB, CB, and SL-V contributed to the funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00268/full#supplementary-material

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REVIEW
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Characterizing the Dysfunctional NK Cell: Assessing the Clinical Relevance of Exhaustion, Anergy, and Senescence

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There is a growing body of literature demonstrating the importance of T cell exhaustion in regulating and shaping immune responses to pathogens and cancer. Simultaneously, the parallel development of therapeutic antibodies targeting inhibitory molecules associated with immune exhaustion (such as PD-1, but also TIGIT, and LAG-3) has led to a revolution in oncology with dramatic benefits in a growing list of solid and hematologic malignancies. Given this success in reinvigorating exhausted T cells and the related anti-tumor effects, there are increasing efforts to apply immune checkpoint blockade to other exhausted immune cells beyond T cells. One approach involves the reinvigoration of "exhausted" NK cells, a non-T, non-B lymphoid cell of the innate immune system. However, in contrast to the more well-defined and established molecular, genetic, and immunophenotypic characteristics of T cell exhaustion, a consensus on the defining functional and phenotypic features of NK "exhaustion" is less clear. As is well-known from T cell biology, separate and distinct molecular and cellular processes including senescence, anergy and exhaustion can lead to diminished immune effector function with different implications for immune regulation and recovery. For NK cells, it is unclear if exhaustion, anergy, and senescence entail separate and distinct entities of dysfunction, though all are typically characterized by decreased effector function or proliferation. In this review, we seek to define these distinct spheres of NK cell dysfunction, analyzing how they have been shown to impact NK biology and clinical applications, and ultimately highlight key characteristics in NK cell function, particularly in relation to the role of "exhaustion."

Keywords: natural killer cells, NK cells, NK dysfunction, NK exhaustion, immune dysfunction

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INTRODUCTION

Reinvigorating exhausted cytotoxic CD8 T cells through checkpoint blockade therapy targeting PD-1 and PD-L1 has led to dramatic benefits in a growing list of solid and hematologic malignancies (Wei et al., 2018). While the understanding of T cell exhaustion has greatly expanded since initial ground-breaking publications by Moskophidis et al. (1993) and Gallimore et al. (1998), critical features have remained constant: (1) exhaustion occurs through persistent antigen

exposure which interferes with standard immune contraction mechanisms and classic T cell memory formation, and (2) subsequent effector function is diminished, seemingly because of the development of the exhausted state (Wherry, 2011; Wherry and Kurachi, 2015). In contrast to this prototypical model of T cell exhaustion (which is critically dependent on chronic and persistent antigen exposure), it is important to acknowledge that other relevant forms of T cell dysfunction exist, namely anergy and senescence, and in these states, different stimuli and pathways are involved producing different manifestations of dysfunction with variable reversibility.

Given this background, the last several years have witnessed significant interest in applying checkpoint blockade therapy to natural killer (NK) cells, primarily to exploit their well-known anti-tumor functions and similarities to CD8 T cells (Narni-Mancinelli et al., 2011; Sun and Lanier, 2011). Yet, while NK and CD8 T cells share many effector traits, the activation, inhibition, and generation of effector functions in NK cells is distinct from cytotoxic T cells, with complex mechanisms involved. Although there are many similarities between cytotoxic T cells and NK cells, leading many to hypothesize that NK cells could be targeted for reversal of "exhaustion" because of these similarities, the certainty of this approach remains undefined. In this review, we summarize the evidence for NK exhaustion as well as other states of dysfunction (including anergy and senescence) and discuss how these dysfunctional states are similar and different, highlighting how critical differences exist between NK and T cell dysfunctional states with implications for clinical application.

NK CELLS AND ANTI-TUMOR EFFECTS

Since the initial descriptions of NK cell activity nearly 50 years ago (Cudkowicz and Stimpfling, 1964; Cudkowicz and Bennett, 1971) and subsequent identification of a distinct NK lymphocyte population (Kiessling et al., 1975), NK cells have been pursued as an anti-tumor therapy due to their ability to kill transformed cells in an MHC-unrestricted manner. This defining characteristic of NK cells contrasts with the antigen specificity of cytotoxic CD8 T cells, which mediate MHC-restricted killing following antigen presentation and T cell priming. Additionally, loss of MHC-I expression occurs in multiple malignancies and has been associated with T cell immune evasion (Garrido et al., 1976, 2016; Restifo et al., 1993; Algarra et al., 1997; Garcia-Lora et al., 2001; Carretero et al., 2008). However, MHC-I downregulation has also been shown to activate NK cells via the "missing-self" hypothesis (Kärre, 2008). These features of NK targeting further support the concept of using NK cells therapeutically to augment tumor killing, especially since T cells are often rendered ineffective by cancer immunoediting, antigen loss variants, and MHC-I downregulation (Mittal et al., 2014). However, despite this paradigm that MHC-I positive tumor cells can be targeted by cytotoxic T cells and MHC-I negative tumor cells can be eliminated by NK cells, the clinical benefit of NK cell-based therapy has overall been modest, especially for solid tumors, and true breakthrough successes have been limited (Suen et al., 2018; Miller and Lanier, 2019).

Clinical trials using NK cells for the treatment of hematologic and solid malignancies have been ongoing for decades (Suen et al., 2018; Hu et al., 2019; Miller and Lanier, 2019). More recent efforts have focused on the *ex vivo* activation and expansion of peripheral NK cells using antigen presenting cells transfected with co-stimulatory ligands and membrane-bound cytokines to achieve high numbers of cells for adoptive therapy (Fujisaki et al., 2009b; Somanchi et al., 2011; Denman et al., 2012). These approaches were developed with the idea that NK cells produced in this way are highly functional, and greater numbers are needed *in vivo* to obtain a measurable anti-tumor effect. In addition to the massive expansion using these feeder-line approaches, these *ex vivo* NK cells are highly activated as shown by cytotoxicity assays against a range of tumor cell lines *in vitro* (Fujisaki et al., 2009b; Garg et al., 2012). However, despite impressive *in vitro* data on NK cytotoxicity using feeder-line expansion, expanded NK cells using these techniques tend to lose function quickly *in vivo* post-adoptive transfer, consistent with the relatively disappointing results of clinical trials irrespective of whether autologous or allogeneic NKs are used (Suen et al., 2018). Results like these have prompted interest in testing other NK sources, such as *in vitro* activated and expanded NK cell lines (e.g., NK-92) as a lower cost, consistent source of allogeneic cells which may overcome barriers to maintaining activation of NK cells following transfer *in vivo*. However, despite similarly impressive pre-clinical data showing high cytotoxicity and significant anti-tumor effects *in vitro* (Klingemann et al., 2016; Suck et al., 2016), results from clinical trials using NK-92 cells have also been modest (Arai et al., 2008; Tonn et al., 2013). Importantly, these discrepancies between *in vitro* and *in vivo* function of NK cells highlight several key observations which likely underlie their unrealized/disappointing clinical potential, namely that continuous stimulation with cytokines, or target cell activation results in acute increases in effector function but at the same time creating a state of cytokine/activating-signal dependence which then leads to rapid loss of function and survival if these activating/stimulatory signals are taken away. These fundamental observations about NK cell dysfunction post-adoptive transfer have led to intense investigation into strategies to reverse NK dysfunction *in vivo* by several different mechanisms, including overexpression of co-stimulatory molecules, pharmacologic doses of stimulatory cytokines, and combination with checkpoint blockade inhibitors (Miller and Lanier, 2019). As use of checkpoint blockade therapy is ubiquitous and increasingly being applied to NK-based therapy, a critical assessment of the extent and mechanisms of NK dysfunction, including exhaustion, is warranted. Techniques utilized in the expansion and activation of NK cells (i.e., cytokines, feeder line co-culture, co-stimulatory molecules) may give rise to heightened activation, but also dysfunction, and further may lead to NK cells "addicted" to supraphysiologic stimulatory signals that can never be safely reproduced in a human recipient following adoptive cell transfer. These dysfunction pathways likely impact the success (or failure) of NK-based clinical trials, and a better understanding of the spectrum of NK dysfunction pathways will allow for improved clinical application of NK cells, including how and when NK

cells might respond to checkpoint blockade therapy to reverse NK "exhaustion."

DEFINING NK CELL DYSFUNCTION

Dysfunctional NK cells are frequently identified by decreased expression of typical NK effector functions in a NK population of interest (such as tumor-infiltrating NK cells) compared to those of a control population (such as circulating NK cells in the peripheral blood) from the same host (Carrega et al., 2008; Carlsten et al., 2009). In general, readouts for NK effector function include *in vitro* cytotoxicity assays against target cells as well as IFNγ and granzyme B production. As these characteristics are generic markers of a dysfunctional NK cell, different states of NK dysfunction, such as anergy and exhaustion, become blurred because there is no established NK phenotype for these dysfunctional states (**Figure 1**). A further challenge in defining the etiology and spectrum of NK cell dysfunction lies in the fact that a thorough understanding of NK ontogeny and maturation remain ill-defined in many cases. And although there are increasing articles and reviews investigating NK cell exhaustion, there remains controversy as to whether NK cells even undergo exhaustion (as opposed to other dysfunction processes), and if so, the phenotypic markers that define an exhausted NK cell. This is in contrast to T cell development and maturation states which are well-characterized and annotated (Crotty and Rafi, 2004; Koch and Radtke, 2011). These knowledge gaps in some of the basic understanding of NK cell biology (Caligiuri, 2008) add difficulty to defining how, why and when NK cell dysfunction occurs.

Induced T cell dysfunction by either exhaustion, anergy or senescence is believed (in most cases) to protect the host from adverse autoimmune disease or immunopathology (Schwartz, 2003; Blank et al., 2019). This reinforces the well-known principle in immunology that the host must balance the anti-microbial/anti-tumor effects of immune defense with the risks of immunopathology from an unrestrained immune response. As virtually all human autoimmune disease is either B- or T-cell mediated (Davidson and Betty, 2001; Marrack et al., 2001; Rosenblum et al., 2015), the need for induced NK cell dysfunction to limit autoimmune disease is much less clear, with conflicting evidence suggesting both helpful and harmful roles of NK cells in autoimmunity (Schleinitz et al., 2010). Distinct from autoimmunity, there does seem to be toxicities associated with highly activated NK cells as recently described following a clinical trial of adoptively transferred *ex vivo* activated NK cells (Shah et al., 2015), suggesting that mechanisms for activation-induced NK dysfunction may be beneficial in regards to limiting host toxicities.

NK CELL EXHAUSTION

Identification and reversal of NK "exhaustion" is a current active field of investigation at both the basic and translational level (**Table 1**). In contrast to seminal studies on T cell exhaustion which have focused on viral models and human patients with chronic viral infections, studies investigating NK cell exhaustion have focused primarily on identifying dysfunctional tumoral-associated NK cells and applying strategies to reverse NK exhaustion to augment anti-tumor effects (da Silva et al., 2014; Beldi-Ferchiou et al., 2016; Seo et al., 2017; Zhang et al., 2018). However, the contribution of NK cells to the tumor immune infiltrate is generally considered to be low (Whiteside and Parmiani, 1994; Halama et al., 2011; Melero et al., 2014), and the clinical relevance of intra-tumoral NK cells remains incompletely characterized (Pagès et al., 2010), highlighting the need for a more thorough understanding of how NK cells home to diverse solid tumors. In the study from Halama et al., the authors set out to compare the NK and T cell infiltrate in colorectal cancer specimens (Halama et al., 2011). Using NKp46 to detect NK cells via IHC, the authors showed that NK cells were substantial in normal colonic mucosa, but limited within adjacent adenomas or carcinomas. A similar trend was detected in liver metastases, which had significantly less infiltrating NK cells that adjacent normal liver. Furthermore, the limited NK infiltrate detected had no correlation with tumor cell HLA class I expression, suggesting that other mechanisms contribute to the paucity of NK cell infiltration (Halama et al., 2011).

Regardless of the mechanism by which dysfunction has occurred, there must be a decrease in some effector function to label a cell as "dysfunctional." For NK cells, this includes reductions in the expression of IFNγ (as shown either by intracellular staining by flow cytometry or by ELISA detection of secreted IFNγ), granzyme B, TNFα, CD107a (marker of degranulation), antibody dependent cell-mediated cytotoxicity (ADCC) via CD16, or decreased target cell cytotoxicity assays. In addition to decreased effector function, there should also be a concordant, subset specific increase in an exhaustion-associated marker (PD-1, TIGIT, TIM-3, LAG-3, etc.). Some of the more prominent studies examining NK cell exhaustion have focused of cancer patients including NK cells from the tumor microenvironment (Benson et al., 2010; da Silva et al., 2014; MacFarlane et al., 2014; Beldi-Ferchiou et al., 2016; Seo et al., 2017; Vari et al., 2018; Zhang et al., 2018; Sun et al., 2019) and in the context of chronic viral infections or prolonged pro-inflammatory cytokines (Wiesmayr et al., 2012; Felices et al., 2018; Alvarez et al., 2019; Zhang et al., 2019). Common to these reports is the identification of decreased NK effector function to first identify NK cells as exhausted–most notably IFNγ by flow cytometry detection. These cells are then shown to have increased expression of an established exhaustion-associated marker.

As PD-1 is the prototypical T-cell exhaustion marker, many studies have identified an association of PD-1 expression on NK cells with exhaustion. However, expression of PD-1 by NK cells remains controversial, and other studies have demonstrated TIGIT and to a lesser extent TIM-3 and LAG-3 to be more critical NK exhaustion markers. One of the early studies investigating PD-1 expression on NK cells was in pediatric post-transplant lymphoproliferative disease (PTLD) (Wiesmayr et al., 2012). This study noted that peripheral NK cells in patients with PTLD were phenotypically and functionally distinct from healthy donors or patients with asymptomatic EBV viremia. In these PTLD patients, there was decreased NKp46 and NKG2D expression, while PD-1 expression was increased compared to healthy

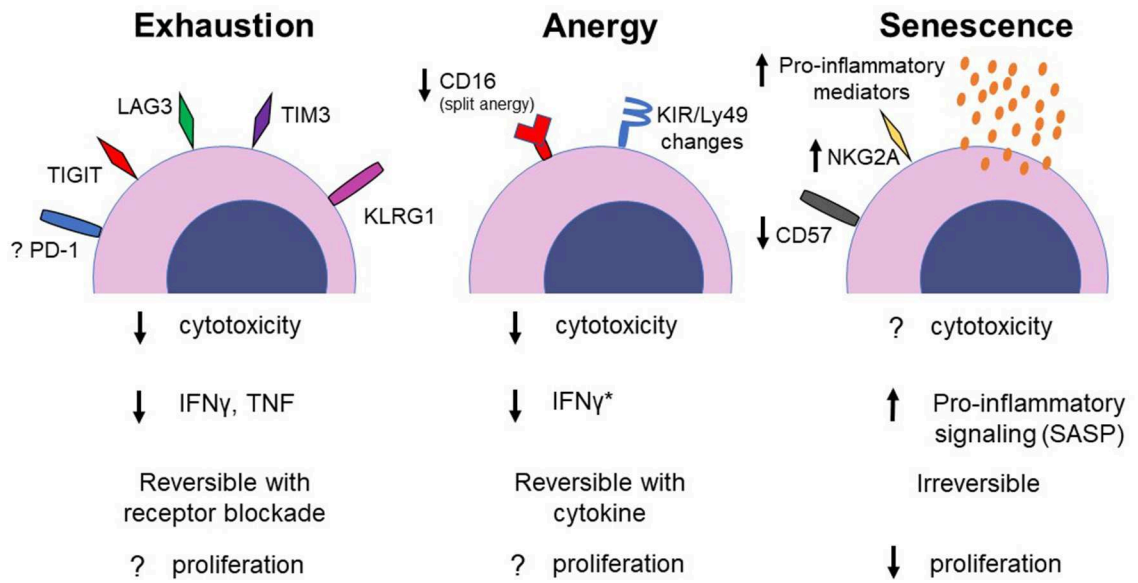

FIGURE 1 | Proposed phenotype and functional changes of NK cells under different dysfunctional states. Specific pro-inflammatory mediators associated with SASP in NK cells have not been determined. *IFNγ production has been shown to increase in split anergized NK cells. SASP, senescence-associated secretory phenotype.

controls and asymptomatic EBV carriers. The authors also showed functional differences between NK cells derived from PTLD patients compared to healthy controls (decreased CD107a, IFNγ), with augmentation of NK function in PTLD patients when they were treated with anti-PD-1 *in vitro*. However, the authors did not assess for differences in PD-1+ vs. PD-1- NK subsets, which is important since one would expect PD-1 negative NK cells in PTLD patients to function similarly to those of healthy donors. More so, evaluating the effects of anti-PD-1 therapy on PD-1+ and PD-1- subsets would be important to confirm the specificity of these findings.

Another important issue that the study by Wiesmayr et al., and other similar studies highlight is the challenge of determining an NK population to be "positive" when flow cytometric gating is used and does not delineate distinct populations. This can lead to wide variability in reported expression because small changes in flow cytometry gating dramatically alter the percent positive population. Additional studies in human cancer patients (Benson et al., 2010; Vari et al., 2018) have also shown PD-1 expression on NK cells, although interpretation of these studies may be limited by a wide variation in PD-1 expression in healthy controls (Vari et al., 2018) and a later finding that a novel antibody putatively identifying PD-1 on NK cells in multiple myeloma patients (Benson et al., 2010) appears to bind a separate receptor rather than PD-1 (Miller and Lanier, 2019).

Using murine models, Hsu et al., identified PD-1 expression on intra-tumoral NK cells, although these studies did not correlate PD-1 expression on NK cells with dysfunction (Hsu et al., 2018). The authors did observe significant variability in PD-1 expression across tumors (and even within replicates of the same tumors). For example, in RMA-S tumors (MHC-I negative lymphoma) PD-1 expression reached as high as 70%, while in RMA tumors (MHC-I positive lymphoma) expression varied between 20–50%, 0–60% in CT26 (colon cancer), 0–20% in B16 (melanoma), <5% in C1498 (AML), and 0–25% in 4T1 (breast). Hsu et al., also demonstrated significant anti-tumor effects when checkpoint blockade therapy targeting PD-1/PD-L1 pathway was administered *in vivo*. Although Hsu et al. do not directly address whether PD-1+ NK cells represent an "exhausted" subset and no comparison of the functional capabilities between PD-1+ vs. PD-1- intra-tumoral NK cells is shown, an earlier report from this group using similar mouse tumor models classified intra-tumoral NK cells as "anergic" (Ardolino et al., 2014) based on decreased expression of CD107a and IFNγ pre- and post-cytokine stimulation *in vivo*. Importantly, the authors did not observe NK cell anergy in MHC-I expressing tumors, suggesting that active inhibitory signaling via MHC-I and Ly49 receptors prevents exhaustion through tempering or tuning of activating signals. Interestingly, although PD-1 expression was variable but detectable by flow cytometry on intra-tumoral NK cells in this analysis (Hsu et al., 2018), there was no evidence of PD-1 expression on splenic NK cells, suggesting tissue specific effects. Moreover, these results are in contrast to a separate report from Quatrini et al., showing PD-1 expression restricted to splenic NK cells during MCMV infection, with no evidence of PD-1 expression from NK cells isolated from other organs or tissues. Importantly, this study uncovered a link between glucocorticoid signaling and the immune response to infection by NK cells.

TABLE 1 | Summary of key studies examining NK cell exhaustion marker expression in different contexts and populations.

References	Species	Population	Exhaustion marker(s)	Key findings and significance	
Benson et al. (2010)	Human	Multiple Myeloma	PD-1	HD: 1.4% ± 0.35 MM: 64% ± 4.4	[a]First paper to show NK PD-1.
Wiesmayr et al. (2012)	Human	PTLD	PD-1	HD: 14% ± 6 PTLD: 36% ± 24	Variable expression of PD-1 on NK cells in healthy and PTLD patients.
da Silva et al. (2014)	Human	Melanoma	Tim-3 PD-1	Tim-3 HD: ∼20-90% MD: ∼25-90% PD-1 HD: <2% MD: <2%	NK PD-1 absent in healthy and melanoma patients. Proposes role for Tim-3 in mediating NK exhaustion in advanced melanoma.
MacFarlane et al. (2014)	Human	RCC	PD-1	MFI: RCC>HD in $CD56^{dim}$ subset	Links NK PD-1 and disease stage, but low PD-1 MFI overall, and differences only compared to healthy donors.
Beldi-Ferchiou et al. (2016)	Human	Kaposi's Sarcoma	PD-1	HD: 0.5% ± 0.08 KS: 4% ± 0.8	Low PD-1 expression overall which could be explained by subjective nature of flow cytometry gating.
Pesce et al. (2017)	Human	CMV+/CMV- healthy adults	PD-1	NK PD-1 0–10%, higher in CMV+	Links NK PD-1 to CMV+ serostatus, although 25% of PD-1- donors were CMV+.
Hsu et al. (2018)	Mouse	Intra-tumoral	PD-1	[b]NK PD-1 0-70%	1st study linking anti-tumor effects of anti-PD-1 therapy to direct NK cell effects. PD-1 expression on NK cells only observed in tumors, and expression levels highly variable across and within tumors.
Lieberman et al. (2018)	Human	Healthy donors	PD-1	Pre-activation ∼5% Post-activation ∼50%	PD-1 on more functional NK cells, not exhausted NK cells and only after 12 days of maximal activation.
Quatrini et al. (2018)	Mouse	MCMV infection	PD-1	MFI: MCMV>UI (spleen only)	1st paper linking tissue specific expression of PD-1 on NK cells to glucocorticoids and neurohormonal axis. Unlike Hsu paper, PD-1 expression only observed on splenic NK cells.
Zhang et al. (2018)	Human Mouse	Intra-tumoral	TIGIT PD-1	[c]Mouse tumor-infiltrating NK: TIGIT: 50–80% PD-1: <10%	1st paper showing influence of TIGIT on intra-tumoral NK cell function. Unlike Hsu paper, PD-1 expression on intra-tumoral NK cells consistently <10% across cell lines (CT26, B16, 4T1).
Alvarez et al. (2019)	Mouse	MCMV infection Cytokine treated	PD-1 TIGIT Tim-3	PD-1 [d]Control: <10% Acute: <10% Chronic: <10% TIGIT Control: ∼10% Acute: 25–40% Chronic: 10–25% Tim-3 Control: <10% Acute: 15–30% Chronic: <10%	PD-1 low on NK cells from control and stimulated mice. TIGIT and Tim-3 correlate with activation, but not maintained with chronic stimulation. Suggests NKG2D important in exhaustion phenotype.

[a] *PD-1 therapeutic antibody used in study later found to bind receptor distinct from PD-1.*
[b] *Variable across tumor cell lines.*
[c] *Human intra-tumoral NK cell TIGIT expression 10-80%.*
[d] *Untreated mice.*
PTLD, post-transplant lymphoproliferative disease; RCC, renal cell carcinoma; CMV, cytomegalovirus; MCMV, murine cytomegalovirus; TILs, tumor infiltrating lymphocytes; MFI, mean fluorescence intensity.

Although the authors did not directly link PD-1 expression to NK dysfunction, their findings of increased immunopathology in the spleens of mice with NK-specific PD-1 gene deletion suggested a novel role for NK cells in the neurohormonal response to infection and a physiological adaptation of NK dysfunction (Quatrini et al., 2018).

Other studies have examined exhaustion marker expression on NK cells in murine models and human cancer patients

and observed different results regarding the key mediators of this process. For example, Zhang et al., examined intra-tumoral NK cells from multiple subcutaneous mouse tumor models and identified the co-inhibitory receptor TIGIT (T cell immunoglobulin and ITIM domain) as the critical marker for dysfunctional NK cells (Zhang et al., 2018). In this and other papers, PD-1 expression was minimal on NK cells, including intra-tumoral NK cells. In fact, TIGIT+ NK cells displayed decreased IFNγ, TNF, CD107a, and TRAIL expression consistent with decreased effector function. TIGIT blockade reversed NK dysfunction with superior anti-tumor effects in multiple murine tumor models.

One of the initial descriptions of TIGIT highlighted the ubiquity of TIGIT expression on healthy NK cells and its ability to bind ligands PVR (CD155) and PVRL2 (CD112) (Stanietsky et al., 2009). In this study TIGIT ligation was associated with decreased NK cytotoxicity. These ligands can be expressed on tumor cells and elicit an inhibitory signal to tumor-infiltrating NK cells (Sanchez-Correa et al., 2019). TIGIT+ NK cells may also be inhibited within the tumor microenvironment by MDSCs expressing the cognate ligands (Sarhan et al., 2016), thus making TIGIT a potentially prominent inhibitory receptor through various mechanisms. Earlier work on TIGIT and T cells showed that TIGIT expression did not have cell-intrinsic effects (Yu et al., 2009), but was based on CD226 interactions and ligand binding (Johnston et al., 2014). It has not been definitively determined if TIGIT expression marks intrinsically dysfunctional NK cells or if ligand binding is required for inhibitory effects, however expression of the inhibitory receptor TIGIT appears prominent both on human and mouse NK cells with potentially important clinical benefits.

Other investigators examining NK cell exhaustion have identified other markers of exhausted NK cells distinct from PD-1 and TIGIT. For example, a study in melanoma patients found a correlation between peripheral NK expression of TIM-3 and disease stage (da Silva et al., 2014), suggesting cancer progression and greater burden of disease induced NK exhaustion via increasing TIM-3 expression. Notably, these authors observed limited to no expression of PD-1 on NK cells ($\leq$2%) and found no difference in PD-1 expression between melanoma patients and healthy donors. A more recent study utilizing CyTOF to analyze infiltrating immune cells in non-small cell lung cancer patients similarly detected virtually no PD-1 expression on intra-tumoral NK cells from 20 separate donors (Datar et al., 2019). Taken together, these studies highlight discrepancies in the evidence for and against exhaustion marker expression in NK cells, including PD-1. In addition, since these studies focus on cell surface marker expression (since this is usually viewed as mechanism to target NK cells therapeutically), there is less in-depth assessment of how expression of these markers is contributing to NK function or dysfunction and whether expression of these markers is adaptive, maladaptive, or potentially both.

Indeed, some studies have examined how stimulation of NK cells can both augment function and induce dysfunction, often simultaneously. For example, Alvarez et al. (2019) evaluated the occurrence of NK cell exhaustion after chronic stimulation (using viral and cytokine models) and proposed a paradigm of NK cell exhaustion similar to T cell/LCMV exhaustion. Specifically, the authors noted that prolonged cytokine exposure with IL-15 for >5 days or following MCMV infection stimulated sustained NK cell proliferation which then lead to decreased Ki67, IFNγ, and granzyme B expression. These exhausted NK cells were characterized by increased expression of KLRG1, decreased expression of cytotoxicity trigger NKG2D, and decreased expression of transcription factor Eomes. In keeping with discrepancies in identifying consistent markers of NK exhaustion, Alvarez et al., detected <5% expression of PD-1 on murine NK cells. Similarly, the finding of decreased expression of Eomes on exhausted NK cells was also discrepant to the classic phenotype of exhausted CD8+ T cells where Eomes expression is increased (Buggert et al., 2014).

Cytokine-based induction of NK dysfunction was also investigated by Felices et al. (2018) who detected differences in human NK cell responses when exposed to either continuous or intermittent IL-15. Though the authors did not define a clear phenotype of exhausted NK cells, they did identify significant differences in expression of CD107a, IFNγ, and NK cytotoxicity. The authors also showed that continuous IL-15 increased NK proliferation and decreased survival, secondary to alterations in NK cell metabolism induced by cytokine exposure which were partially mitigated by mTOR inhibition. While continuous IL-15 exposure appeared to induce an exhausted state in the work by Felices et al., combinations of IL-12, IL-15, and IL-18 were able to generate cytokine-induced memory NK cells (Cooper et al., 2009; Romee et al., 2012). It is currently unclear how the different cytokines act in concert to generate a functionally improved NK cell, while acting individually they appear to induce dysfunction. This suggests significant context-dependent effects from cytokine exposure as well as a narrow window between augmentation of function and induction of dysfunction.

Notably, IL-15, in addition to other cytokines (Cooper et al., 2009; Romee et al., 2012), also has been linked to the generation of memory-like NK cells and is necessary for the *de novo* generation of NK cells (Caligiuri, 2008). However, key papers have also highlighted the detrimental effects of IL-15 on NK cell malignant degeneration in the context of prolonged pro-inflammatory exposure. For example, Fehniger et al., showed that IL-15 transgenic mice develop fatal leukemia from NK and/or CD8 T cell infiltration (Fehniger et al., 2001) and *in vitro* studies from the same group observed that LGL leukemia can be induced from prolonged culture of human NK cells with IL-15, though this was on the order of >6 months exposure (Mishra et al., 2012). While neither of these studies investigated NK exhaustion or dysfunction in these contexts, they do provide evidence for the detrimental effects of prolonged cytokine exposure and the resulting dysfunction that can be attributed to exhaustion when examined under the appropriate lens.

Similar to the paradigm that T cell PD-1 expression can represent both early activation and exhaustion following prolonged antigen exposure depending on the kinetics of TCR engagement (Ahn et al., 2018), it has also been proposed that PD-1 expression on NK cells may delineate the most activated NK cells following *in vitro* stimulation (Lieberman et al., 2018). In these experiments, human NK cells were expanded

using membrane bound-IL-15 and 4-1BBL transfected K562 cells supplemented with rhIL-2 and shown to upregulate PD-1. The PD-1+ NK subset (∼30–70% by day 12, regardless of $CD56^{bright}$ or $CD56^{dim}$ expression) was also shown to have increased expression of the activation marker CD69. Along with the increase in PD-1 expression seen over 12 days of *in vitro* culture, these authors also observed decreased Tbet expression and increased Eomes expression on NK cells as shown by RNA analysis (although PD-1 mRNA was not assessed). Importantly, the interpretation of flow cytometric results such as those by Lierberman et al., using median fluorescence intensity (MFI) assessed at different time points is difficult. Unlike using the endpoint of percent positive cells (for marker expression) compared to control populations using fluorescence minus one (FMO) or isotype antibodies, the use of MFI as a readout of marker expression is susceptible to changes in light scattering properties across conditions and reagents (Vitale et al., 1989; Zamai et al., 1998). As a result, comparing MFI across time and experiments can introduce error as differences in treatment, internal controls and/or flow cytometric parameters may significantly alter the baselines and variance among samples. This is especially true as NK cells increase in size and granularity with activation (Zarcone et al., 1987).

Though controversies exist in defining exhausted NK cells and identifying exhaustion-specific markers on NK cells, there is a lack of investigation into the beneficial role of NK exhaustion. With limited evidence for direct NK-mediated autoimmunity, it is more likely that upregulation of exhaustion markers may serve to limit toxicity under certain highly activating conditions or limit immunopathological effects mediated by other cell types. This has been recently examined in the context of viral hepatitis and autoimmune cholangitis. In the viral hepatitis study, it was determined that liver resident NK cells express PD-L1 to bind and inhibit T cells (via PD-1) to limit immunopathology from anti-viral T cells, while leading to decreased viral clearance and persistence (Zhou et al., 2019). Similarly, a study investigating autoimmune cholangitis showed that liver resident NK cells inhibit CD4 T cells to limit the severity of autoimmune cholangitis, and loss of liver resident NK cells worsens the disease (Zhao et al., 2019). Distinct from immunopathology, however, PD-L1 has also been shown to be induced on NK cells by tumors and augmentable by anti-PD-L1 therapies for an anti-tumor effect (Dong et al., 2019). While only PD-L1 on NK cells has been implicated in these studies, it is probable that other exhaustion markers may be upregulated on NK cells to limit NK-specific toxicities, or to indirectly limit T cell-mediated immunopathology.

OTHER DYSFUNCTIONAL STATES

In recent years immune exhaustion has received the most attention due to the ability to identify exhausted T cells (based primarily on PD-1 expression) and the ability to antagonize PD-1 signaling using therapeutic antibodies with well-publicized clinical benefits. However, other states of cellular dysfunction have also been described and characterized, notably anergy and senescence, as well as deprivation and suppression, which are critical to immune cell function.

ANERGY

In the broadest sense, T-cell anergy represents a state of intrinsic functional inactivation (Schwartz, 2003). Schwartz (who provided some of the initial descriptions of T-cell anergy) divided the term into two categories—clonal anergy and adaptive tolerance. Both states are characterized by decreased proliferation and decreased IL-2 production, but clonal anergy derives from insufficient activation and appears to occur in mature T cells, while adaptive tolerance occurs secondary to insufficient co-stimulation of naïve T cells (Schwartz, 2003; Chiodetti et al., 2006). Further evaluation of these anergic states identified distinct biochemical pathways associated with clonal anergy and adaptive tolerance, respectively (Chiodetti et al., 2006). Despite these differences, it is critical to understand that both states appear to have evolved as a tolerance mechanism aimed at limiting autoimmunity, thus attempts to reverse anergy (as has been pursued for exhaustion) are liable to prove detrimental while potentially also yielding minimal benefit.

Should NK cells follow the same paradigm set forth by T cells, then anergy may occur following an insufficient activating signal (adaptive tolerance) or following a strong stimulus without adequate co-stimulation (clonal anergy). A sequential signaling model for NK cells analogous to naïve T-cell activation was recently proposed by Vidard et al., Here, the authors described a three-signal sequence required for maximal NK cell activation and proliferation (Vidard et al., 2019). The authors showed that maximal NK proliferation required NK-aAPC contact, CD137 (4-1BB) activation, and cytokine (IL-2, IL-15, IL-21) signaling. Once removed from maximally activating conditions, cytokine alone was capable of maintaining cytotoxic function against target cells as evidenced both by target cell lysis and NK cell IFNγ production (Vidard et al., 2019). Hypothesizing a similar mechanism to T cells, NK cells would thus become "anergic" if CD137 signaling is absent at the time of NK-aAPC contact, which may be the case when NK cells are within the tumor microenvironment where NK cells contact MHC-I negative tumor cells and may receive IL-2 and/or IL-15 cytokine signaling, without ligation of CD137, which is typically provided by antigen presenting cells. This sequence of events is supported by Ardolino et al., who showed reversal of NK cell anergy on intra-tumoral NK cells when exogenous IL-12 and IL-18 was given systemically (Ardolino et al., 2014). Overall, although these data support the concept of NK cell anergy via inadequate 3-signal activation, it is difficult to differentiate reversal of NK anergy in these models from *de novo* activation of resting NK cells by cytokine exposure, a process that has been well-established for the generation of lymphokine activated killer (LAK) cells (activated NK and CD8 T cells) and successfully used in clinical trials (Rosenberg, 1985, 2014; Rosenberg et al., 1985, 1987) as well as for generation of cytokine-induced memory NK cells, as discussed above.

Also comparable to T cell anergy (Otten and Germain, 1991), other groups have identified "split anergy" in NK cells

(characterized by simultaneous loss of one specific function, with corresponding gain of a separate effector function) (Jewett and Bonavida, 1996; Jewett et al., 1996, 1997, 2006; Tseng et al., 2015). As per the extensive work by Jewett and Bonavida, NK cell "split anergy" occurs following ligation of CD16 and is typically characterized by the subsequent loss of CD16 expression with gain of cytokine secretion abilities. The beneficial effects of NK split anergy appear to be increased control of cancer stem cells (CSCs) through induction of target cell differentiation (Tseng et al., 2014, 2015), which is consistent with other studies that have observed NK cell targeting of CSCs (Ames et al., 2015; Luna et al., 2017).

The advent of cytokine therapy and other monoclonal-based immunotherapeutics for cancer has created a situation for T cells that may have otherwise never been encountered in nature—that is out of sequence signal 3. This novel scenario and its downstream consequences were previously investigated by multiple labs independently and a novel, anergy-like T-cell dysfunction was identified (Urban and Welsh, 2014; Sckisel et al., 2015). To date, these adverse effects have not been identified in NK cells, and pro-inflammatory cytokines have consistently been shown to enhance NK effector function and proliferation (Biron et al., 1999), though not to a maximal effect as illustrated by Vidard et al. (2019). However, key aspects of anergy have been observed/invoked in the context of licensing as NK cells develop additional immunoregulatory mechanisms to balance appropriate target recognition with tolerance. In this context, these features of anergy likely have host benefits in the context of NK cell maturation and function and highlight another key difference between NK cells and T cells as resting NK cells can kill target cells provided appropriate positive signals are provided and steady-state inhibitory signals are removed (Orr and Lanier, 2010). Interestingly, in studies investigating the topic of NK tolerance, cytokines previously described in NK memory and anergy studies (such IL-12 and IL-18) were also capable of restoring NK function in unlicensed NK cells (Orr and Lanier, 2010), and unlicensed (tolerant, hyporesponsive) NK cells were shown in a related study to predominate in the NK response to MCMV (Orr et al., 2010). These findings regarding overlap between NK tolerance and anergy add complexity to our understanding of whether NK dysfunction is helpful or harmful to the host.

SENESCENCE

The universal phenomenon of replicative senescence (and cellular aging) appears related to shortening of telomeres, the eventual recognition of genomic DNA as double-strand breaks, the implementation of repair machinery, and the ultimate arrest of the cell cycle to halt replication and prevent compounding of genomic instability (Campisi, 2013). Senescent T cells are observed in both aged humans and after prolonged *in vitro* culture (Spaulding et al., 1999), and they are phenotypically characterized by decreased CD28 expression (Effros et al., 1994). Functionally, senescent T cells have decreased replicative ability (Spaulding et al., 1999), are associated with increased production of pro-inflammatory cytokines TNFα and IL-1 (Dayan et al., 2000), several proteases (Callender et al., 2018), and reduced apoptosis (Spaulding et al., 1999). In contrast to other forms of immune cell dysfunction, senescence appears to be a universal byproduct of prolific replication and not a direct result of antigen-specific stimulation or other stimulatory or inhibitory conditions. Characterization of NK senescence has not been specifically elucidated, but given the universality of cellular senescence, it is presumed that senescent NK cells are also associated with a pro-inflammatory senescence-associated secretory phenotype (SASP) and decreased proliferative capacity. As senescence results from continued proliferation, techniques to generate high numbers of NK cells for therapy may induce senescence and promote dysfunction.

Understanding the lifespan of the cell is critical to gaining an understanding of NK senescence, though this is challenging as the lifespan of an NK cell is not clearly defined. Estimates of the *in vivo* half-life of murine NK cells are ∼7–10 days (Yokoyama et al., 2004), and possibly <10 days in humans (Nayar et al., 2015), though this view has been expanded by advances in cell identification and barcoding techniques in non-human primates showing unique developmental pathways of NK subsets (Wu et al., 2014) and prolonged persistence (months) of specific NK clones (Wu et al., 2018). *In vitro,* this can be markedly manipulated, but the limit of healthy, normal human NK cells to grow in culture appears to be ∼15 weeks (Fujisaki et al., 2009a). In mice, *in vitro* proliferation of healthy NK cells is ∼7–10 days before apoptosis-associated changes occur, though NK cells derived from *P53* knockout mice have been cultured for over 1 year under specific conditions (Karlhofer et al., 1995). Fujisaki et al. (2009a) augmented this limit by culturing healthy human NK cells in the presence of the transfected K562 cell line bearing membrane bound 4-1BBL and IL-15, supplemented with 10 IU/mL IL-2. Using this system, healthy NK cells were able to undergo 15 weeks of culturing and 20 population doublings before losing replicative ability and dying despite continued stimulation. When NK cells were transfected with the *TERT* gene, replicative ability was restored, and cells could now be cultured almost 160 weeks with continued cytotoxicity. Transfected NK cells, however, were not able to grow autonomously in NSG mice and still eventually developed senescence *in vitro*, though at a much later time point compared to the non-transfected population (Fujisaki et al., 2009a). Additional studies using similar transfected K562 cells with membrane bound IL-21 (rather than IL-15) showed superior expansion of human NK cells compared to membrane bound IL-15 K562 cells (Denman et al., 2012). NK cells expanded with the IL-21-bearing K562 cell line exhibited increased telomere length which may be related to differences in STAT signaling and *TERT* regulation. These studies highlight that under ideal, highly activating and stimulatory conditions, healthy human NK cells are most limited by senescence at much later time points than believed to occur *in vivo*, but not by other mechanisms of dysfunction (i.e., exhaustion). The reason for this is unclear as prior reports would suggest that continuous cytokine exposure can induce NK cell exhaustion. It is critical to note that *ex vivo* expansion using the transfected K562 cell line utilizes membrane bound

ligands, particularly 4-1BBL, which is not present in other studies utilizing exogenous cytokine alone. Although membrane-bound ligands may be more physiologic, it does not reflect the clinical usage of exogenous cytokine (IL-2 or IL-15) that may induce distinct immunologic effects. Perhaps these supraphysiologic conditions overcome any inhibitory mechanisms NK cells attempt to implement or alternatively there are no ligands present to bind and send inhibitory signals to the NK cells.

The phenotype of senescent NK cells has also been investigated using a similar *in vitro* expansion system based on transfected K562 cells bearing membrane bound 4-1BBL and IL-21 supplemented with 100 U/mL IL-2 (Streltsova et al., 2018). These authors noted that following prolonged culture for 2–8 weeks, NK cell expression of inhibitory receptor NKG2A was increased and maturation marker CD57 was lost. However, there was no difference in IFNγ production or NK cell cytotoxicity against target cells between CD57 positive and negative subsets, and it was unclear if there was a proliferation difference between the CD57 subsets, which would be a hallmark of senescent cells. In prior work evaluating the CD57 subsets, Lopez-Verges et al., identified critical differences between these cell populations when differentiating the mature CD3-CD56^{dim} NK cells between CD57+ and CD57- (Lopez-Vergès et al., 2010). The authors found that CD57+ NK cells proliferated less when exposed to either cytokine or target cells, although the CD57+ NK cells had greater expression of IFNγ and greater cytotoxicity with CD16 stimulation. The authors also observed that IL-2 induced CD57 expression on ∼30% of NK cells after 5 days in culture. With the co-culture NK expansion system (transfected K562-mbIL15-41BBL feeder cells with IL-2), they also showed that the CD57- subset exhibited increased proliferation compared to the CD57+ NK cells (Lopez-Vergès et al., 2010). These findings suggest that over the course of extended *ex vivo* NK expansion the more proliferative CD57- NK cells will disproportionately expand and outcompete the CD57+ population and result in a CD57- NK cell product. Given these results, a definitive phenotype of NK senescence has not been achieved and functional consequences of NK senescence are not clearly established.

The *in vivo* implications of aging on NK-cell senescence and resultant function has also been reviewed (Hazeldine and Lord, 2013) with hypothesized consequences such as increased viral infections, decreased anti-microbial functions and increased malignancies. Gounder et al., showed in healthy humans an age-dependent loss of total peripheral lymphocytes, but relative increases in the NK cell compartment, suggesting that NK cells were less susceptible to the effects of aging, particularly senescence. However, NK cells from younger donors expanded to a greater extent than those from older donors using IL-2-based *in vitro* stimulation, highlighting that NK cells were still susceptible to age-related dysfunction (Gounder et al., 2018). Murine studies have also shown age-related differences in NK cells (Beli et al., 2014; Nair et al., 2015), with one study showing decreased total and mature NK cells in the peripheral tissues in older mice aged 15–18 months, and an accumulation of mature NK cells within the bone marrow of these aged mice compared to younger mice aged 6–8 weeks (Beli et al., 2014).

Separate from their well-established anti-viral and anti-tumor effects, NK cells are also prominent within the gravid uterus early in gestation (Moffett-King, 2002) and senescence may have a beneficial role. These unique uterine NK cells have a proposed role in remodeling uterine vasculature to augment fetal circulation (Rajagopalan and Long, 2012; Rätsep et al., 2015). There is also evidence that this effect is mediated by persistent signaling and induction of a senescent phenotype in NK cells (Rajagopalan and Long, 2012). In their study, Rajagopalan and Long showed that persistent NK cell signaling via CD158d interactions with HLA-G induced a DNA damage response pathway which lead to senescent NK cells with a senescence-associated secretory phenotype and resultant effects on vascular remodeling (Rajagopalan and Long, 2012). This contrasts with the hypothesis that senescence is strictly the end-result of cellular replication and suggests that the induction of a senescent phenotype may be intentional and programmed in specific situations, such as providing marked benefit to the host and fetus during gestation.

DEPRIVATION, INACTIVATION, AND SUPPRESSION

The essential role for cytokines in the survival and maintenance of NK cells has been well-established (Carson et al., 1997; Lindemann et al., 2003). Moreover, withdrawal of cytokine IL-15 has been shown to lead to rapid NK cell apoptosis within 24 h of withdrawal (Huntington et al., 2007). For NK cells activated and expanded using the co-culture system (feeder cells with cytokine typically IL-2 at 100 IU/mL), it seems likely that NK cells undergo rapid apoptosis following adoptive cell transfer into a host that does not provide these robust activating signals *in vivo* which occur *in vitro*. These phenomena are similar to cytokine deprivation which has been proposed to be the critical mechanism for regulating T cell contraction following effector T cell expansion (Vella et al., 1998; Strasser and Pellegrini, 2004; Yajima et al., 2006; Fischer et al., 2008). Though this hypothesis has been challenged (Prlic and Bevan, 2008), it clearly highlights the critical role for cytokine deprivation in shaping immune cell compartments. As NK cells are also intensely dependent on cytokines for survival, proliferation, and activation signals, it is likely that cytokine deprivation plays a major role in regulating NK cells and may be responsible for the underwhelming results of clinical trials using *ex vivo* activated and expanded NK cells.

In particular, clinical trials with *ex vivo* expanded NK cells have shown limited NK engraftment following adoptive transfer. For example, in a phase II trial using IL-2 activated allogeneic NK cells transferred into lymphodepleted recipients with recurrent ovarian or breast malignancies ($n = 20$), post-transfer subcutaneous IL-2 administration (10^7 units, 3×/week for 2 weeks) led to *in vivo* NK expansion in only a single patient (expansion defined as ≥100 donor NK cells/μL whole blood at day 14). In this patient, adoptively transferred NK cells reached 40% of the lymphocyte pool by day 7 post-transfer, but then decreased to 4% of the lymphocyte pool by day 14 (Geller et al., 2011). Similar results regarding loss of donor NK

cells were observed in a more recent study in AML patients (Romee et al., 2016). Allogeneic, haploidentical NK cells were activated *ex vivo* with IL-12, IL-15 and IL-18 then transferred into patients with AML. Patients received subcutaneous IL-2 (10^6 IU/m^2 every other day for six doses), and a similar loss of donor NK cells was observed, with most recipients having undetectable donor NK cells by days 14–21 post-transfer. The duration of NK engraftment appear even more challenging for NK-92-based clinical trials as reported from a small trial for hematologic malignancies (Williams et al., 2017). Of the patients who were evaluated for post-transfer NK-92 detection, most transferred cells were lost after 15 min, and circulating NK-92 cells were undetectable by 6 h post-transfer. These trials underscore the common challenge of maintaining NK cell engraftment and persistence following adoptive cell transfer of pre-activated or expanded NK cells that appear dependent on cytokines. Future directions for limiting the *in vivo* dependence of NK cells of supraphysiologic levels of cytokine may include methods to "wean" activated NK cells from cytokine prior to transfer, inhibitory blockade *in vivo* or genetically engineering expanded NK cells to make IL-15 or other stimulatory cytokines.

Inactivation of NK cells in specific contexts can alter functional readouts that are used for identification of dysfunctional states. This can be the case for the activating immunoreceptor NKG2D, a key mediator of NK-cell target recognition (Raulet, 2003). Initially shown on NK and T cells to be activated through stress-inducible MICA (Bauer et al., 1999), NKG2D recognizes MHC-I-related ligands that are rarely expressed on normal cells, but upregulated on "stressed" cells, resulting from viral infections or transformation (Raulet, 2003). These ligands, which are present in both human and mouse, are also able to induce functional changes on the NK cell upon NKG2D binding. When expressed on stressed cells, these ligands act as a target for NK cell killing, but these ligands have also shown to be shed from tumors to act as a decoy for NKG2D-targeting. These shed NKG2D ligands have been shown to negatively affect the effector function of both T cells (Groh et al., 2002) and NK cells (Song et al., 2006). In the work by Song et al., the authors detected soluble NKG2D ligands from gastric cancer cell lines that caused loss of NKG2D expression on NK cells, leading to a decrease in NK target cell lysis in cytotoxicity assays (Song et al., 2006). The influence of soluble NKG2D ligands has been complicated by further work showing an opposite effect in knockout and transgenic mouse models (Deng et al., 2015). In their work, Deng et al., found that shed MULT1 increased NKG2D expression on NK cells and increased effector function resulting in *in vivo* rejection of tumor. These results complicate interpretation of intra-tumoral NK cell function as these shed NKG2D ligands clearly alter cytotoxic capability, a critical readout for NK cell function.

Lastly, another mechanism which mediates NK dysfunction is by extrinsic suppressive signals that NK cells may be exposed to, particularly in the tumor microenvironment. The effects of TGF-β on NK suppression has been extensively described (Batlle and Massagué, 2019) and is one of the most well-established NK inhibitory cytokine/growth factors in the tumor microenvironment (Bellone et al., 1995; Bergmann et al., 1995; Zaiatz-Bittencourt et al., 2018). In fact, inhibition of NK cells by TGF-β has been shown in multiple pre-clinical models (mouse and human, *in vitro* and *in vivo*) (Li et al., 2009; Zaiatz-Bittencourt et al., 2018). These effects may greatly confound data examining intra-tumoral NK cell dysfunction as the extensive direct and indirect effects of TGF-β may be difficult to control for. Additionally, the source of TGF-β can be diverse (tumor, Tregs, macrophages, MDSCs, etc.) and the heterogeneity of infiltrating immune cells could proportionately alter TGF-β concentrations within tumor models and increase NK cell dysfunction. Additional work has shown the impact of MDSCs on NK cell function (Sarhan et al., 2016). Specifically, this group showed that the memory-like adaptive human NK cells express lower TIGIT and are thus less susceptible to MDSC-mediated inhibition through CD155/TIGIT binding. These data highlight the diverse mechanisms of NK dysfunction in the tumor microenvironment and the interplay between suppressive cells, inhibitory receptors, and NK effector function, and the challenges in identifying the etiology of NK dysfunction.

NK CELLS IN ADAPTIVE IMMUNITY

While the classical definition of NK cells as non-T, non-B lymphoid cells of the innate immune system (Vivier et al., 2011) remains true, it has become increasingly evident that NK cells also possess specific traits of the adaptive immune system such as antigen specificity and memory recall (Sun et al., 2009; Vivier et al., 2011; Cerwenka and Lanier, 2016). The capacity of NK cells to recognize specific antigens (e.g., m157 in murine CMV) and display enhanced function when specific antigens are re-encountered, suggest, circumstantially at least, that immune checkpoints may also be operative in NK cells in order to limit autoimmunity and immunopathology, as this is one of the key proposed evolutionary roles for T cell exhaustion. However, the well-defined sequential activation steps required for generation of effector and memory populations in T cells are unidirectional in nature (Smith-Garvin et al., 2009), and T cells require antigen presentation and priming to occur before they are capable of an effector response. In contrast, NK cells are capable of target cell killing from a baseline state, given the presence of adequate activating signals and lack of inhibition, and unlike T cells they can more easily alternate between resting and activated states. Thus, the plasticity and potential for bidirectionality among resting and activated states likely impacts the expression of checkpoint/inhibitory markers (Lanier, 2008), and since NK cells are shorter-lived immune cells that can oscillate between resting and activated states, the benefit of exhaustion marker upregulation on these cells seems less necessary for appropriate immuno-regulation.

MEMORY NK CELLS AND DYSFUNCTION

In addition, the lifespan of NK cells is critically important to the debate regarding NK exhaustion as only long-lived immune cells would theoretically be worth the evolutionary investment of synthesizing and upregulating exhaustion markers to regulate

chronic immune stimulation. Compared to T cells, the lifespan of an NK cell is proposed to be much shorter, although this topic remains an area of significant debate due to differences between humans and mouse models. Fundamental advances in the understanding of NK cell biology have been generated from mouse models (Sungur and Murphy, 2013), and while these advances have improved understanding of human NK cells, there are critical species differences that limit the application of murine data to human application, opening the door for novel immunocompetent models to study NK biology (Park et al., 2016; Canter et al., 2017). One of the most critical differences between mouse and human NK cells concerns the lifespan of an NK cell and the putative, long-lived "memory" NK cell. Early evidence for a memory response by NK cells was put forth by O'Leary et al. (2006) who identified contact hypersensitivity to a hapten upon re-exposure 4 weeks after the initial sensitization. This "recall" response was maintained in T- and B-cell deficient mice ($Rag2^{-/-}$) but lost in mice devoid of all lymphocytes ($Rag2^{-/-}Il2rg^{-/-}$), thereby implicating NK cells in the mechanism. This concept was further developed in studies evaluating the influence of NK cells on early anti-viral responses and post-vaccine re-exposure responses (Horowitz et al., 2010a,b, 2012). Initial data showed the importance of NK cells in responding to the early malaria infection (within 12–18 h), responses which were dependent on CD4-derived IL-2 (Horowitz et al., 2010b). This phenomenon was exploited in post-vaccine NK recall responses which were also dependent on IL-2 produced from antigen-specific memory CD4 T cells (Horowitz et al., 2010a). These data were corroborated in humans immunized against malaria (Horowitz et al., 2012). The concept of intrinsic memory NK cells was expanded in a seminal paper by Sun et al. (2009) who showed an antigen specific memory response of Ly49H+ NK cells after murine cytomegalovirus (MCMV) infection, including after adoptive transfer of MCMV-exposed Ly49H+ NK cells into MCMV non-exposed recipients. The authors demonstrated that these transferred NK cells remained in the recipient for up to 70 days and could respond better than naïve NK cells following anti-NK1.1 stimulation *in vitro* with increased IFNγ expression. Similarly, adoptive transfer of Ly49H+ memory NK cells into DAP12-deficient neonatal mice also produced improved survival among the recipient mice following MCMV infection *in vivo*. These observations from murine studies indicate that long-lived memory NK cells occur in specific settings, and these memory NK cells can exhibit superior effector function without evidence for dysfunction as might be suspected for a longer-lived immune effector cell. In fact, although studies addressing this question are relatively limited to date, there are currently no reports demonstrating increased susceptibility to dysfunctional states (exhaustion, anergy, or senescence) in murine memory NK cells.

The proposed human correlate to the mouse Ly49H+ memory NK cell is the "adaptive" NK cell characterized by NKG2C expression which is proposed to expand following human CMV exposure and recognize target cells via binding of HLA-E (Gumá et al., 2004; O'Sullivan et al., 2015). Work by Jeff Miller's group at U. of Minnesota has showed that allogeneic hematopoietic cell transplant (HCT) recipients who experience CMV reactivation have a preferential expansion of NKG2C+ NK cells, with increased effector functions of this subset (Foley et al., 2012b). An additional study showed upregulation of NKG2C expression following HCT into CMV seropositive recipients (Foley et al., 2012a). However, the importance of NKG2C as a marker of memory NK cells has been challenged by observations that humans who carry homozygous null mutations for NKG2C do not appear to have any demonstrable viral-specific consequences in their immune responses from loss of NKG2C expression, although compensatory mechanisms have been proposed (Liu et al., 2016). Given the interaction between NK cells and CMV, Pesce et al., examined NK cell phenotype among CMV seropositive and seronegative subjects. They showed that only NK cells from CMV seropositive individuals expressed PD-1 (∼25% of cells) vs. 0% in seronegative subjects (Pesce et al., 2017). While the authors noted several phenotypic and functional differences between PD-1+ and PD-1- NK cells from healthy donors, it was notable in this study that 199 of 200 healthy donors had PD-1 expression ≤10%. Other groups have also examined NKG2C+ expression as a surrogate for long-lived adaptive NK cells. Merino et al., reported that NKG2C+ NK cells upregulate checkpoint receptors PD-1 and LAG-3 following prolonged NKG2C agonist activity (Merino et al., 2019). In their study, the authors showed that IL-15 combined with anti-NKG2C signaling led to LAG-3 upregulation, and the LAG-3+NKG2C+ NK cells were less functional as determined by decreased IFNγ expression and diminished cytotoxicity following co-culture with the erythroleukemia cell line and prototypical NK target, K562. Notably, these endpoints of dysfunction are the same features classically associated with T cell exhaustion. Also of note, in contrast to the results obtained by Pesce et al. (2017), Merino et al., detected no PD-1 expression on NKG2C+ or NKG2C- cells at baseline. And while anti-NKG2C and IL-15 led to increased PD-1 expression on the NKG2C+ NK cells only, it appears the NK2GC+ NK cells only represented ≤5% of the total NK cells in the *in vitro* assay (Merino et al., 2019).

Another important point is that the results from murine and human studies investigating memory/adaptive NK cells appear to show conflicting evidence regarding the extent of NK dysfunction and exhaustion. Data from mouse models suggests that memory NK cells exhibit superior responses compared to naïve NK cells as seen by increased IFNγ and improved survival with infection, suggesting a lack of dysfunction or exhaustion. In contrast, data from human studies suggest that adaptive NK cells are the principal NK cell subset susceptible to exhaustion as seen by increased PD-1 and LAG-3 expression and decreased functional effects. Ultimately, NKG2C+ NK cells appear to be a minority of peripheral NK cells and are lacking in humans with homozygous null mutations, with no apparent adverse effects. However, the therapeutic potential of expanded NKG2C+ NK cells remains to be determined, as CMV exposure is known to alter the KIR repertoire with long-lasting alterations in the inhibitory and activating KIR profile (Béziat et al., 2013).

Apart from antigen-induced memory NK cells, other recent studies suggest that human (Romee et al., 2012) and mouse (Cooper et al., 2009) memory NK cells can be induced solely by cytokine exposure, and that these cytokine-induced memory NK cells can be exploited to elicit meaningful anti-leukemia effects in cancer patients (Romee et al., 2016). Notably, however, although

an NK "memory" phenotype is postulated as the mechanism of action in a clinical trial using cytokine-stimulated memory NK cells, this conclusion is limited by data showing that transferred NK cells following *ex vivo* cytokine expansion (and "memory" formation) rapidly disappear from the recipients' peripheral blood by day 7–14 post-transfer (Romee et al., 2016). While the concept of memory NK cell induction by cytokine signaling alone is intriguing and warrants further investigation, an examination of the significance of this subset of NK cells is hindered by observations showing that this NK cell subset does not persist beyond a maximum of 21 (Romee et al., 2012) or 22 (Cooper et al., 2009) days post-transfer. Although exhaustion or other manifestations of dysfunction may underlie their limited lifespan *in vivo* (as observed with other NK cellular therapies), a formal assessment for exhaustion or dysfunction parameters have not been performed.

CONCLUSION

Characterizing and differentiating between anergy, exhaustion and senescence has led to critical discoveries in the biology of T cell dysfunction, most notably the reversal of exhaustion with blocking antibodies which can lead to dramatic clinical anti-tumor benefits. As the same paradigm is being applied to NK cells through application of checkpoint blockade therapy, including both PD-1 and PD-L1 inhibitors, it is critical to delineate when, how, and why anergy, exhaustion and senescence of NK cells occurs in order to better understand their complex biology and thus fully realize the potential of NK-based therapies.

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SJ, WM, and RC conceptualized the manuscript, reviewed the literature, wrote, and edited the final paper.

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NK Cells Regulate CD8$^+$ T Cell Mediated Autoimmunity

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Elucidating key factors that regulate immune-mediated pathology *in vivo* is critical for developing improved strategies to treat autoimmune disease and cancer. NK cells can exhibit regulatory functions against CD8$^+$ T cells following viral infection. Here we show that while low doses of lymphocytic choriomeningitis virus (LCMV-WE) can readily induce strong CD8$^+$ T cell responses and diabetes in mice expressing the LCMV glycoprotein on β-islet cells (RIP-GP mice), hyperglycemia does not occur after infection with higher doses of LCMV. High-dose LCMV infection induced an impaired CD8$^+$ T cell response, which coincided with increased NK cell activity during early time points following infection. Notably, we observed increased NKp46 expression on NK cells during infection with higher doses, which resulted in an NK cell dependent suppression of T cells. Accordingly, depletion with antibodies specific for NK1.1 as well as NKp46 deficiency (*Ncr1$^{gfp/gfp}$* mice) could restore CD8$^+$ T cell immunity and permitted the induction of diabetes even following infection of RIP-GP mice with high-dose LCMV. Therefore, we identify conditions where innate lymphoid cells can play a regulatory role and interfere with CD8$^+$ T cell mediated tissue specific pathology using an NKp46 dependent mechanism.

Keywords: CTL, LCMV, IFN-α, autoimmunity, pathology

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INTRODUCTION

For decades, efforts have been made to understand ways to promote the induction of CD8$^+$ T cell immunity as an avenue to improve tumor immune therapy, promote viral clearance, or treat autoimmune diseases. Many factors are known to influence T cell activation and function, such as the maturation status of antigen-presenting cells (APC) the expression of co-inhibitory

or co-stimulatory molecules and the cytokine microenvironment. Although immunotherapeutic strategies targeting co-inhibitory molecules have revolutionized cancer treatment (Pardoll, 2012; Ribas and Wolchok, 2018), it is clear that additional work is required to better define the criteria for strong immune responses *in vivo* in order to further develop and/or refine existing immunotherapies.

Various immune cell populations, such as regulatory T cells have been shown to impact $CD8^+$ T cell responses (Mempel et al., 2006). Studies have also demonstrated that innate lymphoid cells including $NK1.1^+$ cells in mice or $CD56^+$ cells in humans have displayed immune-regulatory functions and can play an important role in limiting $CD8^+$ T cell responses (Crome et al., 2013). ILCs/NK cells regulate $CD8^+$ T cell anti-viral immunity (Su et al., 2001; Lu et al., 2007; Lang et al., 2012; Waggoner et al., 2012), and $CD8^+$ T cell antitumor immunity (Iyori et al., 2011; Iraolagoitia et al., 2016; Crome et al., 2017; Picard et al., 2019).

NK cell activity is orchestrated by a wide variety of activating and inhibiting receptors on NK cells. For example, elevated NKG2D-Ligand expression on activated T cells may trigger their susceptibility to NK cell regulation, presumably by binding to NKG2D activating receptors on NK cells (Rabinovich et al., 2003; Lang et al., 2012). Furthermore, type I interferon (IFN-I) can suppress expression of ligands for the activating NK cell receptor NKp46 (Crouse et al., 2014). Hence, IFN-I is important to protect anti-viral T cells against NK cell mediated attack (Crouse et al., 2014; Xu et al., 2014). IFN-I can induce expression of MHC-I and MHC-Ib molecules, such as Qa-1b, which bind to inhibitory NK cell receptors and reduce NK cell mediated regulation of anti-viral T cells (Xu et al., 2014, 2017). Furthermore, lack of the inhibitory NK cell receptor, 2B4, is associated with increased NK regulatory activity and limited T cell immunity during infection (Waggoner et al., 2010). Moreover, NK cells may target $CD4^+$ T cells for killing and thus prevent T cell help to cytotoxic T cells (Waggoner et al., 2012).

We have used the RIP-GP model to dissect the events that are required for the activation of $CD8^+$ effector T cell function that is sufficient to induce tissue destruction *in vivo*. In this model, transgenic expression of the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) is restricted to the β-islet cells of the pancreas via the rat insulin promotor (RIP). In this system, $CD8^+$ T cell mediated destruction of the islets can be induced by LCMV infection, which lead to hyperglycemia and diabetes in these animals. Similarly, mature dendritic cells pulsed with LCMV-GP peptides can also trigger the expansion of tissue specific LCMV-GP reactive T cells, which recognize and destroy β-islet cells expressing LCMV-GP (Ohashi et al., 1991; Dissanayake et al., 2011; Lin et al., 2011). Furthermore, IFN-I increases MHC-I expression on β-islet cells during infection, which is critical for T cell infiltration into the pancreas and diabetes progression (Lang et al., 2005, 2009).

In the present study, we found that RIP-GP mice infected with a high dose of LCMV showed a modest reduction in $CD8^+$ T cell immune responses and did not develop diabetes. High dose, but not low dose LCMV infection resulted in rapid NK cell activation and expression of NKp46 after infection. Accordingly, depletion of NK cells or NKp46 deficiency in mice restored $CD8^+$ T cell immunity and induction of autoimmunity in animals infected with high doses of LCMV. These studies demonstrate that NK cells can significantly limit $CD8^+$ T cell responses by NKp46 *in vivo* and have a profound impact on tissue destruction.

RESULTS

Infection With a High Dose of LCMV Limits the Induction of Diabetes

In order to determine whether different infectious doses of LCMV can influence the induction of $CD8^+$ T cell mediated immune pathology, RIP-GP mice were infected intravenously with the LCMV WE strain ranging from 10^3 to 10^5 plaque-forming units (PFU) per mouse. Consistent with previous observations, all mice infected with low dose LCMV (10^3 PFU) developed hyperglycemia, and subsequently diabetes, after ∼10 days (Ohashi et al., 1991, 1993). Surprisingly however, only a fraction of the animals infected with an intermediate dose (10^4 PFU), and very few of the mice infected with a high dose of LCMV (10^5 PFU) became diabetic (**Figure 1A**, **Supplementary Figure 1A**).

Previous work has shown that induction of diabetes in RIP-GP mice is dependent on $CD8^+$ T cell infiltration and destruction of pancreatic islets (Ohashi et al., 1991). Consistent with glycemic measurement, the pancreatic islets of mice infected with 10^3 PFU of LCMV exhibited strong $CD8^+$ and $CD4^+$ T cell infiltration, compared to the limited infiltration after 10^5 PFU of LCMV (**Figure 1B**). MHC-I expression levels on islet cells were similar in animals infected with a high dose and low dose LCMV (**Supplementary Figure 1B**), indicating that limited $CD8^+$ T cell infiltration and lack of diabetes induction in high dose infection was likely not due to differential islet MHC-I expression (Ohashi et al., 1993; Lang et al., 2005), but may be related to altered T cell function.

Infection With High Dose LCMV Generates Limited Numbers of Virus-Specific $CD8^+$ T Cells

In order investigate the mechanism in which high dose LCMV impairs the induction of diabetes in this model, we analyzed $CD8^+$ T cell responses following infection with different LCMV doses. LCMV-gp33 tetramer analysis in peripheral blood revealed that a significantly higher proportion of virus specific $CD8^+$ T cells were present in the peripheral blood of low dose LCMV infected mice (**Figure 2A**). These effects were not restricted to the gp33 epitope as the percentage of $CD8^+$ T cells recognizing the nucleoprotein-derived np396 epitope of LCMV was also increased following low dose infection (**Supplementary Figure 2A**). Next we evaluated the effect of LCMV dosage on the capacity of virus-specific $CD8^+$ and $CD4^+$ T cells to produce effector cytokines following *in vitro* LCMV-peptide re-stimulation. We found the proportion of TNF-α (**Figure 2B**), IFN-γ (**Figure 2C**), and IL-2 (**Figure 2D**) producing $CD8^+$ T cells was 3–5-fold lower in high dose LCMV infected mice compared with mice infected with low dose LCMV (**Figures 2B–D**). Similar changes were observed for the

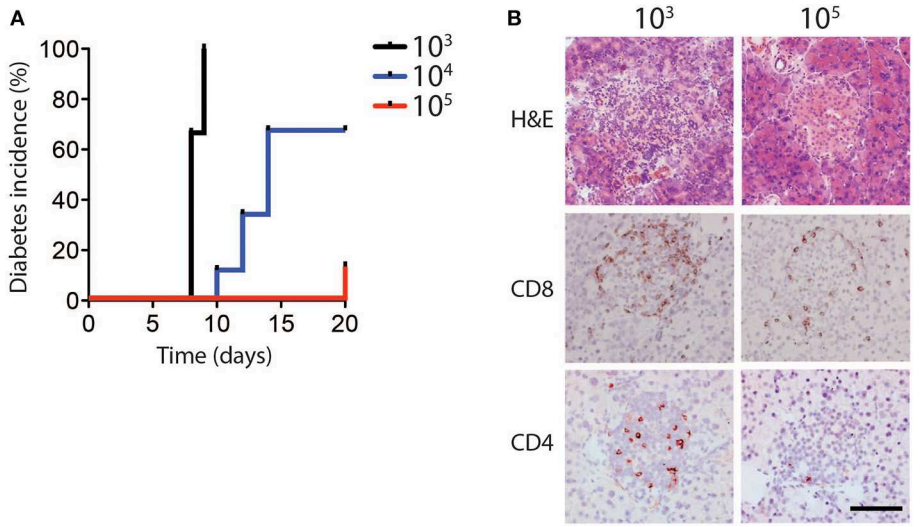

FIGURE 1 | Diabetes incidence depends on dose of LCMV infection. **(A,B)** RIP-GP mice were infected with 10^3, 10^4, or 10^5 PFU of LCMV WE. **(A)** Percent incidence of diabetes following LCMV infection at indicated doses was determined by glycaemia measurement ($n = 8$–9). **(B)** Immunohistochemistry of sections from snap frozen pancreas samples harvested from RIP-GP mice infected with 10^3 and 10^5 PFU of LCMV showing H&E, anti-CD8, and anti-CD4 staining. One representative image of $n = 3$–5 is shown (scale bar = 50 μm).

T cells specific for the LCMV-nucleoprotein- derived epitope (**Supplementary Figure 2B**). Furthermore, the proportion of $CD4^+$ T cells producing IFN-γ in response to the LCMV-derived immune-dominant MHC-II restricted helper cell epitope, GP-61, was also reduced in high dose vs. low dose infected mice (**Figure 2E**). Consistently, direct *ex-vivo* examination of T cell cytotoxicity revealed reduced cytotoxic killing capacity of T cells derived from mice infected with high-dose LCMV compared to T cells from mice inoculated with low dose LCMV (**Figure 2F**).

We next examined potential mechanisms to explain the observed reduced T cell activity and inability to induce diabetes following high dose LCMV infection. We examined the kinetics and the pattern of viral replication in mice infected with different doses of LCMV. Spleen viral titers at day 4, 6, and 12 post-infection were similar in low dose compared to high dose LCMV infected mice (**Figure 2G**). All mice had cleared LCMV by day 12 after infection from the spleen (**Figure 2G**). Thus, although induction of LCMV-specific $CD8^+$ T cells was reduced in high dose LCMV infected mice and did not result in destruction of pancreatic islets *in vivo*, T cell immunity was still sufficient to potently eliminate the virus.

We next examined whether viral inoculum dose resulted in differential levels of LCMV in the pancreas. Replicating LCMV could not be measured in the pancreas at all-time points tested after either low or high dose LCMV infection (**Supplementary Figure 3A**). Immune-histologic staining for the LCMV nucleoprotein readily detected virus infected cells in the marginal zone of the spleen 3 days after low or high dose LCMV infection (**Supplementary Figure 3B**). However, infected cells could not be detected in the pancreas at any of the time points examined (**Supplementary Figure 3C**). Thus, we speculated that it is unlikely that the observed differences in tissue destruction in low dose vs. high dose LCMV infected mice are due to differential accumulation of LCMV antigen in the pancreas.

NK Cells Limit the Generation of $CD8^+$ T Cells Following High Dose LCMV Infection

Next, we examined whether the LCMV doses had an impact on the induction of NK cytotoxicity using YAC-1 target cells. NK cell mediated cytotoxicity was augmented early after high dose LCMV infection when compared with low dose infected animals (**Figure 3A**), which rapidly declined consistent with previous reports (Xu et al., 2017). Forty-eight hours after infection with LCMV high dose, NK cells exhibited higher expression of activation markers, such as CD11b, CD27, and 4-1BB when compared to low dose infected mice (**Figures 3B,C**, **Supplementary Figure 4**). To examine whether NK cells from high vs. low dose infected mice were able to directly kill $CD8^+$ T cells, NK cells were harvested from infected animals and co-incubated with T cells *in vitro*. We observed reduced T cell numbers in the presence of NK cells obtained from 10^6 PFU infected animals when compared to NK cells from 10^3 PFU infected mice (**Figure 3D**). Next we examined potential mechanisms by which increased NK cell activity was observed after high dose infection. NK cells showed upregulation of the NK cell activating receptor NKG2D during LCMV infection (**Figures 3E,F**). However, the activating NK cell receptor NKp46 was selectively up-regulated following infection with high dose of LCMV in contrast to low dose infection (**Figures 3E,G**). Accordingly, NK cell mediated suppression of T cells was not detected when $Ncr1^{gfp/gfp}$ NK cells were used compared to WT NK cells (**Figure 3H**). Therefore, NK cells from mice infected with high dose LCMV were able to kill T cells more readily than NK cells from low dose infected mice via an NKp46 dependent mechanism.

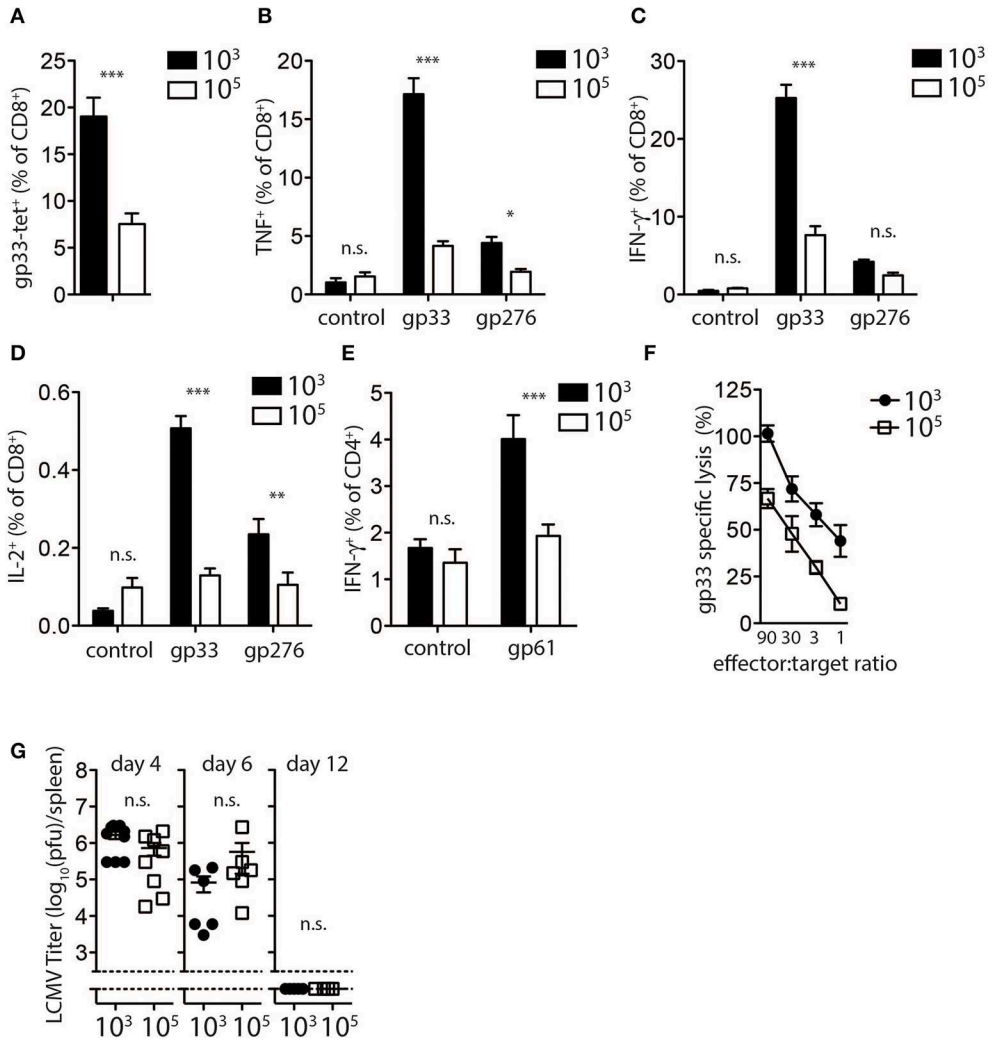

FIGURE 2 | Expansion and function of virus specific CD8$^+$ T cells depends on infectious dose of LCMV. **(A–E)** C57Bl/6 mice were infected with 10^3 and 10^5 PFU of LCMV-WE. T cells were analyzed on day 8 post-infection by flow cytometry or ^{51}Cr release assay. **(A)** LCMV specific CD8$^+$ T cells in blood samples were measured by tetramer staining. ***indicates $p < 0.001$, $n = 8$. **(B)** TNF-α production in CD8$^+$ T cells after re-stimulation for 5 h with LCMV specific peptides determined by intracellular staining (***indicates $p < 0.001$, *indicates $p < 0.05$, $n = 9$–11). **(C)** Intracellular IFN-γ levels measured in CD8$^+$ T cells of from single cell suspended splenocytes after re-stimulation with LCMV specific epitopes (***indicates $p < 0.001$, n.s. indicates not significant, $n = 7$–8). **(D)** Intracellular IL-2 levels measured in CD8$^+$ T cells of from single cell suspended splenocytes after re-stimulation with LCMV specific epitopes (***indicates $p < 0.001$, **indicates $p < 0.01$, n.s. indicates not significant, $n = 5$). **(E)** IFN-γ production in CD4$^+$ T cells after re-stimulation with the LCMV specific epitope gp61 measured by intracellular staining (***indicates $p < 0.001$; n.s. indicates not significant, $n = 5$). **(F)** Percent gp33 specific lysis determined by ^{51}Cr release from labeled EL-4 target cells following incubation at the indicated splenocyte (effector)/target cell ratios (effector target ratio) ($n = 6$, left panel). **(G)** Virus titers were analyzed in spleen tissue at the indicated time points after LCMV infection by plaque assay ($n = 3$–6).

NK Cells Prevent Diabetes Induction Following High Dose LCMV Infection

While low dose LCMV infection induced autoimmunity in RIP-GP mice, high doses of LCMV failed to induce hyperglycemia (**Figure 1A**). In this model, evidence demonstrated that high dose LCMV infection activates NK cells to limit autoreactive CD8$^+$ T cell responses. We next tested whether direct elimination of NK cells might be sufficient to alleviate the block in diabetes induction following high dose LCMV infection of RIP-GP mice. RIP-GP mice were treated with the NK-cell depleting antibody against NK1.1 and infected with high doses of LCMV-WE. All NK cell-depleted RIP-GP mice developed diabetes following high dose LCMV infection, whereas NK cell competent high dose LCMV infected RIP-GP mice remained euglycemic (≤14 mM) or showed significantly delayed hyperglycemia (**Figures 4A,B**). NK cell depletion did not change the initial virus replication in spleen or pancreas tissues nor the IFN-I production in the sera when compared to non-NK cell depleted control animals (**Supplementary Figure 5**).

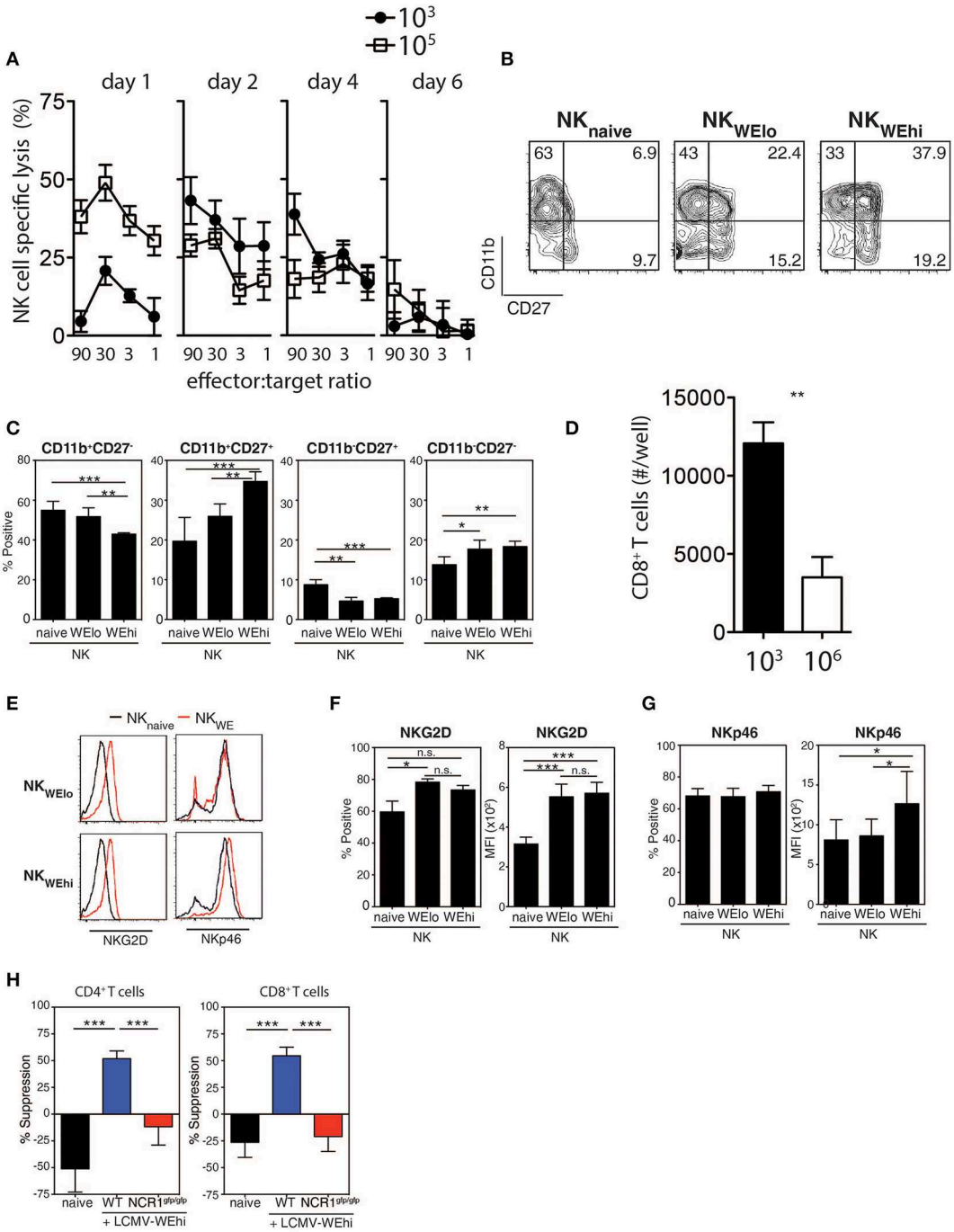

FIGURE 3 | NK cells are activated during high dose LCMV infection. C57Bl/6 mice were infected with 10^3 or 10^5 PFU of LCMV WE at day 2 post-infection. **(A–C)** NK cell cytotoxicity was determined by ^{51}Cr release assay on Yac-1 target cells ($n = 4$). **(B,C)** CD11b and CD27 expression were determined on NK cells ($n = 7$). **(D)** NK cells were harvested from animals infected with 10^3 or 10^6 PFU of LCMV. Following coincubation with T cells, CD8$^+$ T cell number was assessed 48 h later ($n = 3$–5). **(E–G)** C57Bl/6 mice were infected with 10^3 or 10^5 PFU of LCMV WE. NKG2D and the NKp46 expression level was measured on NK cells from spleen tissue at day 2 post-infection. **(H)** NK cells were harvested from WT or NCR1$^{gfp/gfp}$ animals infected with 10^6 PFU of LCMV. Following coincubation with CD4$^+$ T cells or CD8$^+$ T cells, T cell suppression was determined ($n = 4$–13) (*indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$).

We hypothesized that increased NK cell activity was triggered by the NK cell activating receptor NKp46. NKp46 ligands are suppressed on antigen specific T cells through IFN-I signaling, which renders IFNAR deficient T cells susceptible toward NK cell mediated attack (Crouse et al., 2014). Consistent with this hypothesis, when we adoptively transferred WT LCMV-GP

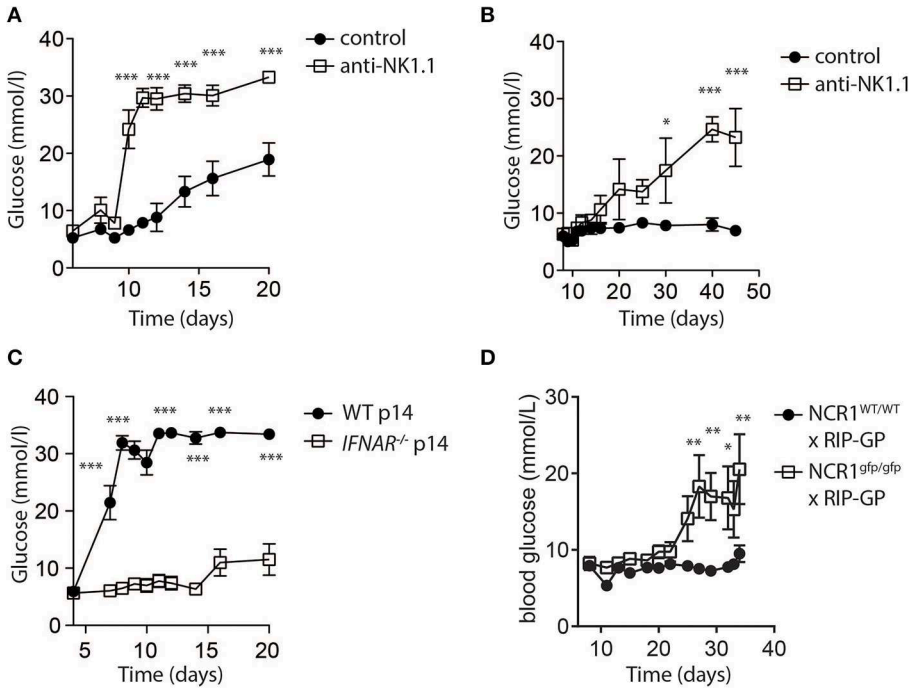

FIGURE 4 | NK cells control CD8$^+$ T cell induced autoimmune disease. **(A)** RIP-GP mice were infected with 10^5 PFU of LCMV. One group (open squares) was treated with anti-NK1.1 on d-3 and d-1. Blood glucose levels were analyzed at indicated time points ($n = 3$–9, ***indicates $p < 0.001$; n.s. indicates not significant). **(B)** Mice were treated as in **(A)** but infected with $3x10^5$ PFU of LCMV ($n = 3$). **(C)** Equal amount of negatively sorted T cells from *P14*$^+$ and *IFNAR*$^{-/-}$*P14*$^+$ mice were transferred into RIP-GP mice and infected with 10^5 PFU of LCMV. Blood glucose levels were monitored at different time points ($n = 6$). **(D)** RIP-GP/NCR1$^{wt/wt}$ and RIP-GP/NCR1$^{gfp/gfp}$ were infected with 10^5 pfu of LCMV. Blood glucose levels were monitored at different time points ($n = 4$) (*indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$; n.s. indicates not significant).

specific T cells, the induction of diabetes in RIP-GP mice occurred following high dose infection due to increased antigen specific T cell immunity (**Figure 4C**). However, in absence of IFNAR on LCMV specific T cells, diabetes was not induced (**Figure 4C**) because the T cells express high levels of NKp46, leading to the activation of NK cells which in turn limits survival of LCMV-specific T cells. Accordingly, when we infected *Ncr1*$^{gfp/gfp}$ X RIP-GP animals, the induction of diabetes was restored compared to WT RIP-GP controls, due to the lack of NKp46 expression which resulted in impaired induction of NK activity (**Figure 4D**). Taken together, these data indicate that NK cells inhibit the LCMV-gp specific CD8$^+$ T cell response and prevent the induction of diabetes via the NK cell activating receptor NKp46.

NK Cells Limit Anti-viral T Cell Immunity During High Dose Infection

Previous reports suggest that NK cell depletion improves T cell immunity during chronic viral infection (Lang et al., 2012; Waggoner et al., 2012). To investigate whether NK cells were responsible for the reduced generation and function of GP-specific CD8$^+$ T cells in high dose LCMV infected mice, we depleted NK cells *in vivo* by administration of the depleting NK1.1 antibody before low or high dose LCMV infection. NK cell depletion rescued TNF-α and IFN-γ production in CD8$^+$ T cells in mice infected with high dose LCMV, but did not alter CD8$^+$ T cell function in low dose LCMV infected mice (**Figure 5A**). Notably, we observed a significant increase in CD4$^+$ T cell mediated IFNγ production following NK cell depletion in mice infected with low and high doses of LCMV (**Figure 5B**). Moreover, we observed increased CD8$^+$ T cell immunity following infection of NKp46 deficient mice with LCMV high dose when compared to WT animals (**Figure 5C**). Taken together we concluded that NK cells limit antigen specific T cell immunity and accordingly prevent establishment of autoimmune diabetes.

DISCUSSION

Here, we examined the effect of the infectious dose of LCMV on CD8$^+$ T cell function and the induction of autoimmunity. Unexpectedly, RIP-GP mice infected with a high dose of LCMV showed limited induction of diabetes, whereas mice infected with low doses of virus developed diabetes within 2 weeks. The impaired induction of diabetes following high dose LCMV infection was associated with reduced numbers of circulating GP-specific CD8$^+$ T cells, and correspondingly reduced cytokine production and cytotoxic function. Importantly, high dose LCMV infection provided the appropriate microenvironment that led to the induction of regulatory NK cells. This was demonstrated by the ability of NK cells from high dose infected

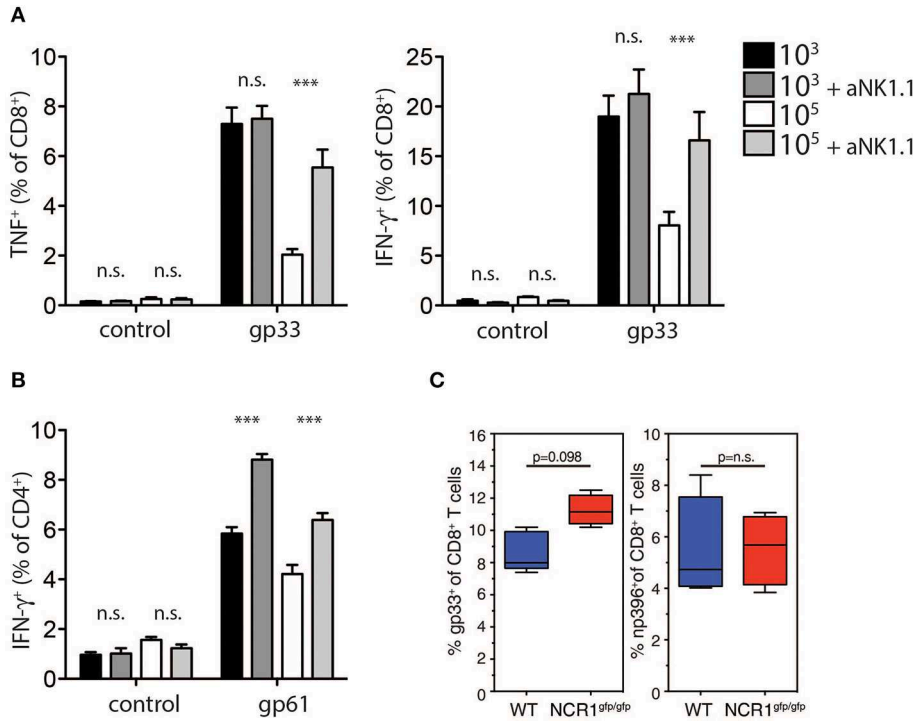

FIGURE 5 | NK cell depletion restores T cell immunity in high dose infected animals. **(A,B)** RIP-GP and anti-NK1.1 treated RIP-GP mice were infected with 10^3 or 10^5 PFU of LCMV-WE **(A)** TNF-α production in $CD8^+$ T cells after LCMV specific peptide restimulation for 5 h was determined by intracellular staining (left panel, ***indicates $p < 0.001$, *indicates $p < 0.05$, $n = 6$). Right panel: intracellular IFN-γ levels were measured in $CD8^+$ T cells after restimulation with the indicated LCMV specific epitopes (***indicates $p < 0.001$, n.s. indicates not significant, $n = 6$) **(B)** IFN-γ production in $CD4^+$ T cells after restimulation with the LCMV specific epitope gp61 was measured by intracellular staining (left panel, ***indicates $p < 0.001$; n.s. indicates not significant, $n = 5$). **(C)** RIP-GP × $NCR1^{wt/wt}$ and RIP-GP × $NCR1^{gfp/gfp}$ animals were infected with 10^5 pfu of LCMV. Tetramer-$gp33^+$ and tetramer-$np396^+$ T cells were determined in spleen tissue (one of three independent experiments were shown $n = 4$–5).

mice to kill activated $CD8^+$ T cells, while NK cells from low dose infected mice could not kill $CD8^+$ T cells. Blocking this regulatory capacity of NK cells by either NK cell depletion *in vivo* or limiting the activation of NK cells using NKp46 deficient mice led to the induction of diabetes after infection with high dose virus. This demonstrates that NK cells regulate $CD8^+$ T cell effector function *in vivo* against tissue specific antigens.

It has been proposed that NK cells play a regulatory function during human autoimmune diseases. Specifically, impaired NK cell activity was associated systemic onset of juvenile rheumatoid arthritis (JRA) and macrophage activation syndrome when compared to pauciarticular and polyarticular JRA (Grom et al., 2003; Villanueva et al., 2005). Consistently, within individuals suffering from type I diabetes after Coxsackie virus infection, the number of β cells in islets where increased when NK cells were present (Dotta et al., 2007; Lehuen et al., 2010). In animal models, NK cells may also mediate protective effects of CFA (complete Freund's adjuvant) in non-obese diabetic mice (Lee et al., 2008). Moreover, decreased NK cells promote the development of autoimmunity in C57BL/6 lpr mice (Takeda and Dennert, 1993). Our data indicate that infection with a higher virus dose may enhance the regulatory function of NK cells in order to prevent T cell mediated autoimmunity. NK cells are known to prevent autoimmunity during autoimmune encephalitis (Poggi and Zocchi, 2014). In patients, expansion of $CD56^{bright}$ NK cells correlate with disease regression during therapy with daclizumab (Bielekova et al., 2006). Accordingly, NK cells inhibit pathologic disease progression during murine experimental autoimmune encephalitis (Zhang et al., 1997). Furthermore, blockade of the inhibitory NK cell receptor NKG2A alleviates the pathologic score by reducing autoreactive T cell immunity (Leavenworth et al., 2010). Considering our data, a high dose viral infection exhibits enhanced NK cell activation and up-regulates the activating NK cell receptor NKp46, alleviating autoreactive T cell immunity. Low dose infections however, may not result in regulatory NK cell functions thus allowing autoimmunity to happen.

NK cells are regulated by a variety of inhibitory and activating receptors. Previous studies indicate that NK cell regulatory ligand activity is clinically relevant for human infection. For instance, homozygous expression of the NK cell inhibitory receptor gene KIR2DL3 and its ligands, the HLA-C group 1 alleles, correlate positively with clearance of HCV (Khakoo et al., 2004). Conversely, the NK cell activating receptor gene KIR2DS3 is associated with elevated transaminases and persistence of seropositive HCV infection (Paladino et al., 2007). Based on our

observations, it may be insightful to investigate the prevalence of $CD8^+$ T cell mediated autoimmune diseases in patients with altered expression of NK regulatory receptors, such as KIR2DL3 and KIR2DS3. Furthermore, it may partially explain HLA driven predisposition to autoimmune disorders by altered $CD8^+$ T cell functions in individuals with HLA haplotypes with variable activity toward inhibiting receptors on NK cells. Patient studies are warranted to investigate the scope of the mechanism we have uncovered in this report.

MATERIALS AND METHODS

Mice, Virus, and Monitoring of Diabetes

C57BL/6J mice were obtained from The Jackson Laboratory. RIP-GP mice (C57Bl/6 background) have been previously described (Ohashi et al., 1991). $NCR1^{gfp/gfp}$ mice have been previously described (Glasner et al., 2018). Wild-type LCMV (WE strain) was originally obtained from F. Lehmann-Grube (Weidt et al., 1995). To generate viral stocks, viruses were grown in L929 cells for 48 h and subsequently titrated as described previously (Battegay et al., 1991). Diabetes progression in RIP-GP mice was assessed by monitoring blood glucose levels following infection with 10^3, 10^4, or 10^5 plaque-forming units (PFU) of wild-type LCMV-WE. A mouse was considered diabetic when its blood glucose reached 14 mM. We measured blood glucose using Contour glucometers (Bayer, Leverkusen). Viral titers in different organs were determined by plaque assays on MC57 cells, as previously described (Battegay et al., 1991). All mice were maintained under specific pathogen-free conditions at the Ontario Cancer Institute Animal Resource Center following institutional guidelines, or in accordance with LANUV under German laws for animal protection.

Cytolytic T Lymphocyte Assay

Splenocytes from LCMV-infected mice were incubated for 5 h at 37°C in 96-well plates with EL-4 target cells previously loaded with LCMV-specific peptide and labeled with 400 μCi/ml ^{51}Cr (Perkin Elmer). Eighty microliters of the culture supernatant was counted from each well using a Wallac Wizard counter (Perkin Elmer). Maximal release was induced by adding 100 μl of 1M HCl to target cells. Percent specific lysis was calculated as (c.p.m. sample release – c.p.m. spontaneous release)/(c.p.m. maximal release – c.p.m. spontaneous release) × 100. For NK cell-mediated killing, we followed the same protocol using YAC cells as targets. For NK cell – T cell assay, T cells were isolated from a naive mouse following stimulation with plate bound anti-CD3 plus soluble anti-CD28 (eBiosciences) antibodies in the presence of NK cells isolated from mice infected at the indicated doses. T cells were counted after 72 h.

Flow Cytometry Analysis

For tetramer staining, splenocytes were stained using phycoerythrin-labeled MHC class I tetramer GP33/H-$2D^b$, or NP396/H-$2D^b$ for 15 min at 37°C, prior to the addition of an antibody specific for CD8 (BD PharMingen) for 20 min at 4°C, as previously described (Lang et al., 2013). For the analysis of IFN-γ, IL-2, and TNF-α intracellular expression, splenocytes were collected from mice 8 days after LCMV infection. The cells were restimulated *ex-vivo* in 96-well round-bottom plates (10^6 cells/well) in Iscove's medium supplemented with 10% FCS and in presence of Brefeldin A (Pharmingen) and LCMV-specific MHC-I epitope peptides at a concentration of 10^{-7} M. After 5 h at 37°C, the cells were harvested, washed once with FACS buffer, and surface-stained with an allophycocyanin-Cy7-labeled anti-CD8 antibody. After washing, cells were stained for intracellular cytokines using the cytofix/cytoperm kit accordingly to manufacturer's instructions (Pharmingen). Cells were analyzed using a FACSCalibur equipped with Cellquest software (Becton Dickinson, San Jose, CA) or a FACS Canto equipped with DiVa software. The NKG2D tetramer was performed like previously described (Jamieson et al., 2002). NK cells were determined by anti-NK1.1 and anti-CD3 staining. All NK cell receptor antibodies and all NK cell receptor ligand antibodies were obtained from eBioscience.

Immunohistochemistry

Freshly removed pancreata were immersed in phosphate-buffered saline (PBS) and snap-frozen in liquid nitrogen. For the staining of cell differentiation markers, frozen tissue sections (8 μm thick) were cut using a cryostat and stained as previously described (Lang et al., 2008) with primary rat monoclonal antibodies to CD8, CD4, MHC class I.

ELISA

Sera IFN-α level was determined according to Invitrogen IFN alpha Mouse ELISA Kit's instruction.

Statistical Analysis

Data are expressed as mean ± S.E.M. Statistical significant differences between two different groups were analyzed using student's *t*-test. Statistical difference between several groups was tested using one-way ANOVA with additional Bonferoni or Dunnett test. Statistically significant differences between groups in experiments involving more than one analysis time point were calculated using two-way ANOVA (repeated measurements). $p < 0.05$ were considered as statistically significant.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Ontario Cancer Institute Animal Resource Centre, Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV).

AUTHOR CONTRIBUTIONS

PL and SC performed experiments and wrote the paper. HX, KL, LC, ED, MG, AP, VP, RW, TH, AE, and DM performed

experiments. HC, AD, MR, DH, and TM provided reagents and discussed the data. PO initiated the study and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00036/full#supplementary-material

Supplementary Figure 1 | Different infectious doses of LCMV affect diabetes induction but does not affect MHC-I upregulation in pancreatic islet cells. **(A)** RIP-GP mice were infected with 10^3, 10^4, or 10^5 PFU of LCMV WE. Glycemia measurements are shown for individual mice following indicated doses of LCMV (percent incidence of diabetes for same experiment shown in **Figure 1A** for all doses). **(B)** C57Bl/6 mice were infected with 10^3, 10^4, or 10^5 PFU of LCMV. MHC-I specific immunohistochemistry staining of snap frozen pancreas samples are shown. One representative of $n = 3$ is shown (scale bar = 50 μm).

Supplementary Figure 2 | LCMV-NP specific $CD8^+$ T cells responses are impaired after high dose infection compared to low dose infection. C57Bl/6 mice were infected with 10^3 or 10^5 PFU of LCMV-WE. 8 days post-infection, **(A)** np396-tetramer$^+$$CD8^+$ T cells were determined and **(B)** splenocytes were restimulated with the LCMV specific peptide np396 followed by measurement of intracellular IL-2 (left panel), TNF-α (middle panel), and IFN-γ (right panel) levels by flow cytometry (***indicates $p < 0.001$, $n = 5$).

Supplementary Figure 3 | LCMV replication can be detected in the spleen but not in the pancreas after infection with 10^3 or 10^5 PFU LCMV. **(A–C)** C57Bl/6 mice were infected with 10^3 or 10^5 PFU of LCMV WE. **(A)** Virus titers in pancreas tissue were measured at the indicated time points following LCMV infection by plaque assay **(B,C)** Immunohistochemistry staining for the nucleoprotein of LCMV (clone: VL-4) was performed on sections obtained 3 days following LCMV infection in spleen **(B)**, and pancreas **(C)** sections (one representative image of $n = 3$ mice is shown, scale bar = 50 μm).

Supplementary Figure 4 | 4-1BB is upregulated in NK cells from high dose infected animals. **(A–D)** C57Bl/6 mice were infected with 10^3 or 10^5 PFU of LCMV WE. The expression of various surface markers and transcriptional factors indicated were determined in NK cells 2 days after infection (*indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, $n = 3$–4).

Supplementary Figure 5 | Similar early virus replication and normal IFN-I production in the presence or absence of NK cells. Control or NK cell depleted mice were infected with 10^3 or 10^5 PFU of LCMV WE. **(A)** At day 2 post-infection virus titer in spleen and pancreas tissues were measured ($n = 4$). **(B)** IFN-I level from sera at day 1 and day 2 post-infection was quantified (*indicates $p < 0.05$, ***indicates $p < 0.001$, $n = 4$).

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Regulation of NK-Cell Function by HLA Class II

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Natural Killer (NK) cells were initially described as part of the innate immune system and characterized by their ability to lyse malignant and virus-infected cells. The cytolytic function of NK cells is tightly controlled by activating and inhibitory receptors expressed on the cell surface. Ligands that interact with a variety of NK-cell receptors include the human leukocyte antigen (HLA) molecules, and the regulation of NK-cell function by HLA class I molecules is well-established. Earlier studies also suggested a role of HLA class II molecules in regulating NK cell activity; yet, interactions between HLA class II molecules and NK cell receptors have not been well-characterized. We recently identified a subset of HLA-DP molecules that can serve as ligands for the natural cytotoxicity receptor NKp44 and activate NK cells. This novel receptor-ligand interaction provides a potential mechanism to explain the strong associations of HLA-DP molecules with HBV infection outcomes, graft-vs.-host disease and inflammatory bowel disease. Furthermore, it adds a new mechanism for NK-cell crosstalk with immune cells expressing HLA class II molecules. In this perspective article, we discuss the potential implications of NK cell receptor interactions with HLA class II molecules for the regulation of immune responses.

Keywords: natural killer cells, HLA class II, HLA-DP, immune cross-talk, HBV

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HLA CLASS II MOLECULES CAN SERVE AS LIGANDS FOR NK CELL RECEPTORS

The functional activity of Natural Killer (NK) cells is regulated by the expression of inhibitory and activating receptors, many of which interact with human leukocyte antigen (HLA) molecules. HLA class I (HLA-I) molecules have been well-characterized as ligands for the NK cell receptor group of killer-cell immunoglobulin like receptors (KIR) (Jost and Altfeld, 2013). The specificity of KIR-HLA-I interactions as well as the influence of peptides presented by HLA-I on KIR-binding has been extensively studied (Vales-Gomez et al., 1998; Moesta et al., 2008; Fadda et al., 2011; Rahim et al., 2014; Guethlein et al., 2015; Holzemer et al., 2015; O'Connor et al., 2015; Garcia-Beltran et al., 2016; Chapel et al., 2017; Naiyer et al., 2017). HLA-I complexes consist of a polymorphic α-chain and a conserved chain, termed β2-microglobulin, and present intracellularly-derived peptides on the cell surface. HLA-I molecules are expressed on the surface of all nucleated cells. In contrast, the expression pattern of HLA class II molecules (HLA-II) is mainly restricted to antigen-presenting cells under homeostatic conditions (Muhlethaler-Mottet et al., 1997; Ting and Trowsdale, 2002). However, also non-hematopoietic cells have been shown to express HLA-II molecules after exposure to IFN-γ (Herkel et al., 2003; Stevanovic et al., 2013). HLA-II molecules consist of two polymorphic chains, a α- and a β-chain, and mainly present exogenous-derived peptides. Surface-expressed HLA-II complexes classically interact with $CD4^+$ T cells; however

HLA-II recognition has also been described for CD8$^+$ T cells, especially in the context of chronic virus infections (Heemskerk et al., 2001; Rist et al., 2009; Ranasinghe et al., 2016). Earlier studies suggested a regulation of NK cell activity not only by HLA-I but also HLA-II molecules (Jiang et al., 1996; Lobo et al., 1996). In particular, reduced cytolytic activity of NK cells has been reported after co-incubation with HLA-DR$^+$ target cell lines in comparison to non-HLA-II-expressing target cell lines (Jiang et al., 1996). Which NK cell receptors are involved in the recognition of HLA-DR and subsequent inhibition of NK cells remains unknown. The authors suggested a "missing-self" hypothesis not only for HLA-I but also HLA-II molecules and further discussed the possibility of specific NK cell populations not only recognizing HLA-DR, but also HLA-DQ and HLA-DP molecules (Jiang et al., 1996).

We recently identified a subset of HLA-DP molecules as ligands for the activating NK cell receptor NKp44 (**Figure 1**). The interaction between NKp44 and HLA-DP was dependent on the HLA-DP allotype and further modulated by the peptide presented by HLA-DP molecules (Niehrs et al., 2019), reminiscent of KIR binding to HLA-I. KIR molecules have been crystallized in complex with HLA-I molecules, and these structures clarified how KIR-HLA-I interactions are modulated by the presented peptide, in particular by the C-terminal amino acids of the peptide (Maenaka et al., 1999; Boyington et al., 2000; Fan et al., 2001; Liu et al., 2014). NKp44 has not yet been crystallized in a ligand-bound state and a crystal structure of NKp44 in complex with HLA-DP will help to further elucidate the factors that determine binding of NKp44 to HLA-DP. In contrast to previous studies, we detected activation of NK cells after NKp44-binding to HLA-II. Since we observed differential binding of HLA-DP to NKp44 in a peptide-dependent manner, we cannot exclude that NKp44 is able to bind to other HLA-II molecules during malignancies or infection, where these molecules present a different peptide reservoir. NKp44 has been described as an activating NK cell receptor, but also has an inhibitory splice isoform, NKp44-1 (Cantoni et al., 1999). This inhibitory splice form of NKp44 is the predominant form in decidua tissue (Siewiera et al., 2015), and engagement of HLA-DP molecules by tissue-resident NK cells might result in a different functional activity than by peripheral blood NK cells. In addition, NKp44 has been described to be expressed on diverse cell types, including innate lymphoid cells (ILCs) and plasmatocytoid dendritic cells (pDCs), and the binding of NKp44-expressing cells to HLA-DP might therefore result in different functional outcomes. NKp44$^+$ pDCs within the tonsils for example have displayed reduced production of IFN-α after encountering an NKp44 ligand (Fuchs et al., 2005). Furthermore, there is the possibility of an inhibitory counterpart to NKp44, binding to different HLA-II molecules and inhibiting NK cell activity, similar to what has been described for activating and inhibitory KIR molecules.

LAG-3, a homolog of the CD4 molecule, has been shown to interact with HLA-II molecules (**Figure 1**) and is also expressed on activated NK cells (Baixeras et al., 1992; Huard et al., 1995). There are conflicting results regarding the regulation of NK cell function by LAG-3 via HLA-II. Studies in mice described an inhibition of NK cell activity after binding to HLA-DR molecules (Miyazaki et al., 1996), while later studies, using primary human NK cells, did not observe an effect on NK cell activity toward several HLA-II expressing target cell lines after blocking the LAG-3 receptor (Huard et al., 1998). Recent studies described a peptide-dependent interaction of LAG-3 with the HLA-II complex, and furthermore showed a functional inhibition of CD4$^+$ T cells upon ligand engagement of LAG-3 (Maruhashi et al., 2018). Yet, an inhibition of the interaction between CD4 and HLA-II molecules by LAG-3 has not been observed (Maruhashi et al., 2018). In addition to HLA-II molecules, the liver-secreted protein fibrinogen-like protein 1 (FGL1) has been recently identified as a ligand for LAG-3 (Wang et al., 2019). FGL1 can be overexpressed on tumor cells and blocking of FGL1-LAG-3 interactions led to an increased immune activity (Wang et al., 2019). Interaction of LAG-3 with FGL1 was HLA-II-independent, indicating that LAG-3 might not be restricted to HLA-II recognition but interact with a spectrum of different cellular ligands. A second molecule described to be expressed on NK cells and to interact with HLA-II molecules is FCRL6 (Schreeder et al., 2010). FCRL6 reporter cell lines interacted with HLA-DR molecules, yet, whether FCRL6 is able to recognize a broad spectrum of HLA-DR molecules or only specific allotypes has not been determined (Schreeder et al., 2010). FCRL6 possess an intracellular immunoreceptor tyrosine-based inhibition motif (ITIM), implying that FCRL6 transmits inhibitory signaling cascades. A recent study identified high levels of FCRL6 on NK cells in HLA-II$^+$ solid tumor environment, and expression of HLA-DR molecules on the surface of K562 cells inhibited the cytotoxic function of FCRL6$^+$ NK-92 cells (Johnson et al., 2018). Interestingly, FCRL6 is down-modulated on the surface of NK cells upon exposure to IL-2 and IL-15 (Wilson et al., 2007), in contrast to NKp44, which expression is

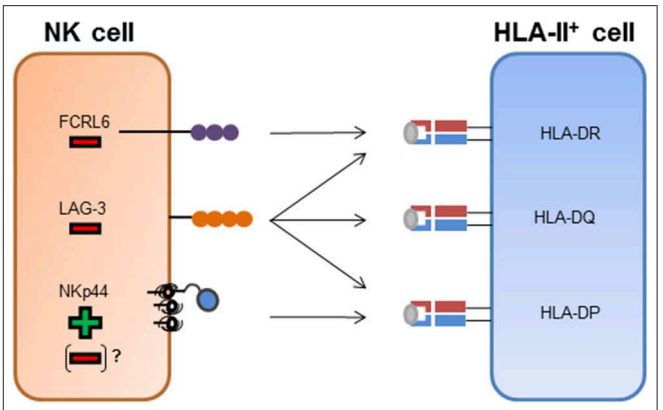

FIGURE 1 | NK cell receptor interactions with HLA class II molecules. FCRL6$^+$ NK cells have been shown to interact with HLA-DR molecules. The binding of FCRL6 to HLA-DR molecules inhibits NK cell function. LAG-3 has been described to bind to HLA-II molecules and has been attributed an inhibitory function after engagement of HLA-II molecules. NKp44 has been described to bind to a subset of HLA-DP molecules and transmit activating signals after binding. Inhibitory splice isoforms of NKp44 expressed on tissue-resident NK cells or NKp44 expression on other innate immune cells might transmit inhibitory signaling after engagement of HLA-DP molecules.

induced after stimulation with these cytokines. The conflicting modes of transcriptional regulation of FCRL6 and NKp44 and their opposite effects on NK cell activity might implicate FCRL6 as a potential inhibitory counterpart to NKp44 in the context of HLA-II ligand recognition (**Figure 1**).

NK cells are part of the innate immune system, but also play an important role in regulating adaptive as well as innate immune responses. NK cells have been shown to regulate immune cell responses of T cells (Waggoner et al., 2011; Cook et al., 2014; Crouse et al., 2015), antigen-presenting cells (Andrews et al., 2005; Moretta et al., 2005; Alter et al., 2010; Altfeld et al., 2011; Michel et al., 2012), and indirectly B cells (Rydyznski et al., 2015, 2018), all of which express HLA-II molecules. In addition, NK cells themselves are able to express HLA-II molecules (Sedlmayr et al., 1996; Erokhina et al., 2018; Costa-Garcia et al., 2019), implicating that a potential interaction between HLA-II and NK cell receptors can not only occur in *trans* but also in *cis*. The identification of HLA-II molecules as ligands for NK cell receptors now provides a possible molecular mechanism to investigate the immune cross-talk between NK cells and HLA-II-expressing immune cells, and the implications for immune responses against malignant cells and pathogens. Furthermore, a variety of non-hematopoietic cells have been described to express HLA-II molecules after exposure to IFN-γ (Kambayashi and Laufer, 2014). These "atypical" antigen-presenting cells might also represent potential targets for innate immune cell receptors recognizing HLA-II, especially under inflammatory conditions.

HLA-II MOLECULES IN MALIGNANCIES AND AUTO-INFLAMMATORY DISEASES

HLA-II molecules have been associated with the outcome of a variety of malignancies, auto-inflammatory and infectious diseases. The identification of innate immune cell receptors interacting with HLA-II now provides additional mechanisms to explain these disease associations, and can potentially lead to new therapeutic strategies. Anti-PD-1 immunotherapy has proven substantial success in the treatment of cancer patients (Page et al., 2014; Zou et al., 2016). Yet, not all patients respond to anti-PD-1 immunotherapy and some develop resistances (Kleponis et al., 2015). The level of HLA-II expression within the tumor environment can predict patient responses toward anti-PD-1 immunotherapy (Johnson et al., 2016). Interestingly, high FCRL6 expression has been detected on NK cells within HLA-II$^+$ solid tumors, and blocking of FCRL6 increased the functional response of NK cells as well as T cells toward HLA-DR$^+$ tumor cells (Johnson et al., 2018). In addition, FCRL6 levels were elevated at relapse within patients that progressed under anti-PD-1-therapy (Johnson et al., 2018). Therefore, the authors suggested the possibility of a combined immune checkpoint inhibitor treatment, targeting both PD-1 and FCRL6, to boost cytotoxic immune cell responses. Within certain tumors, such as colorectal carcinomas, high HLA-II expression has been associated with a favorable clinical outcome (de Bruin et al., 2008; Sconocchia et al., 2014). Induction of HLA-II expression on tumor cells has been attributed to IFN-γ exposure (de Bruin et al., 2008), indicating that the tumor microenvironment and infiltrating immune cells contribute to a favorable clinical outcome (Galon et al., 2006). However, these studies focused on T cell responses and did not exploit a possible role of innate immune cells in tumor progression. Thus, the newly identified HLA-II-NKp44 interaction might possibly contribute to the favorable prognosis of certain high HLA-II-expressing tumors.

One of the major risk factors for the development of graft-vs.-host disease (GvHD) are different HLA-DP allotypes between donor and recipient. Furthermore, in particular a single nucleotide polymorphism (SNP) within the HLA-DP β-chain that determines the expression levels of HLA-DP is associated with GvHD (Petersdorf et al., 2015), with high HLA-DP expression levels in the recipient being associated with a higher risk of developing GvHD (Petersdorf et al., 2015). The gut is one of the first sites where a GvHD response evolves, and serves as a diagnostic marker for the prognosis of GvHD. Recent studies described the expression of MHC-II molecules on the surface of intestinal epithelial cells (IECs) within the ileum of mice upon IFN-γ exposure (Koyama et al., 2019). The gut microbiota contributed to the induction of HLA-II expression, and HLA-II molecules were absent in the ileum of germ-free mice. The exposure of IECs to microbes and consequently IFN-γ secretion was essential for HLA-II expression. Interestingly, IFN-γ secretion during the course of GvHD within the murine gut was not only detected by CD4$^+$ T cells but also type 1 innate lymphoid cells (ILC1s) (Koyama et al., 2019). HLA-II expression has also been described by human gut enteroid organoids after IFN-γ exposure (Koyama et al., 2019; Wosen et al., 2019), indicating that a similar mechanism might apply for the development of GvHD within humans. Which specific receptor-ligand interactions trigger IFN-γ secretion of ILC1s has to be determined, but intraepithelial IFN-γ producing ILC1s have been previously described within the tonsils and gut mucosa. Here, the secretion of IFN-γ was higher within the NKp44$^+$ cell population (Fuchs et al., 2013), indicating that IFN-γ-secretion can be triggered by an NKp44-dependent mechanism.

Furthermore, specific HLA-II molecules represent risk factors for development of inflammatory bowel disease (Goyette et al., 2015). In particular, the HLA-DR β-chain HLA-DRB1*01:03 and the HLA-DP α-chain HLA-DPA1*01:03 have been associated with a higher risk of manifesting Crohn's disease. HLA-DPA1*01:03 interacts with a variety of HLA-DP β-chains, such as HLA-DPB1*04:01, to form HLA-DP401 molecules. HLA-DP401 is one of the most frequent allotypes within the Caucasoid population (Sidney et al., 2010) and interacts strongly with NKp44 (Niehrs et al., 2019). However, in patients developing Crohn's disease a reduced fraction of NKp44-expressing mucosal NK cells has been described, and high IFN-γ production contributing to disease development has been attributed to NKp46$^+$ NK cells (Takayama et al., 2010), indicating an NKp44-independent mechanism. Nevertheless, ILC1s have also been shown to be high producers of IFN-γ during Crohn's disease (Bernink et al., 2013; Fuchs et al., 2013), contributing substantially to pathogenesis. Whether IFN-γ secretion by NKp44$^+$ ILC1s is linked with recognition of HLA-II molecules needs to be determined. In conclusion, certain HLA-II molecules

have been associated with a variety of auto-inflammatory diseases and malignancies, and NCR$^+$ innate cell interactions with HLA-II molecules might provide additional molecular mechanisms underlying these disease associations.

HLA-II MOLECULES IN HEPATITIS B INFECTION

Despite existence of an effective vaccine, hepatitis B virus (HBV) infection remains one of the major global health problems with more than 200 million chronically infected people (Schweitzer et al., 2015). Risk factors for the development of chronic HBV include a lack of functional Th1 cytokine responses during the acute phase of infection (Penna et al., 1997) as well as genetic factors, with SNPs within the HLA-DP region representing the main genome-wide genetic determinant for development of chronic HBV infection throughout different ethnic populations (Kamatani et al., 2009; Thomas et al., 2012). Importantly, a SNP in the 3′ untranslated region of the HLA-DP β-chain has been linked to HLA-DP surface expression levels, and HBV persistence and clearance, respectively. Low-expressed HLA-DP variants, e.g., HLA-DPB1*04:01 and HLA-DPB1*02:01, have been described to be protective while highly-expressed variants, e.g., HLA-DPB1*03:01 and HLA-DPB1*06:01, have been associated to a higher risk of developing chronic HBV (Thomas et al., 2012). The identification of binding of the NK cell receptor NKp44 to a subset of HLA-DP molecules might provide an additional molecular mechanism for the described disease association with HBV. We observed a functional interaction between NKp44 and HLA-DP401, an HLA-DP molecule associated with low surface expression and HBV clearance, but not between NKp44 and HLA-DP301, which is associated with high surface expression and HBV persistence [overview for high and low-expressed variants provided in Fleischhauer (2015)]. In a HLA-II bead-based screening assay, NKp44 interacted mainly with low-expressed HLA-DP β-chains but to some extent also displayed binding to high-expressed HLA-DP variants. Yet, we observed binding of NKp44 to high-expressed HLA-DP variants only in combination with a specific subset of HLA-DP α-chains (Niehrs et al., 2019), indicating that NKp44-binding depended on the combination of both HLA-DP chains, and suggesting that NKp44 binding occurred in close proximity to the peptide binding groove.

Interestingly, HLA-DP401 and HLA-DP201, in addition to being associated with low HLA-DP surface expression, also share a second similarity, namely the amino acid Glycine at position 84 within the HLA-DP β-chain. This amino acid position plays an important role for peptides presented by HLA-DP (Diaz et al., 2003). Recent studies have described low affinity binding of the class II invariant chain peptide (CLIP) to HLA-DP molecules possessing Gly84, and thereby insufficient blocking of the peptide binding groove of these molecules during cellular HLA-II trafficking (Yamashita et al., 2017; Anczurowski et al., 2018). Therefore, HLA-DP molecules carrying Gly84 residues are prone to present endogenous peptides derived from HLA-I peptide processing pathways (Yamashita et al., 2017). While these observations warrant further confirmation, they suggest that certain HLA-DP molecules, such as HLA-DP401 and HLA-DP201, could present intracellularly-produced peptides during HBV infection, and therefore possibly also HBV-derived peptides, which might alter the binding to TCRs of CD4$^+$ T cells as well as NKp44$^+$ NK cells and ILCs. Future studies characterizing the peptide-repertoires presented by HLA-II during HBV infection will provide insights into these potential interactions between NKp44$^+$ immune cells and HBV-infected cells.

In the course of acute HBV infection, IFN-γ secreted by Th1 cells has been described to be associated with self-limitation of the virus infection (Penna et al., 1997) and in addition to Th1 cells, NK cells are also able to secrete IFN-γ upon activation. During the course of HBV infection, IFN-γ has a direct anti-viral activity itself (Guidotti et al., 1999; Xia et al., 2016) but also induces HLA-II expression on hepatocytes, which in turn can function as "atypical" antigen-presenting cells (Herkel et al., 2003). The induction of an NK cell receptor ligand by pro-inflammatory cytokines is reminiscent of the induction of B7-H6, a ligand for the NKp30 receptor, upon exposure to interleukin 1-β or tumor necrosis factor-α (Matta et al., 2013). Another effector cytokine secreted by Th1 cells is interleukin-2 (IL-2), which induces the expression of NKp44 on NK cells, while NKp44 surface expression is absent on resting NK cells (Cantoni et al., 1999). These data suggest a model in which the acute phase of HBV infection induces production of IFN-γ by NK cells and Th1 cells, which also produce IL-2. IFN-γ can trigger expression of HLA-II on infected hepatocytes, while secretion of IL-2 promotes NKp44 expression on NK cells. The simultaneous expression of NKp44 and HLA-DP permits an interaction between NK cells and infected hepatocytes in individuals encoding for HLA-DP401 and other HLA-DP molecules serving as NKp44-ligands, leading to lysis of the HBV-infected cells (**Figure 2A**). In contrast, in HBV-infected individuals encoding for HLA-DP301 or other HLA-DP molecules not serving as NKp44-ligands, IFN-γ and IL-2 secretion can also induce HLA-DP and NKp44 expression, but NKp44$^+$ cells are unable to bind to the expressed HLA-DP molecules (**Figure 2B**). The lack of innate immune cell recognition of HBV-infected cells might contribute to a higher risk for persistent HBV infection and provides a new mechanistic link for the described association between specific HLA-II allotypes and chronic HBV disease.

A POSSIBLE ROLE FOR NKP44-HLA-II INTERACTIONS IN NK CELL MEMORY

A hallmark of the innate immune system is the prompt reaction toward infected and malignant cells without the need of prior antigen-dependent stimulation. However, over the past years, studies have described an antigen-dependent memory function of NK cells (O'Leary et al., 2006; Sun et al., 2009; Paust et al., 2010). Memory NK cells have been mainly characterized by the expression of CD49a and CXCR6, are thought to be enriched in the liver and subsequently migrate to the site of infection (Paust et al., 2010; Peng et al., 2013; Reeves et al., 2015; Nikzad et al., 2019). In addition, NK cell memory has been demonstrated to be inducible by cytokine exposure as well as

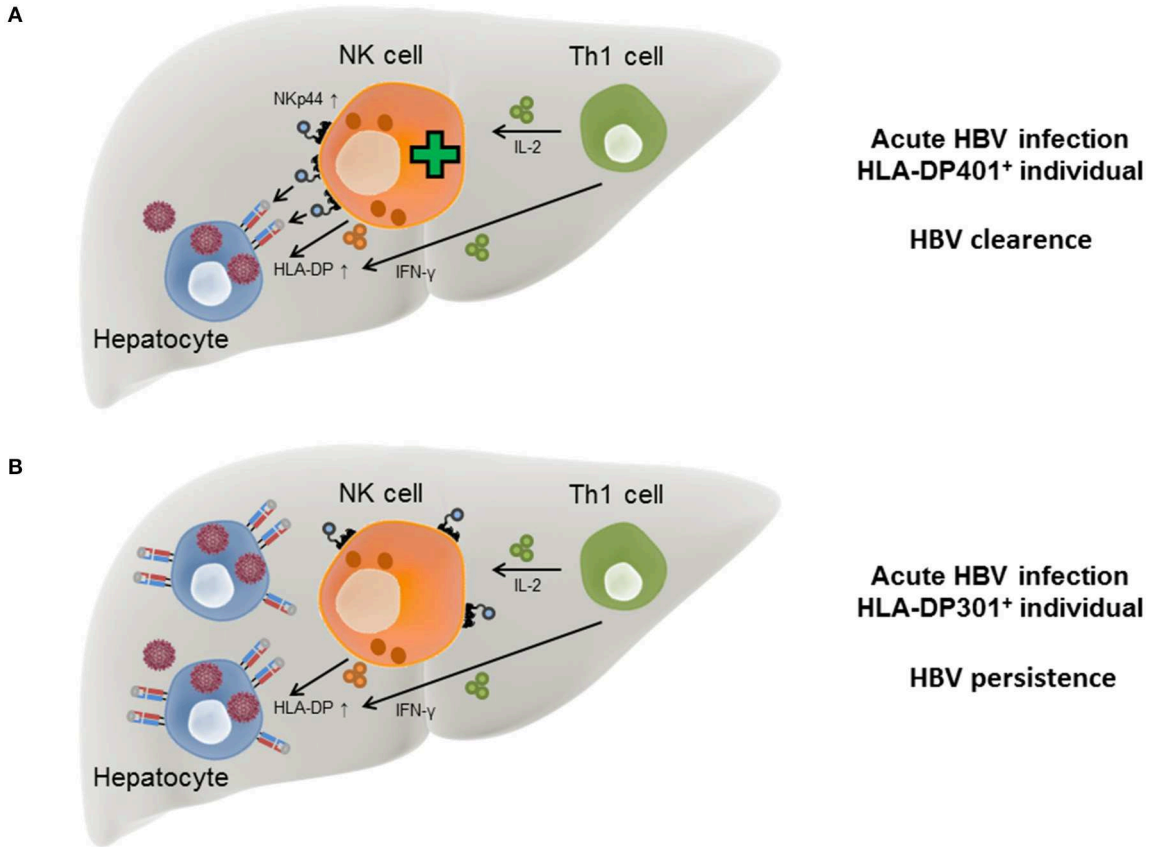

FIGURE 2 | Model for HLA-DP-NKp44 interactions during HBV infection in individuals with different HLA-DP genotypes. During acute HBV infection HLA-DP molecules are upregulated on the surface of human hepatocytes in response to IFN-γ secreted by Th1 and NK cells. NKp44 expression by NK cells is initiated by IL-2 secreted by Th1 cells. In HLA-DP401$^+$ HBV-infected individuals, NKp44 interacts with HLA-DP401 molecules expressed on the surface of infected hepatocytes, contributing to lysis of infected cells and HBV control **(A)**. In HLA-DP301$^+$ HBV-infected individuals, NKp44 is unable to bind to HLA-DP301 molecules expressed on the surface of infected hepatocytes, resulting in inefficient lysis of infected hepatocytes by innate immune cells and higher risk of chronic HBV infection **(B)**.

exposure to tumor cells (Cooper et al., 2009; Pal et al., 2017). It is still unclear which NK cell receptors mediate the observed memory responses. The newly identified interaction between NKp44 and HLA-DP was modulated by the HLA-II presented peptide, and can thus be potentially dependent on the respective antigen, indicating NKp44 as a possible receptor candidate for mediating NK cell memory responses. It is however unlikely, that NKp44 can explain all antigen-specific NK cell memory responses reported to date. Yet, NKp44-HLA-DP interactions might provide a first hint toward a possible mechanism mediating NK cell memory responses.

CONCLUDING REMARKS

Interactions between HLA-II molecules and innate immune cells including NK cells are poorly understood. However, regulation of NK cell activity by HLA-II molecules in complex with specific pathogenic and cellular-derived peptides might help to better explain described associations between certain HLA-II allotypes and distinct outcomes of infectious as well as auto-inflammatory diseases or malignancies. Notably, the induced expression of HLA-II molecules by IFN-γ also on non-hematopoietic cells favors an interaction with NK cell receptors under inflammatory conditions. Future studies will have to determine the extent by which different NK cell receptors might interact with HLA class II molecules during physiologic conditions and in disease settings, and how these receptor-ligand interactions influence NK cell function and disease outcomes.

AUTHOR CONTRIBUTIONS

AN wrote the manuscript. MA revised and edited the manuscript.

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Conflict of Interest: AN and MA filed a patent application (EP18174760.1), regarding the therapeutically use of anti-NKp44 antibodies for the treatment and or prevention of graft-vs.-host disease.

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ORIGINAL RESEARCH
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Distinct Human NK Cell Phenotypes and Functional Responses to *Mycobacterium tuberculosis* in Adults From TB Endemic and Non-endemic Regions

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Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), which leads to an estimated 1. 5 million deaths worldwide each year. Although the immune correlates of protection against Mtb infection and TB disease have not been well-defined, natural killer (NK) cells are increasingly recognized as a key component of the innate immune response to Mtb and as a link between innate and adaptive immunity. In this study, we evaluated NK cell phenotypic and functional profiles in QuantiFERON-TB (QFT)$^{+}$ and QFT^{-} adults in a TB endemic setting in Kisumu, Kenya, and compared their NK cell responses to those of Mtb-naïve healthy adult controls in the U.S. We used flow cytometry to define the phenotypic profile of NK cells and identified distinct CD56^{dim} NK cell phenotypes that differentiated the Kenyan and U.S. groups. Additionally, among Kenyan participants, NK cells from QFT^{+} individuals with latent Mtb infection (LTBI) were characterized by significant downregulation of the natural cytotoxicity receptor NKp46 and the inhibitory receptor TIGIT, compared with QFT^{-} individuals. Moreover, the distinct CD56^{dim} phenotypic profiles in Kenyan individuals correlated with dampened NK cell responses to tumor cells and diminished activation, degranulation, and cytokine production following stimulation with Mtb antigens, compared with Mtb-naïve U.S. healthy adult controls. Taken together, these data provide evidence that the phenotypic and functional profiles of NK cells are modified in TB endemic settings and will inform future studies aimed at defining NK cell-mediated immune correlates that may be protective against acquisition of Mtb infection and progression to TB disease.

Keywords: *Mycobacterium tuberculosis*, LTBI, NK cells, innate immunity, phenotype

INTRODUCTION

Infection with *Mycobacterium tuberculosis* (Mtb) can lead to development of active tuberculosis (TB) disease, which is currently the leading cause of death in the world due to a single infectious agent (WHO Publication, 2018a). The vast majority of individuals infected with Mtb remain asymptomatic and are considered to have latent Mtb infection (LTBI). Approximately one quarter of the global population is estimated to harbor Mtb infection (Houben and Dodd, 2016), with 10 million individuals developing active TB disease each year (WHO Publication, 2018a). The only currently licensed TB vaccine, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), provides variable efficacy, ranging from 0 to 80%, against pulmonary TB disease in adults (Andersen and Scriba, 2019).

Both innate and adaptive immunity, including Mtb-specific T cell and antibody (Ab) responses, are clearly important in maintaining control of Mtb (Lu et al., 2016; Simmons et al., 2018), although the precise immune correlates of protection to Mtb infection have not been well-defined. Natural killer (NK) cells are increasingly recognized as a key component of the innate immune response to Mtb and as a link between innate and adaptive immunity (Gabrielli et al., 2016; Choreno Parra et al., 2017). IFN-γ production by NK cells activates antimicrobial effector functions of macrophages, which is essential for control of Mtb; furthermore, secretion of granulysin by NK cells can kill intracellular Mtb when delivered by perforin (Stenger et al., 1998; Lu et al., 2014). Studies of Mtb infection in T cell-deficient mice indicated that NK cell-mediated IFN-γ production contributes significantly to inhibition of bacterial replication (Feng et al., 2006). Mtb can bind directly to TLR2 on NK cells (Esin et al., 2013) as well as the natural cytotoxicity receptor (NCR) NKp44 (Esin et al., 2008). Human NK cells can lyse Mtb-infected macrophages *in vitro* via interactions with c-type lectins and NCRs expressed on NK cells (Korbel et al., 2008), and suppress growth of Mtb in infected monocytes (Yoneda and Ellner, 1998; Brill et al., 2001). IL-22 production by NK cells inhibits intracellular growth of Mtb *in vitro* by enhancing phagolysosomal fusion (Dhiman et al., 2009). NK cells are recruited to the lung in patients with active TB disease (Portevin et al., 2012). However, NK cells circulating in peripheral blood of patients with pulmonary TB disease exhibit decreased IFN-γ production capacity (Bozzano et al., 2009; Garand et al., 2018), which is partially restored following anti-TB treatment (Nirmala et al., 2001), thus suggesting an association between NK cell functional capacity and bacterial load. Moreover, longitudinal cohort studies have indicated that progression to active TB disease is preceded by a decline in the frequency of circulating NK cells, which is restored following successful treatment for active TB (Roy Chowdhury et al., 2018), thus providing further evidence of an important role for NK cells in Mtb infection and TB disease in humans.

NK cell activity is tightly regulated through a sophisticated network of numerous germline-encoded activating and inhibitory receptors (Bryceson et al., 2006), the variegated expression of which generates heterogenous populations of NK cells with high diversity (Horowitz et al., 2013; Strauss-Albee et al., 2014). Moreover, NK cell surface marker expression changes in the settings of inflammation, infection and cancer, and increasing evidence indicates NK cells can differentiate into distinct subsets with specialized functions, referred to as "adaptive" NK cells (Tesi et al., 2016; Freud et al., 2017). Adaptive NK cells in humans have been defined most clearly in the context of human cytomegalovirus (HCMV) infection, which has been associated with expansion of distinct NK cell subsets and enhanced responsiveness to virally-infected cells in an antibody-dependent manner (Wu et al., 2013; Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015). Antigen-specific NK cells have also been described in simian immunodeficiency virus (SIV)-infected and vaccinated rhesus macaques (Reeves et al., 2015). In humans, infection with HCMV and other viruses leads to expansion of NK cell subsets with adaptive features expressing CD57 and the activating receptor NKG2C (Guma et al., 2004; Bjorkstrom et al., 2011; Lopez-Verges et al., 2011; Beziat et al., 2013). Adaptive NK cells in HCMV infection are characterized by downregulation of receptors such as NKp30 and NKp46 (Guma et al., 2004), downregulation of the transcription factors PLZF and IKZF2 and loss of intracellular adaptor signaling molecules (Lee et al., 2015; Schlums et al., 2015). Downregulation of these molecules has been associated with pronounced changes in DNA methylation patterns (Lee et al., 2015; Schlums et al., 2015), thus clearly demonstrating pathogen-induced epigenetic reprogramming as a mechanism driving the generation of adaptive NK cells.

In addition to HCMV, NK cells with adaptive features have been described in other chronic human infections, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), and Epstein-Barr virus (EBV) (Paust et al., 2017). The potential for persistent bacterial infections, such as Mtb, to promote adaptive diversification of NK cell is less clear. In a mouse model of TB, vaccination with BCG induces memory-like NK cells producing IFN-γ, which provide protection against challenge with Mtb (Venkatasubramanian et al., 2017). In humans, recent studies in South Africa indicate that BCG revaccination of individuals with LTBI boosts BCG-reactive NK cell responses for at least 1 year after revaccination (Suliman et al., 2016). NK cells from patients with active TB disease exhibit decreased expression of the activating NCRs NKp30 and NKp46 (Bozzano et al., 2009), a phenotype consistent with changes in adaptive NK cell phenotypic profiles in HCMV infection (Guma et al., 2004).

While increasing evidence from animal models and humans indicates that NK cells can differentiate into distinct subsets with specialized functions, it currently remains unclear if Mtb exposure and infection modifies NK cell phenotypic and functional signatures, and if so, how Mtb-associated changes in the NK cell repertoire may impact acquisition of Mtb infection and/or progression to active TB disease. To test the hypothesis that Mtb exposure and infection are associated with distinct NK cell phenotypic and functional profiles, we recruited a cohort of HIV-negative, Mtb-infected and uninfected adults in a TB endemic setting in Kisumu, Kenya and a cohort of Mtb-naïve, healthy adults in the U.S., a non-TB endemic setting. We performed a comprehensive analysis of NK cells from individuals in these cohorts and identified distinct $CD56^{dim}$ NK cell phenotypic profiles that differentiated Kenyan adults

from U.S. adults. Moreover, we demonstrated that $CD56^{dim}$ phenotypic profiles correlated with dampened NK cell responses to MHC class I-devoid cells and diminished reactivity to Mtb antigen stimulation in Kenyan adults.

MATERIALS AND METHODS

Study Participants

Kenya Cohort: Blood samples were collected from individuals ≥18 years of age enrolled at the Kenya Medical Research Institute Clinical Research Center in Kisumu, Kenya. Study participants included adults with a normal chest x-ray and no symptoms of TB disease and no previous history of diagnosis or treatment for active TB disease. Mtb infection status was evaluated by QuantiFERON®-TB Gold In-Tube (QFT; Qiagen). Individuals with a positive QFT result (TB Antigen-Nil ≥0.35 IU/ml) were defined as having LTBI. Individuals with a TB Antigen-Nil response <0.35 IU/ml were defined as healthy controls (QFT^-). Serologic testing for HIV antibodies was done for all individuals using the Diagnostic Kit for HIV (1+2) Antibody V2 (*KHB*® Shanghai Kehua Bio-engineering Co., Ltd). All participants enrolled for the study were seronegative for HIV. HCMV seropositivity in healthy Kenyan adults is 97% (Njeru et al., 2009), thus participants enrolled in Kisumu are presumed to be HCMV seropositive. *U.S. healthy controls*: Blood samples were collected from healthy adults enrolled at the Emory Vaccine Center in Atlanta, GA. U.S. healthy adult controls were U.S.-born, had not been vaccinated with BCG, and had no history of exposure to TB. All U.S. healthy controls were seropositive for HCMV IgG antibodies, as measured using the Cytomegalovirus IgG ELISA kit (Abnova).

Ethics Statement

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All subjects provided written informed consent for participation in the study, which was approved by the Kenya Medical Research Institute Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

PBMC Isolation

Blood samples from all participants were collected in sodium heparin tubes for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated via density gradient centrifugation, cryopreserved, and stored in LN_2 until use. Cryopreserved PBMCs were thawed in a 37°C water bath and resuspended in 10 ml RPMI 1640 (Corning) with deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich) and washed twice with RPMI 1640. Cells were then suspended in R10 (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) and used in phenotypic and functional NK cell assays described below.

NK Cell Phenotyping

Thawed PBMCs were washed in PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 min at room temperature. Cells were washed with PBS and surface stained for 30 min in the dark at room temperature with anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD56 Brilliant Violet (BV) 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-NKG2A PE (Beckman Coulter; IM329IU), anti-NKG2D BV 421 (BioLegend; 1D11), anti-NKp30 Alexa Fluor 647 (BioLegend; P30-15), anti-NKp46 PE-Cy7 (BioLegend; 9E2), and anti-CD57 FITC (BioLegend; HCD57). After incubation with conjugated antibodies, cells were washed with PBS and fixed with 2% paraformaldehyde (PFA).

A second phenotyping panel was designed to measure the NK cell expression of intracellular markers. PBMCs were stained with Zombie NIR™ Fixable Viability Dye and surface stained for 30 min at room temperature with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), and anti-TIGIT PE (BioLegend; A15153G). Cells were washed in PBS and suspended in FoxP3 Fixation Buffer (eBioscience) for 30 min on ice. After fixation, cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained with anti-granzyme B PE-CF594 (BD; GB11), anti-perforin PE-Cy7 (BD; BD48), and anti-granulysin Alexa Fluor 488 (BD; RB1) for 30 min in the dark at room temperature. Finally, cells were washed with FoxP3 Staining Buffer and resuspended in PBS.

Stimulation and Staining of NK Cells

NK cell responses to target cells were evaluated using K562 and p815 tumor cell lines (ATCC). Just prior to use in NK cell stimulation experiments, p815 cells were incubated with rabbit anti-mouse polyclonal lymphocyte serum (Cedarlane) for 30 min to coat the cells with Ab. Ab-coated p815 cells were then washed with R10 before being added to PBMCs. Donor PBMCs were incubated with K562 or Ab-coated p815 cells at an effector to target ratio of 10:1. PBMCs incubated in R10 media alone served as a negative control. CD107a PE-Cy7 (BioLegend; H4A3), brefeldin A (5 μg/ml; Sigma-Aldrich), and monensin (5 μg/ml; BioLegend) were added to each sample at the beginning of stimulation. Cells were incubated at 37°C degrees for 5 h.

NK cell responses to Mtb antigens were evaluated by stimulation of PBMCs with 10 μg/ml of *Mycobacterium tuberculosis* H37RV derived cell wall, cell membrane and whole cell lysate (obtained from BEI Resources, NIAID, NIH; catalog numbers NR14828, NR14831, and NR14822, respectively). PBMCs were incubated in R10 with anti-CD107a PE-Cy7 (BioLegend; H4A3), recombinant human IL-2 (100 U/ml; NIH AIDS Research and Reference Reagent Program, Catalog #136), and Mtb antigens for 24 h at 37°C and 5% CO_2. Brefeldin A (5 μg/ml; Sigma-Aldrich) and monensin (5 μg/ml; BioLegend) were added at the final 5 h of incubation. PBMCs incubated in R10 media with anti-CD107a and IL-2 alone served as a negative control. For cytokine neutralization experiments, PBMCs were incubated with purified NA/LE mouse anti-human IL-12 (p40/p70) (BD; C8.6), purified mouse anti-human IL-18 (R&D Systems; 125-2H), or purified NA/LE mouse IgG1 κ isotype

control (BD; 107.3) for 15 min prior to addition of Mtb antigens, as described above.

Following stimulation of PBMCs with either target cell lines or Mtb antigens, cells were washed with PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 min. Cells were washed with PBS and surface stained with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD158a FITC (BioLegend; HP-MA4), anti-CD158b FITC (BioLegend; DX27), and anti-CD158e1 FITC (BioLegend; DX9) for 30 min at room temperature in the dark. Stained cells were washed with PBS and fixed with FoxP3 Fixation Buffer (eBioscience) for 30 min on ice. Cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained intracellularly with anti-CD69 PerCP-Cy5.5 (BioLegend; FN50), anti-IFN-γ BV 480 (BD; B27), anti-TNFα Alexa Fluor 647 (BioLegend; MAB11), and anti-IL-22 PE (BioLegend; 2G12A41) for 30 min at room temperature in the dark. Cells were washed in PBS and prior to acquisition on a BD LSRII flow cytometer.

Flow Cytometry and Data Analysis

Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed with FlowJo software (v9.6; BD). Compensation was performed using single-stained anti-mouse Ig,κ beads (BD Bioscience). Single cells were identified by plotting forward scatter height and forward scatter area. Lymphocytes were identified by plotting forward scatter height and side scatter height. Viable lymphocytes were identified by low expression of NIR viability dye. NK cells were identified as live lymphocytes negative for CD3, CD14, CD19, and positive for the NK lineage markers CD56 and/or CD16. NK cells were further stratified based on intensity of the expression of CD56 ($CD56^{dim}$, $CD56^{bright}$, and $CD56^{neg}$ NK cells).

Data Analysis and Statistics

A minimum of 1,000 NK cells was acquired in each panel for each individual. Expression of functional markers by NK cells (CD69, IFN-γ, CD107a, TNF-α, and IL-22) were analyzed after subtraction of background expression in the negative control condition. A non-parametric Mann-Whitney test was used to compare differences between two groups. Differences between 3 groups were evaluated using a non-parametric Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. Correlations were evaluated using Spearman's rank-order correlation. $P < 0.05$ were considered significant.

RESULTS

Study Participants

Blood samples were collected from 61 participants in Kisumu, Kenya and from 9 Mtb-naïve healthy adult controls in Atlanta, GA (U.S.) (**Table 1**). Participants enrolled in Kenya were stratified by QFT result: QFT^+ (considered to have LTBI, $n = 31$) and QFT^- ($n = 30$). The majority of participants from Kisumu were household contacts of an active TB patient within 2 years prior to study enrollment (20/31 QFT^+ individuals [64.5%] and 21/30 QFT^- individuals [70%]). All three groups were similar with regard to age and sex characteristics, with the exception that U.S. healthy controls were older than Kenya QFT^- participants.

TABLE 1 | Characteristics of study participants.

Participant group	*n*	Age, y[a] (IQR)	Sex (% male)	QFT, IFN-γ IU/ml[b] (IQR)
Kenya QFT^+	31	32 (23–53)	26	8.84 (3.22–10.00)
Kenya QFT^-	30	24 (21–33)	37	0.00 (0.00–0.05)
U.S. Healthy Controls	9	43 (33–60)[c]	22	N/A

[a] *Value denotes median age in years.*
[b] *Value denotes median.*
[c] *$p < 0.05$, compared with Kenya QFT^-.*
IQR, interquartile range.

NK Cells in QFT^+ and QFT^- Kenyan Adults Are Characterized by Increased Proportions of $CD56^{neg}$ NK Cells

Redistribution of NK cell subsets, including expansion of the $CD56^{neg}$ subset, has been described in the setting of chronic viral infections (Bjorkstrom et al., 2010). To determine if the frequency and distribution of NK cell subsets are modified in the setting of Mtb infection, we used flow cytometry to measure NK cells directly *ex vivo* in PBMCs from all three participant groups (**Figure 1A**). The frequency of total NK cells in the lymphocyte population was similar between the three participant groups (**Figure 1B**). As expected, $CD56^{dim}$ NK cells constituted the dominant subset of NK cells in all participant groups (**Figure 1C**). There were no significant differences in the proportions of CD56 subsets between QFT^+ and QFT^- Kenyan groups, thus indicating that Mtb infection does not significantly modify the frequency and distribution of NK cells subsets in peripheral blood. However, both QFT^+ and QFT^- Kenyan adults had significantly higher proportions of $CD56^{neg}$ NK cells, compared with Mtb-naïve healthy adult controls in the U.S. (**Figure 1C**).

$CD56^{dim}$ NK Cells Exhibit Distinct Phenotypic Profiles in Kenyan and U.S. Adults

Given that NK cell surface marker expression can change in the setting of infection (Freud et al., 2017), we performed flow cytometry to evaluate expression of 10 phenotypic markers expressed by NK cells in each of the three participant groups (**Figure 2A**). Since CD56 subsets have distinct transcriptional profiles (Collins et al., 2019), we determined the phenotypic profiles of $CD56^{dim}$ and $CD56^{bright}$ NK cells subsets separately. There was a progressive increase in expression of the differentiation marker CD57 by $CD56^{dim}$ NK cells from U.S. healthy controls to QFT^- and QFT^+ Kenyan individuals. By contrast, NKG2A, NKp30, and NKp46 were progressively decreased on $CD56^{dim}$ NK cells from U.S. healthy controls to QFT^- and QFT^+ Kenyan individuals (**Figure 2B**). While the cytotoxic molecules perforin and granulysin were expressed at similar levels among the three groups, expression of granzyme B

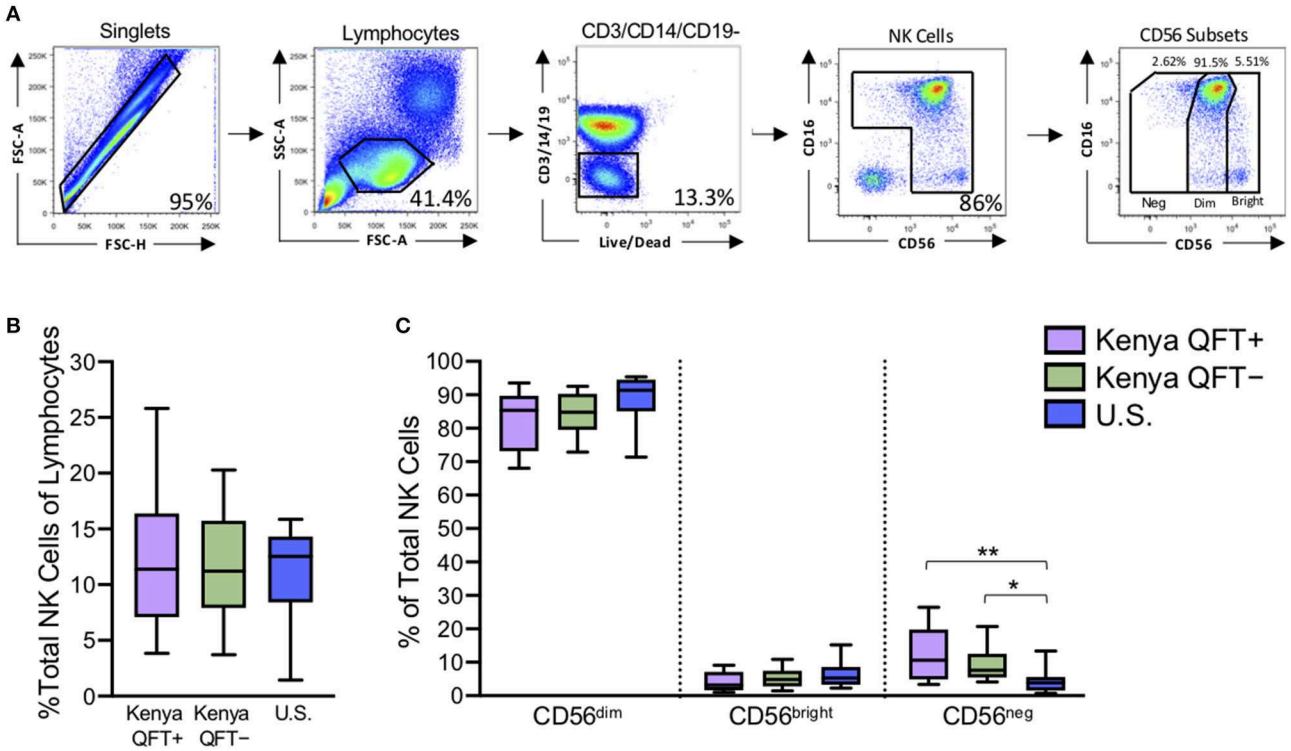

FIGURE 1 | Kenyan adults exhibit higher proportions of CD56^{neg} NK cells, compared with U.S. adults. Flow cytometry was used to identify NK cell subsets in PBMCs from Kenyan adults ($n = 31$ QFT$^+$; $n = 30$ QFT$^-$) and U.S. adult healthy controls (U.S., $n = 9$). **(A)** Flow cytometry gating strategy for NK cells and CD56 subsets (CD56^{neg}, CD56^{dim}, CD56^{bright}). **(B)** Frequency of total NK cells as a percentage of lymphocytes. **(C)** Frequency of CD56 subsets as a proportion of total NK cells. Boxes in **(B,C)** represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *$p < 0.05$ and **$p < 0.01$.

was markedly increased in both groups of Kenyan participants, compared with U.S. healthy controls. Moreover, expression of the inhibitory receptor TIGIT was significantly higher on CD56^{dim} NK cells from Kenyan participants, compared with U.S. healthy controls. Similar phenotypic differences were also found when evaluating the phenotype of the total NK cell population in the three participant groups (**Figure S1**).

While there were substantial differences in the phenotypic profile of CD56^{dim} NK cells between Kenyan and U.S. adults, we also identified phenotypic markers that were expressed at significantly different levels between QFT$^+$ and QFT$^-$ Kenyan adults, with lower expression of both NKp46 and TIGIT on CD56^{dim} NK cells from QFT$^+$ individuals, compared with QFT$^-$ individuals (**Figure 2B**). These data indicate that human Mtb infection may be associated with downregulation of specific receptors expressed by circulating CD56^{dim} NK cells.

By contrast with CD56^{dim} NK cells, fewer differences were found among the three participant groups when evaluating the phenotypic profiles of CD56^{bright} NK cells. Although expression of NKG2D was similar among the groups on CD56^{dim} NK cells, NKG2D expression was significantly higher on CD56^{bright} NK cells from QFT$^+$ and QFT$^-$ Kenyan adults, compared with U.S. healthy controls (**Figure 2C**). Importantly, similar to CD56^{dim} cells, expression of NKp46 was also significantly lower on CD56^{bright} NK cells from Kenyan QFT$^+$ individuals, compared with QFT$^-$ individuals. PCA of expression of the 10 phenotypic markers by CD56^{dim} cells from the three groups indicated that U.S. healthy controls can be clearly differentiated from Kenyan individuals. By contrast, PCA of the same 10 phenotypic markers by CD56^{bright} NK cells in the same individuals does not clearly distinguish the participant groups (**Figure 2D**).

Taken together, these data indicate substantial differences in CD56^{dim} NK cell phenotypic profiles distinguish Kenyan and U.S. adults. Moreover, these data also indicate that among Kenyan adults residing in a TB endemic environment, expression levels of the natural cytotoxicity receptor NKp46 and the inhibitory receptor TIGIT are further modified in QFT$^+$ adults with LTBI, compared with QFT$^-$ adults.

CD56^{dim} NK Cells From Kenyan Adults Have Dampened Responses to Tumor Cells, Compared With U.S. Adults

Through receptor dependent mechanisms, NK cells recognize tumor and virus infected cells that downregulate MHC class I expression as an immune escape mechanism (Paul and Lal, 2017). Given the substantial differences in CD56^{dim} NK cell phenotype in the three participant groups, we next evaluated

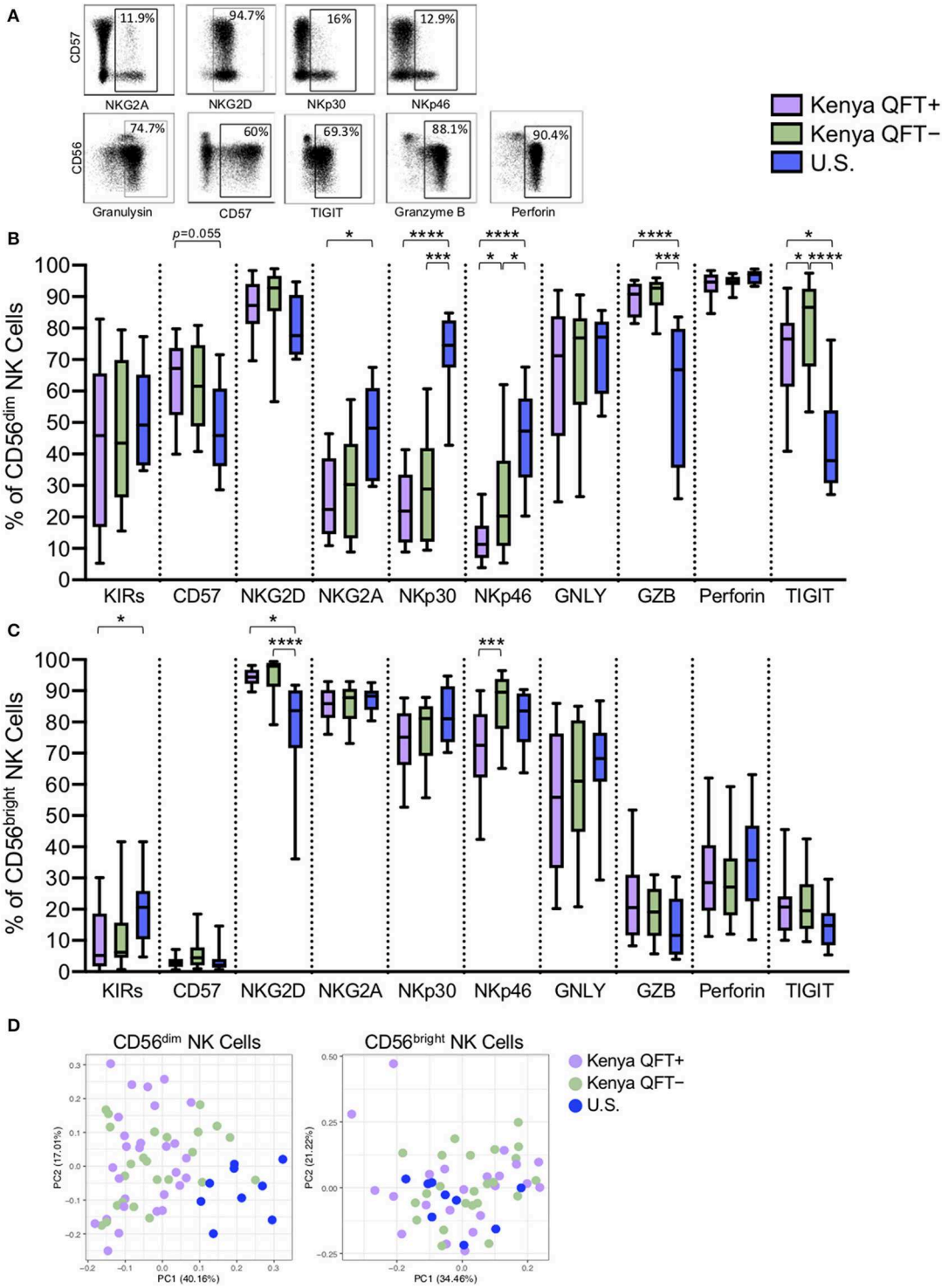

FIGURE 2 | CD56^{dim} NK cells exhibit distinct phenotypes in Kenyan and U.S. adults. Flow cytometry was used to evaluate the phenotype of NK cells in PBMCs from each of three participant groups. **(A)** Representative flow plots from a Kenyan QFT^{+} individual demonstrating expression of the indicated phenotypic markers; plots are shown gated on total NK cells. **(B)** Frequency of CD56^{dim} NK cells expressing each phenotypic marker. **(C)** Frequency of CD56^{bright} NK cells expressing each phenotypic marker. GNLY, granulysin; GZB, granzyme B. **(D)** Principal component analysis (PCA) of expression of the 10 phenotypic markers on CD56^{dim} NK cells (left panel) and CD56^{bright} NK cells (right panel) from each of the three participant groups. Boxes in **(B,C)** represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups in **(B,C)** were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. $^{*}p < 0.05$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

the functional capacity of CD56^{dim} NK cells to respond to the generic NK cell targets of MHC class I-devoid K562 tumor cells and Ab-coated p815 cells (**Figure 3A**). CD56^{dim} NK cells from U.S. healthy adults had a generally more robust response to stimulation with K562 cells, as measured by CD69, CD107a, and IFN-γ expression (**Figure 3B**), compared with Kenyan adults. In addition, Boolean analysis of all three markers indicated increased co-expression of two or three markers by CD56^{dim} NK

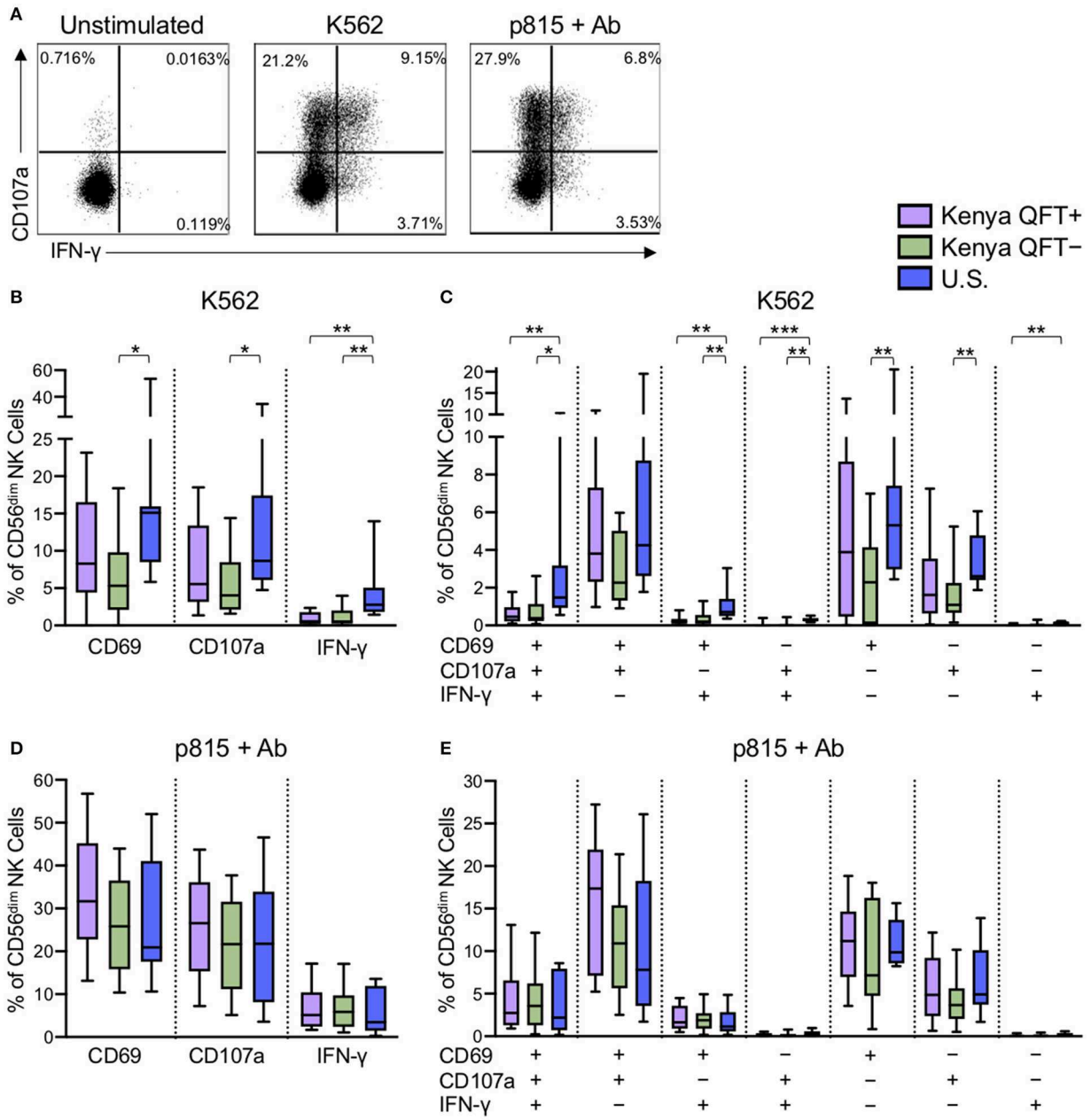

FIGURE 3 | NK cells from Kenyan adults have dampened responses to MHC class I-devoid target cells, but similar ADCC responses, compared with U.S. adults. PBMCs were stimulated for 5 h with either MHC class I-devoid K562 cells or with Ab-coated p815 cells. NK cells were evaluated by flow cytometry for expression of CD69, CD107a, and IFN-γ. **(A)** Representative flow plots from a U.S. healthy control of CD107a and IFNγ production by CD56^{dim} NK cells following stimulation with K562 cells or Ab-coated p815 cells. **(B,D)** Expression of CD69, CD107a and IFN-γ by CD56^{dim} NK cells after stimulation with K562 cells **(B)** or Ab-coated p815 cells **(D)**. **(C,E)** Boolean analysis of co-expression patterns of CD69, CD107a, and IFN-γ by CD56^{dim} NK cells after stimulation with K562 cells **(C)** or Ab-coated p815 cells **(E)**. Data in **(B–E)** are shown from 53 Kenyan individuals ($n = 26$ QFT$^+$ and $n = 27$ QFT$^-$) and 9 U.S. healthy controls. Frequencies in **(B–E)** are shown after subtraction of background expression in the unstimulated control condition. Boxes in **(B–E)** represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

cells from U.S. healthy controls, compared with Kenyan adults (**Figure 3C**). Interestingly, among Kenyan adults, $CD56^{dim}$ NK cells from QFT^{+} individuals with LTBI co-expressed CD69 and CD107a at higher levels following stimulation with K562 cells, compared with QFT^{-} individuals, although this did not maintain statistical significance after correction for multiple comparisons (**Figure 3C**). No significant differences in CD69, CD107a, or IFN-γ expression were observed by $CD56^{dim}$ NK cells from the three groups following stimulation with Ab-coated p815 target cells (**Figures 3D,E**). Taken together, these data indicate that a high pathogen burden environment in Kenya is associated with reduced NK cell reactivity to MHC class I-devoid cells, whereas Ab-mediated activation of NK cells is maintained at similar levels to those seen by NK cells from U.S. healthy controls.

Differential Reactivity of $CD56^{dim}$ NK Cells to Mtb Antigen Stimulation in Kenyan and U.S. Adults

The above data indicated that NK cell phenotype and functional responses to generic stimuli are impacted by Mtb infection status, as well as high (Kenya) vs. low (U.S.) pathogen burden settings. We next sought to determine if Mtb infection modifies NK cell reactivity to Mtb antigen stimulation. Thus, we stimulated PBMCs from each of the participant groups with Mtb cell wall antigen for 24 h in the presence of IL-2, followed by flow cytometry for expression of CD69, CD107a, IFN-γ, TNF-α, and IL-22 (**Figure 4A** and data not shown). $CD56^{dim}$ NK cells expressed very low levels of TNF-α and IL-22 following stimulation of PBMCs with Mtb cell wall antigens (median <0.1% of $CD56^{dim}$ NK cells in each group; data not shown). However, Mtb antigen stimulation induced upregulation of CD69 and IFN-γ expression by $CD56^{dim}$ NK cells, as well as degranulation, as measured by surface expression of CD107a. Of note, there were marked differences in $CD56^{dim}$ NK cell reactivity to Mtb antigens in healthy adults from the U.S. vs. Kenya, with U.S. healthy controls expressing significantly higher levels of CD69 and CD107a following stimulation with Mtb antigens, compared with QFT^{+} and QFT^{-} individuals from Kenya (**Figure 4B**).

We next evaluated Mtb cell wall-induced co-expression of CD69, CD107a and IFN-γ by $CD56^{dim}$ NK cells. The frequency of $CD56^{dim}$ NK cells expressing CD69, either alone or in combination with CD107a and/or IFN-γ, was consistently higher in U.S. healthy controls, compared with Kenyan adults (**Figure 4C**). These data suggest a heightened level of Mtb antigen-induced activation, as measured by upregulation of CD69, by NK cells from Mtb-naïve, healthy adults in the U.S., compared with Kenyan adults in a TB-endemic environment. Interestingly, among Kenyan adults, the frequency of Mtb antigen-induced CD69 single-positive $CD56^{dim}$ NK cells was higher in QFT^{-} individuals, compared with QFT^{+} individuals, although this difference did not remain statistically significant following correction for multiple comparisons (**Figure 4C**).

To determine if our results of NK cell reactivity to Mtb cell wall were reproducible with other Mtb antigen preparations, we also stimulated PBMCs with Mtb cell membrane and Mtb whole cell lysate. Similar to stimulation with Mtb cell wall, stimulation of PBMCs with Mtb cell membrane and whole cell lysate antigens induced higher expression of CD69 and CD107a by $CD56^{dim}$ NK cells from Mtb-naïve U.S. healthy adults, compared with Kenyan adults (**Figure S2**). Similar to our findings with Mtb cell wall, among Kenyan adults, the frequency of Mtb cell membrane induced $CD56^{dim}$ NK cells co-expressing CD69 and CD107a was higher in QFT^{-} individuals, compared with QFT^{+} individuals (**Figure S2D**), although this did not maintain statistical significance following correction for multiple comparisons. Overall, these data indicate that $CD56^{dim}$ NK cell responses to Mtb antigens are characterized predominately by upregulation of CD69 and CD107a expression, and that $CD56^{dim}$ NK cells from Mtb-naïve adults express significantly higher frequencies of these markers, compared with Kenyan adults. Moreover, these data suggest that the capacity to restimulate NK cells with Mtb antigens *in vitro* may be further dampened in individuals with LTBI, compared with QFT^{-} individuals from the same TB endemic environment.

Ex vivo Phenotype of $CD56^{dim}$ NK Cells Correlates With Functional Reactivity to Mtb Antigens

NK cells can directly recognize pathogens through antigen interaction with NK cell receptors (Esin et al., 2008; Li et al., 2013). To better define the relationship between NK cell receptor expression *ex vivo* and functional responses to Mtb antigens, we generated a correlation matrix of $CD56^{dim}$ NK cell receptor expression *ex vivo* and the frequency of $CD56^{dim}$ NK cells expressing CD69, CD107a, and IFN-γ following stimulation with Mtb cell wall *in vitro* (**Figure 5**). We focused our analysis of phenotypic markers to those markers that were expressed at significantly different levels among the participant groups (see **Figure 2A**): NKG2A, NKp30, NKp46, granzyme B, and TIGIT. Among the phenotypic receptors, there was a strong positive correlation between expression of granzyme B and TIGIT, and between NKG2A, NKp30, and NKp46. There were also strong inverse correlations between granzyme B and NKG2A, NKp30, and NKp46 (**Figure 5A**). By contrast, analysis of $CD56^{bright}$ NK cells by a similar correlation matrix approach revealed no relationship between granzyme B expression and NKG2A, NKp30, and NKp46 on $CD56^{bright}$ cells (**Figure 5B**).

As expected, the frequencies of Mtb-induced NK cells expressing CD69, CD107a, and IFN-γ correlated positively with each other (**Figures 5A,B**). The frequency of $CD69^{+}$ $CD56^{dim}$ cells following stimulation with Mtb antigen correlated positively with *ex vivo* frequencies of NKG2A, NKp30, and NKp46, and inversely with *ex vivo* frequencies of granzyme B and TIGIT (**Figure 5A**). Furthermore, Mtb-induced expression of CD107a by $CD56^{dim}$ cells correlated inversely with *ex vivo* granzyme B expression, while Mtb-induced induction of IFN-γ correlated positively with NKp46 (**Figure 5A**). No significant correlations were found between *ex vivo* NK cell expression of CD57, KIRs, or granulysin and the NK cell responses to Mtb antigens *in vitro* (data not shown).

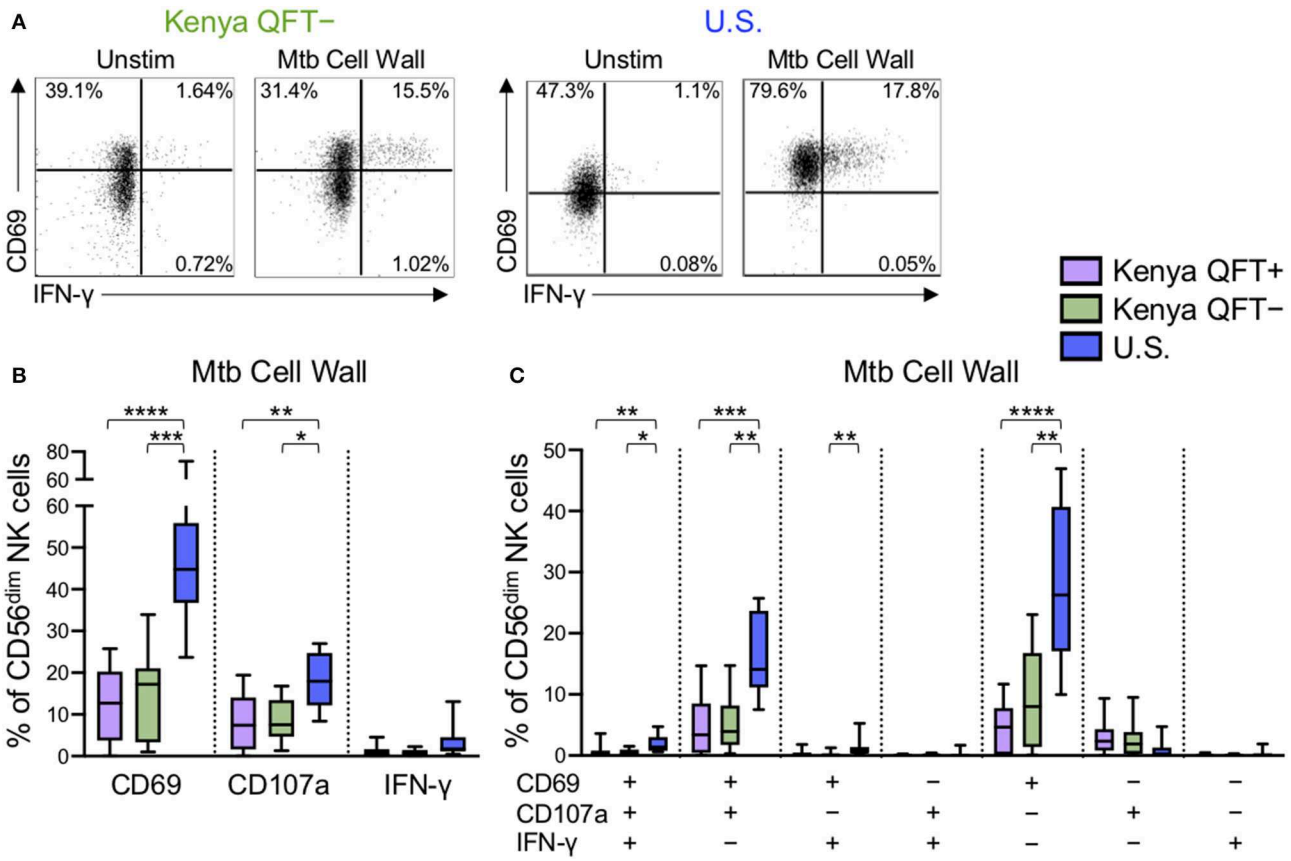

FIGURE 4 | Differential reactivity of CD56^{dim} NK cells to Mtb antigen stimulation in Kenyan adults and U.S. healthy controls. PBMCs were stimulated with Mtb cell wall antigen for 24 h in the presence of 100 U/ml IL-2; PBMCs incubated with IL-2 alone, in the absence of Mtb antigen, served as a negative control (Unstim). **(A)** Representative flow plots of CD69 and IFNγ expression by CD56^{dim} NK cells from a QFT^{-} Kenyan donor and a U.S. healthy donor. **(B)** Expression of CD69, CD107a, and IFN-γ measured on CD56^{dim} NK cells after stimulation with Mtb cell wall antigen. **(C)** Boolean analysis of co-expression patterns of CD69, CD107a, and IFN-γ expression by CD56^{dim} NK cells after stimulation of PBMCs with Mtb cell wall. Data in **(B,C)** are shown from 51 Kenyan donors ($n = 26$ QFT^{+}, $n = 25$ QFT^{-}) and 9 U.S. healthy controls. Frequencies in **(B,C)** are shown after subtraction of background expression by CD56^{dim} NK cells in the presence of media with IL-2 alone. Boxes in **(B,C)** represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$.

By contrast with CD56^{dim} NK cells, significant correlations between *ex vivo* CD56^{bright} phenotype and Mtb antigen-induced CD69, CD107a, and IFN-γ expression were limited to TIGIT, with *ex vivo* expression of TIGIT correlating inversely with all three effector molecules by CD56^{bright} cells following Mtb antigen stimulation (**Figure 5B**). Unlike CD56^{dim} cells, there were no positive correlations between *ex vivo* receptor expression by CD56^{bright} NK cells and CD56^{bright} reactivity to Mtb antigen stimulation. Taken together, these data identify NK cell phenotypic markers that correlate with functional NK cell responses to Mtb antigens *in vitro*. In addition, these results highlight the differences in reactivity by CD56^{bright} and CD56^{dim} NK cell subsets to Mtb antigen stimulation.

NK Cell Reactivity to Mtb Antigens Is Partially Dependent on IL-12 and IL-18

NK cells express a number of cytokine receptors that allow them to become activated in proinflammatory environments. It has been well-described that NK cells become activated through signaling mediated by IL-12 and IL-18 produced by activated monocytes and dendritic cells (DCs) (Fehniger et al., 1999; Son et al., 2001; Ferlazzo et al., 2004; Chaix et al., 2008; Chijioke and Munz, 2013; Leong et al., 2014). To better define the mechanism of NK cell activation by Mtb antigens, we stimulated PBMCs from each participant group with Mtb antigens in the presence of neutralizing Abs to either IL-12, IL-18, a combination of IL-12 and IL-18 together, or an isotype control (**Figure 6A**). Neutralization of IL-12 alone did not significantly impact Mtb-induced expression of CD69 or CD107a (**Figures 6B,C**), although Mtb-induced expression of IFN-γ was significantly reduced by IL-12 neutralization (**Figure 6D**). Neutralization of IL-18 alone resulted in a significant reduction in the frequencies of CD56^{dim} NK cells expressing CD69, CD107a, and IFN-γ following Mtb antigen stimulation (**Figures 6B–D**). CD56^{dim} NK cell responses to Mtb antigens were further diminished by simultaneous neutralization of IL-12 and IL-18 together

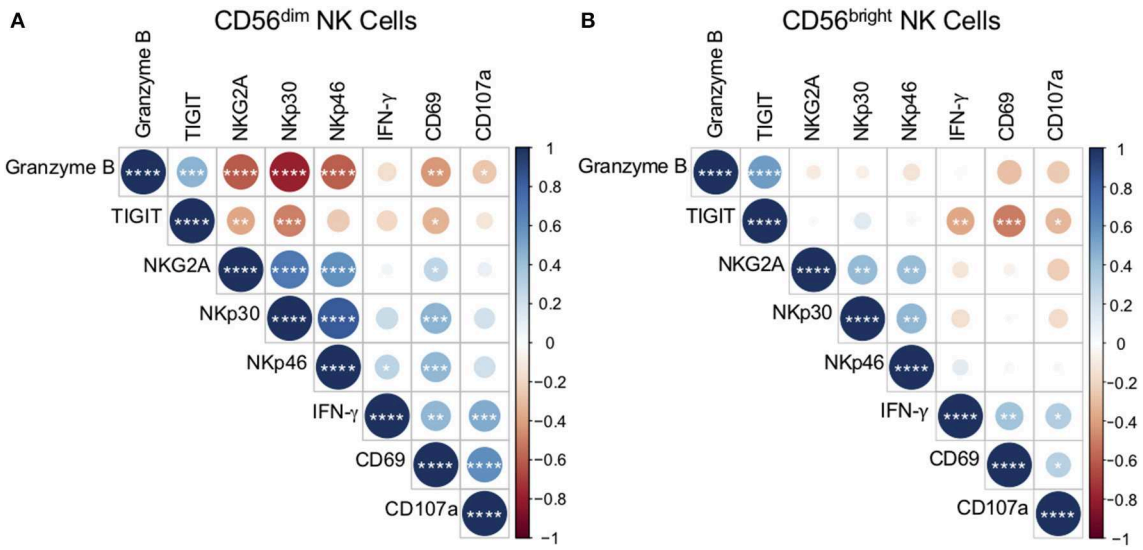

FIGURE 5 | *Ex vivo* phenotype of CD56^{dim} NK cells correlates with reactivity to Mtb cell wall. The phenotype of CD56^{dim} and CD56^{bright} NK cells in PBMCs *ex vivo* was determined as described in **Figure 1**. Expression of CD69, CD107a, and IFN-γ by CD56^{dim} and CD56^{bright} NK cells following stimulation with Mtb cell wall was determined as described in **Figure 4**. Correlations between CD56^{dim} **(A)** and CD56^{bright} **(B)** NK cell phenotype and Mtb cell wall-induced effector function were evaluated using a non-parametric Spearman rank correlation. Correlograms were generated using the corrplot function in R. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Phenotypic markers and effector molecules within the CD56^{dim} plot were ordered using the Ward method of hierarchical clustering. The order was then applied to the CD56^{bright} data. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$;and $^{****}p < 0.0001$.

(**Figures 6B–D**). Overall, these data suggest that CD56^{dim} NK cell reactivity to Mtb antigens, regardless of Mtb exposure or infection status, is at least partially mediated by indirect mechanisms in which Mtb antigens induce cytokine production by other cells present within PBMCs, which in turn induce expression of CD69, CD107a and IFN-γ by NK cells.

DISCUSSION

In this study we evaluated the phenotypic and functional profiles of NK cells from QFT^{+} and QFT^{-} adults residing in a TB-endemic region in western Kenya, and compared those with NK cell profiles in Mtb-naïve, healthy adults from the U.S. We demonstrated that CD56^{dim} NK cells from Kenyan adults have distinct phenotypic profiles and attenuated responses following stimulation with MHC class I-devoid target cells and Mtb antigens, compared with U.S. healthy adult controls. Furthermore, within Kenyan adults, we found evidence of significant downregulation of NKp46 and TIGIT expression on CD56^{dim} cells from QFT^{+} individuals with LTBI, compared with QFT^{-} individuals. Moreover, we demonstrated that NK cell responses to Mtb antigen stimulation are inversely associated with *ex vivo* expression of granzyme B and TIGIT. We further characterized NK cell reactivity to Mtb antigen stimulation and determined that NK cell reactivity to Mtb is dependent, at least in part, on IL-12 and IL-18.

NK cells in the blood of humans can be divided into three distinct populations based on expression of CD56 (Cooper et al., 2001). While CD56^{dim} NK cells are highly cytolytic and CD56^{bright} cells secrete large amounts of cytokines, CD56^{neg} NK cells have diminished cytolytic activity and cytokine production capacity (Bjorkstrom et al., 2010). Previous studies have indicated that CD56^{neg} NK cells are expanded in individuals with chronic viral infections such as HIV and hepatitis C virus (Hu et al., 1995; Mavilio et al., 2005; Gonzalez et al., 2008, 2009). Despite similar frequencies of total NK cells within the lymphocyte population, we found that QFT^{+} and QFT^{-} Kenyan adults have increased proportions of CD56^{neg} cells within the total NK cell population, compared with U.S. healthy adult controls. The increased proportion of CD56^{neg} NK cells observed in adults from Kenya, a high pathogen burden setting that is endemic for malaria, helminths, and TB (Odiere et al., 2011; WHO Publication, 2018a,b), could be an indication of NK cell activation resulting from an accumulation of pathogen exposures over time.

NK cell activity is regulated through expression of various activating and inhibitory receptors (Bryceson et al., 2006), the varied expression of which generates heterogenous populations of NK cells with high diversity (Horowitz et al., 2013; Strauss-Albee et al., 2014). The most compelling evidence for pathogen-induced differentiation of NK cells with unique phenotypic and functional properties has come from studies of CMV infection (Paust et al., 2017). The prevalence of HCMV infection in healthy Kenyan adults approaches 100% (Njeru et al., 2009), thus it was not feasible to evaluate NK cell profiles in our Kenyan groups according to HCMV status. Given the near universal prevalence of HCMV infection in Kenyan adults, we selectively enrolled HCVM^{+} U.S. adults as controls, to

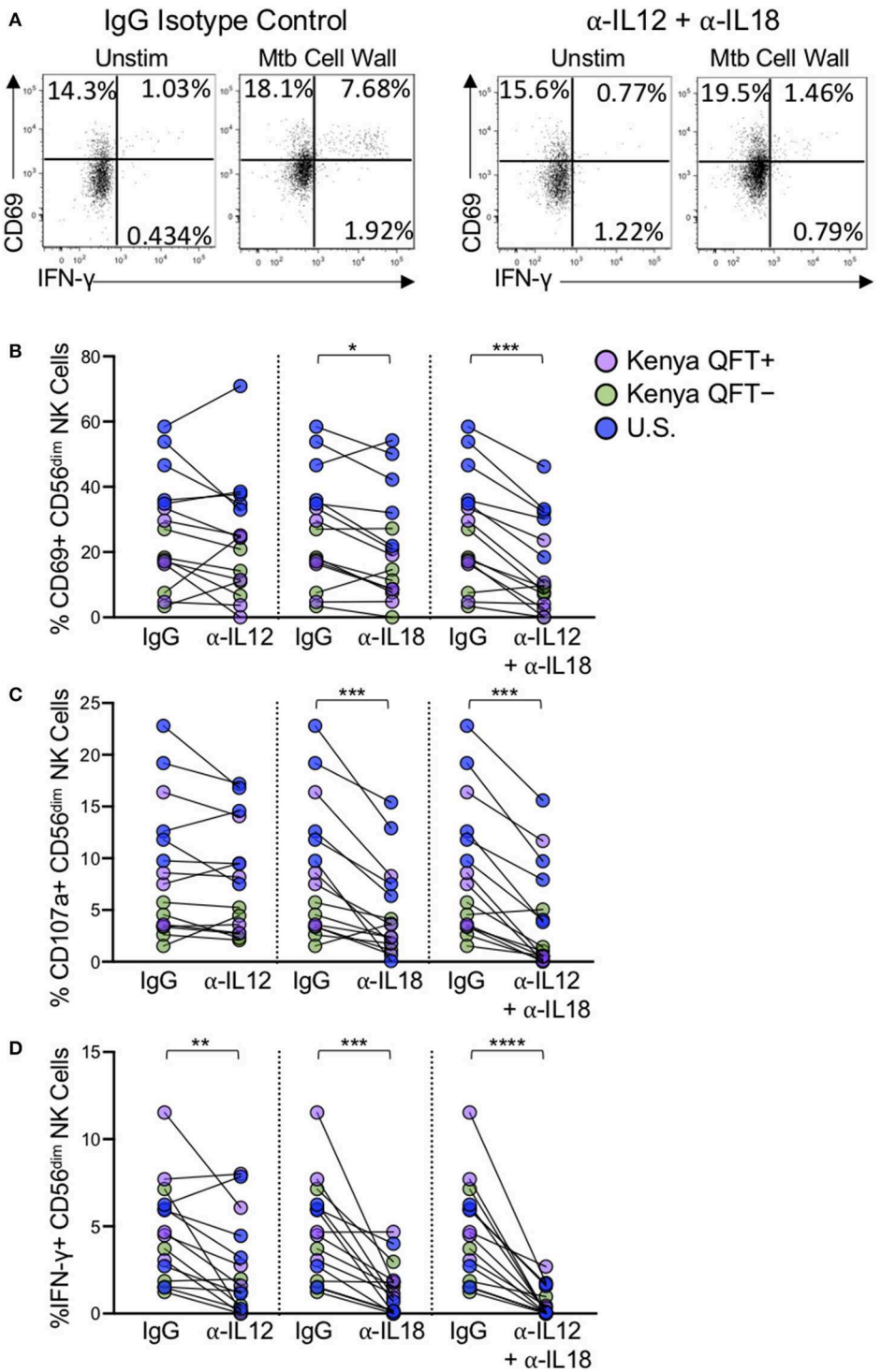

FIGURE 6 | NK cell reactivity to Mtb antigen stimulation is partially dependent on IL-12 and IL-18. PBMCs were stimulated with Mtb cell wall, as described in **Figure 4**, in the presence of anti-human IL-12 Ab, anti-human IL-18 Ab, anti-IL-12, and anti-IL-18 Abs together, or an IgG1 isotype control. **(A)** Representative flow plots of PBMCs from a Kenya QFT^{+} individual stimulated with Mtb cell wall in the presence of either anti-IL-12 + anti-IL-18 Abs or an IgG1 isotype control. **(B–D)** CD69, CD107a, and IFN-γ expression, respectively, by CD56^{dim} NK cells in PBMCs stimulated with Mtb cell wall in the presence of anti-IL12, anti-IL-18, or anti-IL-12 + anti-IL-18 together. Data from 15 individuals are shown in **(B–D)** ($n = 5$ Kenya QFT^{+}, $n = 5$ Kenya QFT^{-}, $n = 5$ U.S. healthy controls). Frequencies of Mtb cell wall-stimulated CD56^{dim} NK cells in **(B–D)** are shown after subtraction of background expression in the unstimulated negative control condition. Differences between the IgG1 isotype control and anti-IL-12/IL-18 neutralizing Abs were determined using a Wilcoxon matched-paired signed rank test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$.

minimize potential differences in NK cell profiles between groups that may be attributed to HCMV infection. Thus, although all study participants were HCMV$^+$, we still observed marked differences in NK cell phenotype between Kenyan and U.S. adults, particularly in the CD56^{dim} subset. CD57, a marker of differentiation on NK cells (Lopez-Verges et al., 2010), was expressed at higher levels on CD56^{dim} cells from Kenyan adults, compared with U.S. adults, thus suggesting an increased level of NK cell differentiation in adults residing in a TB-endemic region in Kenya. Expression of CD57 and NKG2C has been proposed as a marker of adaptive NK cells (Lopez-Verges et al., 2011). Unfortunately, we did not evaluate expression of NKG2C in our study participants, thus we are unable to determine if NKG2C$^+$ cells are also expanded within this CD57$^+$ subset of CD56^{dim} NK cells in Mtb-infected and exposed Kenyan adults.

We also observed marked downregulation of NKp30 and NKp46 expression by CD56^{dim} NK cells in QFT$^+$ and QFT$^-$ Kenyan adults, compared with U.S. adults. Downregulation of both NKp30 and NKp46 has been described in HCMV infection (Guma et al., 2004), yet here, among our HCMV$^+$ participant groups, we observed progressive downregulation of NKp46 from the highest expression levels in Mtb-naïve U.S. adults, to intermediate levels in QFT$^-$ Kenyan adults and finally the lowest NKp46 expression in QFT$^+$ Kenyan adults. In addition, we observed significant downregulation of NKp46 expression on CD56^{bright} cells in QFT$^+$ individuals with LTBI, compared with QFT$^-$ Kenyan adults, thus providing further evidence of Mtb infection-associated changes in NKp46 expression across NK cell subsets. A previous study also reported significant downregulation of NKp30 and NKp46 expression by NK cells from patients with active TB disease, compared with Mtb-uninfected healthy adults (Bozzano et al., 2009). NKp46 has been implicated as a receptor mediating NK cell lysis of Mtb-infected monocytes (Vankayalapati et al., 2002, 2005; Garg et al., 2006), thus downregulation of NKp46 could lead to impaired NK cell lysis of Mtb-infected cells. Although we did not evaluate NK cells from patients with active TB disease in the present study, future studies directly comparing NKp46 expression levels across a spectrum of Mtb exposure, infection and disease are warranted to further define the relationship between NKp46 expression and control of Mtb.

In addition to NKp30 and NKp46, significant differences in expression of NKG2A, granzyme B, and TIGIT were also observed by CD56^{dim} NK cells from Kenyan adults, compared with U.S. adults. Among Kenyan adults, TIGIT expression was downregulated on CD56^{dim} cells from QFT$^+$ individuals, compared with QFT$^-$ individuals. Although no previously published studies have comprehensively evaluated TIGIT on NK cells in the setting of human Mtb infection, increased TIGIT expression on NK cells has been reported in studies of HIV-infected individuals, compared with HIV-uninfected individuals (Yin et al., 2018; Motamedi et al., 2019; Vendrame et al., 2020), indicating that TIGIT expression on NK cells may be modulated in the setting of chronic infection. Moreover, the level of expression of TIGIT on human NK cells has been associated with NK cell functional heterogeneity in healthy adults (Wang et al., 2015), and blockade of TIGIT has been reported to increase NK cell effector functions (Stanietsky et al., 2009; Wang et al., 2015; Zhang et al., 2018), thus providing rationale for exploring TIGIT as a potential immunotherapeutic target. Future studies will be required to more clearly define the role of TIGIT expression on NK cells in human Mtb infection.

Our analysis of NK cell functional capacity revealed dampened IFN-γ production to K562 tumor cells in QFT$^+$ and QFT$^-$ Kenyan adults, compared with U.S. healthy adults. These results are consistent with the functional profile of adaptive NK cells, which have been reported to exhibit diminished IFN-γ production to tumor cells, compared with conventional NK cells (Hwang et al., 2012; Beziat et al., 2013). By contrast with dampened responses to tumor cells, adaptive NK cells have been reported to have enhanced Ab-dependent responses, compared with conventional NK cells (Lee et al., 2015; Schlums et al., 2015). We measured Ab-dependent NK cell responses in our participant groups using Ab-coated p815 cells and did not find statistically significant differences in CD56^{dim} NK cell responses, as measured by expression of CD69, CD107a, and IFN-γ. Using a cytotoxicity assay, a previous study of adolescents in South Africa indicated enhanced NK cell killing of Ab-coated p815 cells in QFT$^+$ adolescents, compared with QFT$^-$ adolescents (Roy Chowdhury et al., 2018). Although we observed a trend of higher frequencies of CD69$^+$CD107a$^+$ NK cells following stimulation with Ab-coated p815 cells in QFT$^+$ adults, compared with QFT$^-$ Kenyan adults and Mtb-naïve controls, this difference did not reach statistical significance. We did not measure actual killing of Ab-coated target cells in our study and it is currently unclear whether or not QFT$^+$ Kenyan adults have enhanced Ab-dependent cellular cytotoxicity (ADCC) responses, compared with QFT$^-$ Kenyan adults.

Although adoptive transfer studies in mouse cytomegalovirus (MCMV) infection have demonstrated the capacity of adaptive NK cells to mediate protective immunity against viral challenge (Sun et al., 2009), definitive evidence for Ag-specific adaptive NK cells in humans has been challenging. A previous study in South Africa reported that BCG revaccination of individuals with LTBI boosts BCG-reactive NK cell responses after revaccination (Suliman et al., 2016). We initially hypothesized that QFT$^+$ adults with LTBI would display enhanced NK cell reactivity to Mtb antigen stimulation, compared with QFT$^-$ and Mtb-naïve adults. Contrary to our initial hypothesis, we observed the lowest reactivity to Mtb antigen stimulation by NK cells from QFT$^+$ Kenyan adults, a result consistent across multiple Mtb antigen preparations (cell wall, whole cell lysate, and cell membrane). These data suggest that NK cells from QFT$^+$ individuals with LTBI may either be more tolerized to Mtb or have a higher threshold by which they become activated.

A previous study of healthy adults in the U.K. reported that heterogeneity in NK cell responses to BCG was associated with KIR haplotype (Portevin et al., 2012), thus suggesting a potential contribution of host genetics to NK cell reactivity to mycobacteria. Our analysis of KIR expression was limited to CD158 (KIR2DL1/S1/S3/S5), CD158b (KIR2DL2/L3), and CD158e1 (KIR3DL1); furthermore, we did not perform KIR genotyping of the participants in our cohort, thus the potential

contribution of KIR haplotype to NK cell responses to Mtb in our cohorts remains uncertain.

To better understand the relationship between NK cell phenotype *ex vivo* and functional reactivity to Mtb antigens, we performed a correlation matrix analysis and found that $CD56^{dim}$ NK cell activation to Mtb antigens, as measured by CD69 upregulation, is inversely correlated with *ex vivo* expression of granzyme B and TIGIT, and positively correlated with expression of NKG2A, NKp30, and NKp46. These data suggest that elevated $CD56^{dim}$ expression of granzyme B and TIGIT *ex vivo* could be indicative of attenuated NK cell responses to mycobacteria. Interestingly, the only receptor that was found to correlate positively with Mtb antigen-induced NK cell IFN-γ production was NKp46, which is significantly downregulated on both $CD56^{dim}$ and $CD56^{bright}$ subsets in QFT^+ individuals. Of note, only NKp46 expression by $CD56^{dim}$ cells, and not $CD56^{bright}$ cells, correlated positively with IFN-γ production following stimulation with Mtb antigen. Downregulation of NKp46 by NK cells in QFT^+ individuals with LTBI could be a potential mechanism contributing to dampened reactivity of NK cells to Mtb antigen stimulation, although it remains to be determined what role NKp46 may play in mediating NK cell activation to Mtb.

The positive correlation between NK cell reactivity to Mtb antigen stimulation and *ex vivo* expression of NKG2A, NKp30 and NKp46 by $CD56^{dim}$ cells, and not $CD56^{bright}$ cells, could be reflective of the higher proportions of $CD56^{dim}$ NK cells, compared with $CD56^{bright}$ NK cells, thus facilitating more robust detection of NK cell responses to Mtb antigens due to the higher starting number of $CD56^{dim}$ cells than $CD56^{bright}$ cells in PBMCs. Given the distinct transcriptional programs of $CD56^{dim}$ vs. $CD56^{bright}$ NK cells (Collins et al., 2019), it is also likely that there are other receptors on $CD56^{bright}$ cells that we did not evaluate in this study that may be associated with NK cell reactivity to Mtb. Future studies in which $CD56^{dim}$ and $CD56^{bright}$ NK cells are sorted from PBMCs prior to Mtb antigen stimulation, as well as receptor blockade studies, will help further elucidate the contribution of each NK cell subset and receptors that are directly involved in NK cell reactivity to Mtb.

The precise mechanisms by which NK cells recognize Mtb remains an open area of investigation. We evaluated NK cell responses by stimulating PBMCs with Mtb antigens in the presence of IL-2, thus both direct and indirect mechanisms could potentially contribute to NK cell activation. Monocytes are also present in PBMCs and can recognize bacterial antigens through pathogen-associated molecular pattern molecules and produce several inflammatory mediators including IL-12 and IL-18, which are potent stimulators of NK cells (Denis, 1994; Son et al., 2001; Leong et al., 2014). Our experiments with IL-12 and IL-18 neutralizing Abs indicated that the combined blockade of IL-12 and IL-18 signaling substantially inhibited NK cell IFN-γ production to Mtb antigens. NK cell degranulation, as measured by CD107a expression, and activation, as measured by CD69 upregulation, are also significantly reduced by combined IL-12 and IL-18 blockade, although not entirely abolished. These results suggest that at least part of the NK cell reactivity measured in these assays may be due to indirect mechanisms of NK cell activation whereby stimulation of PBMCs with Mtb antigens induces cytokine production by other cell populations, such as monocytes and DCs, which in turn activates NK cells. In addition, stimulation with Mtb antigens can induce cytokine production by γδ T cells and CD1-restricted T cells (Kabelitz et al., 1990, 1991; Porcelli et al., 1992; Ulrichs et al., 2003; Montamat-Sicotte et al., 2011), which could also influence NK cell activation. In addition to cytokines, direct interaction with monocytes and DCs can regulate NK cell activity (Fernandez et al., 1999; Brill et al., 2001; Gerosa et al., 2002; Schierloh et al., 2005). NK cells from QFT^+, QFT^-, and Mtb-naïve controls reacted in a similar manner to neutralization of IL-12 and IL-18, thus suggesting a common mechanism of NK cell activation by Mtb antigens, regardless of Mtb infection or exposure status. Due to limited cell availability, we were not able to conduct Mtb stimulation experiments on purified populations of NK cells to better define the capacity of Mtb antigens to directly activate NK cells. Further studies are warranted to stimulate purified NK cells with Mtb, or deplete distinct subsets from Mtb-stimulated PBMCs, such as monocytes, DCs or T cells, to inform the receptors and pathways by which Mtb activates NK cells.

There are several limitations to our study, including the use of flow cytometry to evaluate a discrete number of NK cell phenotypic markers. Future studies conducting transcriptional profiling by RNA sequencing of purified populations of NK cells from individuals across a spectrum of Mtb infection and disease states will be necessary to more comprehensively define NK cell signatures associated with human Mtb infection. Another limitation in this study was that we analyzed NK cells circulating in the peripheral blood, and it remains unknown whether distinct populations of NK cells with unique phenotypic and functional profiles are present in the lungs of Mtb-infected individuals. Additionally, we stimulated PBMCs with complex Mtb antigen preparations, rather than individual Mtb antigens, and it is unknown which Mtb antigens mediate direct or indirect activation of NK cells. It is also important to note that while Kenya is a TB-endemic country, it is also endemic for other infections, including malaria and helminths (Salgame et al., 2013), which are not endemic in the U.S. While our Kenyan subjects did not have concurrent malaria or helminth infection at the time of blood sample collection for our study, it is possible that they had previous malaria and/or helminth infections, which may have contributed to shaping the NK cell repertoire. Thus, markedly different pathogen burdens and environmental exposures likely contribute to intrinsic differences in NK cell profiles between Kenyan and U.S. adults. Lastly, we enrolled Mtb-naïve healthy adult controls in the U.S. who are seropositive for HCMV to more closely match the HCMV prevalence in the adult population in Kenya. However, we do not know the duration of HCMV infection in the U.S. and Kenya participant groups; it is possible that HCMV was acquired at a younger age in the Kenyan participants, compared with U.S. controls, which could potentially contribute to shaping the phenotypic and functional profiles of circulating NK cells due to longer duration of infection. Given the low prevalence of LTBI among U.S. born individuals (Mancuso et al., 2016), we were not able to compare NK cell profiles between QFT^+ and QFT^- U.S. born adults, as

we did among Kenyan adults. Future studies comparing NK cell profiles between QFT^+ and QFT^- individuals in a low pathogen burden, non-TB endemic environment, as well as longitudinal studies of the same individual before and after Mtb infection, will be important to better define the direct contribution of Mtb infection to NK cell phenotypic and functional profiles.

In conclusion, we performed a comprehensive analysis of phenotypic and functional profiles of NK cells from QFT^+ and QFT^- adults in a TB-endemic region in Kenya and found distinct phenotypes of $CD56^{dim}$ NK cells in these individuals, compared with Mtb-naïve healthy adult controls. Furthermore, the *ex vivo* expression of specific markers by $CD56^{dim}$ NK cells correlated with NK cell reactivity to Mtb antigen stimulation. There is growing appreciation that a subset of individuals who are highly exposed to infectious TB do not develop LTBI and remain persistently TST^- and/or QFT^- (Chapman and Dyerly, 1964; Houk et al., 1968; Muecke et al., 2006; Morrison et al., 2008), thus suggesting the innate immune response may be capable of clearing the bacteria in some individuals (Simmons et al., 2018). Our results identify specific differences in $CD56^{dim}$ NK cell phenotype and function in QFT^+, QFT^-, and Mtb-naïve individuals and inform future studies aimed at defining NK cell correlates that may be protective against acquisition of Mtb infection and progression to TB disease.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kenya Medical Research Institute Scientific and Ethics Review Unit and Emory University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CD, LH, and GA contributed conception and design of the study. LH, JK, JO, LS, and JT performed experimental work. CD, LH, SO, FO, AC, and NG contributed to execution and oversight of experimental work, participant recruitment and enrollment, and study database management. CD and LH contributed to data interpretation, statistical analyses, and drafted the manuscript. All authors approved the final 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/fcimb.2020.00120/full#supplementary-material

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REVIEW
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Memory and Memory-Like NK Cell Responses to Microbial Pathogens

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NK cells are cytotoxic lymphocytes that provide systemic defense against pathogens and malignancy. Although historically considered cells of the innate immune system, NK cells are now known to be capable of memory or memory-like immune responses in certain settings. Memory NK responses were initially reported over a decade ago in studies involving mouse models of cytomegalovirus infection and delayed-type hypersensitivity reactions to chemical haptens and viral antigens. Since then, a growing body of literature suggests that memory or memory-like NK cell responses may occur in a broader range of immunological settings, including in response to various viral and bacterial infections, and some immunization protocols. Memory-like NK cell responses have also now been reported in humans and non-human primates. Here, we summarize recent studies demonstrating memory or memory-like responses by NK cells in settings of infection and immunization against infectious agents.

Keywords: natural killer cells, memory NK cells, memory-like NK cells, adaptive NK cells, infection

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INTRODUCTION

Natural killer (NK) cells are cytotoxic lymphocytes specialized for immunological defense against malignant cells and intracellular pathogens. NK cells possess a repertoire of germline-encoded activating and inhibitory receptors that recognize self– and/or microbe–encoded molecules expressed on the surface of host cells (Vivier et al., 2011). Specific receptors include the killer cell immunoglobulin-like receptors (KIRs) in humans, the Ly49 receptors in mice, and natural cytotoxicity receptors (NCRs) and NKG2 receptors in both species. Major histocompatibility complex class I (MHC-I) molecules, which are present on most healthy host cells, are key ligands for inhibitory NK receptors, whereas stress-induced and/or pathogen-encoded molecules upregulated on malignant or infected cells are important ligands for activating NK receptors. NK cells also express Fcγ receptors that engage the Fc portion of antibodies, triggering NK cell activation and antibody-dependent cellular cytotoxicity (ADCC). Overall, NK cell activation is modulated by the balance of signals received through inhibitory and activating receptors. Once activated, NK cells secrete cytolytic molecules, such as perforin and granzymes, to directly lyse target cells. In addition to their cytotoxic functions, activated NK cells are an important source of chemokines and pro-inflammatory cytokines, particularly IFN-γ, which serve to shape and amplify the overall immune response.

NK cells have historically been considered innate lymphocytes, owing to their rapid effector responses even in the absence of prior antigen exposure and their lack of somatically rearranged

antigen receptors. NK cells share many developmental and functional attributes with Group 1 "helper" innate lymphoid cells (ILC1s), although their development from distinct progenitor cell populations under homeostatic conditions suggests that they are indeed distinct lineages (Vivier et al., 2018). Notwithstanding the traditional designation of NK cells as innate immune cells, studies over the past decade provide compelling evidence that some NK cell responses exhibit features of adaptive immunity, including clonal-like expansion of antigen-specific effector cells and generation of long-lived memory populations capable of enhanced recall responses. Here, we will use the term "memory" to describe enhanced (or otherwise reprogrammed) responses that are cell-intrinsic, long-lasting, and antigen-specific; "memory-like" will describe responses in which any of the three latter criteria are lacking or unknown.

Antigen-specific memory responses by NK cells were first recognized over a decade ago in independent studies in mice involving murine cytomegalovirus (MCMV) infection (Sun et al., 2009) and delayed-type hypersensitivity reactions to chemical haptens and viral antigens (O'Leary et al., 2006; Paust et al., 2010). Since then, a growing body of literature suggests that memory-like NK cell responses may occur in response to a broader range of viral, bacterial, and possibly even eukaryotic pathogens. Moreover, memory-like NK responses are not limited to experimental mouse models, but have now been described in humans and non-human primates in settings of infection and immunization. Here, we provide an up-to-date overview of studies on adaptive NK cell responses to microbial infections in mice, humans, and non-human primates.

MEMORY AND MEMORY-LIKE NK CELL RESPONSES TO VIRAL INFECTION

Cytomegalovirus (CMV)

NK cells provide critical host defense against viral pathogens, particularly herpesviruses and papillomaviruses (Orange, 2002). Individuals with deficiencies in functional NK cells are highly susceptible to recurrent, systemic, and even life-threatening herpesvirus infections, and to severe consequences of papillomavirus infections (Biron et al., 1989; Orange, 2002; Etzioni et al., 2005; Notarangelo and Mazzolari, 2006; Mace et al., 2013). NK cells also confer protection against herpesvirus infections in mice. In some mouse strains (e.g., C57BL/6 mice), resistance to the herpesvirus, murine CMV (MCMV), is mediated by a subset of NK cells bearing the NK activating receptor, Ly49H, which recognizes the virus-encoded glycoprotein, m157, expressed on the surface of MCMV-infected cells (Brown et al., 2001; Arase et al., 2002; Smith et al., 2002). MCMV infection triggers the activation and proliferative clonal-like expansion of Ly49H$^+$ NK cells. The Ly49H$^+$ NK cell response to MCMV is reminiscent of a virus-specific CD8$^+$ T cell response, with effector cell expansion peaking at ∼day 7 post-infection and a subsequent phase of programmed cell death leading to the removal of most but not all MCMV-experienced Ly49H$^+$ NK cells (Sun and Lanier, 2011). Those that remain form a small, long-lived pool of memory cells that can provide superior protection against secondary challenge with MCMV (Sun et al., 2009). MCMV-induced memory NK cells are predominantly Ly6C^{hi}DNAM1^{lo}CD27$^-$CD11b^{hi}KLRG1^{hi}, and arise from a pool of KLRG1^{lo} cells present within the effector Ly49H$^+$ population (Sun et al., 2009; Nabekura et al., 2014; Kamimura and Lanier, 2015). Effector cell expansion and memory cell generation are dependent on the Ly49H receptor and its viral ligand, m157, consistent with a *bona fide* antigen-specific memory response (Sun et al., 2009).

Since the initial description of MCMV-specific NK cell memory over a decade ago, our understanding of the molecular pathways that shape this response has advanced significantly. These advances, which have been comprehensively summarized in several recent reviews (Rapp et al., 2018; Brillantes and Beaulieu, 2019), include findings of positive regulators of memory cell formation, such as IL-12, the costimulatory molecule DNAM1, the transcription factors STAT4, T-bet, Eomes, Runx1, and CBF-β, the microRNA miR-155, and the pro-mitophagy proteins BNIP3/BNIP3L, as well as negative regulators such as the pro-apoptotic molecule, Bim. Effector-to-memory NK cell differentiation is accompanied by transcriptional and epigenetic alterations, such as increased abundance of *Ly6c1* (encoding Ly6C) and *Gzmb1* (encoding Granzyme B) transcripts and enhanced chromatin accessibility at the *Prf1* (encoding Perforin-1) locus, which support the distinct phenotype and functions of memory NK cells (Bezman et al., 2012; Lau et al., 2018).

Analogous to the MCMV-specific memory responses demonstrated in mice, memory-like NK cell populations have also been described in humans with a history of human cytomegalovirus (HCMV) infection. Compared to their HCMV-seronegative counterparts, HCMV-seropositive individuals harbor an expanded population of NK cells expressing the receptor complex comprised of CD94 and NKG2C (Guma et al., 2004). NKG2C$^+$ NK cells transferred into patients in settings of hematopoietic stem cell transplantation expand and mount potent IFN-γ responses during HCMV reactivation (Foley et al., 2012a,b). These expanded NKG2C$^+$ cells have a unique surface phenotype, with preferential expression of the maturation marker, CD57, and of inhibitory immunoglobulin-like transcript 2 (ILT2) and KIRs, but reduced expression of the NCRs, NKp30 and NKp46, the intracellular signaling proteins, SYK and EAT-2, and the transcription factor, PLZF (Guma et al., 2004; Lopez-Verges et al., 2011; Schlums et al., 2015). HCMV-seropositive individuals also harbor more FcεRIγ$^-$ NK cells—many but not all of which are NKG2C$^+$–that exhibit enhanced ADCC functionality and IFN-γ production following exposure to antibody-coated target cells (Hwang et al., 2012; Zhang et al., 2013; Lee et al., 2015). The unique phenotype and function of these HCMV-expanded NK cells are mirrored by epigenetic modifications at regulatory regions for the genes encoding FcεRIγ, IFN-γ, EAT-2, and PLZF (Luetke-Eversloh et al., 2014; Lee et al., 2015; Schlums et al., 2015). For example, loss of silencing DNA methylation marks at a conserved non-coding sequence (CNS) upstream of the *IFNG* promoter correlates with enhanced IFN-γ production by activated memory-like NK cell populations (Luetke-Eversloh et al., 2014). Conversely, increased

methylation at the FcεRIγ and EAT-2 gene loci correlates with reduced expression of these proteins in memory-like cells (Schlums et al., 2015). These findings suggest that epigenetic reprogramming is an important mechanism underlying the altered functionality of memory-like NK cell populations.

Although the specific NK receptor-ligand interaction(s) that drive expansion of NKG2C$^+$ NK cells during HCMV infection remain incompletely understood, the response is thought to be HCMV-specific. In humans, acute HCMV infection or reactivation is associated with the selective expansion or re-expansion of NKG2C$^+$ NK cells (Lopez-Verges et al., 2011; Foley et al., 2012a,b). Likewise, CMV infection drives the selective expansion of NKG2C$^+$ NK cells in rhesus macaques (Ram et al., 2018). Co-culture of human peripheral blood lymphocytes with HCMV-infected fibroblasts *in vitro* has been reported to drive the selective expansion of NKG2C$^+$ NK cells in some (Guma et al., 2006), although not all studies (Newhook et al., 2017).

Recent work demonstrated that NKG2C$^+$ NK cells are responsive to the HCMV-derived peptide, UL40, in complex with the non-classical MHC-I molecule, HLA-E, a known NKG2C ligand (Hammer et al., 2018). Similar to their response to HCMV infection *in vivo*, NKG2C$^+$ NK cells stimulated with UL40-HLA-E *in vitro* proliferated, downregulated expression of FcεRIγ, and lost DNA methyl marks at the *IFNG* regulatory region CNS1. These effects were highly sensitive to the UL40 peptide sequence, as minor HCMV strain-specific differences in the sequence altered both the magnitude and quality of the response (Hammer et al., 2018).

Altogether, the studies described above suggest that HCMV-specific memory NK cells exist within the NKG2C$^+$ NK cell compartment in HCMV-seropositive individuals. However, the role of the NKG2C receptor itself in formation of memory-like NK cells during HCMV infection is not completely clear. In one study, HCMV-exposed *NKG2C*$^{-/-}$ individuals were reported to have fewer CD94$^+$ NK cells and, in individuals <10 years old, fewer CD57$^+$ NK cells overall. In the younger cohort (<10 years old), anti-HCMV antibody titers were also higher, possibly reflecting poorly controlled HCMV infection (Goodier et al., 2014). In contrast, another study reported that HCMV-exposed *NKG2C*$^{-/-}$ individuals maintain an expanded population of NK cells that, although missing NKG2C expression, retained other memory-associated phenotypic and functional characteristics, including enhanced IFN-γ secretion and a demethylated *IFNG* CNS1 region (Liu et al., 2016). Moreover, *NKG2C*$^{-/-}$ NK cells transferred in the context of clinical transplantation therapies were shown to rapidly expand and mature in response to HCMV infection (Della Chiesa et al., 2014). Thus, while the specific role of NKG2C remains to be fully clarified, it is possible that receptors other than, or in addition to, NKG2C support the differentiation of memory-like NK cells during HCMV infection.

Additional evidence that the NKG2C$^+$ NK cell response in HCMV-infected individuals is pathogen-specific comes from studies showing that NKG2C$^+$ NK cells do not expand during other herpesvirus infections. For example, neither Epstein-Barr virus (EBV) nor herpes simplex virus (HSV)-2 infections are associated with selective expansion of NKG2C$^+$ NK cells (Bjorkstrom et al., 2011b; Hendricks et al., 2014). Furthermore, although expansion of NKG2C$^+$ NK cells has been described in people infected with hepatitis B virus, hepatitis C virus, chikungunya virus, and hantavirus, this phenomenon appears to be largely restricted to HCMV-seropositive individuals (Brunetta et al., 2010; Bjorkstrom et al., 2011a; Petitdemange et al., 2011; Beziat et al., 2012). Expansion of NKG2C$^+$ NK cells in these settings may reflect antibody-driven proliferation of the FcεRIγ$^-$ population that is abundant in HCMV-seropositive individuals, consistent with studies showing that FcεRIγ$^-$ NK cells expand *in vitro* following exposure to target cells infected with HCMV, HSV-1, or influenza, but only when virus-specific antibodies are also present (Lee et al., 2015).

Epstein-Barr Virus (EBV)

EBV is a gammaherpesvirus that latently infects most adults worldwide. EBV typically gains entry through the tonsils and establishes long-term residence in B cells, where it is associated with various B cell malignancies, including Burkitt's lymphoma, Hodgkin's disease, and post-transplant lymphoproliferative disorder (PTLD) (Gru et al., 2015). NK cells are thought to play an important role in controlling EBV-induced B cell transformation (Strowig et al., 2008). Human tonsillar NK cells with a NKG2A$^+$CD94$^+$CD56^{bright} phenotype were shown to restrict the transformation of autologous B cells exposed to EBV *in vitro,* an activity that was dependent on both IFN-γ and the NCR family activating receptor, NKp44 (Strowig et al., 2008; Lunemann et al., 2013; Jud et al., 2017). In pediatric patients with acute symptomatic EBV infection (also known as infectious mononucleosis), NKG2A$^+$CD56^{dim}KIR$^-$ NK cells were shown to undergo selective expansion and persist in peripheral blood at elevated frequencies for many months (Azzi et al., 2014). They also degranulated with greater frequency than their NKG2A$^-$CD56^{dim}KIR$^+$ and CD56^{bright} counterparts following exposure to B cells infected with actively replicating (although not latent) EBV (Azzi et al., 2014). Similarly, in humanized mice, NKG2A$^+$ NK cells have been shown to persist at elevated frequencies in the blood for many weeks after EBV infection (Chijioke et al., 2013). Another group reported that human NKG2A$^+$ NK cells were also more responsive to lymphoblastoid cell lines latently infected with EBV (Hatton et al., 2016). Collectively, these findings raise the possibility that the NKG2A$^+$ compartment may harbor EBV-specific memory-like NK cells in individuals with a history of EBV infection.

Herpes Simplex Virus (HSV)

Studies in mice suggest that infection with the alphaherpesvirus, HSV-2, may also elicit protective memory-like NK responses. Prior infection of *Rag1*$^{-/-}$ mice with an attenuated strain of HSV-2 was shown to confer resistance to a later lethal dose of HSV-2, notwithstanding the absence of adaptive T and B cells in these animals. NK cells were required for this resistance, as NK cell depletion during the secondary challenge neutralized the protective effect of prior HSV-2 exposure (Abdul-Careem et al., 2012). NK cells from HSV-2-primed mice also exhibited enhanced functionality *in vitro*, producing more IFN-γ than NK cells from naïve mice when exposed to lysate from HSV-2-infected Vero cells, but not lysate from uninfected control

cells (Abdul-Careem et al., 2012). Nevertheless, this enhanced functionality was relatively short-lived (∼30 days), in contrast to the months-long memory responses elicited by MCMV infection (Sun et al., 2009; Abdul-Careem et al., 2012).

Varicella Zoster Virus (VZV)

VZV, another common alphaherpesvirus and the causative agent of chickenpox and shingles, was recently suggested to promote memory-like NK responses in humans. Individuals with a history of chickenpox during childhood were shown to mount robust delayed-type hypersensitivity reactions to intradermally injected VZV antigens (Nikzad et al., 2019). This response involved the selective recruitment of actively degranulating NK cells to the VZV antigen injection site, but not to saline-injected control sites, although the contribution of T and B cells to these responses could not be excluded by the study design. The majority of the VZV-recruited NK cells were $CD56^{hi}$ and more frequently expressed markers associated with tissue-residency, including CXCR6, NKG2D, CD69, and CD62L (Nikzad et al., 2019).

HIV/SIV

The lentivirus, human immunodeficiency virus (HIV), causes acquired immunodeficiency syndrome (AIDS) in humans. Early studies in mice were the first to demonstrate that immunization with HIV antigens could elicit HIV-specific memory NK cell responses (Paust et al., 2010). In these studies, *Rag1*$^{-/-}$ mice were immunized with viral-like particles (VLPs) containing HIV antigens. Transfer of liver, but not splenic, NK cells from immunized donors into naive *Rag2*$^{-/-}$*Il2rg2*$^{-/-}$ recipients resulted in NK-mediated recall responses that were antigen-specific, occurring only in recipients rechallenged with HIV antigens but not antigens from other viruses such as influenza (Paust et al., 2010). More recently, vaccination of BLT (human bone marrow, liver, and thymus) humanized mice with HIV antigens was shown to elicit human NK-mediated recall responses that were both vaccination-dependent and antigen-specific (Nikzad et al., 2019).

Consistent with findings in mouse studies, HIV-specific memory NK cell responses have also been demonstrated in rhesus macaques infected with or vaccinated against simian immunodeficiency virus (SIV) and simian HIV (SHIV) (Reeves et al., 2015). NK cells from infected or vaccinated, but not naive, macaques were shown to lyse target cells pulsed with the HIV Gag or Env antigens. Lysis was antigen-specific, as the NK cells did not kill target cells pulsed with mismatched antigens. Notably, HIV-specific recall responses persisted for up to 5 years after vaccination, underscoring the longevity of these memory NK cell responses in macaques (Reeves et al., 2015). NK killing of antigen-pulsed targets was dependent, at least in part, on functional signaling through the NKG2A and NKG2C receptors, although whether these receptors mediate direct or indirect recognition of HIV antigens remains unclear (Reeves et al., 2015). Intriguingly, studies involving human NK cells indicate that the human KIR, KIR2DL1, can recognize HIV peptides on infected cells in an HLA-dependent manner (Alter et al., 2011). Moreover, $KIR3DS1^{+}$ and $KIR3DL1^{+}$ NK cells have been shown to selectively expand during acute HIV infection in individuals that express HLA-B Bw4801(Alter et al., 2009). Collectively, these findings suggest that investigation of HIV-specific memory NK responses in humans will be an important focus for future studies.

Vaccinia Virus

Memory-like NK cell responses to vaccinia virus, a member of the Poxviridae family, have been suggested in studies involving mice and macaques. Adoptively transferred $CD90^{+}$ liver NK cells from mice previously infected with vaccinia were more effective than NK cells from naïve donors at protecting *Rag1*$^{-/-}$ recipients against lethal vaccinia challenge (Gillard et al., 2011). A complementary study in macaques examined NK cell responses elicited by prime-boost immunization protocols involving the modified vaccinia virus Ankara strain. NK cells from "boosted" macaques had higher expression of molecules associated with cytotoxicity, homing, adhesion, and maturity than NK cells from macaques that received the priming challenge only. Importantly, these changes were long-lived, persisting for months after the initial priming event, consistent with a possible memory-like phenotype (Palgen et al., 2019).

Influenza Virus

Influenza vaccination in humans was shown to increase the frequency of circulating $CD56^{dim}$ NK cells for >1 month post-vaccination (Dou et al., 2015). These $CD56^{dim}$ NK cells had lower surface expression of NKp46 than their $CD56^{bright}$ counterparts and produced more IFN-γ when restimulated *in vitro* with inactivated influenza (Dou et al., 2015). Enhanced IFN-γ production was dependent on functional NKp46 signaling at the time of restimulation, possibly reflecting a role for NKp46 in recognition of influenza antigens as previously suggested (Mandelboim et al., 2001; Dou et al., 2015). Similar observations of enhanced effector responses by peripheral blood NK cells after influenza vaccination were also reported by a separate group (Goodier et al., 2016). In this study, NK cells evaluated up to 3 months after vaccination produced more IFN-γ and degranulated better when restimulated *in vitro* with inactivated influenza and low-dose IL-12 and IL-18. This enhanced functionality was partially dependent on Type I interferon signaling. The latter observation led to the suggestion that Type I interferons elicited by vaccination might alter the activation threshold of NK cells in immunized individuals (Goodier et al., 2016), consistent with prior findings that exposure to pro-inflammatory cytokines, such as IL-12, IL-18, and IL-15, can program long-lasting, albeit antigen-independent, memory-like features in NK cells (Cooper et al., 2009; Romee et al., 2012).

Analogous to findings in humans, mice immunized with sublethal doses of influenza virus were shown to harbor a population of $CD49a^{+}DX5^{-}$ NK cells that expressed high levels of the memory-associated markers, Ly6C and KLRG1 (Li et al., 2017). Adoptively transferred liver, but not lung, $CD49a^{+}DX5^{-}$ NK cells from influenza-vaccinated mice protected naive mice against a secondary challenge with lethal doses of virus (Li et al., 2017). Similarly, Rag1-deficient mice immunized with VLPs containing influenza antigens were shown to harbor liver-resident memory NK cells capable of protective, virus-specific recall responses (Paust et al., 2010).

MEMORY-LIKE NK CELL RESPONSES TO BACTERIAL PATHOGENS

Mycobacteria Species

Recent studies in mice and humans indicate that NK cells may mediate memory-like responses to *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB). Human NK cells expressing the T cell memory marker, CD45RO, were shown to accumulate in the pleural fluid of TB patients. Compared to CD45RO$^-$ NK cells, these CD45RO$^+$ NK cells produced more IFN-γ and were more cytotoxic against tumor cells when re-stimulated with IL-12 *in vitro* (Fu et al., 2011). They also produced more IL-22 and IFN-γ when co-cultured with autologous monocytes infected with Bacillus Calmette-Guerin (BCG), a live-attenuated mycobacterium routinely used to vaccinate humans against TB (Fu et al., 2016). Indeed, revaccination of individuals with BCG has been shown to boost the frequency of BCG-reactive NK cells in the peripheral blood for at least a year after revaccination (Suliman et al., 2016).

Similarly, in mice, BCG inoculation expanded a subset of CD27$^+$KLRG1$^+$ NK cells that underwent selective re-expansion in response to a subsequent challenge with Mtb. Transfer of CD27$^+$ NK cells from BCG-primed, but not mock-immunized, mice into naïve animals conferred partial protection against Mtb infection, reducing overall bacterial burdens in the lung. This protection was specific to the CD27$^+$ compartment—transferred CD27$^-$ NK cells from BCG-primed donors failed to protect recipients from Mtb—and correlated with the enhanced proliferative and IFN-γ responses by transferred CD27$^+$ NK cells during Mtb infection. Mechanistically, generation of memory-like NK cells following BCG inoculation required the presence of T cell-derived IL-21 at the time of primary BCG infection (Venkatasubramanian et al., 2017).

In addition to, or perhaps instead of, mycobacterial-specific memory responses, BCG immunization may also confer antigen-independent memory-like features in NK cells, possibly through reprogramming mechanisms involving pro-inflammatory cytokine stimulation, as described in other settings (Cooper et al., 2009; Romee et al., 2012). Indeed, NK cells from BCG-immunized patients were shown to mount enhanced effector responses when restimulated not only with mycobacterial, but also with fungal antigens *in vitro* (Kleinnijenhuis et al., 2014). Additionally, BCG-vaccinated mice were shown to be more resistant than naïve animals to heterologous challenge with the fungal pathogen, *Candida albicans*. Heterologous protection was maintained in vaccinated SCID mice, which lack T and B cells but possess functional NK cells, but was lost in mice lacking all lymphocytes, suggesting that memory-like NK cells contributed to protection in these studies (Kleinnijenhuis et al., 2014).

Ehrlichia Species

Memory-like NK cell responses were recently described in mice infected with the intracellular bacterial pathogen, *Ehrlichia muris*. Prior work had established that mice infected with a non-lethal dose of *E. muris* were resistant to a later challenge with the more virulent *Ehrlichia* strain, *Ixodes ovatus Ehrlichia* (IOE) (Thirumalapura et al., 2008, 2009). A follow-up study demonstrated that NK cells were critical for this resistance, as *E. muris*-primed mice depleted of NK cells prior to secondary IOE challenge rapidly succumbed to infection (Habib et al., 2016). Moreover, transferred NK cells from *E. muris*-primed donor mice, but not NK cells from naïve or IOE-primed donors, were capable of protecting *Rag2*$^{-/-}$*Il2rg2*$^{-/-}$ recipients against a high dose *E. muris* challenge. Protection was maintained even in *Rag2*$^{-/-}$*Il2rg2*$^{-/-}$ recipients co-treated with anti-CD4 antibodies, ruling out a protective role for contaminating CD4$^+$ T cells that may have been co-transferred with the NK cells (Habib et al., 2016).

MEMORY-LIKE NK CELL RESPONSES TO EUKARYOTIC PATHOGENS

Plasmodium falciparum is a unicellular protozoan parasite that causes malaria in humans. At specific stages of its life cycle, *P. falciparum* infects red blood cells (RBCs), which display parasite-derived proteins on their surface that facilitate vascular adhesion and sequestration. Recent studies identified a unique population of FcεRIγ$^-$ NK cells that mediates killing of *P. falciparum*-infected RBCs via ADCC when antibodies that bind *P. falciparum* antigens on the RBC surface are also present (Arora et al., 2018; Hart et al., 2019). These FcεRIγ$^-$ NK cells were found to be expanded in malaria-exposed individuals, and their relative abundance correlated with reduced parasitemia and resistance to clinical symptoms of malaria (Arora et al., 2018; Hart et al., 2019). Similar to the memory-like NKG2C$^+$ population that expands during CMV infection, the FcεRIγ$^-$ population in malaria-exposed subjects largely lacked PLZF expression and exhibited heightened ADCC functionality. However, these FcεRIγ$^-$ NK cells were not uniformly NKG2C$^+$, and their frequency was similar in both HCMV-seropositive and –seronegative subjects, suggesting they are at least partially distinct from CMV-expanded adaptive NK cells (Hart et al., 2019). Whether the FcεRIγ$^-$ population contains genuine *Plasmodium*-specific memory cells, or even non-specific cytokine-induced memory-like cells, remains to be determined.

Of note, evidence of adaptive NK cell responses was found to be conspicuously absent in mice infected with a related apicomplexan parasite, *Toxoplasma gondii*. Although NK cells were important for protection against secondary *T. gondii* challenge, this role was notably independent of any cell-intrinsic differences in functionality. NK cells from mice previously infected with *T. gondii* were comparable to those from naïve mice with respect to longevity and their failure to protect *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice from *T. gondii* challenge (Ivanova et al., 2019).

MEMORY NK CELLS AS VACCINE TARGETS

Current vaccination protocols largely target B and T cells, with the general goal of generating high titers of neutralizing antibodies. However, vaccine strategies that target memory NK

responses may be useful in boosting protection, especially against microbes that evade control by neutralizing antibodies. The feasibility of harnessing NK cells in this manner is supported by findings of antigen-specific memory NK responses against diverse classes of antigens, ranging from distinct viral proteins to small chemical haptens (Geary and Sun, 2017). In addition, the enhanced ADCC functionality of some memory-like NK populations, e.g., the FcεRIγ$^-$ populations observed in CMV-exposed individuals (discussed in CMV section above), suggests a possible role for adaptive NK cells in boosting the efficacy of vaccine-induced antibodies.

Notwithstanding the potential utility of adaptive NK cells in providing long-lasting immunity, the development of vaccines that elicit pathogen-specific NK responses will be aided by a better understanding of how NK cells gain specificity for diverse antigens in light of their fixed receptor repertoire. To date, little is known about the specific microbial antigen and cognate NK receptor pairs that support NK memory. A notable exception is the recognition of m157 by Ly49H. The gene encoding Ly49H appears to have arisen out of a DNA recombination event involving the inhibitory receptor, Ly49I, which binds MHC class I (Brown and Scalzo, 2008). Given that m157 is a structural mimic of MHC class I (Adams et al., 2007), it may have originally evolved to suppress NK cell function through engagement of Ly49I. Indeed, the activating effects of m157 are limited to mouse strains that evolved to express Ly49H rather than Ly49I (Arase et al., 2002; Smith et al., 2002). Thus, the role of m157 and Ly49H interactions in memory NK responses likely reflects a specific process of co-evolution between MCMV and its mouse host, rather than a general mechanism by which NK cells acquire specificity to diverse antigens. Nevertheless, memory responses against another MCMV-encoded glycoprotein, m12 were recently described for ILC1s (Weizman et al., 2019) and other Ly49 receptors have been shown to recognize MCMV antigens, e.g. recognition of MCMV m04 in the context of H2-D^k in MA/MyJ mice (Kielczewska et al., 2009). Thus, the Ly49H memory NK response to m157 is not likely an isolated phenomenon. Whether and how NKG2 receptors contribute to antigen-specific NK responses to HIV antigens in macaques; why NKG2C$^+$ and NKG2A$^+$ populations are associated with memory-like responses to HCMV and EBV in humans, respectively; and whether the KIR3DS1$^+$ and KIR3DL1$^+$ NK cells that expand during HIV infection in humans become memory cells are important and open questions in the field. Until we better understand the specific NK receptors and cognate antigens involved in NK memory, vaccination with inactivated or attenuated pathogens may remain the most viable option to maximize the likelihood of generating pathogen-specific responses.

Another important consideration in developing vaccines to target NK cells is related to the finding that unique tissue-resident or tissue-specific populations can contribute to adaptive NK responses (Paust et al., 2010; Nikzad et al., 2019). Understanding how to target these populations might be beneficial in generating protective immunity against pathogens that gain entry through or colonize specific tissues. Toward this end, humanized mouse models might be particularly useful in the experimental interrogation of human tissue-resident NK cells not readily obtained from blood.

NK CELL MEMORY: A T CELL PERSPECTIVE

NK cells share many functional properties with cytotoxic CD8$^+$ T cells, and recent studies have highlighted notable similarities in memory cell differentiation as well. As described above, mouse Ly49H$^+$ NK cells activated during MCMV infection exhibit distinct activation, expansion, contraction, and memory phases that are similar to classical antiviral CD8$^+$ T cell responses. Notably, the signals and molecular pathways that regulate these specific phases also share similarities. For example, the "three-signal" model of T cell activation—TCR activation via antigen (signal 1), co-stimulation (signal 2), and inflammation (signal 3)—has clear parallels in NK cell activation during MCMV infection, with Ly49H:m157 engagement providing signal 1, costimulatory receptors such as DNAM1 providing signal 2, and pro-inflammatory cytokines such as IL-12 providing signal 3. With respect to Signal 1, recent studies suggest that its strength and/or duration not only impacts T cell memory, but also NK cell memory responses (Snook et al., 2018; Adams et al., 2019; Grassmann et al., 2019; Li et al., 2019). Specifically, high-avidity Ly49H^{hi} NK cells were shown to be more proliferative and to preferentially form memory cells following MCMV infection, whereas low-avidity Ly49H^{lo} cells became the principal IFN-γ producers (Adams et al., 2019; Grassmann et al., 2019). Avidity-based memory cell programming may provide a mechanism to ensure that the memory pool is comprised of cells with increased specificity or binding to viral ligand. Other common molecular regulators of T and NK memory responses include the cytokine IL-15 and Bcl-2 family proteins, such as pro-apoptotic Bim and anti-apoptotic Bcl-2 and Mcl-2, which act in balance to ensure proper effector cell apoptosis and memory cell survival during the contraction phase (Grayson et al., 2000; Min-Oo et al., 2014).

Intriguingly, differential expression of the activating receptor, KLRG1, by effector T and NK cells has been shown to distinguish cells with effector vs. memory cell potential at early stages of a viral infection. Among activated T cells, high KLRG1 expression marks short-lived effector cells (SLECs) and low KLRG1 expression marks memory precursor effector cells (MPECs). Similarly, for Ly49H$^+$ NK cells activated during MCMV infection, KLRG1$^+$ cells appear to reflect terminally differentiated effector cells, whereas KLRG1$^{low/-}$ cells may preferentially seed the memory compartment, analogous to T cell SLECs and MPECs (Kamimura and Lanier, 2015). For T cells, MPECs give rise to central memory (T_{cm}) or effector memory (T_{em}) T cells, which preferentially home to lymphoid organs or remain in circulation, respectively. Whether a similar "division of labor" occurs among Ly49H$^+$ memory NK cells remains unknown, although recently developed single-cell genomics and lineage-tracing tools may prove useful in addressing this question.

TABLE 1 | Memory and memory-like NK cell populations in mice, humans, and non-human primates.

Pathogen/Microbial Antigen	Species	Memory cell markers	Select references
MCMV	Mouse	$Ly49H^{+}Ly6C^{hi}KLRG1^{hi}$ $CD43^{hi}DNAM1^{-/lo}CD27^{lo}$ $CD11b^{+}$	Sun et al., 2009; Nabekura et al., 2014; Kamimura and Lanier, 2015
HCMV	Human	$NKG2C^{+}CD94^{+}CD57^{+}ILT2^{+}$ $NKp46^{lo}NKp30^{lo}SYK^{lo}EAT\text{-}2^{lo}$ $PLZF^{lo}Fc\varepsilon RI\gamma^{lo}$	Guma et al., 2004, 2006; Lopez-Verges et al., 2011; Beziat et al., 2012; Foley et al., 2012a,b; Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015
EBV	Human	$NKG2A^{+}KIR^{-}CD56^{dim}$	Chijioke et al., 2013; Azzi et al., 2014; Hatton et al., 2016
HSV-2	Mouse		Abdul-Careem et al., 2012
VZV	Human	$CD56^{hi}CXCR6^{+}$	Nikzad et al., 2019
HIV[a]	Mouse/humanized mouse	$CXCR6^{+}$	Paust et al., 2010; Nikzad et al., 2019
SIV/SHIV	Rhesus macaque		Reeves et al., 2015
Vaccinia virus	Mouse	$CD90^{+}$	Gillard et al., 2011
Influenza virus	Human	$CD56^{dim}NKp46^{lo}$	Dou et al., 2015
Influenza virus	Mouse	$CD49a^{+}CD49b^{-}KLRG1^{hi}Ly6C^{hi}CD62L^{lo}$	Paust et al., 2010; Li et al., 2017
Mycobacterium spp.	Human	$CD45RO^{+}CD27^{+}$	Fu et al., 2011, 2016; Venkatasubramanian et al., 2017
Mycobacterium spp.	Mouse	$CD27^{+}$	Venkatasubramanian et al., 2017
Ehrlichia spp.	Mouse		Habib et al., 2016
Plasmodium falciparum (?)	Human	$PLZF^{lo}Fc\varepsilon RI\gamma^{lo}$	Hart et al., 2019

[a] *Mice were immunized with HIV antigens Gag and/or Env.*

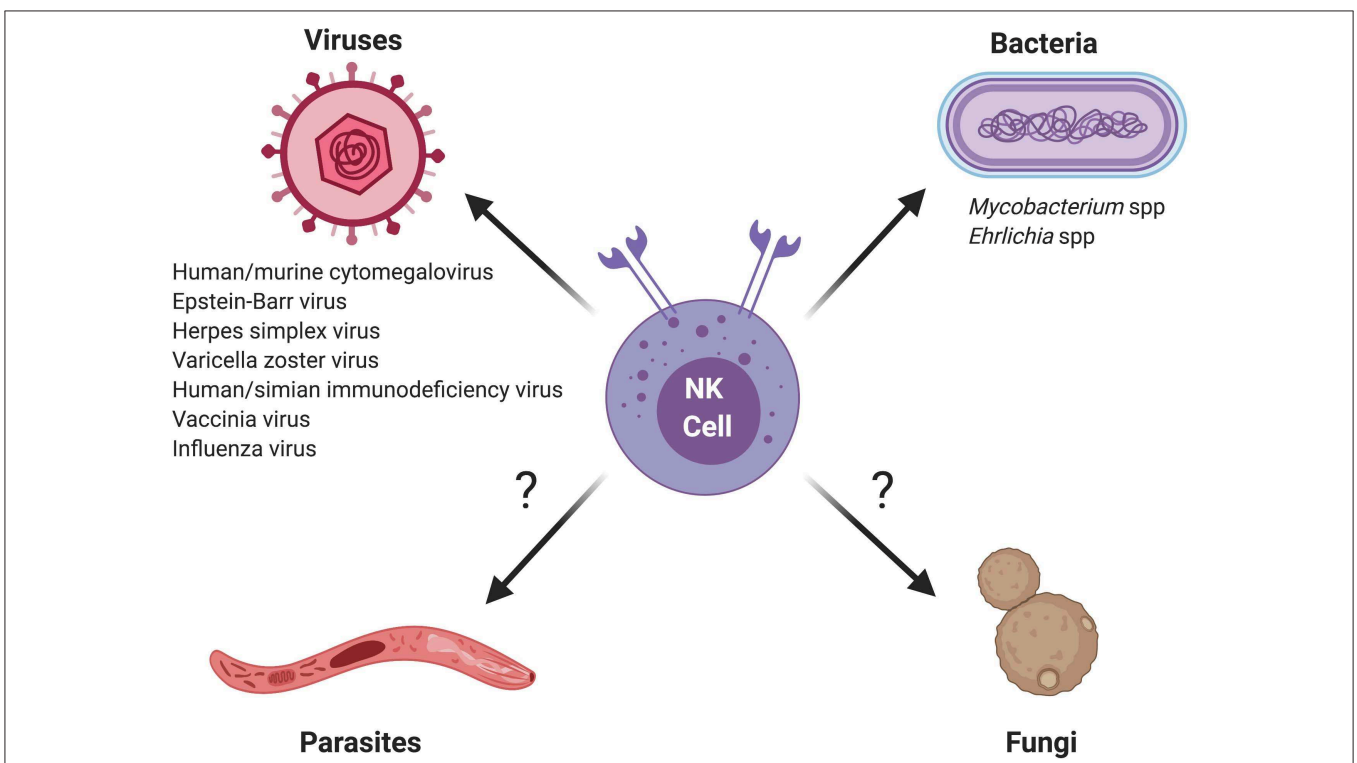

FIGURE 1 | Schematic of memory and memory-like NK cell responses against different classes of microbial pathogens. Question marks indicate eukaryotic pathogens for which memory or memory-like responses by NK cells require further investigation.

CONCLUDING REMARKS

In summary, a growing body of evidence suggests that NK cells have the capacity to mount memory or memory-like responses to a diverse range of viral and bacterial pathogens, and possibly even eukaryotic pathogens such as *Plasmodium falciparum* (**Table 1** and **Figure 1**). It will be interesting to see whether future studies uncover memory NK responses against other eukaryotic organisms, and in particular the various fungal pathogens controlled by NK-mediated host defenses (reviewed in Ogbomo and Mody, 2016; Mody et al., 2019).

In some experimental models, memory NK responses are clearly pathogen-specific. In others, inflammatory signals that arise during infection or immunization protocol may reprogram NK cells, such that subsequent responses to both specific and non-specific stimuli are enhanced or altered. Indeed, exposure to pro-inflammatory cytokines is known to imprint long-lived memory-like features in both human and mouse NK cells (Cooper et al., 2009; Romee et al., 2012). The collective body of literature suggests that individuals likely harbor multiple, distinct pools of memory of memory-like NK populations, which may act independently or cooperatively to confer long-term immunity against different pathogens. Future studies are needed to understand the common and unique features of these various memory-like populations, as well as the molecular and epigenetic pathways that program them. Ultimately, a better understanding of NK cell memory could inform clinical efforts to harness their capabilities in vaccination strategies or cellular immunotherapies, particularly in settings where memory responses by T cells and/or B cells are inadequate to prevent infection or malignancy.

AUTHOR CONTRIBUTIONS

MB and AB wrote the manuscript and designed the Figure and Table.

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A New Hope for CD56^{neg}CD16^{pos} NK Cells as Unconventional Cytotoxic Mediators: An Adaptation to Chronic Diseases

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Natural Killer (NK) cells play an essential role in antiviral and anti-tumoral immune responses. In peripheral blood, NK cells are commonly classified into two major subsets: CD56^{bright}CD16^{neg} and CD56^{dim}CD16^{pos} despite the characterization of a CD56^{neg}CD16^{pos} subset 25 years ago. Since then, several studies have described the prevalence of an CD56^{neg}CD16^{pos} NK cell subset in viral non-controllers as the basis for their NK cell dysfunction. However, the mechanistic basis for their cytotoxic impairment is unclear. Recently, using a strict flow cytometry gating strategy to exclude monocytes, we reported an accumulation of CD56^{neg}CD16^{pos} NK cells in *Plasmodium falciparum* malaria-exposed children and pediatric cancer patients diagnosed with endemic Burkitt lymphoma (eBL). Here, we use live-sorted cells, histological staining, bulk RNA-sequencing and flow cytometry to confirm that this CD56^{neg}CD16^{pos} NK cell subset has the same morphological features as the other NK cell subsets and a similar transcriptional profile compared to CD56^{dim}CD16^{pos} NK cells with only 120 genes differentially expressed (fold change of 1.5, $p < 0.01$ and FDR<0.05) out of 9235 transcripts. CD56^{neg}CD16^{pos} NK cells have a distinct profile with significantly higher expression of *MPEG1* (perforin 2), *FCGR3B* (CD16b), *FCGR2A,* and *FCGR2B* (CD32A and B) as well as *CD6*, *CD84*, *HLA-DR*, *LILRB1/2*, and *PDCD1* (PD-1), whereas Interleukin 18 (IL18) receptor genes (*IL18RAP* and *IL18R1*), cytotoxic genes such as *KLRF1* (NKp80) and *NCR1* (NKp46), and inhibitory *HAVCR2* (TIM-3) are significantly down-regulated compared to CD56^{dim}CD16^{pos} NK cells. Together, these data confirm that CD56^{neg}CD16^{pos} cells are legitimate NK cells, yet their transcriptional and protein expression profiles suggest their cytotoxic potential is mediated by pathways reliant on antibodies such as antibody-dependent cell cytotoxicity (ADCC), antibody-dependent respiratory burst (ADRB), and enhanced by complement receptor 3 (CR3) and FAS/FASL interaction. Our findings support the premise that chronic diseases induce NK cell

modifications that circumvent proinflammatory mediators involved in direct cytotoxicity. Therefore, individuals with such altered NK cell profiles may respond differently to NK-mediated immunotherapies, infections or vaccines depending on which cytotoxic mechanisms are being engaged.

Keywords: natural killer cells, CD56^{neg}CD16^{pos} subset, endemic Burkitt lymphoma, malaria, epstein-barr virus, transcription profile

INTRODUCTION

Natural Killer (NK) cells are crucial mediators of innate immune responses against virally infected and malignant cells (Herberman et al., 1975; Kiessling et al., 1975; Trinchieri and Santoli, 1978). NK cell function depends on a balance between activation and inhibition signals triggered by multiple surface receptors engaged during their surveillance of host cells (Long et al., 2013). NK cells were originally defined as CD3^{neg}CD56^{pos} cells and represent 10 to 15% of lymphocytes in peripheral blood (Robertson and Ritz, 1990). CD56 is a Neural Cell Adhesion Molecule 1 (NCAM-1) involved in cell-to-cell and cell-to-matrix interactions (Lanier et al., 1991) and its expression varies with NK cell maturation. Of the peripheral NK cells, ∼10% are CD56^{bright} NK cells which are essential for pro-inflammatory cytokine production (Cooper et al., 2001) particularly when they also express CD62L, an adhesion/homing molecule (Cichocki et al., 2016) and are less cytotoxic (Jacobs et al., 2001); whereas CD56^{dim} NK cells comprise ∼90% of NK cells in healthy adults and have low cytokine production but high cytotoxic capacity (Cooper et al., 2001; Jacobs et al., 2001). CD56^{dim} NK cells are polyfunctional and play either an immunoregulatory role as canonical CD56^{dim} NK cells characterized as CD62L^{neg}CD57^{pos} Eomesodermin^{pos} (Eomes) Promyelocytic Leukemia Zinc Finger^{pos} (PLZF) (Cichocki et al., 2016) or are considered adaptive NK cells which do not express PLZF or FcRγ (also referred to as an Immunoreceptor Tyrosine-based Activation Motif (ITAM)-bearing transmembrane adapter protein). Adaptive NK cells are involved in immunosurveillance with induction of cytotoxic granules (perforin and granzymes) upon engagement with CD16, NKG2C or activating Killer Immunoglobulin-like Receptor (KIR) (Hwang et al., 2012; Schlums et al., 2015; Tesi et al., 2016). Recently, adaptive CD56^{dim} NK cells were associated with protection from *Plasmodium falciparum* (*Pf*) malaria (Hart et al., 2019). *Pf*-exposed individual had a higher frequency of FcRγ^{neg} adaptive CD56^{dim} NK cells and displayed increased antibody-dependent cellular cytotoxicity (ADCC) against *Pf*-infected red blood cells (Hart et al., 2019).

CD56^{neg}CD16^{pos} NK cells were discovered 25 years ago in Human Immunodeficiency Virus (HIV) patients (Hu et al., 1995) and has been shown to expand during other chronic infections, such as Hepatitis C Virus (HCV), especially those who failed treatment (Mavilio et al., 2003, 2005; Gonzalez et al., 2009; Prada et al., 2013) and more recently in human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) co-infected elderly individuals (>60 years of age) (Müller-Durovic et al., 2019). Compared to CD56^{pos} NK cells, CD56^{neg} NK cells have been portrayed as "dysfunctional" because of lower expression of cytotoxic receptors such as NKp46 and NKp30, low cytokine production as well as reduction of natural cytotoxicity (Mavilio et al., 2005; Müller-Durovic et al., 2019). More recently, we reported a dramatic expansion of CD56^{neg}CD16^{pos} NK cells in African children chronically/repeatedly infected with *Plasmodium falciparum* malaria and in those who were diagnosed with endemic Burkitt lymphoma (eBL) (Forconi et al., 2018). Proteomic analyses showed similarities between CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells (Voigt et al., 2018) thus supporting the classification of this subset as NK cells. Since CD56^{neg}CD16^{pos} NK cells are extremely low in American/European healthy adults (**Supplemental Figure 1**), most studies have focused on characterizing the function and therapeutic potential of CD56^{bright} and CD56^{dim} NK cell subsets. However, it appears that healthy adults from western Kenya also have a significant proportion of CD56^{neg}CD16^{pos} NK cells, similar to children chronically/repeatedly infected with *Pf*-malaria (**Supplemental Figure 1**). Besides the emerging evidence associating this NK cell subset with chronic infections, the development and function of CD56^{neg}CD16^{pos} NK cells have only begun to be explored.

Endemic BL is an Epstein-Barr virus (EBV) associated, aggressive pediatric cancer that occurs in regions of equatorial Africa with high *P. falciparum* transmission, i.e., holoendemic malaria (Burkitt, 1962). EBV is a herpesvirus which has evolved to evade immune clearance in order to establish a life-long, asymptomatic infection within immunocompetent individuals (Schmiedel and Mandelboim, 2017). Children residing in malaria holoendemic areas, where eBL incidence is high, are usually infected by EBV before 2 years of age (Piriou et al., 2012). At the same time these children are repeatedly infected with *P. falciparum* which in turn induces episodes of viral reactivation resulting in higher EBV loads (Moormann et al., 2005; Piriou et al., 2012; Reynaldi et al., 2015). *P. falciparum* malaria is postulated to diminish EBV-specific immune surveillance as a component of eBL etiology, a cancer common in children aged 5–9 years (Moss et al., 1983; Whittle et al., 1984; Moormann et al., 2007, 2009; Njie et al., 2009; Snider et al., 2012; Chattopadhyay et al., 2013; Parsons et al., 2016). NK cells have been independently shown to help control both of these infections, killing EBV-infected B cells during adolescent acute infectious mononucleosis (AIM) (Azzi et al., 2014) and malaria-infected red blood cells (Horowitz et al., 2010; Wolf et al., 2017). However, little is known about NK cell function during EBV

and malaria co-infections and their role in protection against eBL pathogenesis.

In order to further clarify similarities and differences between CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells we performed histology staining, bulk RNA sequencing and protein expression profile validation by flow cytometry using fluorescence-activated cell sorting (FACS) of NK subsets of peripheral blood mononuclear cells (PBMCs) isolated from children who had life-long exposure to *P. falciparum* infections and were diagnosed with eBL.

METHODS

Study Population and Ethical Approvals

Ethical approval was obtained from the Scientific and Ethics Review Unit (SERU) at the Kenya Medical Research Institute (KEMRI) and the Institutional Review Board at the University of Massachusetts Medical School, Worcester, USA. Written informed consent was obtained from adults and from parents of minor study participants. Healthy children and adults were recruited at a rural health center in Kenya. Inclusion criteria for children were EBV sero-positivity, HIV-negative and born to HIV-negative mothers. Inclusion criteria for Kenyan and American adults was HIV-negative status. Children with suspected eBL were enrolled at Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) in Kisumu, Kenya. Two independent pathologists confirmed diagnosis by cyto-pathology and May-Grunwald Giemsa staining. Tumor samples were further characterized by transcriptome and mutational profiling to confirm eBL diagnosis (Kaymaz et al., 2017). This cancer is more prevalent in male compared to female children, with a peak-age incidence ranging from 5 to 9 years old (Buckle et al., 2016), and at the time of this study, we only had sufficient samples from male eBL children. Therefore, baseline peripheral blood samples were used from 8 male eBL children before induction of chemotherapy. However, we have previously shown that both male and female eBL patients have significantly elevated frequencies of CD56^{neg}CD16^{pos} NK cells (Forconi et al., 2018).

ddPCR to Quantify EBV Load

For each patient, 500 μl of blood was collected in an EDTA microtainer tube. After 5 min of centrifugation, 200 μl of plasma was separated from the blood cell pellet and replaced by an equivalent amount of 1X PBS, pH 7.4. Using the whole blood DNA extraction kit from Qiagen, DNA was isolated from the PBS resuspended blood cell pellet and total DNA concentration was measured by NanoDrop (Thermo Fisher Scientific). We used digital droplet PCR (ddPCR) to determine EBV load in each sample by amplifying EBV BALF5 and human β-actin gene, using primers and probes shown in **Table 1**. The duplex ddPCR reactions were prepared in a total volume of 20 μl which contained 10 μl of ddPCR Supermix for probes (No UTP) (Bio-Rad Laboratories), and 2 sets of each primer and probe combination (0.9 μM of primers and 0.25 μM of probes). The BioRad Automated Droplet Generator (AutoDG) (Bio-Rad Laboratories) was used to ensure consistent droplet generation. After the ddPCR reaction, the number of positive and negative droplets were counted by the Bio-Rad QX200TM Droplet reader and EBV viral loads quantified as copies/ng human DNA.

TABLE 1 | Droplet Digital PCR (ddPCR) EBV and Human Primer and Probe Sequences.

	Sequence 5′-3′
EBV-BALF5 FP	GAAGCCCTCTGGACTTCCATG
EBV-BALF5 RP	CCCTGTTTATCCGATGGAATG
EBV-BALF5 Probe	FAM -TGTACACGCACGAGAAATGCGCCT-BHQ-1
Human β-actin FP	GCTCATGGCAAGAAAGTGCTC
Human β-actin RP	GCAAAGGTGCCCTTGAGGT
Human β-actin Probe	HEX-AGTGATGGCCTGGCTCACCTGGAC-BHQ

FP, Forward primer; RP, Reverse primer.

Multiplex Suspension Bead-Based Serology Assay

To measure IgG antibody levels in the plasma fraction, we used a Luminex bead-based suspension assay as previously published (Cham et al., 2009; Forconi et al., 2018). In brief, antibodies to Viral Capsid Antigen (VCA) and Epstein-Barr nuclear antigen 1 (EBNA1) (gift from Jaap Middeldorp, Cyto-Barr) were used to determine EBV seropositivity (Middeldorp and Herbrink, 1988; van Grunsven et al., 1994). Previous *P. falciparum* exposure was determined using recombinant proteins to blood stage malaria antigens: apical membrane antigen 1 (AMA1) and merozoite surface protein (MSP1) (gifts from Sheetji Dutta, Evelina Angov, and Elke Bergmann from the Walter Reed Army Institute of Research). Briefly, 200 μg of each antigen or bovine serum albumin (BSA) (Sigma-Aldrich) were coupled to carboxylated beads microspheres (BioRad) and then incubated with plasma samples from our study participants, followed by incubation with biotinylated anti-human IgG diluted 1:1000 and streptavidin (BD Pharmingen) diluted 1:1000 following manufacturer's instructions. Antigen-specific fluorescence values were quantified on a BioPlex 200 multi-analyte analyzer with subtraction of fluorescence values obtained with BSA-conjugated beads for each patient. Results are reported as median fluorescence intensity (MFI) after acquisition of a minimum of 50 beads per antigen.

Fluorescence-Activated Cell Sorting (FACS) of NK Subsets

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation and cryopreserved until use (viability > 97%). PBMCs were thawed at 37°C in complete media composed of RPMI, 10% heat-inactivated Fetal Bovine Serum (MilliporeSigma), 2 mM L-glutamine, 1X Penicillin/Streptomycin and 10 mM HEPES (Invitrogen). NK cell subsets were then isolated using a 16-color BD FACS 2-ARIA II cell sorter at the UMASS Flow Cytometry Core. The gating strategy (**Figure 1**) used the following antibody/fluorochrome combinations identified by the Resource Identification Portal number RRID to isolate NK subsets: CD16-BV510 (RRID: AB_2562085) and CD56-PECy7 (RRID: AB_399970) with the

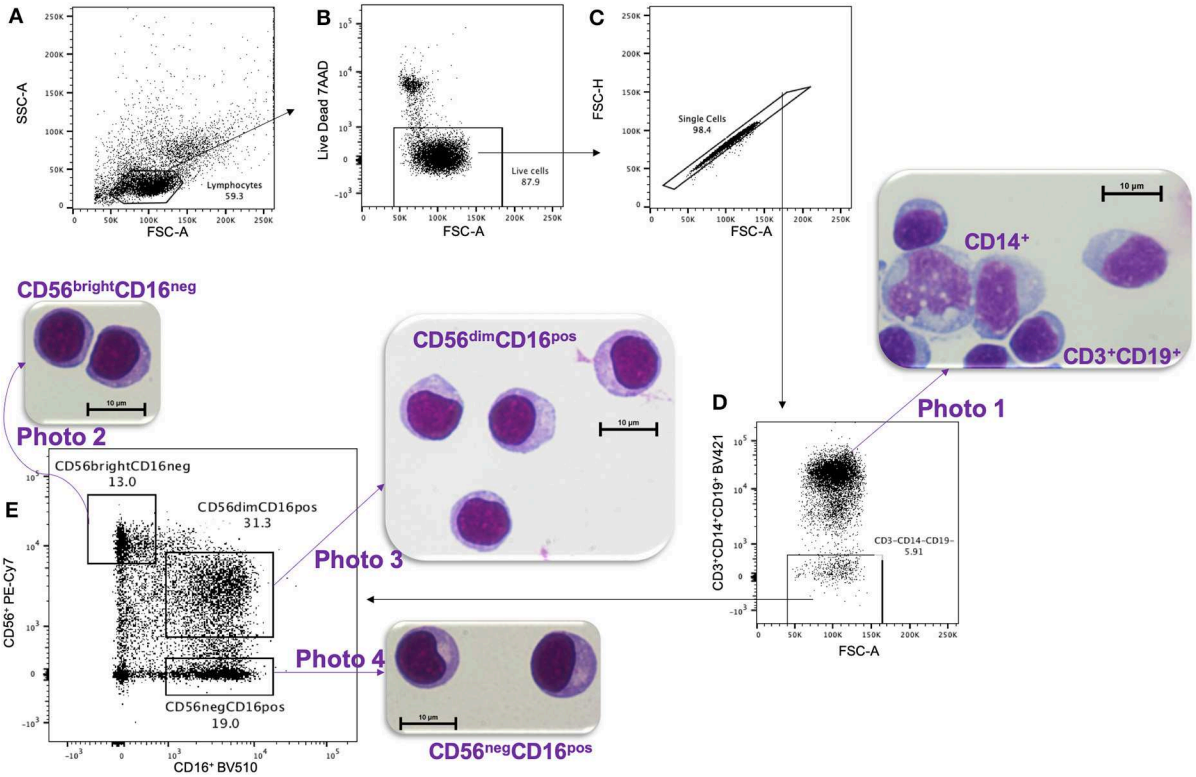

FIGURE 1 | Gating strategy for FACS and HandE staining. PMBCs were gated on **(A)** lymphocytes size determined by SSC-A vs. FSC-A cytoplots, then **(B)** live, and **(C)** single cells were selected. **(D)** A Dump channel was used to eliminate CD3$^+$, CD14$^+$, and CD19$^+$ cells. These cells were stained purple using hematoxylin and eosin (HandE), as shown in **photo 1**. Then **(D)** CD3$^-$CD14$^-$CD19$^-$ cells were **(E)** sorted based on CD56 vs. CD16 expression and the three isolated NK cell subsets were stained with HandE: **photo 2** CD56^{bright}CD16^{neg}, **photo 3** CD56^{dim}CD16^{pos}, and **photo 4** CD56^{neg}CD16^{pos}.

exclusion of dead cells by 7'AAD (BD Pharmingen, Cat#559925) and dump channel for cells expressing either CD3-BV421 (RRID: AB_10962690), CD14-BV421 (RRID: AB_2563296), or CD19-BV421 (RRID: AB_11142678). In order to compare cell morphology between CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cell subsets, we also collected the CD56^{bright}CD16^{neg} NK cells and CD3$^+$CD14$^+$CD19$^+$ cells.

Morphology by Light Microscopy

To examine morphology, live-sorted cells from each subset were cytospun on a slide and stained with hematoxylin and eosin (H&E) following Hema 3^{TM} Stat Pack kit instructions (Fisherbrand Cat#122-911). Hematoxylin stains the nucleus deep blue-violet whereas eosin stains the elastin/collagen/reticular fibers of the cell pink. Slides were imaged on Nikon microscope Eclipse E400 with ocular lens 10x and 100x objectives using the DS-Qi1MC and Digital Sight DS-U3 (Nikon) camera system and NIS-Element BR version 4.20 software. Images were analyzed by a clinical pathologist and transfusion specialist at UMass Medical School to determine cell types.

RNA Sequencing

Live-FACS sorted NK cell subsets (CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos}) were immediately collected in 4°C 2× Buffer TCL with (2%) β-mercaptoethanol (Qiagen). Total RNA was isolated and strand-specific ribosomal RNA-depleted sequencing libraries were generated using the standard protocol of SMARTer Stranded Total RNA-Seqv2 Pico input kit (Takara Bio). Given the small quantity of total RNA that was obtained from the ∼5,000 NK cells sorted for each subset, the depletion of abundant rRNA was performed after cDNA synthesis using probes specific to mammalian rRNA. Sequencing libraries were purified using XP Ampure magnetic beads (Beckman Coulter Inc.) after each reaction step. Final libraries were amplified using SeqAmp DNA polymerase, and qualities and concentrations were measured with a Bioanalyzer Agilent High sensitivity DNA kit. Samples were sequenced on an Illumina HiSeq 4000 (Illumina, Inc.), obtaining depths of 10–20 million paired-end 50 bp reads for each NK cell subset sequenced. Sequencing files were deposited in the NCBI's database of Genotypes and Phenotypes (dbGaP) with accession number phs001282.v2.p1.

Differential Gene Expression Analysis

Differential gene expression was performed using standard methods. Sequence reads were first checked for quality using FastQC (Andrews, 2014) and sorted by sample based on the unique sample indexes identified by Novobarcode (Novocraft Technologies). Residual Illumina 3'-end adaptor sequences and

template switching oligos introduced during the cDNA synthesis were trimmed using Cutadapt (Martin, 2011). Paired reads were then aligned to a transcriptome index built by RNA-Seq by expectation-maximization (RSEM) (Li and Dewey, 2011) using Gencode annotation version 19 for protein coding transcripts and hg19 genomic sequence. RSEM calculated strand-specific expected read counts for each gene and gene expression count matrices for each NK cell subset were generated for downstream statistical analyses that were performed with R software (https://www.R-project.org/) (version 3.5.3).

Differential gene expression analysis between NK cell subsets (CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos}) was performed using R package edgeR (Robinson et al., 2010) which implements a Trimmed mean of M-values (TMM) normalization and a negative binomial approach (Robinson and Oshlack, 2010). We removed from the analysis all genes for which all counts per million (CPM) values were lower than 5 cpm. To control multiple testing, we applied the Benjamini-Hochberg procedure (BH) with threshold for statistical significance set at an adjusted p-value < 0.01 and false discovery rate (FDR) < 0.05.

Validation by Flow Cytometry

PBMCs from 3 eBL children (2 of them were also used for RNA-sequencing) and 3 healthy Kenyan children were thawed and FACS live-sorted, as described above. In addition, we included the following antibodies: CD62L-PerCP5.5 (RRID: AB_2239105), DNAM1-BV711 (RRID: AB_2738956), TIM3-BV650 (RRID: AB_2565829), PD-1-APC-Fire750 (RRID: AB_2616721) and granzyme B-APC (RRID: AB_1500190); CD32-PerCP5.5 (RRID: AB_2616924) and IL18Ra-APC (RRID: AB_2800828). Data was acquired on a 19-color BD LSRII at the UMASS FACS Core and analyzed using FlowJo v10.6.0, R v3.5.1 and Prism v8.3.0. Non-parametric two-tailed paired Wilcoxon t-test was used and a p-value <0.05 was considered significant.

gProfiler

The genes identified to be significantly differentially expressed (p-value < 0.01 and FDR < 0.05) between the NK cell subsets were further analyzed using the gProfiler software (https://biit.cs.ut.ee/gprofiler) (Raudvere et al., 2019) to explore the potential functional consequences and associated pathways. gProfiler was run using g:GOSt ordered query with Bonferroni correction and a threshold of 0.01 equating to a p-unadjusted $< 10.E\text{-}16$.

RESULTS

Characterization of the Samples

Initial samples at diagnosis prior to chemotherapy from 8 eBL children were used for this study with a median age of 9.5 years (**Table 2**). Serology for the two pathogens associated with eBL was assessed and all children were seropositive for both EBV and *P. falciparum*. Survival outcomes varied with five eBL children (62%) being long-term (>2 year) survivors and three (38%) in-hospital deaths. Half of the children had low EBV loads (median 0.5 EBV copies/ng of human DNA) and half had high EBV loads (median 23.3 copies/ng of human DNA). No child in our study had lymphopenia with a median absolute lymphocyte count (ALC) of 3.5×10^5 lymphocytes per μl blood (Shapiro et al., 1998). Finally, the percentage of each NK subset was assessed within the total NK cell population. Consistent with our previous study (Forconi et al., 2018), eBL children had elevated CD56^{neg} CD16^{pos} NK cell subset, with a median of 30.3%.

TABLE 2 | Patients characteristics.

	eBL ($n = 8$)
Age†:	9.5 [6.5–11.75]
Sex: (Male)	8
Hemoglobin (g/dl)†	10.80 [9.75–11.55]
ALC: (10^3 lymphocytes per μl)†	346.8 [214.1–418.4]
Serology in Median Fluorescence Intensity (MFI): EBNA1†	8,848 [6,595–24,668]
VCA†	28,114 [15,487–31,459]
MSP-1 (3D7)†	27,785 [22,058–29,767]
AMA-1†	26302 [21,173–31,008]
Survivors:	5 (62%)
Non-survivors:	3 (38%)
Low EBV viral load: (eBL $n = 4$)† EBV copies/ng human DNA	0.5 [0.11–0.7]
High EBV viral load: (eBL $n = 4$)† EBV copies/ ng human DNA	23.35 [7.47–245.1]
Tumor localization	
Jaw:	3 (38%)
Abdomen:	5 (62%)
% CD56^{dim} within NK cells†:	56.6 [34.55–67.1]
% CD56^{neg} within NK cells†:	30.30 [24.23–57.1]

†*Median [25–75% interquartile].*

Morphology of CD56^{neg}CD16^{pos} Cells Similar to Other NK Subsets

After live-sorting, an aliquot of each cell subset was fixed and stained by H&E in order to assess their morphology (**Figure 1**). All three NK cell subsets CD56^{bright}CD16^{neg} (**Figure 1**, photo 2), CD56^{dim}CD16^{pos} (**Figure 1**, photo 3) and CD56^{neg}CD16^{pos} (**Figure 1**, photo 4) had similar morphology by microscopy, round in shape, ∼10 μm in size and with a prominent nucleus typical of lymphocyte histology. Importantly, these cells differed morphologically compared to CD14^{+} monocytes (**Figure 1**, photo 1), which were larger (averaging 15–18 μm in diameter), ameboid in appearance, had a lighter cytoplasm, and unilobar nucleus. This morphological comparison confirms that CD56^{neg}CD16^{pos} NK cells are visually indistinguishable from other NK cell subsets and are clearly not monocytes.

Validating the Purity of Sorted NK Cell Subsets

To check the purity of the sorted CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cell subsets, we explored the normalized expression values [transcripts per million (TPM)] of each gene, to assess if any of the classical monocyte or neutrophil gene signatures were detected on the bulk RNA-seq data of the sorted cells. From this interrogation, we identified one of the CD56^{neg}CD16^{pos} NK samples as having higher expression of classical monocyte genes (CD14 and CD33) (**Supplemental Figure 2**), suggestive of trace monocyte contamination. Thus, this sample was excluded from further analysis leaving us with 7 CD56^{neg}CD16^{pos} and 7 CD56^{dim}CD16^{pos} NK subsets for downstream differential expression analysis. All remaining samples lacked expression of CD14, CD33, and CD34 (**Supplemental Figure 3A**). In addition, we found that neither NK cell subset expressed neutrophil-associated genes, such as CD66b *(CEACAM8), ARG1, MPO, ABCA13, CA1, IFIT1B, CRISP3, LCN2, BPI, CNTNAP3B,* and *PAD4* (**Supplemental Figure 3B**). However, other neutrophil-associated genes, such as the integrin family genes *ITGAL* (CD11a), *ITGAM* (CD11b), *ITGAD* (CD11d), and *ITGB2* (CD18) which are involved in innate immunity (Roberts et al., 2016) and can be expressed by NK cells were observed in both the CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cell subsets (**Supplemental Figure 3B**). In addition, we show that *PTPRC* (CD45) was highly expressed in both the CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cell subsets (logCPM = 11.37, **Figure 2**). CD45 is known to be expressed on hematopoietic cells and more highly expressed on lymphoid cells compared to myeloid cells based on BioGPS and MyGene.info organizing online gene-centric information (Wu et al., 2013). This criteria further supports the premise that CD56^{neg}CD16^{pos} cells are lymphocytes.

CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK Cells Transcriptome Expression Profiles

Using purified CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cell subsets we compared their gene expression profiles and identified 536 genes that were differentially expressed (p-value<0.01 and FDR 0.05) among a combined total of 9,235 genes (**Supplemental Figure 4**, **Supplemental Table 1**). Of the differentially expressed genes, 120 had a log Fold Change (logFC) >1.5, with 73 genes downregulated and 47 genes upregulated in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells. Each sample showed appropriate expression based on their flow sorting: CD56^{dim}CD16^{pos} vs. CD56^{neg}CD16^{pos} suggesting minimal cross-contamination. To further characterize the CD56^{neg}CD16^{pos} cell subset, we examined the expression of genes that define and distinguish NK cells from other cell types (**Figures 2A,B**, and **Supplemental Table 1**). Innate Lymphoid Cells (ILCs) have been categorized into 5 Groups: NK cells that differ based on developmental trajectories from ILC of Group 1 (ILC1) yet both display type 1 immunity, Group 2 (ILC2) able to produce type 2 cytokines, Group 3 (ILC3) defined by their capacity to produce IL-17A and IL-22 and lymphoid tissue-inducer cells (LTi) involved in the creation of secondary lymphoid organ (Vivier et al., 2018). ILC2s are also defined by their expression of *GATA3*, *BCL11B,* and GFI1 which we found to be expressed at similar levels within CD56^{neg}CD16^{pos} relative to the CD56^{dim}CD16^{pos} NK cells (**Figure 2C**). However, amphiregulin (*AREG*) which is expressed by ILC2 group, appeared slightly downregulated in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells (logFC of −1.32, p-value = 2.24E-06 and FDR of 0.0001). Regarding ILC3 genes, *AHR* was similarly expressed in both NK cell subsets but neither CD56^{neg}CD16^{pos} nor CD56^{dim}CD16^{pos} cells expressed *CSF2* and *RORC*, considered classical ILC3 genes. Together, the transcriptional profile for CD56^{neg}CD16^{pos} cells conform to neither Group 2 nor 3.

CD56^{neg}CD16^{pos} NK Cells Display a Transcriptome Signature Reminiscent of Adaptive NK Cells

Even though 98.7% of genes shared expression between the two NK cell subsets, the differentially expressed genes shed light on functional differences between these two cell populations. Applying our NK-centric transcriptomic analysis, we show that both CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells express activation and maturation markers such as *KLRD1* (CD94), CD96, and *B3GAT1* (CD57) (**Figure 2B**). However, CD56^{neg}CD16^{pos} NK cells had lower expression of *CLEC2C* (CD69, logFC of −1.45, p-value = 3.96E-10 and FDR of 7.06E-08) and *CD38* (log CPM of 7.47 with logFC of −0.53, p-value = 0.0005 and FDR of 0.014) relative to CD56^{dim}CD16^{pos} NK cell. In contrast, CD56^{neg}CD16^{pos} NK cells tend to express more *HLA-DRA* (logCPM of 5.78 with logFC of 0.91, p-value = 0.001 and FDR of 0.03) and *CD6* (logCPM of 6.32 with logFC of 0.96, p = 4.84E-06 and FDR of 0.0002) relative to CD56^{dim}CD16^{pos} NK cell. As expected, *NCAM-1* (CD56) was strongly downregulated in CD56^{neg}CD16^{pos} cells compared to CD56^{dim}CD16^{pos} NK cells (logFC of −5.84, p-value = 2.17E-198 and FDR of 2.03E-194). Regarding their ability to proliferate, *MKI67* (Ki67) was not differentially expressed (logFC of 0.42, p-value = 0.18) between the two subsets. Although, we observed higher expression of *SPI1* (PU.1, logFC of 2.65, p-value = 0.0002 and FDR of 0.006) for the CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells. *SPI1* has been suggested to play a role in NK cell proliferation (Sun, 2016).

Besides potential cytotoxic and pro-inflammatory functions, NK cells have been implicated in regulating immunity by killing activated T cells or antigen-presenting cells (Ferlazzo et al., 2002; Waggoner et al., 2011; Crouse et al., 2014). Therefore, we queried the CD56^{neg}CD16^{pos} NK cell transcriptome for immunoregulatory cytotoxic activity. Interestingly, *SELL* (CD62L, logFC of −3.08, p-value = 9.28E-17 and FDR of 1.09E-13) and *ZBTB16* (PLZF, logFC of −2.52, p-value = 2.65E-14 and FDR of 1.24E-11) gene expression was significantly lower in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells, and both *ITGAE* (CD103) and *ITGA*1 (CD49a) were not expressed which suggests that CD56^{neg}CD16^{pos} cells may be a form of adaptive NK cell. This is consistent with

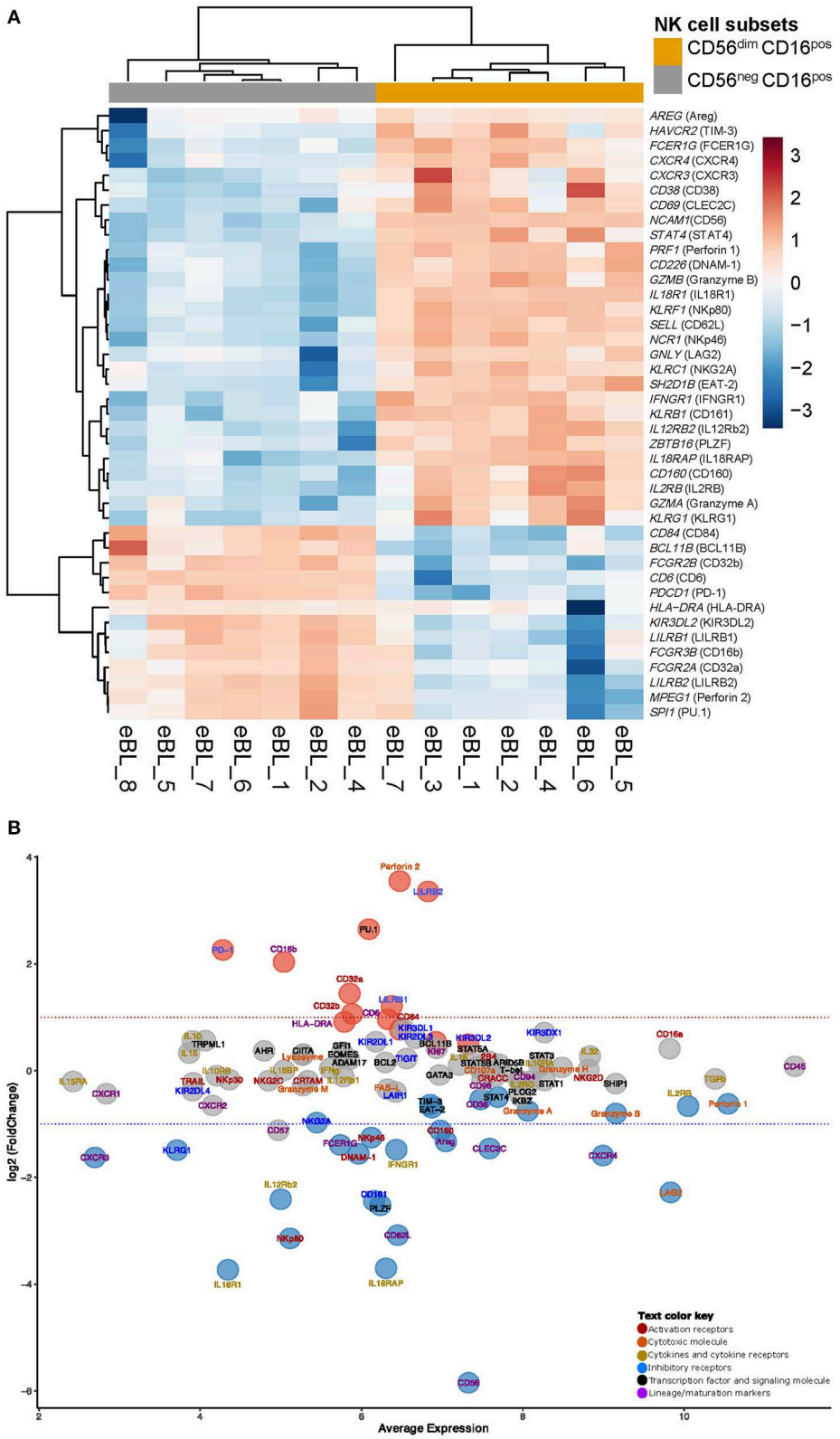

FIGURE 2 | Continued

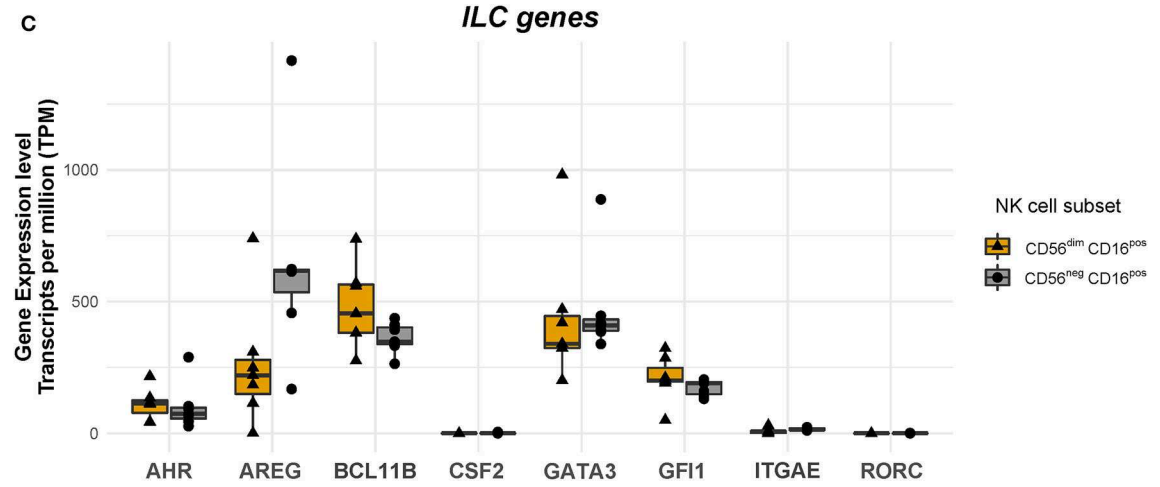

FIGURE 2 | Heatmap and MA plot of selected differentially expressed genes associated with NK cell function. **(A)** Heatmap of selected NK cell genes comparing CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells. The id between the brackets are the protein name for that particulate gene, i.e., gene symbol (protein name), NCAM1 (CD56). **(B)** MA plot of differential expression analysis between CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cell subsets. The MA plot illustrates a log2 fold change of NK specific gene expression in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} cells and the average normalized expression counts of genes expressed by CD56^{neg}CD16^{pos} cells. Protein names are used in the MA plot for ease of comparison to flow data even though these results are for gene expression. The Red dots indicate genes that have significantly higher expression in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells (with BH-adjusted p-value < 0.01 and FDR < 0.05), whereas blue dots indicate genes that have significantly lower expression (with BH-adjusted p-value < 0.01 and FDR < 0.05), and gray dots are genes that are similarly expressed. **(C)** Boxplot of gene expression profiles that define Innate Lymphoid Cells (ILCs): *AHR, AREG, BCL11B, CSF2, GATA3, GFI1, IRGAE, RORC* comparing CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells.

Schlums et. al who characterized adaptive CD56^{dim} NK cells as CD62L^{-}CD103^{-}CD49a^{-}PLZF1^{-} (Schlums et al., 2015). Moreover, *FCER1G* (FCεRγ) was strongly downregulated (logFC of −1.39, p-value = 8.43E-09 and FDR of 1.05E-06, respectively) and to a lesser extent lower levels of *SH2D1B* (EAT-2) (logFC of −0.69, p-value = 4.55E-06, FDR of 0.0002) were observed for CD56^{neg}CD16^{pos} compared to CD56^{dim}CD16^{pos} NK cells. These genes have been correlated with a loss of immunoregulatory cytotoxic activity (Schlums et al., 2015) further supporting the categorization of CD56^{neg}CD16^{pos} NK cells as adaptive NK cells.

CD56^{neg}CD16^{pos} NK Cells Express Higher Levels of *PDCD1* and *LILR* Family Inhibitory Receptors Relative to CD56^{dim}CD16^{pos} NK Cells

To evaluate the therapeutic potential of these two NK cell subsets, we examined the expression of common NK cell inhibitory molecules (**Figure 2B**). Most of the KIR members, *TIGIT* and *LAIR1* were similarly expressed by CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} cells. But interestingly, CD56^{neg}CD16^{pos} NK cells expressed significantly lower *KLRG1* (logFC of −1.48) and *KLRB1* (CD161, logFC of −2.43) compared to the CD56^{dim}CD16^{pos} NK cells, whereas *KLRC1* (NKG2A, logFC of −0.97) and *HAVCR2* (TIM-3, −0.63) were only slightly downregulated in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells. In contrast, *PDCD1* (PD-1, logFC of 2.26) and *LILR* family members, *LILRB1* (logFC of 1.21) and *LILRB2* (logFC of 3.35) were observed to be upregulated within CD56^{neg}CD16^{pos} compared to CD56^{dim}CD16^{pos} NK cell. However, *PDCD1* was weakly expressed within CD56^{neg}CD16^{pos} cells (logCPM of 4) compared to higher expression of *LILRB1* (logCPM of 6). Together these data suggest that CD56^{neg}CD16^{pos} NK cells are not exhausted but do overexpress distinct inhibitory receptors that may pose a challenge to overcome with NK-based immune checkpoint inhibitors.

CD56^{neg}CD16^{pos} NK Cells Express Low NKp80 & NKp46 but High CD16, CD32 and Perforin Relative to CD56^{dim}CD16^{pos} NK Cells

We previously showed that CD56^{neg}CD16^{pos} NK cells were poorly cytotoxic in K562 co-culture assays (Forconi et al., 2018), however at the transcriptional level we observed similar gene expression of some activation (*KLRC2*/NKG2C, *SLAMF7*/CRACC, *TRAIL*, *CRTAM*/CRTAM), co-stimulation (*CD244*/2B4, *KLRK1*/NKG2D) and natural cytotoxic receptors (*NCR3*/NKp30) for these two subsets (**Figure 2B**). Yet, CD56^{neg}CD16^{pos} NK cells expressed less *KLRF1* that codes for the natural cytotoxic receptor NKp80 (logFC−3.14, p-value = 6.02E-16 and FDR of 4.03E-13), *NCR1* (NKp46, logFC −1.25, p-value = 1.13E-09 and FDR of 1.63E-07), *CD226* (DNAM-1, logFC −1.55, p-value = 1.66E-11 and FDR of 4.39E-09) and *CD160* (logFC −1.11, p-value = 1.72E-08 and FDR of 1.87E-06) which might explain in part the loss of cytotoxicity against K562 cells along with the absence of the CD56 adhesion molecule. In contrast, *FCGR3A* (CD16a) is highly expressed on both CD56^{neg}CD16^{pos} NK cells (logCPM of 9.81) and CD56^{dim}CD16^{pos} NK cells. CD56^{neg}CD16^{pos} NK cells expressed higher levels of *FCGR3B* (CD16b) (logFC of 2.03, p-value = 0.0005 and FDR of 0.013), *FCGR2A* (CD32A, logFC of 1.4,

p-value = 3.69E-10 and FDR of 6.78E-08), and *FCGR2B* (CD32B, logFC of 1.06, *p*-value = 7.68E-07 and FDR of 5.25E-05) relative to CD56^{dim}CD16^{pos} NK cells with a slightly elevated expression of *CD84* (logCPM of 6.47, logFC of 0.76, *p*-value = 4.07E-05 and FDR of 0.001), respectively. We were surprised to see significantly elevated expression of cytotoxic molecules such as *MPEG1* (perforin 2) for the CD56^{neg}CD16^{pos} compared to CD56^{dim}CD16^{pos} NK cells (logFC of 3.55, *p*-value = 1.44E-07 and FDR of 1.25E-05). Despite this interesting observation, other genes involved in direct cytotoxicity, such as *LYZ* (lysozyme), *LAMP-1* (CD107a), *FASLG* (FAS-L), *GZMH* and *GZMM* (granzyme H and M) were not differentially expressed between the CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells, supporting their ability to kill target cells by degranulation and through the FAS-L/FAS pathway. CD56^{neg}CD16^{pos} NK cells expressed *PRF1* (perforin 1, logCPM of 10.54, logFC of −0.62), *GZMA* (granzyme A, logCPM of 8.06, logFC of −0.75) and *GZMB* (granzyme B, logCPM of 9.15, logFC of −0.8) these markers were slightly lower for CD56^{neg}CD16^{pos} compared to CD56^{dim}CD16^{pos} NK cells. Another gene related to cytotoxicity *GNLY* (granulysin or LAG2) was significantly downregulated in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells (logCPM of 9.83 and logFC of −2.27). Overall, these data suggest that CD56^{neg}CD16^{pos} NK cells retain cytotoxic potential, albeit mediated through different mechanisms compared to CD56^{dim}CD16^{pos} NK cells.

CD56^{neg}CD16^{pos} NK Cells Express Less IL-2, IL-12, and IL-18 Receptors Relative to CD56^{dim}CD16^{pos} NK Cells

As shown in **Figure 2B**, we found no differences in *IL2RG* gene expression (subunit γ of the IL2 receptor), *IL15RA* (receptor for IL15), *IL12Rb1* (subunit β 1 of the IL12 receptor) between these two NK cell subsets, whereas the other chain of the IL12 receptor (*IL12Rb2,* logFC of −2.4, *p*-value = 7.95E-17 and FDR of 1.06E-13) was found to be significantly downregulated in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells. Significant differences were also observed for *IFNGR1* (logFC of −1.48, *p*-value = 2.41E-14 and FDR of 1.19E-11), and both *IL18R1* (IL18 receptor, logFC of −3.73, *p*-value = 4.65E-16 and FDR of 3.35E-13) and *IL18RAP* (IL18 receptor accessory protein, logFC of −3.7, *p*-value = 2E-23 and FDR of 6.25E-20). However, *IL18BP* which encodes the IL18 binding protein is similarly expressed in both CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells (logFC of 0.007). Interestingly, *CD122* (IL2RB/IL15RB) appeared to be slightly downregulated in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells (logFC of −0.66, *p*-value = 4.85E-05 and FDR of 0.001). These observations suggest that CD56^{neg}CD16^{pos} may be impervious to activation by IL2, IL12, IL15, and IL18 cytokines.

CD56^{neg}CD16^{pos} Cells Do Not Differ From CD56^{dim}CD16^{pos} NK Cells in Cytokine Gene Expression

We assessed the cytokine expression for CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells without any *in vitro* pre-stimulation (**Figure 2B**). We did not observe any differences in basal expression levels for IL10, IL15, IL16, IL32, or IFNγ between the two NK subsets. However, we observed that both NK cell subsets expressed high TGFβ levels (logCPM of 10.38) which suggests an inherent anti-inflammatory role.

Validation of Gene Expression by Flow Cytometry

Most of the genes differentially expressed when comparing CD56^{neg}CD16^{pos} to CD56^{dim}CD16^{pos} NK cells that are highlighted in the RNA-sequencing experiment have already been described at the protein expression level in previous studies (Forconi et al., 2018; Voigt et al., 2018). CD56^{dim}CD16^{pos} compared to CD56^{neg}CD16^{pos} NK cells express less NKp80, IL18Ra, CD161, NKp46, DNAM1 with no differences for CD57, Perforin 1, CD11c, NKG2D, NKG2C and most of the KIRs. We performed additional flow cytometry experiments and confirmed that CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells cluster separately (**Figure 3A**). We also confirmed higher expression of IL18Ra, CD62L, DNAM1, and TIM-3 on the CD56^{dim}CD16^{pos} compared to CD56^{neg}CD16^{pos} NK cells and higher expression of CD32 and PD-1 on the CD56^{neg}CD16^{pos} compared to CD56^{dim}CD16^{pos} NK cells (**Figure 4B**) but no difference in granzyme B expression, thereby validating our RNAseq results.

Biological Processes Enriched Using gProfiler

Because of the low number of genes differentially expressed between CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells, no gene set enrichment was suitable within the GSEA software. However, using the free online tool gProfiler (**Figure 4**), we were able to visualize which biological processes were enriched using the 536 significantly differentially expressed genes. The Manhattan plot shows the most significant biological processes involved are immune system processes, leukocyte activation, cell activation, and immune response. These results are consistent with our previous observations that CD56^{neg}CD16^{pos} NK cells are activated and should be able to drive cytotoxic responses although through alternative pathways to those commonly used by other NK cell subsets.

DISCUSSION

In this study, we have shown that CD56^{neg}CD16^{pos} NK cells share morphological and transcriptional profiles with CD56^{dim}CD16^{pos} NK cells. In fact, CD56^{neg}CD16^{pos} cells expressed multiple NK cell markers including KIRs, NKG2C, NKp30, CD16a, NKG2D, 2B4, CD57, TRAIL, CRTAM and CRACC. Moreover, our previous study highlighted the phenotypic similarities between these two NK cell subsets (Forconi et al., 2018) which was supported by the Voigt et.al proteomic study (Voigt et al., 2018). Together, these findings confirm that CD56^{neg}CD16^{pos} cells are true NK cells. However, we find interesting differences between these two NK cell subsets that might impact functional differences and potential targets that may be potentially harnessed therapeutically to drive NK cell-mediated cytotoxicity.

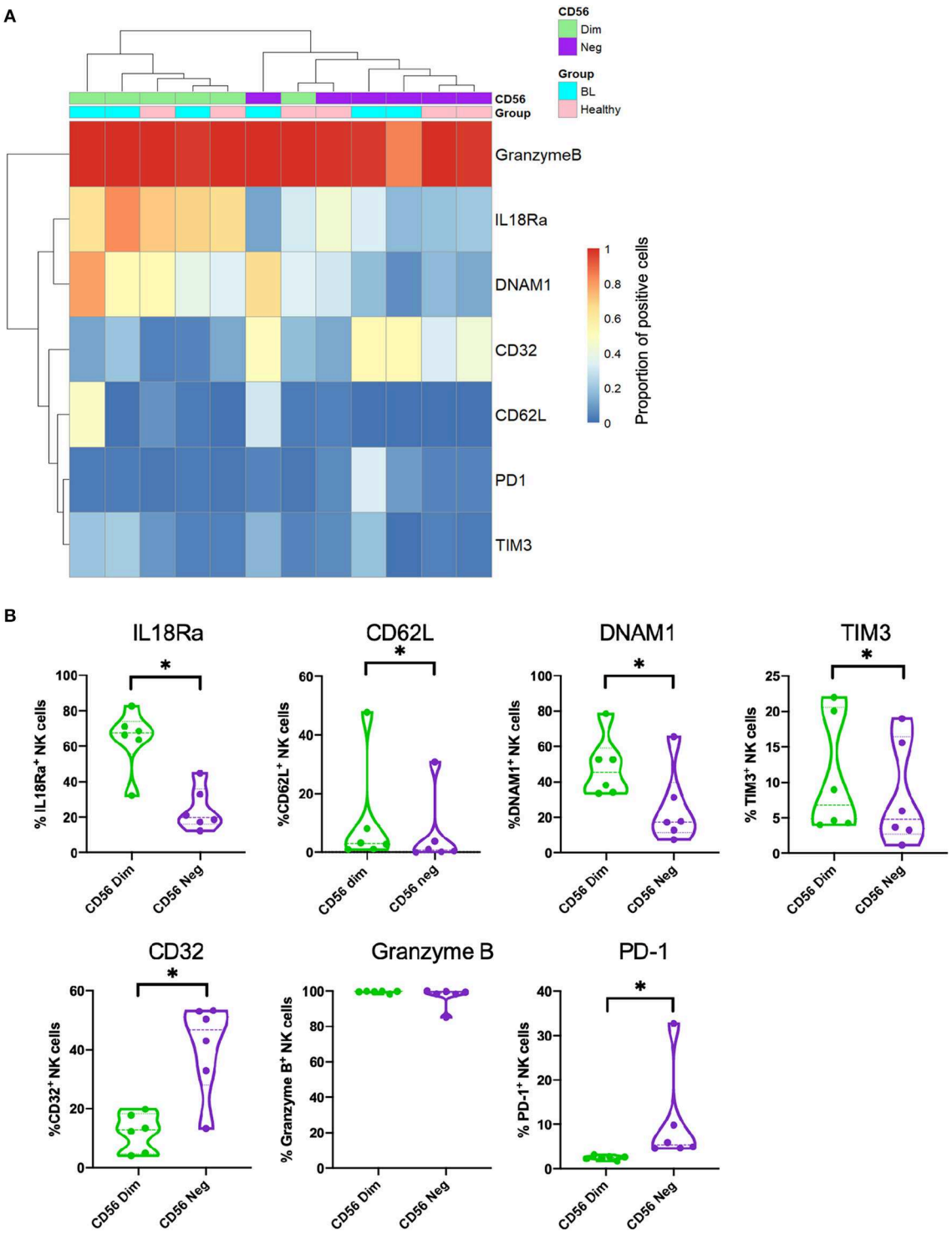

FIGURE 3 | Validation of RNA-sequencing data by flow cytometry. **(A)** Heatmap of markers expressed on both CD56^{dim}CD16^{pos} (in green) and CD56^{neg}CD16^{pos} (in purple) NK cells from eBL (in turquoise) and healthy (in pink) children. **(B)** Violin plots showing the protein expression of IL18Ra, CD62L, DNAM1, TIM3, CD32, granzyme B and PD-1 from both CD56^{dim}CD16^{pos} (in green) and CD56^{neg}CD16^{pos} (in purple) NK cells. *Represents a p-value < 0.05.

First, we showed that CD56^{neg}CD16^{pos} cells expressed a unique inhibitory marker profile with higher *LILR* family and *PDCD1* gene expression. *LILRB1* encodes for LILRB1 transmembrane receptors which contain 4 ITIMs motif in the cytoplasmic tail and is expressed by various immune cells (Zhang et al., 2015). After stimulation by its ligands, various HLA class

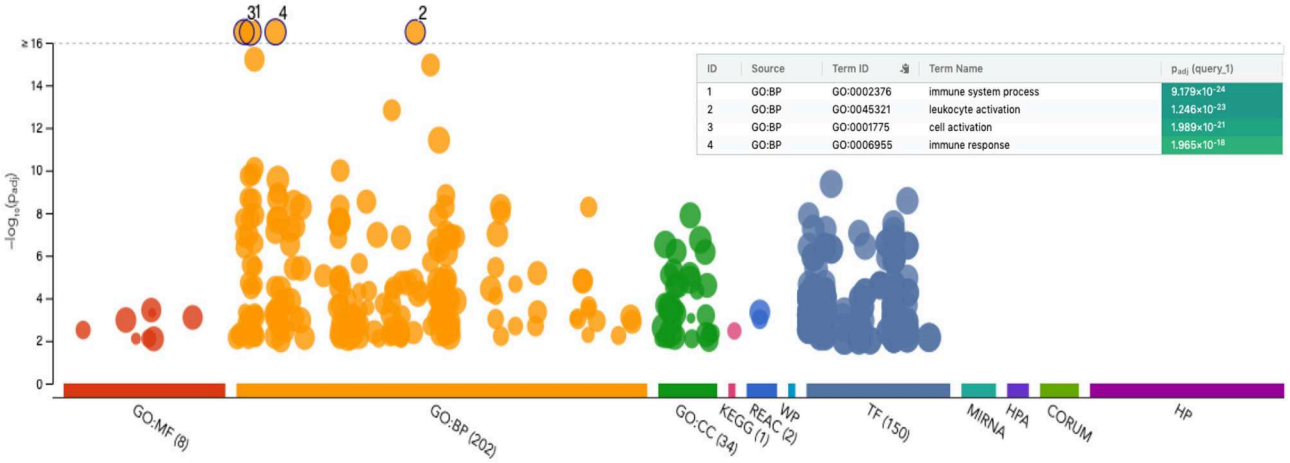

FIGURE 4 | Manhattan plot of functional profiling of the list of upregulated genes from CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} cells. Using the online tool gProfiler and the ordered g:GOSt query, we assessed which biological process (BP) will be linked to the list of 536 significantly differentially expressed genes from CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} cells. The x-axis represents functional terms that are grouped and color-coded by data sources [molecular function (MF), biological process (BP), cell component (CC)]. The y-axis shows the adjusted enrichment *p*-values in negative log10 scale. Adjusted *p*-values g:GOSt used Bonferroni correction and a threshold of 0.01. On the table, adjusted *p*-values were color coded as yellow for insignificant findings to dark blue with highest significance.

I molecules among others, trigger a strong inhibition signal in order to limit inflammatory and cytotoxic responses. Its most efficient ligand is a dimerized HLA-G which was described as up-regulated in some human tumors such as breast cancer (Lefebvre et al., 2002), certain AML (acute myeloid leukemia) (Kang et al., 2015) and cutaneous T cell lymphoma (Urosevic et al., 2004) in which both CD8^{+} T and CD56^{+} NKT cells highly expressed LILRB1 and thereby possibly contributed to tumor immune escape. More recently, LILRB1 blockade was shown to enhance cytotoxic CD8^{+} T cell activity using bispecific T cell engager (BiTE) (Kim et al., 2019) molecules, highlighting the potential of the LILRB1 receptor as an anti-cancer therapeutic target. LILRs are not known to be included in a T cell exhaustion signature (McLane et al., 2019), contrary to other markers such as PD-1, TIM-3, TIGIT, LAG3, CTLA-4, KLRG1, BTLA, CD160, and 2B4. In our study, *PDCD1*, coding for the PD-1 protein, was more highly expressed for CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells even though its expression was very low compared to most other genes of interest (**Figure 2B**). At the proteome level, PD-1 was not differentially expressed across NK cell subsets (Voigt et al., 2018). PD-1 is a popular target for immune checkpoint inhibitors, although insufficient in isolation to determine the extent it plays in T cell exhaustion (Blank et al., 2019; McLane et al., 2019). In this viewpoint article, the authors suggested the potential for adaptation of T cells into an exhaustion profile in order to limit immunopathology during chronic infections (Blank et al., 2019). Similarly, we suggest that CD56^{neg}CD16^{pos} NK cells are an adaptation of CD56^{dim}CD16^{pos} NK cells under conditions of chronic infections or persistent tumor ligand stimulation. In our study of eBL patients, we observed that KLRG1, TIM-3 and CD160 had lower expression in CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells. Moreover, CTLA-4 and BTLA were not expressed at all, which suggests that the CD56^{neg}CD16^{pos} NK subset does not appear to have a more exhausted profile than the other NK cells subsets but they clearly express multiple inhibitory markers which could limit immune responses.

As NK cells function depend on multiple signals triggered by both inhibitory and activation receptors, we assess as well which activation and cytotoxic markers were strongly differentially expressed between CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells. Despite numerous markers similarly expressed (CD6, HLA-DR, CD57, CD84, TRAIL, NKp30, NKG2C, NKG2D, 2B4...), CD56^{neg}CD16^{pos} NK cells showed a strong downregulation of *KLRF1* coding for the cytotoxic receptor NKp80 and to a lesser extent *NCR1* coding for NKp46 relative to CD56^{dim}CD16^{pos} NK cells. A recent study described NKp80 as a marker of NK cell maturity (Freud et al., 2016). In brief, they characterized NKp80^{neg} NK cells from secondary lymphoid tissues as stage 4a of NK cell development, which also included low expression of perforin, T-bet, EOMES, lack of Granzymes A, B and K but higher expression of *RORC2* and *AHR* (features shared with ILC3). Despite the expression of *AHR* in both CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cell subsets, *RORC2* was absent and we didn't observe a significant differential expression of T-bet and EOMES genes. However, Perforin 1, granzyme A and B genes appeared to be slightly downregulated within the CD56^{neg}CD16^{pos} NK cells. Regarding NKp46, we have previously shown that the expression of this cytotoxic receptor is significantly lower for children exposed to *Plasmodium falciparum* (Forconi et al., 2018). These data suggest another role for NKp80 and potentially an adaptation of NKp46 expression within eBL children within the context of malaria, EBV co-infections and the eBL pathogenesis. Despite the less expression of cytotoxic receptors essential in natural direct cytotoxicity, NK cells have other ways to kill target cells involving the presence of IgG antibodies. Interestingly, we observed upregulation of low-affinity Fcγ receptor CD16, the medium-affinity Fcγ

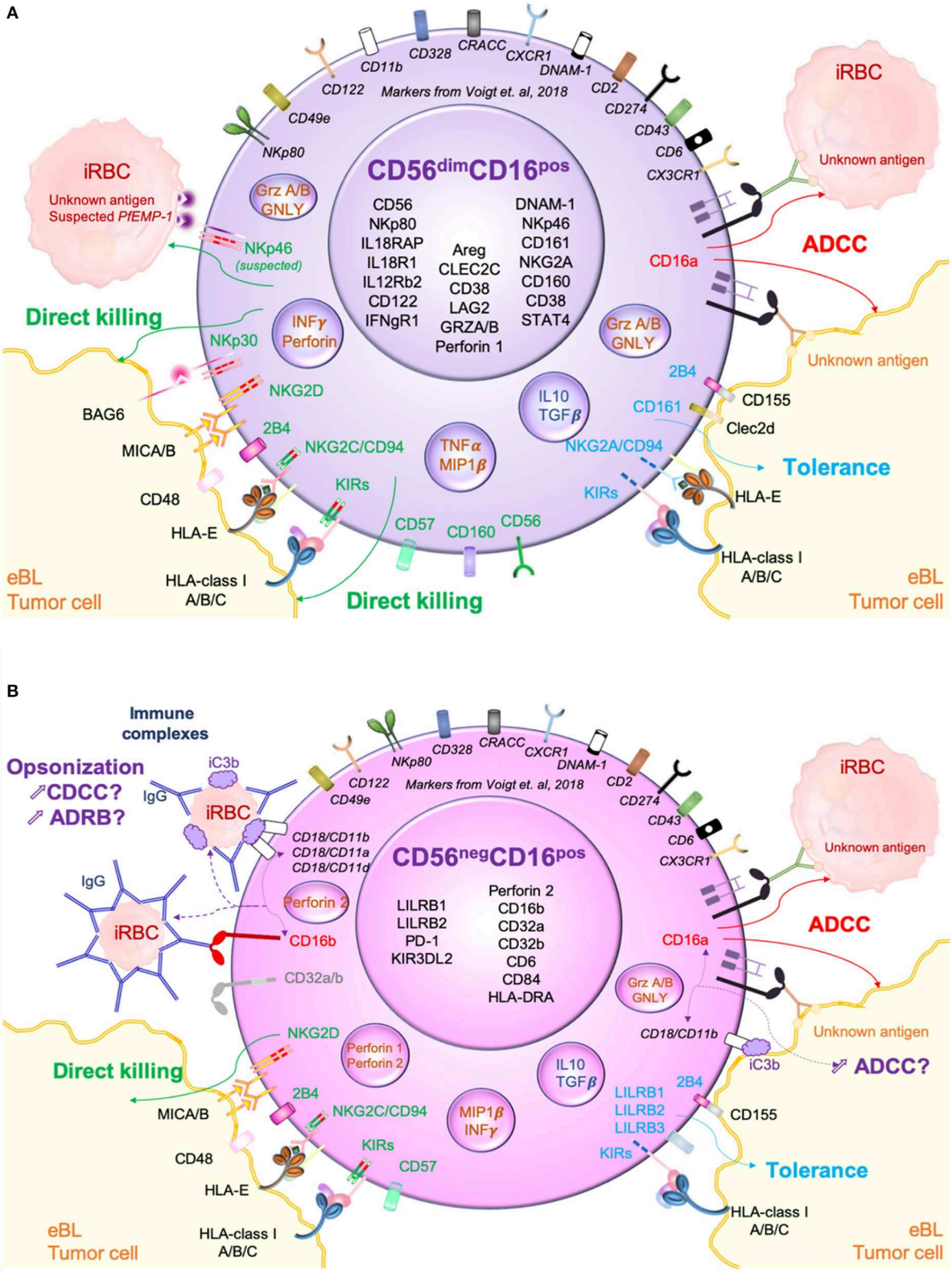

FIGURE 5 | Hypothetical model of CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells cytotoxic pathways against both *Plasmodium falciparum*-infected red blood cells (*Pf*-iRBC) and endemic Burkitt lymphoma (eBL) tumor cells. **(A)** Proposed killing pathways used by CD56^{dim}CD16^{pos} NK cells against iRBCs and eBL tumors primarily *(Continued)*

FIGURE 5 | mediated by natural direct cytotoxicity (direct killing) through the activation of cytotoxic receptors (NKp46, NKp30, NKp80, NKG2D…) but also antibody dependent cell cytotoxicity (ADCC) through CD16a activation. **(B)** Proposed killing pathways used by CD56^{neg}CD16^{pos} NK cells against iRBC and eBL tumors. Due to the low expression of cytotoxic receptors, direct killing appears to be incapacitated however other killing pathways based on recognition of opsonized targets might be enhanced.

receptor CD32 and Perforin 2 genes within the CD56^{neg}CD16^{pos} compared to the CD56^{dim}CD16^{pos} NK cell subset. There are two genes which encode the CD16 protein: *FCGR3A* and *FCGR3B* and they share more than 95% of homology so that common CD16 flow cytometry antibodies cannot distinguish them from one another (Ravetch and Perussia, 1989). However, the expression of these two genes were described as cells-specific and with different functions from CD16b which is a glycophosphatidylinositol (GPI) -anchored molecule without intracellular signaling motifs. In order to measure cells specificity of both CD16a and CD16b, Ravetch's team reconstituted these receptors in transgenic mice (Li et al., 1996). They showed that CD16a is expressed by macrophages and NK cells whereas CD16b is exclusively expressed by neutrophils. In our study we show that CD16a is highly and similarly expressed by CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells, however, CD16b is significantly upregulated within CD56^{neg}CD16^{pos} NK cell subset. CD16a or *FCGR3A* receptor is known as an important mediator of antibody dependent cell cytotoxicity (ADCC), an indirect mechanism used by NK cells and other innate immune cells to kill tumors and infected host cells (Hart et al., 2017; Arora et al., 2018; Victor et al., 2018). CD16b has been implicated as an essential mediator of antibody-dependent respiratory burst (ADRB) by neutrophils and has also been shown to be essential for immune complexes (ICs) but not necessarily involved in phagocytosis (Fossati et al., 2002). A recent study showed that CD16b can also regulate ADCC by neutrophils in competition with CD16a (Treffers et al., 2018), and in fact low copy number variation (CNV) of the gene *FCG3RB* within activated cells might increase ADCC capacity. Our transcriptome analysis was not able to assess CNV although we observed a logCPM of 5.03 for CD16b which is twice less than logCPM of 9.81 for CD16a. It will be important to consider CNV in future experiments in order to clearly determine the role of CD16b within NK cells. Both *FCGR2A* (CD32a) and *FCGR2B* (CD32b) were also more highly expressed by CD56^{neg}CD16^{pos} than CD56^{dim}CD16^{pos} NK cells. These findings suggest that CD56^{neg}CD16^{pos} NK cells may be superior in recognizing antibody opsonized targets. Using recognition of the complement, *CD11b* and *CD18* were found expressed within NK cells from our study population. The heterodimer CD11b/CD18 is called complement receptor 3 (CR3, Mac1 or $\alpha_M\beta_2$). CR3 is a multi-functional receptor which was described as predominantly expressed on myeloid and NK cells (Ross and Vetvicka, 1993; Vorup-Jensen and Jensen, 2018) and involved in NK cell cytotoxicity (Lee et al., 2017). CR3 can interact with Fc receptors for adhesion to immune complexes and therefore enhance cell mediated antibody-dependent cytotoxicity (Ortiz-Stern and Rosales, 2003).

Cytokines also play an essential role in NK cell activation and function. In this study we observed a strong significant downregulation of IL18 receptor (*IL18RAP* and *IL18R1*) as well as IL12RB2, β chain of IL12 receptor within CD56^{neg}CD16^{pos} relative to CD56^{dim}CD16^{pos} NK cells. IL18 and IL12 are both known to be crucial in NK cell activation, therefore, the downregulation of these receptors on CD56^{neg}CD16^{pos} NK cells might impair their activation. However, it was previously shown that both IL18 and IL12 receptors are silenced in adaptive CD56^{dim} NK cells in order to block their ability to produce immunoregulatory cytokines (Schlums et al., 2015), whereas the IL15 receptor (*IL15RA*) is still expressed. We also show that CD56^{neg}CD16^{pos} NK cells are CD62L^{-}CD103^{-}CD49a^{-}PLZF1^{-} with a downregulation of *FCER1G* (FCεRγ) and *SH2D1B* (EAT-2). This phenotype has been correlated with a loss of immunoregulatory cytotoxic activity (Schlums et al., 2015). Similar observations were made for HIV-positive individuals who had broadly reactive neutralizing antibodies (bnAbs), lower expression of IL12 and IL18 receptors and PLZF1 yet with higher expression of CD6 (Bradley et al., 2018), confirming an adaptive-like NK cell phenotype. In addition, HIV-positive individuals with bnABs had a higher proportion of CD56^{neg}CD16^{pos} NK, which was associated with better viral control. Interestingly, a positive correlation was observed between CD56^{neg}CD16^{pos} NK cells and mRNA expression of *RAB11F1P5*, encoding for Rab11 recycling endosome molecule, which was also found to be increased in CD56^{neg}CD16^{pos} NK cells from children in our study. Together, these studies support the development of adaptive CD56^{neg}CD16^{pos} NK cells as adaptation to chronic infections.

In summary, we present a hypothetical model (**Figure 5**) contrasting killing pathways used by CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells against eBL tumor cells as well as *P. falciparum*-infected red blood cells (iRBC). *P. falciparum* has been consistently linked to eBL pathogenesis (Moormann and Bailey, 2016). Here, we hypothesize that continual malaria infections induce NK cell adaptation thereby increasing the prevalence of CD56^{neg}CD16^{pos} NK cells. **Figure 5A** illustrates how CD56^{dim}CD16^{pos} NK cells kill iRBC after direct recognition between NKp46 and its putative ligand, *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) (Wolf et al., 2017). CD56^{dim}CD16^{pos} NK cells also use direct recognition to kill tumor cells by activation of multiple receptors. However, KIRs, NKG2A/CD94 and 2B4 can trigger tolerance and therefore allow immune escape of the tumor cells. Determining which NK cell inhibitory ligands are expressed by tumors is an area of ongoing investigation. Finally, in the presence of antibodies directed against iRBCs and tumor cells neo-antigens, CD56^{dim}CD16^{pos} NK cells can kill both tumor cells and iRBCs through ADCC triggered by CD16a. ADCC against iRBCs has already been well-described by others (Hart et al., 2017; Arora et al., 2018). In contrast, we hypothesize that the main method of killing

for CD56^{neg}CD16^{pos} NK cells is through antibody dependent cytotoxicity (**Figure 5B**). Expression of all CD18/CD11$_{a/b/c}$ as well as CD16a, CD16b, CD32a, CD32b and Perforin 2 suggests a multifaceted involvement of antibody opsonization in the killing capacity of CD56^{neg}CD16^{pos} NK cells. *P. falciparum* induces a broad range of antibodies directed against the many parasite antigens that are able to trigger ADCC and ADRB (Moormann et al., 2019). Another important component for killing target cells is complement. Present in plasma, iC3b can be fixed on iRBCs as well as tumor cells and thereby opsonize the target for innate cells. This immune complex can be recognized by CR3 expressed on NK cells and trigger complement dependent cell-mediated cytotoxicity (CDCC) against the target cells. Moreover, it was shown that CR3 should be able to communicate with the Fcγ receptors (CD16a/b) and therefore be able to enhance ADCC and ADRB functions (Lee et al., 2017). We therefore suspect that ADCC can as well be important against tumor cells if the tumor is expressing surface antigens for antibody opsonization. In contrast, we and others have shown that CD56^{neg}CD16^{pos} NK cells are not well-adapted for natural direct cytotoxicity because of their strong downregulation of multiple cytotoxic and activation receptors. Thus, CD56^{neg}CD16^{pos} NK cell abundance appears to be a refined adaptation influenced by chronic diseases, that focuses NK cell mediated cytotoxicity toward antibody opsonized targets.

As a limitation of our study, bulk RNA-sequencing didn't allow us to assess the copy number variation (CNV) that can impact the role of CD16b on ADCC. CD56^{neg}CD16^{pos} NK cells might use other means to kill target cells such as ADRB or CDCC. These pathways will need to be assessed in functional assay in order to determine which cytotoxic mechanisms are engaged by CD56^{neg}CD16^{pos} NK cells.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI's database of Genotypes and Phenotypes (dbGaP) with accession number phs1282.V2.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Massachusetts Medical School Institutional Review Board and the Scientific and Ethical Review Unit at the Kenya Medical Research Institute. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CF designed research, performed research, analyzed data, and wrote the paper. CO designed research, performed research, analyzed data, and reviewed the paper. PO performed research and reviewed the paper. JO contributed to samples acquisition and reviewed the paper. CM reviewed the paper. JB contributed experimental tools, analyzed data, and reviewed the paper. AM designed research, contributed experimental tools, analyzed data, and reviewed the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00162/full#supplementary-material

Supplemental Table 1 | Genes significantly differentially expressed between the CD56^{neg}CD16^{pos} NK cells and CD56^{dim}CD16^{pos} NK cells (Benjamini-Hochberg procedure (BH) adjusted p-value < 0.01 and false discovery rate (FDR) < 0.05).

Supplemental Figure 1 | CD56^{neg}CD16^{pos} NK cells across population. **(A)** Representative cytoplots of NK cell subsets repartition from PBMCs within healthy American and Kenyan adults as well as healthy and eBL Kenyan children. CD56^{pos} NK cells are gated in red box and CD56^{neg} in blue box. **(B)** Percentage of CD56^{neg}CD16^{pos} NK cells across our different groups: healthy American, healthy Kenyan, healthy children from Nandi (EBV+/*Pf*-), healthy children from Kisumu (EBV+/*Pf*+) and eBL children (EBV+/*Pf*+). ****Represents a p-value $\leq$ 0.0001; ***represents a p-value $\leq$ 0.001; **represents a p-value $\leq$ 0.01. *Represents a p-value < 0.05.

Supplemental Figure 2 | Excluding NK subsets with possible monocyte contamination. Boxplot showing the expression of monocyte associated genes (*CD14*, *CD33,* and *CD34*). We identified one of the sorted CD56^{dim}CD16^{pos} NK cells (highlighted red in the boxplot), to have elevated expression of monocyte marker genes (CD14 and CD33). Elevated expression of these 2 genes could have been due to monocyte contamination during the sorting process of that particular sample. This sample was excluded from all downstream analysis.

Supplemental Figure 3 | Purity of flow sorted CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} cell subsets. **(A)** Boxplot of monocyte gene expression profile *CD14*, *CD33,* and *CD34* genes expression within both CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells. **(B)** Boxplot of neutrophil gene expression profile *ARG1, ARHGEF12, BPI, CA1, CEACAM8, CNTNAP3B, CRISP3, FUT4, IFIT1B, ITGAM, ITGB2, LCN2,* and *MPO* genes expression within both CD56^{neg}CD16^{pos} and CD56^{dim}CD16^{pos} NK cells. ***Represents a p-value $\leq$ 0.00001; *represents a p-value $\leq$ 0.01.

Supplemental Figure 4 | Heatmap of all differentially expressed genes for CD56^{neg}CD16^{pos} compared to CD56^{dim}CD16^{pos} NK cells. Differential gene expression analysis identified 536 genes to be significantly differentially expressed (Benjamini-Hochberg method [BH] adjusted p-value < 0.01 and False Discovery Rate (FDR) < 0.05) between the CD56^{neg}CD16^{pos} and the CD56^{dim}CD16^{pos} NK cell subsets. The heatmap shows hierarchical clustering based on the expression profile of the 536 differentially expressed genes between the two NK cell subsets. The color key indicates the intensity associated with normalized expression values. Of the 536 genes identified to be differentially expressed between these two NK cell subsets, 350 genes were downregulated among the CD56^{neg}CD16^{pos} cells compared to the CD56^{dim}CD16^{pos} and 186 genes were upregulated in the CD56^{neg}CD16^{pos} cells compared to the CD56^{dim}CD16^{pos} cells (**Supplemental Table 1**).

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Delineation and Modulation of the Natural Killer Cell Transcriptome in Rhesus Macaques During ZIKV and SIV Infections

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Natural killer (NK) cells are crucial regulators of antiviral and anti-tumor immune responses. Although in humans some NK cell transcriptional programs are relatively well-established, NK cell transcriptional networks in non-human primates (NHP) remain poorly delineated. Here we performed RNA-Seq experiments using purified NK cells from experimentally naïve rhesus macaques, providing the first transcriptional characterization of pure NK cells in any NHP species. This novel NK cell transcriptomic signature (NK RMtsig) overlaps with published human NK signatures, allowing us to identify new key signaling and transcription factor networks underlying NK cell function. Finally, we show that applying NK RMtsig to an unrelated rhesus macaque cohort infected with SIVmac251 or ZIKV can sensitively detect NK cell repertoire perturbations, thus confirming applicability of this approach. In sum, we propose this NHP NK cell signature will serve as a useful resource for future studies involving infection, disease or treatment modalities in NHP.

Keywords: NK cells, rhesus macaques, RNA-seq, SIV, ZIKV

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INTRODUCTION

Natural killer (NK) cells are some of the earliest responders to viral infections and form an essential component of the anti-viral innate immune response (Alter and Altfeld, 2009; Gandhi et al., 2010). Though they play a substantial role in the regulation of viral infections and nascent neoplasms, these immune cells remain poorly investigated compared to their adaptive counterparts, T cells and B cells. Although NK cells are considered a heterogenous population, they can be broadly classified as cytokine-producing or cytotoxic cells (Manickam et al., 2019). Their functional and phenotypic characterization may also vary depending on tissue localization or disease state allowing NK cells to be versatile in their responses. In many non-human primates (NHP), NK cells are defined as being $CD14^{-}CD20^{-}CD3^{-}CD159A/C^{+}$ and in rhesus macaques the predominant population in blood can be additionally defined by the expression of CD16, whereas in certain tissues CD56 is generally upregulated on the cell surface of NK cells (Reeves et al., 2010; Manickam et al., 2019).

In humans and NHP, NK cells have been shown to play a critical role in controlling HIV and SIV infections mainly by direct targeting of virally-infected cells through activating receptors, or via antibody dependent cell-mediated cytotoxicity (ADCC) (Shieh et al., 2001; Schafer et al., 2015).

In SIV-infected monkeys, activated NK cells with cytolytic functions in peripheral blood correlated with better control of infection (Shieh et al., 2001). During the course of HIV/SIV infection it has been observed that NK cells become dysfunctional, especially during the chronic stages of infection (Alter and Altfeld, 2009; Brunetta et al., 2009; Schafer et al., 2015). The exact mechanisms of how HIV/SIV infection alters NK cell function are still unclear, though evidence suggests this may be a result of broader effects from altered CD4+ T cell levels (Luo et al., 2017). Interestingly, NK cells have also been shown to be capable of eliciting memory recall responses in the context of SIV infection in rhesus macaques (Reeves et al., 2015) and to CMV infection in humans (Foley et al., 2012; Hammer et al., 2018). This may provide an opportunity for developing novel vaccine strategies and as a result it is important to understand the mechanisms regulating NK cell function, especially in the context of viral infection.

To date a small number of studies have been performed in humans to define the transcriptional profile of human NK cells using microarrays or RNA-Seq experiments (Nakaya et al., 2011; Li et al., 2014; Newman et al., 2015; Costanzo et al., 2018; Crinier et al., 2018) whereas in NHP most of the NK cell transcriptional characterization has been carried out using Fluidigm or RT-PCR (Hong et al., 2013). The NK cell transcriptome has been characterized more extensively in mice (Zhou et al., 2017; Crinier et al., 2018), but since the differences between mouse and rhesus are greater than those between rhesus and human we focused on comparing our data with several human datasets for this work. NK cell gene signatures identified in these human studies are currently used as a reference to study NK cells and to screen for the enrichment of NK cell markers in both human and NHP studies. Since NHP are largely used as a model to study human biology and infectious diseases, particularly HIV, it is crucial to define an NHP NK cell signature and to understand how NK cell transcriptional responses in NHP may also change during infection. Therefore, we performed RNA-Seq experiments to define the NK cell transcriptomic identity in NHP and provide a resource for NHP research. To our knowledge, this is the first large scale study to define the transcriptomic profile of NK cells in a rhesus macaque model.

MATERIALS AND METHODS

Ethics Statement

This manuscript utilized banked animal PBMC samples and no new animals were acquired specifically for the analyses described herein. All original animals were housed at the New England Primate Research Center of Harvard Medical School in accordance with the rules and regulations of the Committee on the Care and Use of Laboratory Animal Resources. 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. All studies reported here were performed under protocol #04637 which was reviewed and approved by the Harvard University IACUC. When necessary, macaques were immobilized with ketamine HCl (Parke-Davis) at approximately 10 mg/kg and injected intramuscularly after overnight fasting. Blood samples were collected using venipuncture.

Animals

Experimentally naive male age-matched Indian-origin rhesus macaques were analyzed in this study. All animals were colony-housed at the New England Primate Research Center and were free of simian retrovirus type D and simian T-lymphotropic virus type 1. Blood samples were collected in EDTA-treated tubes, and peripheral mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation over lymphocyte separation media (MP Biomedicals, Solon, OH) and any contaminating red blood cells were lysed using hypotonic ammonium chloride solution. PBMCs were frozen in 90%FBS + 10% DMSO (Sigma) in the LN_2 vapor phase.

Antibodies and NK Cell Sorting

All antibodies were purchased from BD Biosciences unless otherwise specified. For NK cell sorting the following cell antigens were used: CD14 (MøP9), CD20 (L27), CD3 (SP34.2), CD159a (Z199, Beckman Coulter). Additionally, the AQUA viability assay was used to identify viable cells. PBMC were thawed and stained with AQUA viability stain, followed by sorting antibodies as detailed above. NK cells were then sorted into cold R10 using the following gating strategy in order to identify NK cells: $CD14^{-}CD20^{-}CD3^{-}CD159A/C^{+}$ (Reeves et al., 2010; **Supplementary Figure 1**).

RNA Isolation

The sorted NK cells were pelleted (500 × g, for 10 min) and lysed by vortexing for 1 min in cold supplemented RLT buffer (RLT + β-MeOH) using the following ratio: 50 μL cells in R10 and 350 μL RLT buffer, 4°C. Samples were then immediately frozen at −80°C. RNA was extracted from these samples using the RNeasy Micro kit (Qiagen) with on-column DNase digestion. RNA quality was assessed using an Agilent Bioanalyzer.

Library Preparation and RNA-Seq Processing

Five (5) nanograms of total RNA was used as input for cDNA synthesis using the Clontech SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio) according to the manufacturer's instructions. Amplified cDNA was fragmented and appended with dual-indexed bar codes using the NexteraXT DNA Library Preparation kit (Illumina). Libraries were validated by capillary electrophoresis on an Agilent 4200 TapeStation, pooled at equimolar concentrations, and sequenced on an Illumina HiSeq3000 at 100SR, yielding 20–25 million reads per sample. Alignment was performed using STAR algorithm version 2.5.2b (Dobin et al., 2013). Transcripts were annotated using MacaM assembly and annotation of the Indian rhesus macaque genome (Zimin et al., 2014) (http://www.unmc.edu/rhesusgenechip/index.htm#NewRhesusGenome). Transcript abundance estimates were calculated internal to the STAR aligner using the algorithm of htseq-count generating the raw read count table (Anders et al., 2015).

Gene Desirability Score Function

To define our NK cell signature from rhesus macaques, first we converted the raw gene expression matrix into a count per million (cpm) expression matrix in order to normalize for library size differences between samples. Then, we filtered all genes with a cpm count <1 across all animals to ensure each gene is expressed at some minimal level across all animals. This step generated an initial signature of approximately 9,000 genes. Next, we applied the approach implemented in the desiR package (https://cran.r-project.org/web/packages/desirability/desirability.pdf) (Derringer and Suich, 1980) to select for the top NK cell expressed genes. This approach ranks genes using their overall importance across all samples and assigns a desirability score between 0 and 1 to each gene.

For each gene, we defined its average cpm count as $R_i(x)$. A desirability function $D_i(R_i)$ assigns a score between 0 and 1 to all possible values of R_i, with:

$D_i(R_i) = 0$ representing the lowest undesirable value of R_i and $D_i(R_i) = 1$ representing the highest desirable value of R_i.

The desirability function $D_i(R_i)$ varies depending on whether a particular response R_i is to be maximized, minimized or equal to a specific threshold.

Let L, H and T be the lower, upper, and target values, respectively, that are desired for a response R_i, with $L \leq T \leq H$, where L, H and T represent threshold values defined by the user. We implemented a desirability function that maximizes the score assigned to important genes (genes with high average cpm count) and defined the desirability function for each gene as:

$$Di(Ri\,(x)) = \begin{cases} 0 \; if \; Ri(x) < L \\ \frac{(Ri(x)-L)}{(T-L)} \; if \; L \quad \leq \; Ri\,(x) \; \leq T \\ 1.0 \; if \; Ri\,(x) \geq T \end{cases} \qquad (1)$$

Where, $Di(Ri(x))$ is the desirability score for gene x_i.

In order to select L and T parameters, first we plotted the histogram of average cpm count distribution of all genes in our initial signature (9,000 genes) and selected the minimum cut-off L equal to 1 and the maximum cut-off T equal to 6 (**Supplementary Figure 2**). Although the choice of these two parameters may seem random, we selected the values of L and T based on the specific distribution of our data by (1) filtering more genes with low cpm count and (2) setting up a a maximal value T that reflects the inflection point starting from which a gene is considered to be highly significant and assign a score of 1 to all the genes with an average cpm count higher than this maximal threshold. Also, because we did not prioritize only genes with maximal desirability score ($DesiR = 1$) we think these parameters represent an acceptable trade-off to distinguish between high and low desirable genes as shown in **Supplementary Figure 2**. Next, We used the DesiR package and its *d.high* function to assign a score to all genes in our initial signature. This function generated desirability scores ranging from 1 (highly desirable gene) to 0 (not desirable gene). We selected the top genes (5,627 genes) with a desirability score of 0.70 or higher as the final NK cell signature designated by the NK cell rhesus macaque transcriptomic signature NK RMtsig (**Supplementary Table 1**). Although, we used these highly desirable genes for all the analyses conducted in this study, we think that the remaining genes (desirability score <0.70) are also important and need to be considered when screening for the enrichment of NK cell signatures (**Supplementary Table 2**).

Pathways Enrichment Analyses

We used the overlapping test implemented in the GeneOverlap R package (https://github.com/shenlab-sinai/geneoverlap) to assess the overlap of our NHP NK cell signature with published collections of gene sets and pathways (Chaussabel et al., 2008; Liberzon et al., 2011; Nakaya et al., 2011; Newman et al., 2015; Costanzo et al., 2018; Yang et al., 2019). All gene sets and pathways that were enriched with a false discovery (FDR) q value cut-off of 0.05 were selected and the overlapping genes between these significant signatures and our NK RMtsig were used to generate heatmaps and gene networks.

Gene Network Analyses

All gene networks were generated using the DyNet Analyzer tool implemented under Cytoscape version 3.6.0 (https://cytoscape.org). For gene annotation, we used GeneMANIA version 3.3.1 (http://genemania.org), Genecards (https://www.genecards.org), Reactome database and CluGo tool implemented under Cytoscape version 3.6.0. For transcription factors (TFs) enrichment analyses, we used the database pscan (http://www.fiserlab.org/tf2dna_db/) and selected TF targets from humans and NHP studies only.

Statistical Analysis

All the analyses in this paper were generated using the following R packages: limma, corrplot, DESeq2, heatmap.2, pheatmap, circlize, and GeneOverlap available via the Bioconductor web site at https://www.bioconductor.org. RNA-Seq analysis was performed using DESeq2 R package (Love et al., 2014). Correlation plots were generates using the R package corrplot with the following parameters (method=pie, correlation = Spearman, significance p value level sig.level = 0.05 and interval confidence conf.level = 0.95). Microarray data from previously published independent studies of SIV-infected rhesus macaques in blood, LN and FRT and from ZIKV infected rhesus macaques in blood were analyzed using the limma R package as described previously (Barouch et al., 2016; Aid et al., 2017). First, differential gene expression analysis was performed at days 1, 3, 7, and 10 following SIV infection compared to day 0 in blood, LN and FRT tissues and at days 2, 4, 6, and 14 following ZIKV infection in blood. Next, we overlapped our NK RMtsig with genes modulated by SIV or ZIKV and those NK RMtsig Genes that were significantly increased or decreased (Benjamini–Hochberg adjusted $p < 0.05$) following infection.

RESULTS

Transcriptomic Profiling of NK Cells in Rhesus Macaques by RNA-Seq

To provide an unbiased transcriptomic profile of purified NK cells, we performed RNA-Seq experiments using sorted

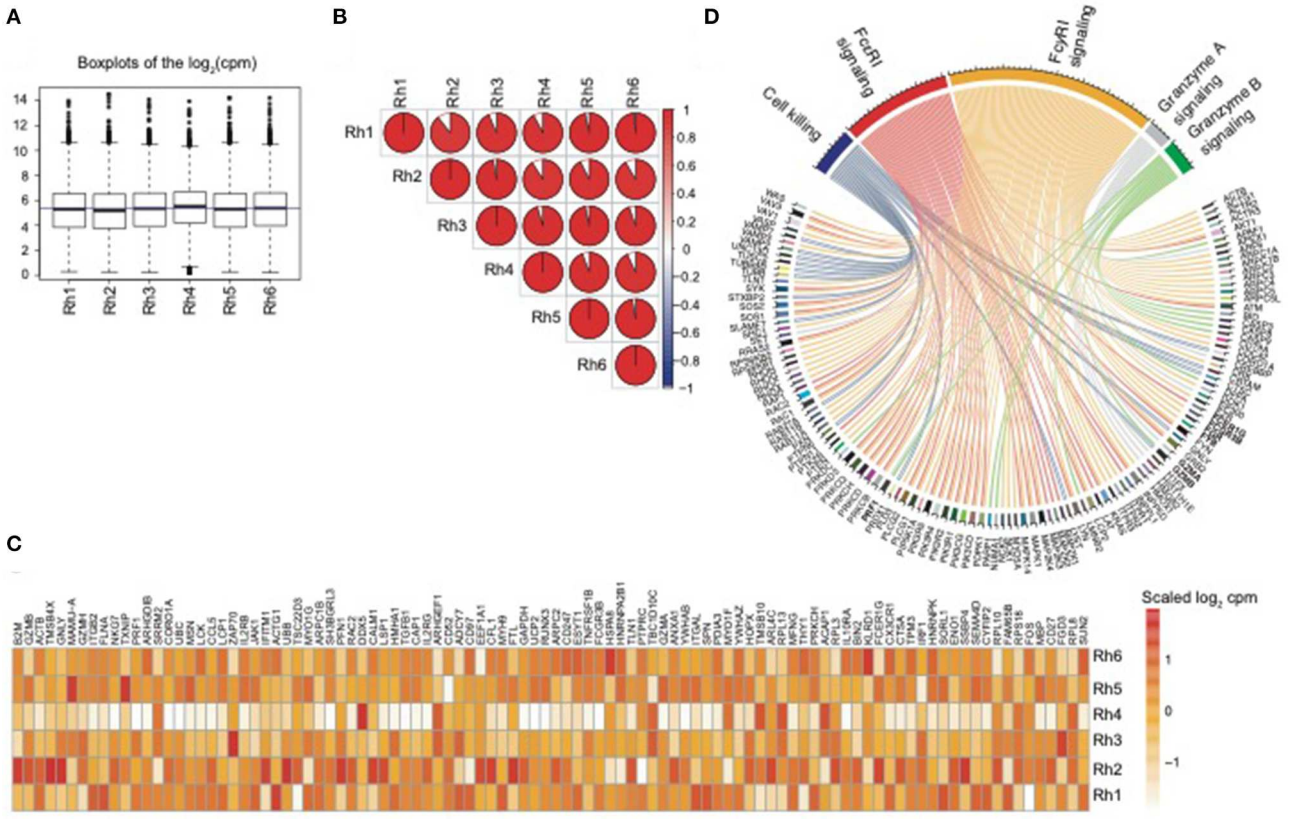


FIGURE 1 | NK RMtsig expression profile is consistent across all animals and shows enrichment of published NK cell markers and signaling pathways. **(A)** Boxplots showing the distribution of the log2(cpm counts) for each sample using the NK RMtsig. The blue horizontal line represents the median (cpm counts) across all animals. **(B)** Correlation plot showing the pairwise Spearman correlation of the NK RMtsig expression across all six animals. Pie charts represent the degree of the Spearman correlation ranging from blue (negative correlation) to red (positive correlation), where full pie corresponds to maximal correlation ($r = 1$ and empty pie represents no correlation ($r = 0$). **(C)** Heatmap representing the scaled $\log_2$ (cpm count) of the top 100 highly expressed gene from the NK RMtsig. **(D)** Circular plot of major NK cell signaling pathways enriched in the NK RMtsig. Pathways were shown in different colors. Genes within each pathway were shown with edges of the same color.

peripheral NK cells from experimentally naive rhesus macaques. NK cells from Indian-origin rhesus macaques were sorted by the $CD14^{-}CD20^{-}CD3^{-}CD159A/C^{+}$ cell phenotype as shown in **Supplementary Figure 1** as previously defined by our group (Reeves et al., 2010). From our analyses we selected the top genes with a desirability score higher than 0.70 to define our NHP NK cell signature (**Supplementary Table 1**). Genes with a desirability score <0.70 are shown in **Supplementary Table 2**. In the remaining sections, we will refer to this as NK cell rhesus macaques transcriptomic signature (NK RMtsig).

In order to assess the overall expression similarity of the NK RMtsig across all animals, we performed a pairwise Spearman analysis correlating the expression of all genes within the NK RMtsig between each pair of animals. We observed that the expression of the NK RMtsig was highly similar across all animals as shown by the boxplots of the cpm count distribution and the Spearman correlation plot (**Figures 1A,B**, **Supplementary Figure 6**). These results suggest little to no difference in the transcriptomic profile, and that the expression of the NK RMtsig was quite uniform with minimal animal-to-animal variation. Next, we tested if our NK RMtsig was enriched in known major NK cell markers. We found that several well-established NK cell genes were among the top genes with high desirability scores including *GZMB, GZMA, NKG7, PRF1, CCL5, KLRD1, KLRC1, KLRC3, KLRF1, KIR2DL4*, and *FCGR3B, IL2RB, EOMES, LAIR1*, and *CD2* (**Figure 1C**, **Supplementary Table 1**). These genes have been well characterized to play roles in NK cell effector immune responses as well as in normal NK cellular differentiation (Costanzo et al., 2018). In order to further characterize our NK RMtsig, we tested for the enrichment of known NK cell signaling pathways in the NK RMtsig. This approach allowed us to assess whether NK RMtsig overlaps with published human NK signaling pathways. We used the overlapping test as described in the Methods section and NK cell molecular signatures from the MSigDB C2 gene sets and the NK cell signatures from the blood transcription modules collection (Chaussabel et al., 2008; Liberzon et al., 2011). While it was not surprising to see many pathways enriched in NK RMtsig, we observed significant enrichment of several key, well-characterized NK cell pathways as determined by the overlapping test significance (FDR < 0.05). These significantly enriched pathways included granzyme B and granzyme A

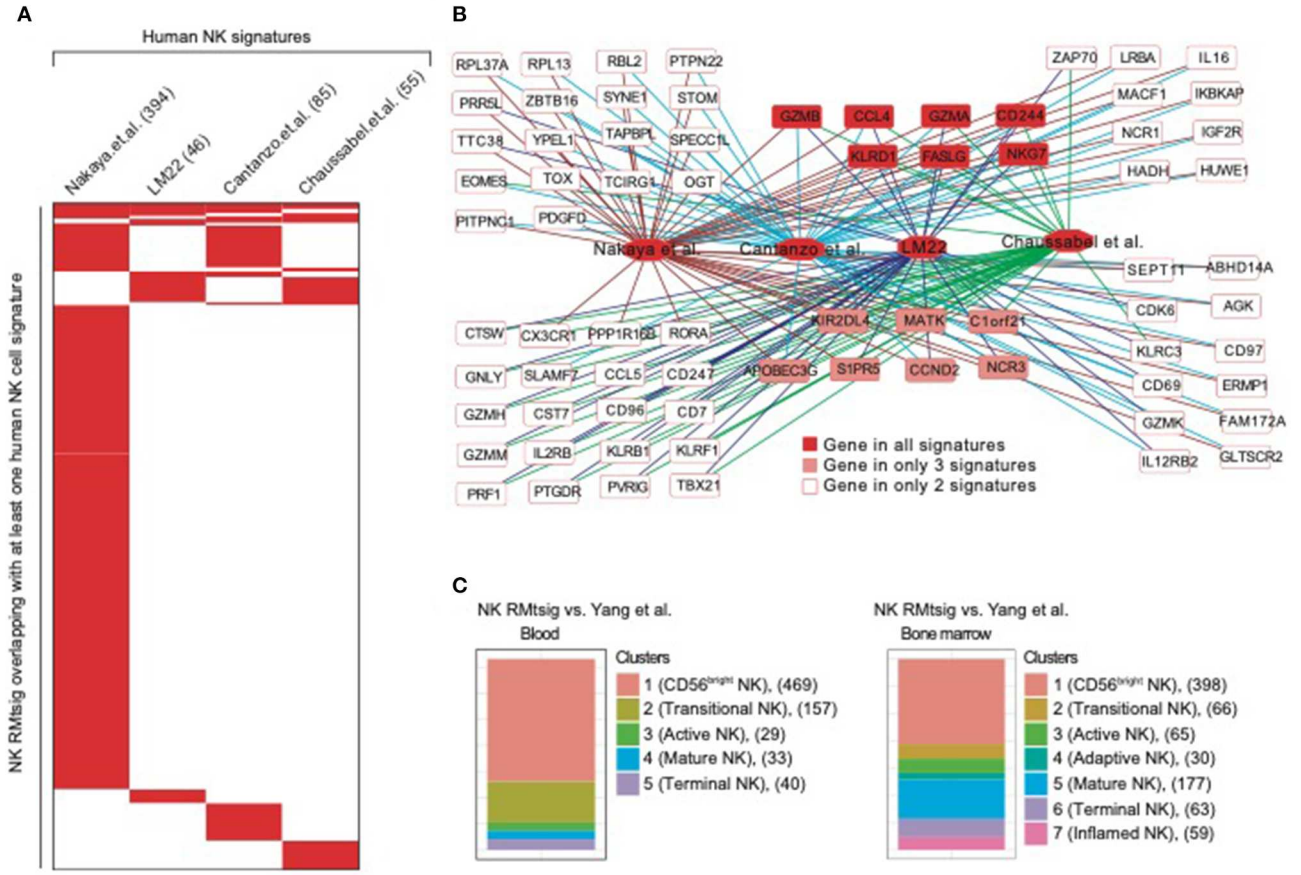


FIGURE 2 | NK cell signature from NHP overlaps with published NK signatures in humans. **(A)** Heatmap showing the overlap between NK RMtsig and published NK signatures from several human studies. Each column represents an NK signature from humans and each row represents a common gene with the NK RMtsig, depicted in red if the gene is present in the human signature or in white if the gene is absent from the human signature. For each human signature, the number of overlapping genes with the NK RMtsig is shown between parentheses. **(B)** Genes Network of NK genes common to both NHP and humans from the signatures shown in panel A. **(C)** Graphical representation of the overlap between the NK RMtsig and NK cell populations signatures derived from human in blood and bone marrow using single cell RNA-Seq experiments.

signaling (*PRF1GZMB, GZMA, BID, CASP3, CASP8*), cell killing (*MAP2K1, TUSC2, TUBB4B, VAMP2, PRDX1*), FcγRI and FcεRI signaling (*FCER1G, FCGRB, FGR, FYB, PIK3R5, PIK3R4, PIK3R1, PIK3R2, VAV3, VAV1, SOS1, SOS2*) (**Figure 1D**). Our results showed that NK RMtsig overlaps significantly with published human NK cell markers and with well-established NK cell signaling pathways.

Genes Expressed in Rhesus Macaque NK Cells Display Significant Overlap With Published Human NK Cell Signatures

To further assess the degree of overlap between NK RMtsig and published NK cell signatures derived from *in vivo* and *in vitro* human studies, we conducted a meta-analysis where we compared NK RMtsig with several human NK cell signatures derived from microarrays or RNA-Seq experiments in humans (**Figure 2**, **Supplementary Table 3**). First, we compared NK RMtsig with the human NK cell module from the LM22 database (Newman et al., 2015). Among the 79 human NK cell genes in the LM22, 46 genes were found in the NK RMtsig (**Figures 2A,B**). These genes include major NK cell markers such as *PRF1, NKG7, CCL5, GZMB, KLRD1, KLRC3, KLRB1, KIR2DL4, CD69*, and TBX21. In a second study, Nakaya and colleagues (Nakaya et al., 2011) conducted a meta-analysis using publicly available microarrays datasets of major PBMC populations, including T cells, B cells, monocytes and NK cells. They analyzed two different NK cell microarray datasets. In the first dataset, NK cells from tonsil were obtained from patients undergoing routine tonsillectomy. In the second dataset, primary NK cells ($CD56^{+}/CD16^{+}$ and $CD3^{-}$) were isolated from PBMC from seven healthy donors. Out of the 1469 NK genes reported in this microarrays analysis, 394 genes overlapped with NK RMtsig (**Figures 2A,B**), including several interferon and cytokine signaling genes. In a third study, Costanzo and colleagues (Costanzo et al., 2018) sorted NK cells ($CD56^{dim/neg}$ and $CD57^{+}NKG2C^{+}$) from PBMC from six healthy donors under various activating conditions. Then gene expression profiling was

FIGURE 3 | NK RMtsig revealed expression of a broad range of innate and adaptive immune pathways. Diagram showing all the significant pathways (FDR q value of the overlapping test < 0.05) enriched within the NK RMtsig. Pathways were grouped into modules of similar or close molecular function. The color gradient represents the gene overlapping FDR q value, ranging from light orange (FDR = 0) to dark red (FDR = 0.05).

performed to define the gene signature of responding NK cells compared to unstimulated NK cells. In this study, the authors reported three NK cell signatures: genes upregulated following IL12 and IL-18 treatment (IL12_IL18 NK, 79 genes), NK cytolytic genes (98 genes) and genes upregulated during NK coculture with K562 (K562 NK, 67 genes). Among these three signatures 41, 33 and 11 genes overlapped with NK RMtsig, respectively, and all include *CCL4*, *BCL2*, *CD244*, *EOMES*, *FALSG*, *IL21R*, *IL2RG*, *NFKB1*, *IL12RB2*, *IL4R*, *IL16*, *TOX and PRDX1* (**Figures 2A,B**). Finally, we assessed the overlap between NK RMtsig with a module of NK cell genes (78 genes) from the molecular signatures database (Chaussabel et al., 2008). Fifty-five genes out of 78 were found in NK RMtsig and include *CX3CR1*, *EOMES*, *NLRC3*, *TIGIT*, *TGFBR3*, *RORA*, *STAT4* (**Figures 2A,B**).

A recent study by Yang and colleagues (Yang et al., 2019) using single cell RNA-Seq of NK cells sorted from human bone marrow and blood, identified the transcriptional profiles of several NK cell populations including (i) CD56 bright, (ii) transitional, (iii) active, (iv) mature and (v) terminally differentiated NK cells. Therefore, we also compared NK RMtsig to these different NK cell populations signatures (**Figure 2C**). In bone marrow, 398 genes in cluster 1 ($CD56^{bright}$ NK), 66 genes in cluster 2 (transitional NK), 65 genes in cluster 3 (active NK), 30 genes in cluster 4 (adaptive NK), 177 genes in cluster 5 (mature NK), 63 genes in cluster 6 (terminal NK) and 59 genes in cluster 7 (inflamed NK) were found in NK RMtsig. Similarly in blood, 469 genes in cluster 1 ($CD56^{bright}$), 157 genes in cluster 2 (transitional), 29 genes in cluster 3 (active), 33 genes in cluster 4 (mature) and 40 genes in cluster 5 (terminally differentiated) were found in NK RMtsig. Together, these analyses showed a significant overlap between NK RMtsig and published human NK cell signatures generated from independent studies, thus confirming the validity of our approach and providing this signature as a sensitive tool to probe both human and NHP data sets.

NK RMtsig Is Enriched in Pathogen-Associated Molecular Pattern Sensing, Metabolism, Cell Cycle and Survival Pathways

Using the same overlapping approach as described above, we tested for the enrichment of additional signaling pathways in NK RMtsig. We found that modules of cell cycle, cell motility and export, interferon and inflammatory responses, metabolism, glycan signaling and antigen presentation and processing including MHC I and MHC II pathways were all significantly enriched in NK RMtsig (**Figure 3**), as were multiple

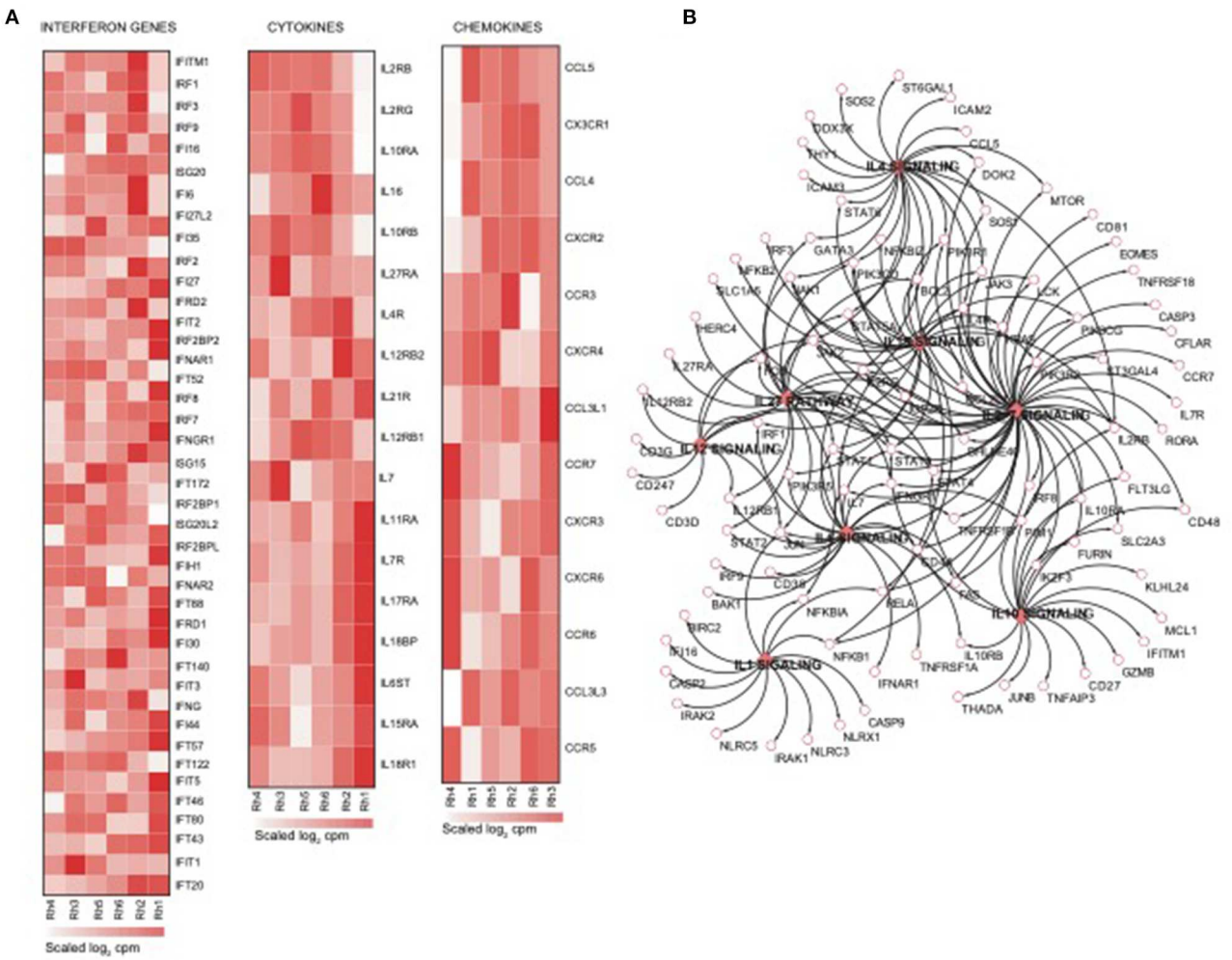


FIGURE 4 | NK cell transcriptomic profile is enriched in interferon genes, cytokines and chemokines and their downstream signaling. **(A)** Heatmaps representing the normalized expression of interferon genes, cytokines and chemokines enriched in the NK RMtsig. Each column represents an individual animal and each row represents a gene. **(B)** Genes network representing the cytokines downstream signaling enriched in NK RMtsig. Genes shared by two pathways or higher were highlighted in full red boxes. Unique genes for each pathway were shown in empty boxes. The red color gradient corresponds to the number of pathways which a specific gene belongs.

interferon, cytokine and chemokine genes (**Figure 4**). Moreover, downstream signaling by cytokines involved in the inflammatory response (*IL-1* signaling pathway), immune inhibition (*IL-10, IL-27* and *TGF-β* signaling pathways), cell activation and proliferation (*IL-2* and *IL-7* signaling pathways) and others were also enriched in NK RMtsig (**Figure 4A**). We also observed that several signaling pathways associated with pathogen recognition and rapid innate immune responses were enriched in NK RMtsig, including TLRs, those shared with other intracellular pathogen-associated molecular pattern sensors as well as the inflammasome (**Figure 5A**). In addition, we observed enrichment in metabolic pathways including signaling by mechanistic target of rapamycin (*mTOR*) and fatty acid metabolism (**Figure 5B**) as well as pathways involved in functional NK cell responses, including protein secretion, synapse formation (**Figure 5C**), and several markers of glycosylation signaling (**Supplementary Figure 3**).

In order to further dissect the NK cell transcriptional programs, we performed an enrichment analysis using the transcription factors (TFs) database pscan (Zambelli et al., 2009). First, we screened for all human TFs and found 332 TFs among NK RMtsig (**Supplementary Table 4).** Several TFs involved in regulating many biological functions were part of NK RMtsig and include interferon regulatory factors (*IRF7, IRF3, IRF8*), Forkhead transcription factor family (*FOXO1, FOXP1*), several transcriptional activator and repressor (*BHLHE40, TOX, CTCF, SP1, SMAD*), TFs involved in proliferation and activation (AP1, NFAT, JUN, FOS, EOMES) and TFs regulating apoptosis (*FOXO3, HIF1A*). Next, we tested the enrichment of TFs downstream targets in the NK RMtsig using the overlapping test

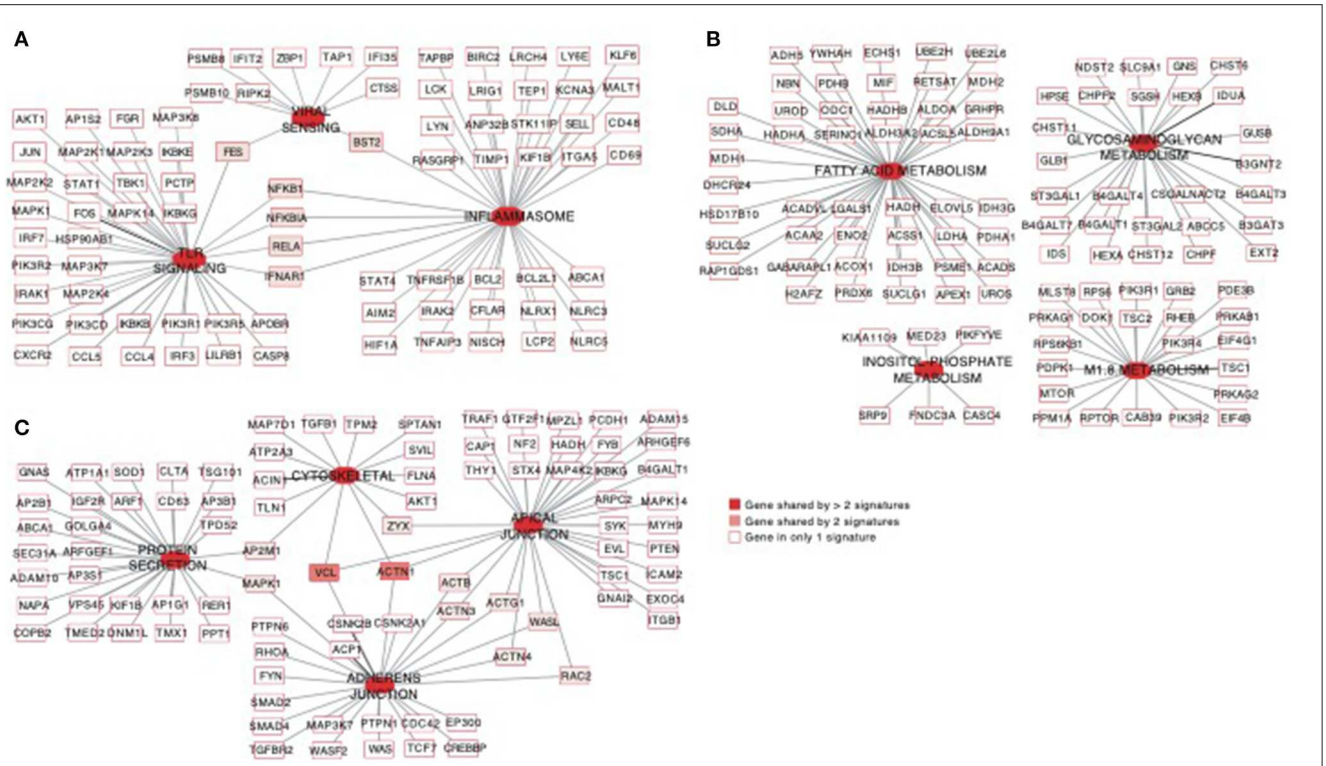


FIGURE 5 | Pathways of viral sensing and TLR signaling, metabolism, cell motility and protein export are enriched in the NK RMtsig. **(A–C)** Gene networks representing pathways of viral sensing, TLR signaling, inflammasome **(A)**, metabolism pathways **(B)** and cell motility and protein export pathways **(C)** enriched in the NK RMtsig. Genes shared by 2 or more pathways were highlighted with full red boxes, where red color gradient represents the number of pathways that share this gene. Unique genes for each pathway were shown in empty boxes.

as described above. We found that several transcription factor targets were enriched in our NK RMtsig including TF regulating interferon responses and inflammation, survival, proliferation and activation signaling (**Figure 6**, **Supplementary Table 4**). Some of the TFs downstream targets identified in NK RMtsig are illustrated in **Figure 6B**. Collectively, these data suggest that at a naive state NK cells are poised for rapid responses that are regulated by common TFs and engage in a complex integrative innate immune networks that include metabolism, cytokines and chemokines, TLR and interferon signaling.

The NHP NK Cell Signature Is Modulated During Acute SIV and ZIKV Infections

As an unbiased test case for whether our NK RMtsig could be applied to retrospective longitudinal infection studies, we decided to use the published longitudinal gene expression signatures from two independent NHP infection studies (Barouch et al., 2016; Aid et al., 2017). In the first study (Barouch et al., 2016), microarray experiments were performed on unfractionated mononuclear cells from rhesus macaques intravaginally infected with SIVmac251 taken on days 1, 3, 7, and 10 after infection and at day 0 (before infection) in blood, lymph node (LN) and female reproductive tract (FRT). In the second study (Aid et al., 2017), mononuclear cells were isolated from peripheral blood at days 2, 4, 6, and 14 after intravenous infection with ZIKA virus (ZIKV) and at day 0 (before infection) followed by microarray experiments. Analysis of the first data set revealed that in blood, several NK RMtsig genes were significantly modulated (adjusted $p < 0.05$) following SIV infection at day 1 (1,051 genes), day 3 (1,883 genes), day 7 (2,002 genes) and day 10 (1,429 genes) as compared to day 0 (**Figure 7A**).

We also observed significant modulation (adjusted $p < 0.05$) following ZIKV infection at day 2 (76 genes), day 4 (742 genes), day 6 (1067 genes) and day 14 (231 genes) relative to the pre-infection time point, day 0 (**Figure 7B**). While we observed some similarities in NK RMtsig genes commonly up or down regulated by SIV and ZIKV, several NK RMtsig were regulated in the opposite direction by SIV and ZIKV (**Figure 7C**). Interestingly, while there were many differences between SIV and ZIKV infection we observed that these up- and down-regulated NK RMtsig genes could be broken down into three phases of modulation: early, intermediate or late responsive genes. The different patterns of modulation ranging from early (day 1 or day 2), intermediate (days 3, 4, 6, or 7), late (day 10 or day 14) or unchanged (similar expression patterns compared to day 0) are shown in **Figures 8A,B**. In addition, our NK RMtsig showed distinct expression patterns in blood, LN and FRT at different time points following SIV infection suggesting an NK cell tissue specific gene expression profile (**Supplementary Figures 4, 5**,

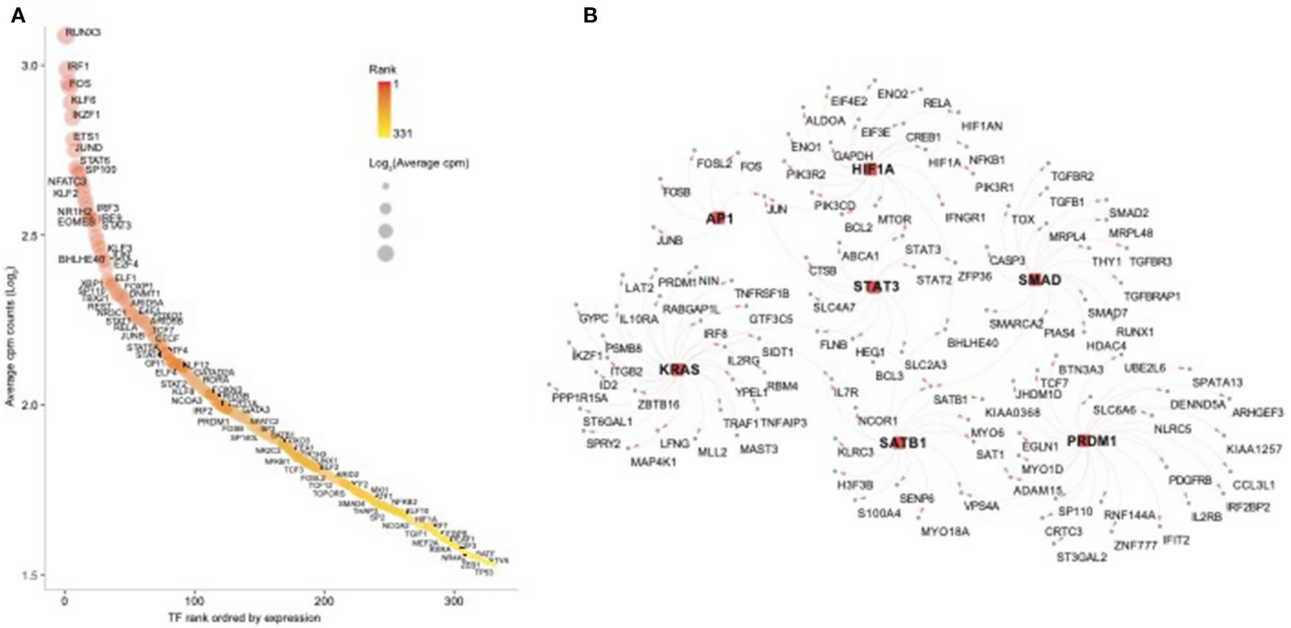
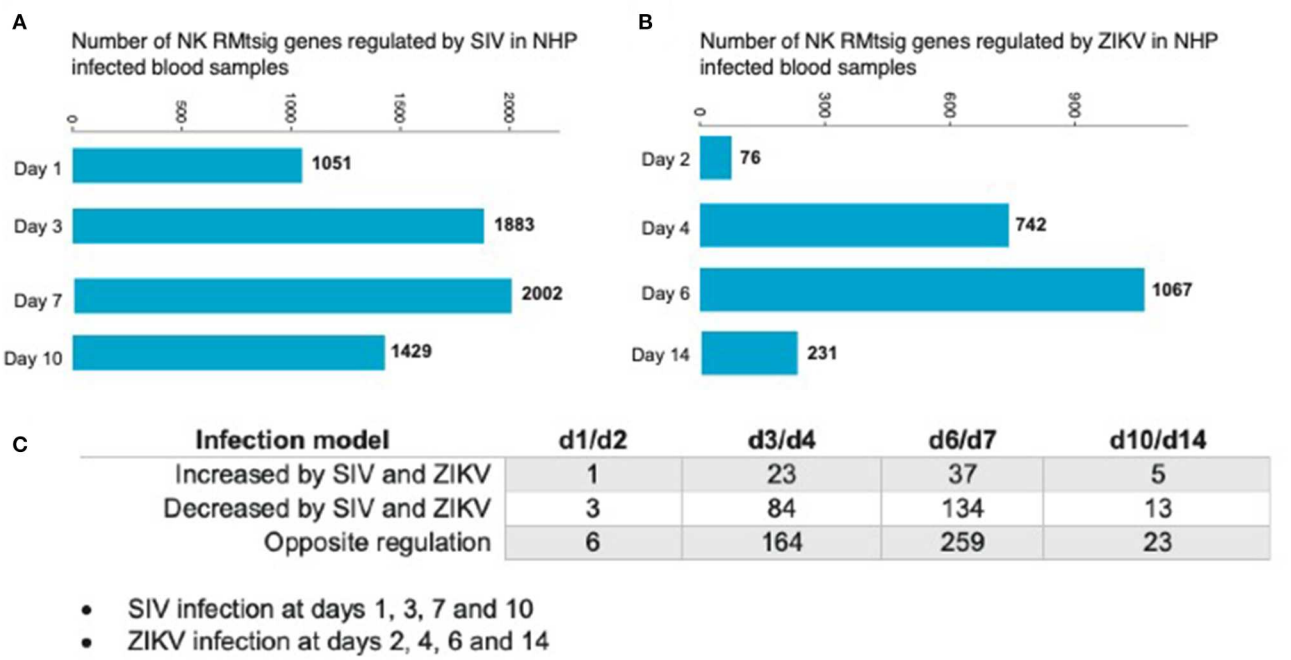


FIGURE 6 | Transcription factors regulating epigenetic modifications, proliferation, cell homeostasis, T follicular helper cell and B cell functions are enriched in the NK RMtsig. **(A)** Scatter plot showing the 332 human transcription factors (TFs) enriched in NK RMtsig. All TFs were ranked using their expression from high to low. X axis represents gene rank and Y axis represents the Log_2 transformation of average cpm count for each TF. Red color gradient and circle size represent TF expression level. Highly ranked TFs were shown in darker red and bigger circles. **(B)** Genes networks representing transcription factors (TFs) targets, selected from panel A, enriched in the NK RMtsig. TF names are shown in the middle with square red nodes, where each TF (red node) is connected to its targets (gray nodes) with red arrows.



Infection model	d1/d2	d3/d4	d6/d7	d10/d14
Increased by SIV and ZIKV	1	23	37	5
Decreased by SIV and ZIKV	3	84	134	13
Opposite regulation	6	164	259	23

FIGURE 7 | NK RMtsig is modulated following SIV and ZIKV infections in two independent NHP cohorts. **(A,B)** Barplots of the number of NK RMtsig genes that are modulated by SIV **(A)** or ZIKV **(B)** at different time points following infection. **(C)** Number of NK RMtsig genes that are commonly increased or decreased by SIV and ZIKV at different time points following infection.

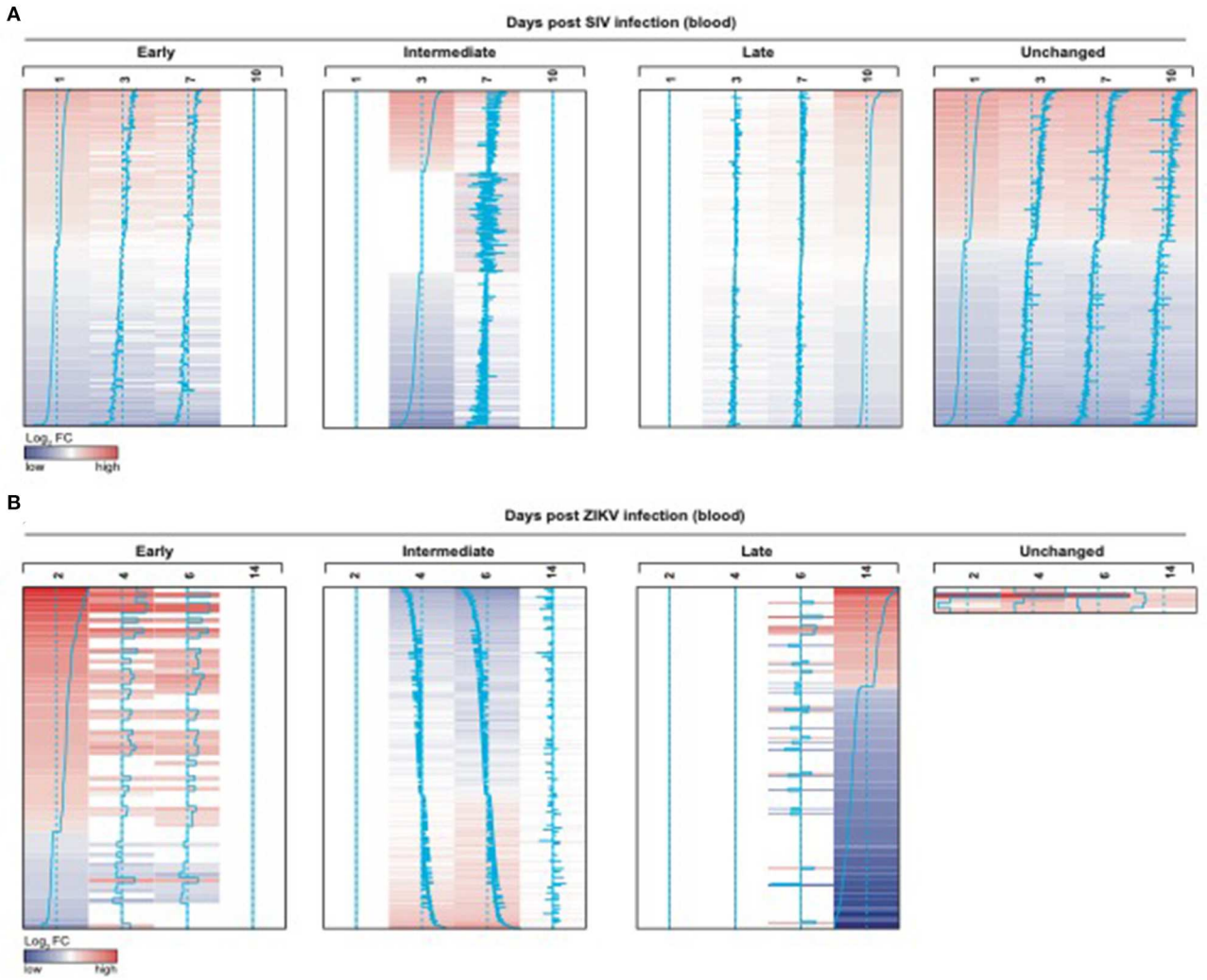


FIGURE 8 | NK RMtsig is modulated following SIV and ZIKV infections in blood from two independent NHP cohorts. **(A,B)** NK RMtsig upregulated (red) or down regulated (blue) genes following SIV infection **(A)** on days 1, 3, 7 and 10 (post-infection) compared to day 0 (before infection) and following ZIKV infection **(B)** on days 2, 4, 6 and 14 (post-infection) compared to day 0 (before infection). Heatmaps represent the $\log_2$ fold change expression of all significant NK RMtsig genes (Adjusted $p < 0.05$) compared to day 0. Genes were grouped using their expression profiles into 4 clusters: early (day 1 or 2), intermediate (days 3, 4, 6, or 7), late (day 10 or 14) or unchanged.

Supplementary Table 5). Together, these results suggest that our NK cell signature RMtsig could be applied to a wide range of infection models in a highly sensitive manner.

DISCUSSION

NHP SIV infection models provide an unmatched resource for modeling HIV/AIDS and have been critical to the development of many therapeutic inteventions and vaccine strategies that are currently under development or in use. Because NK cells play a crucial role in regulating viral infections, and the interest in exploiting them for immunotherapeutics, it is important to understand how NK cells respond to viral infections specifically in NHP. Surprisingly, despite the significant work carried out to characterize cellular responses by NK cells, currently there is no substantial unbiased characterization of the NK cell transcriptome in NHP. Prior to this work, NK cell transcriptional profiles have been assessed via RT-PCR or Fluidigm technologies which rely on pre-identified genes of interest (Hong et al., 2013). As such, we set out to first define the identity of an NK cell transcriptomic profile in NHP using RNA-Seq from bulk sorted NK cells to provide an unbiased assessment of the NK cell transcriptome in rhesus macaques. We sorted and performed RNA-Seq on NK cells from experimentally-naïve rhesus macaques in order to minimize animal-to-animal variability. After processing the raw RNA-Seq data, we implemented a statistical method to assign a desirability score for each gene by selecting the

most highly expressed genes across all animals in order to minimize any confounding individual-specific responses. This was confirmed via the Spearman rank correlation analysis in **Figure 1B**, which shows that indeed, there was a low gene expression variability in the NK RMtsig. While it is possible that our selection criteria for determining the genes included in RMtsig may exclude relevant NK cell genes, we decided to use stringent criteria in order to minimize any animal-specific variability and to maximize inclusion of true, NK-specific genes. Nevertheless we provide the full list of genes in the supplemental data along with their desirability scores to serve as a repository for other investigators to assess their genes of interests, if they are not present in RMtsig. We suggest that in some specific setups and experimental designs, where the overall gene expression levels are low, NK genes with low desirabliry scores could also be of interest.

In order to increase confidence that our sorting strategy and mRNA preparation approach was appropriate to characterize the rhesus macaque NK cell transcriptomic signature we decided to compare our signature with several publicly available, NK-specific datasets from human samples (**Figures 1C,D**, **2**). Our analysis revealed that well-established NK cell markers and NK cell signaling pathways were enriched within the NK RMtsig. Through these comparisons we were able to identify genes shared between NK cells in human and NHP, though there are some caveats that must be considered—including differences in cell and RNA preparation, use of different sequencing platforms, and the markers used to sort for NK cells in all these studies. While we did not observe a complete similarity, we were able to identify significant overlap between our NK RMtsig and NK cell signatures identified in the various human studies (Nakaya et al., 2011; Newman et al., 2015; Costanzo et al., 2018; Yang et al., 2019). For instance, in the LM22 study, Newman and colleagues (Newman et al., 2015) defined a molecular signature consisting of 547 genes that distinguish 22 mature human hematopoietic populations isolated from PBMCs from healthy adults including NK cells ($CXCR3^+CD16^+CD56^+$). While there was substantial overlap between the LM22 NK signature and NK RMtsig, there were 25 genes specific to the LM22 NK signature, including *KIR3DL2*, *KIR2DL1*, *KIR2DS4*, and *KLK1*. These markers were either not expressed in our study (cpm=0 or less than the selected significance cut-off) or were highly variable across all animals. The variable gene expression is also partly unsurprising given that the expression of KIR family members has been shown to be extremely variable across individual macaques (Walter and Ansari, 2015). Interestingly, the top overlapping genes between NK RMtsig and all human NK cell signatures include major NK cell markers and signaling networks (**Supplementary Table 3**), suggesting that our RMtsig is an appropriate signature for rhesus macaque NK cells.

Through this work we identified several signaling mechanisms consistent with NK cell immune responses as evidenced through the enrichment of several cytokines and chemokines, interferon and pathogen sensing pathways (**Figures 3**, **5**). We also observed an enrichment of metabolism, protein secretion, synapse formation and cell survival pathways that support an ability for rapid cellular responses, consistent with perceived NK cell functions (**Figure 5**). Furthermore, several key transcription factors networks that seem to contribute toward the regulation of many of the identified signaling pathways were enriched in our NK RMtsig (**Figure 6**). It would be interesting to follow these gene networks over the course of an infection in order to determine whether the presence of viral infections such as HIV or SIV lead to modulation of these key networks, especially during acute infection, where it appears that NK cells are among the earliest immune cells to respond.

Overall in this work we provide the first transcriptomic characterization of NK cells in rhesus macaques, and the first in a non-human primate species. As a result this RMtsig can now be used to interrogate pre-existing datasets even from unfractionated cells. Indeed, we observed that NK RMtsig genes were up or down regulated following intravaginal SIVmac251 infection in blood, LN and FRT tissues and following ZIKV infection in blood in two independent NHP cohorts (**Figures 7**, **8**, **Supplementary Figures 4, 5**). We anticipate that using RMtsig will provide insight into NK cell responses to disease or future therapeutic interventions through the modulation of the NK RMtsig in unfractionated or NK cell-sorted samples. RMtsig will also serve as a reference point for a "normal" NK cell transcription profile in rhesus macaques. Collectively, this work provides the basis for future NHP infection and disease studies to understand the role of NK cells, and may provide critical insights into informing future human studies of NK cell responses during infection or disease states.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI GEO, accession GSE148290, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148290.

ETHICS STATEMENT

The animal study was reviewed and approved by Harvard Institutional Animal Care and Use Committee and Biomere Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

RR designed the project. DR processed the PBMCs, sorted NK cells and prepared the RNA for RNA-Seq. SB performed RNA-Seq and generated raw data. MA and DR analyzed the data. MA and DB generated and analyzed whole blood, lymph node and female reproductive tract transcriptomic data. MA, DR, and RR wrote the 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/fcimb.2020.00194/full#supplementary-material

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frontiers in Cellular and Infection Microbiology

MINI REVIEW
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NK Cell-Mediated Recall Responses: Memory-Like, Adaptive, or Antigen-Specific?

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Mounting experimental evidence hints to an import role for natural killer (NK) cells in adaptive immune responses to pathogens. NK cells with adaptive features are heterogeneous and belong to different subsets according to their phenotype as well as the nature of their adaptive recall reactions. Three types of adaptive NK cell responses have been described: (i) NK cells with long-lived memory of multiple different haptens and viral antigens were described in murine liver tissue with a possible human counterpart; (ii) infection of human and mouse cytomegalovirus is associated with an expansion of NKG2C$^+$ and Ly49H$^+$ NK cells, respectively, that selectively recognize CMV-encoded peptides thereby facilitating recall responses; (iii) cytokine-stimulated NK cells respond to different stimuli with enhanced production of IFN-γ after re-stimulation. These exciting findings not only support the idea of NK cells with adaptive features, but define a novel field of harnessing memory NK cell subsets for therapeutic strategies.

Keywords: memory NK cells, adaptive immunity, vaccine strategy, recall response, trained immunity

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INTRODUCTION

Immunological memory is a hallmark of adaptive immunity with leukocytes recognizing a previously encountered antigen and facilitating a specific and rapid immune response. Therefore, immunologic memory has the following basic characteristics: (i) enhanced response upon re-challenge to pathogens/antigens that have been encountered before and (ii) long-lived memory cells, that can persist even without the continuous presence of an antigen (Janeway et al., 2001). Immunological memory has been traditionally regarded as a unique feature of the two classical arms of the adaptive immune system, namely B cell-derived antibodies and T cells (Raff, 1973). T cells and B cells use recombinase–mediated recombination of variable gene segments (RAG) to generate a multitude of T and B cell antigen receptors (Bassing et al., 2002). Activation of the receptor by the appropriate antigen triggers clonal selection, differentiation and expansion of short-lived effector cells and long-lived memory cells. In contrast to B and T cells, all other leukocytes, including natural killer (NK) cells, do not express RAG proteins, have a limited repertoire of germline encoded receptors for the identification of target cells and are therefore considered as innate immune cells (Janeway and Medzhitov, 2002). The traditional view included that innate immune cells rapidly respond to pathogens, are critical in controlling viral infections and participate in tumor immunosurveillance in a non-specific fashion, but are unable to differentiate into memory cells (Lanier, 2005, 2007).

The paradigm of B and T cells as the sole carriers of immunological memory has been challenged by accumulating evidence indicating that NK cells respond to certain antigens with features of adaptive immunity. NK cell subsets mediating recall responses are heterogeneous with diverse functional properties. They differ in their phenotype and are currently described in the literature under various terms and concepts, such as adaptive, memory, memory-like and antigen-specific NK cells. In this review we summarize and categorize the current understanding of NK cells with adaptive features.

ANTIGEN-SPECIFIC NK CELL RESPONSES

The first evidence for NK cell-associated recall responses came from mice deficient in T cells and B cells developing antigen-specific immunological memory to haptens, small molecules that form immunogenic adducts with proteins (O'Leary et al., 2006). When mice lacking B and T cells were sensitized by applying the hapten to the skin, they still developed a measurable recall response within a few days. Re-challenge with the hapten that had been applied before induced ear swelling, while application of a different hapten to sensitized mice did not induce a contact hypersensitivity reaction, indicating antigen specificity of this phenomenon (O'Leary et al., 2006). Using antibody depletion, genetic alterations and adoptive transfer of purified NK cell subsets into naïve $RAG^{-/-}\gamma c^{-/-}$ recipients (which lack all endogenous lymphocytes, including NK cells), hapten-specific memory was identified in a subset of NK cells residing in the liver that was absent from the spleen (O'Leary et al., 2006).

So far, different groups provided accumulating evidence that murine NK cells have adaptive immune features against various structurally different haptenized proteins [2,4-dinitro-1-fluorobenzene (DNFB), 4-ethoxy-methylene-2-phenyl-3-oxazalin-5-one (oxazolone) and picryl chloride] (Peng et al., 2013) as well as viral [VSV, influenza, HIV (Paust et al., 2010a), vaccinia virus (Gillard et al., 2011)] and bacterial [Salmonella typhimurium (Kupz et al., 2013)] antigens (**Figure 1A**).

Searching for antigen-specific memory NK cells in other species, Reeves et al. gave evidence for long-lived antigen-specific splenic and hepatic NK cells in SIV-infected rhesus macaques that lysed Gag- and Env-pulsed autologous dendritic cells compared to uninfected macaques. Blocking of the inhibitory NKG2A and activating NKG2C receptors significantly reduced antigen-specific killing, which suggested that these receptors play a role in antigen-specific target cell lysis. However, the contribution of NK cell receptors to antigen-specific responses needs to be clarified. NK cells from vaccinated animals demonstrated antigen-specific recall responses up to 5 years after vaccination (Reeves et al., 2015), indicating that antigen-specificity of NK cells might be relevant in primate species including humans.

Previous studies have long suggested that NK cell responses in humans might be antigen-specific. In HIV-1-exposed seronegative persons, increased NK cell anti-viral functions have been associated with protection (Scott-Algara et al., 2003; Ravet et al., 2007). In addition, potent NK cell responses in uninfected infants of HIV-1-positive mothers were linked with blockage of transmission *in utero* (Tiemessen et al., 2009), which hinted to pre-sensitization to the virus.

Subsequently, phenotypic analyses of human hepatic NK cells were suggestive for NK cell subsets similar to liver-derived NK cells in earlier animal studies (Marquardt et al., 2015; Stegmann et al., 2016). NK cells account for ∼30–40% of all lymphocytes in human livers compared to relatively low NK cells frequencies in the peripheral blood (5–15%) which could be indicatory for a pool of a tissue-resident NK cell subset (Doherty et al., 1999). Human liver-resident NK cells are phenotypically different to blood-derived NK cells with increased expression of the subunit CD49a of the α1β1 integrin receptor. Hepatic $CD49a^{+}$ NK cells resemble an immature phenotype with high expression of CD56, and low-to-absent expression of CD16 and CD57 (Marquardt et al., 2015; Stegmann et al., 2016). This is in contrast to the majority of blood NK cells that are characterized as $CD56^{dim}$, $CD57^{+}$, $CD16^{+}$, Killer Ig-Like Receptor $(KIR)^{+}$ cells and lack CD49a (Bjorkstrom et al., 2010). The heterogeneity of NK cell subsets is also reflected by the expression of T-box transcription factor (T-bet) and Eomesodermin (Eomes). Both transcription factors are crucial for specific developmental stages of NK cells (Gordon et al., 2012; Collins et al., 2017). NK cells isolated from human peripheral mononuclear cells are $T\text{-}bet^{hi}$ and $Eomes^{low}$ in spite of hepatic NK cells expressing low levels of T-bet (Knox et al., 2014; Stegmann et al., 2016). However, their function could not be directly linked to memory until a recent study demonstrated antigen-specific recall responses of NK cells in a humanized mouse model. These NK cells exhibit a phenotype similar to memory NK cells in blisters of individuals after re-exposure with peptides of varicella zoster virus (Nikzad et al., 2019). The observations of this study support mouse models of antigen re-challenge suggesting liver-resident NK cells to be capable to elicit antigen-specific recall responses in effector sites such as the skin. According to a recently published study human blood-derived NK cells exhibit antigen-specific cytotoxicity upon vaccination against or infection with hepatitis B (Wijaya et al., 2020). However, it is unclear whether (i) there is a well-defined subset of NK cells that is distinct in function and phenotype and (ii) this NK cell subset originates in the liver and appears in the blood stream *en route* to effector sites, as proposed previously (Paust et al., 2010b). Among NK cell lineages, liver-resident and skin-infiltrating NK cells appear to be highly related (Sojka et al., 2014). If liver NK cells differentiate from circulating precursor or have the ability to maintain and proliferate on site from progenitors that seeded in embryogenesis still needs to be proven (Peng et al., 2013; Cuff et al., 2016). Certain chemokine receptors have been shown to be important for homeostasis of hepatic NK cells. CXCR6 and CCR5 are mostly found on human liver NK cells and are largely absent from peripheral NK cells (Hudspeth et al., 2016; Stegmann et al., 2016). The corresponding ligands CXCL16, CCL3, and CCL5 are highly expressed by Kupffer cells, T cells, NK cells and endothelial cells on liver sinusoids (Heydtmann et al., 2005; Hudspeth et al., 2016). Additionally, hepatic $CD56^{bright}$ NK cells can migrate in response to CCL3, CCL5, and CXCL16 (Hudspeth et al., 2016). However, direct proof of a liver-effector site axis and the molecular

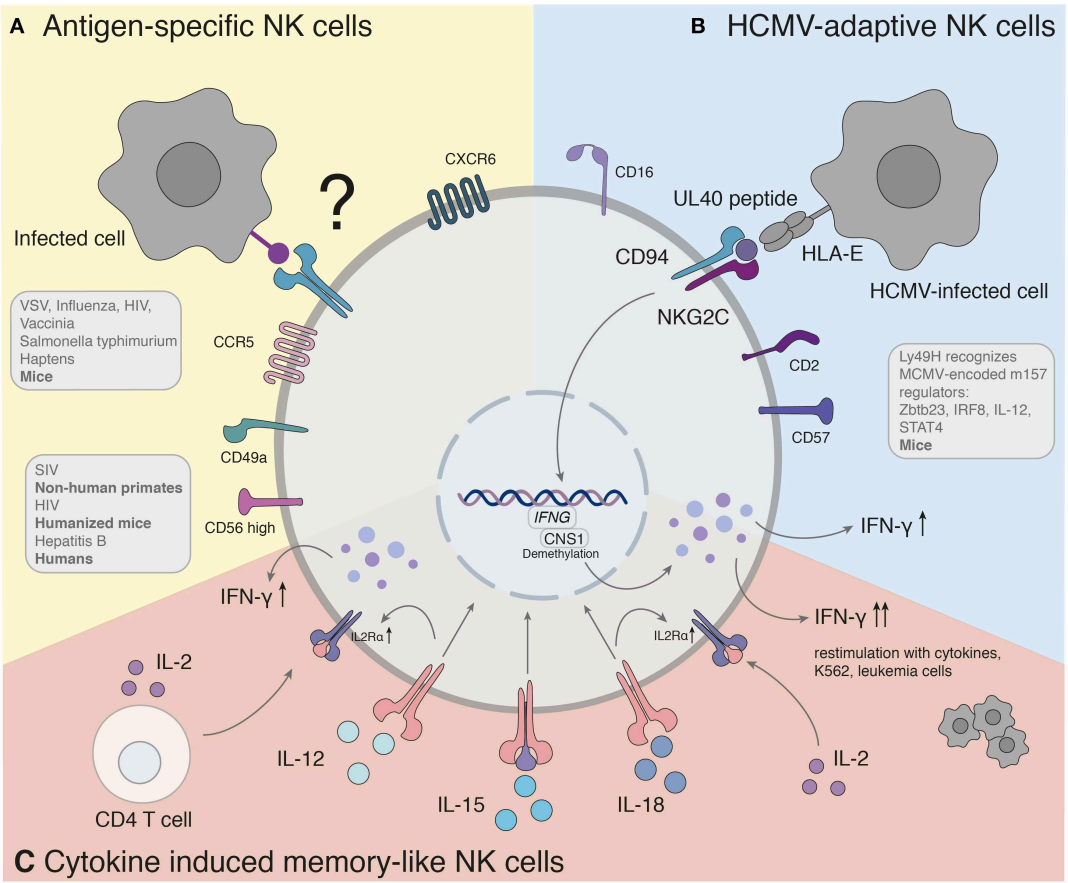

FIGURE 1 | Antigen-specific recall responses of NK cell subsets. **(A)** Studies hint to the presence of a human counterpart of antigen-specific NK cells. The molecular mechanism of antigen-specific NK cells has yet to be found. **(B)** Adaptive NK cells in response to HCMV recognize variable UL40 peptides of different HCMV strains through NKG2C/CD94. **(C)** Cytokine induced memory-like NK cells activated with IL-12, IL-15, and IL-18 produce significantly more IFN-γ when re-stimulated with a non-specific stimulus. IFNG, Interferon-γ; CNS1, conserved non-coding sequence 1.

mechanism of recognition of various antigens remain to be identified.

ADAPTIVE NK CELLS IN CMV INFECTION

Recognition of target cells by NK cells is regulated through a variety of activating and inhibitory receptors. Ly49H is responsible for direct recall responses and subsequent resistance of mouse cytomegalovirus infection (MCMV) in certain strains, including C57BL/6 mice. As an activating receptor, Ly49H can engage the MCMV-encoded cell-surface glycoprotein m157 (Brown et al., 2001; Arase et al., 2002; Smith et al., 2002) (**Figure 1B**). Upon infection with MCMV, Ly49H$^+$ NK cells undergo rapid clonal proliferation followed by a contraction phase, which was not observed in other viral infections (Daniels et al., 2001; Dokun et al., 2001). Adoptive transfer of Ly49H$^+$ NK cells 50 days after infection was capable to induce a robust secondary expansion und enhanced effector function upon re-challenge in naïve mice (Sun et al., 2009). The transcription factor Zbtb23 is upregulated in NK cells upon MCMV infection and crucial as regulator for the proliferation machinery of MCMV-adaptive NK cells by antagonizing the anti-proliferative factor Blimp-1 (Beaulieu et al., 2014). IL-12 receptor-deficient NK cells fail to expand after MCMV infection and do not mediate protection after re-challenge (Sun et al., 2012), indicating that proinflammatory cytokine signaling is required for the generation of adaptive NK cells toward MCMV. Subsequently, IL-12 and STAT4 promote epigenetic remodeling of the IRF8 locus and therefore upregulation of IRF8. Furthermore, IRF8 is associated with the proliferative burst of adaptive NK cells (Adams et al., 2018).

NKG2C is the human counterpart of the murine Ly49H (Sun et al., 2011) (**Figure 1B**). The infection of human cytomegalovirus is associated with an increased expression of HLA-E binding and activating the NK cell receptor NKG2C (Guma et al., 2004). An expansion of NKG2C$^+$ NK cells was observed during CMV infection (Guma et al., 2006a), similar to the response of Ly49H$^+$ NK cells in MCMV. NKG2C$^+$ NK cells of CMV-seropositive donors persist at higher frequencies and exhibit enhanced activity in response to CMV reactivation compared with NKG2C$^+$ NK cells from seronegative donors

(Lopez-Verges et al., 2011; Foley et al., 2012). In the case report of a 3 year-old patient with $T^-B^+NK^+$ severe combined immunodeficiency (SCID), $NKG2C^+$ NK cells were the sole provider of protective immunity against CMV after significant expansion of IFN-γ-producing $CD16^+CD94^-NKG2C^+$ NK cells (Kuijpers et al., 2008). NK cells with adaptive features to HCMV have been furthermore identified in peripheral blood, liver and lung of seropositive individuals (Guma et al., 2004; Beziat et al., 2012; Marquardt et al., 2015, 2019). Notably, a similar expansion of $NKG2C^+$ NK cells in HCMV-seropositive individuals has also been observed during infections with other pathogens, such as the Hantavirus (Bjorkstrom et al., 2011), HIV (Guma et al., 2006b), EBV (Saghafian-Hedengren et al., 2013) and malaria (Hart et al., 2019).

Besides NKG2C expression, HCMV-adaptive NK cells display a distinct receptor profile with a more mature and differentiated phenotype, including upregulation of CD57 and decreased expression of the inhibitory receptor NKG2A. This subset also expresses killer cell immunoglobin-like receptor (KIR) specific for self-MHC (Guma et al., 2004; Beziat et al., 2012; Schlums et al., 2015). Recent studies reveal stable alterations of transcriptional programs of HMCV-adaptive NK cells. This includes epigenetic imprint of the INFG locus, which drives IFN-γ expression by HCMV-adaptive NK cells (Luetke-Eversloh et al., 2014). In a cohort of 196 healthy adults, HCMV seropositivity was correlated with a lack of FcεRγ, SYK, and EAT-2 expression in $CD56^{dim}$ NK cells of the peripheral blood which correlated with a downregulation of the transcription factor ZBTB16 (Zinc Finger and BTB Domain Containing 16). ZBTB16 was shown to bind the promotors of these genes (Schlums et al., 2015). Preferential expansion upon HCMV was largely confined to FcRγ-deficient NK cells in an antibody-dependent fashion (Lee et al., 2015). The mechanism of HCMV-adaptive $NKG2C^+$ NK cells in the blood of patients with a history of HCMV infection was ultimately proven to depend on the recognition of UL40 encoded HCMV peptides, which stabilize and load on HLA-E by NKG2C (Hammer et al., 2018; Rolle et al., 2018). Interestingly, $NKG2C^+$ NK cells were able to differentially discriminate HCMV strains encoding for variable UL40 peptides.

There is emerging interest in the metabolic regulation of NK cell cytotoxicity in viral infections including adaptive immune functions. In general, naïve NK cells rely on glucose fueled by oxidative phosphorylation (Gardiner, 2019). Inhibition of glycolysis, however, decreases the clearance of MCMV-infected murine target cells by $Ly49H^+$ NK cells indicating that NK cells require glycolysis for cytotoxicity during viral infection (Mah et al., 2017). Studies with KIR^+ educated blood-derived NK cells showed increased rates of glycolysis compared to uneducated NK cells even in a resting state (Pfeifer et al., 2018; Schafer et al., 2019). It was furthermore reported that $CD56^{bright}$ NK cells express high levels of the glucose uptake receptor Glut1 and have therefore increased rates of glucose intake compared with $CD56^{dim}$ NK cells (Keating et al., 2016). The elevated glycolytic metabolism of $CD56^{bright}$ NK cells would consequently support the energy demands of increased interferon-gamma (IFN-γ) production in the event of an immune response. In a cohort of HCMV seropositive individuals, $NKG2C^+CD57^+$ NK cells exhibited increased oxidative and glycolytic metabolic profiles compared to seronegative donors. Moreover, this NK cell subset of HCMV-experienced individuals also demonstrated enhanced mitochondrial membrane potential, higher expression of genes associated with the mitochondrial ATP synthase production and electron transport chain (Cichocki et al., 2018).

However, studies give hints to the existence of NKG2C-independent pathways of HCMV-adaptive NK cell differentiation (Muntasell et al., 2016) or emphasize a contribution of certain activating KIRs in addition to NKG2C (Beziat et al., 2012). Della Chiesa et al. studied patients with hematological malignancies after umbilical cord blood transplantation of donors carrying a homozygous deletion of the NKG2C gene. Although NKG2C was missing, they described an expansion of $CD56^{dim}NKG2A^-KIR^+$ NK cells in response to CMV infection (Della Chiesa et al., 2014). These reports may be an indicator of possibly different mechanism other than NKG2C being able to facilitate recall responses against CMV.

CYTOKINE-INDUCED MEMORY-LIKE NK CELLS

In 2009, Cooper et al. demonstrated that murine NK cells activated with cytokines produce significantly more IFN-γ when re-stimulated after up to 22 days of prior activation with IL-12, IL-15, and IL-18. Pre-activated NK cells elicit an enhanced effector function against tumor cell lines. It was further shown that memory-like "trained" features are passed on to the next non-activated generation of NK cells (Cooper et al., 2009). Since the observed adaptive responses arise as a result of non-specific activation, the term cytokine-induced memory-like (CIML) NK cell was formed. CIML NK cells display some hallmarks of adaptive immunity, as they are long-lived, exhibit enhanced anti-tumor as well as anti-viral immune responses, and have certain epigenetic modifications.

Several features of CIML NK cells hold true for humans (summarized in **Figure 1C**). Analogous to NK cells of mice, human NK cells activated with cytokines were described to increase IFN-γ production after re-stimulation with cytokines, K562, or leukemia cells (Romee et al., 2012, 2016). The mechanisms underlying the effect of these cytokines are incompletely understood. Studies of the contributions of each cytokine (IL-12, IL-15, and IL-18) demonstrated a synergistic effect in degranulation, IFN-γ, TNF-α, and CCL3 production. At the same time IL-15 alone or in combination exhibited the highest cytotoxicity against K562 (Terren et al., 2018). The NK cell intrinsic ability for IFN-γ production coincided with demethylation of the conserved non-coding sequence (CNS) 1 in the *Ifng* locus, enhancing transcription of *Ifng* and was additionally maintained through IL-2 production of $CD4^+$ T cells (Ni et al., 2016). The enhanced NK cell proliferation and cytotoxicity of CIML NK cells arose due to a prolonged expression of IL2Rα (CD25) resulting in a high responsiveness of IL-2 receptor stimulation (Leong et al., 2014). In addition, IL-12 and IL-18 were shown to upregulate the IL2Rα chain sensitizing NK cells to IL-2 stimulation (Lee et al., 2012). In NK cells

the upregulation of the mammalian target rapamycin complex 1 (mTORC1) pathway was associated with the acquisition of NK cell effector functions (Donnelly et al., 2014). IL-15 and IL-18 can activate mTORC1, which plays a key role for glycolytic reprogramming and facilitated upregulation of glycolytic enzymes in NK cells (Marcais et al., 2014; Almutairi et al., 2019).

These features of CIML NK cells represent a potential approach for translational immunotherapy. CIML NK cells promoted enhanced anti-tumor activity after adoptive transfer in a melanoma xenograft model (Ni et al., 2016). Adoptive transfer of donor NK cells demonstrated to mediate graft-vs.-leukemia effects while suppressing acute graft-vs.- host disease in a murine model of allogeneic hemopoietic stem cell transplantation (Song et al., 2018). Results from a first-in-human phase 1 clinical trial with adoptively transferred CIML NK cells exhibited increased effector functions against leukemia targets leading to favorable clinical responses and remissions in a subset of AML patients (Romee et al., 2016).

CONCLUSION

The findings summarized in this review suggest that NK cells are able to fundamentally change the way they respond to later activation, resulting in features which, in certain subsets, fulfill the requirements of immunologic memory or, at least, a trained immune response. The discovery of adaptive NK cells gives rise to many questions. For instance, what are the precise mechanism of development of different subsets of NK cells? What molecular pathways underlie the specificity to a variety of antigens? Which factors drive NK cell activation, proliferation and generation of immunological memory? How are long-lived memory NK cells maintained? The paradigm shift of NK cells from classical innate killers to sophisticated immune cells possessing attributes of both entities has just begun. Further investigations into NK cell memory will have major implications in vaccination or immunotherapeutic approaches.

AUTHOR CONTRIBUTIONS

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Targeting Natural Killer Cells for Improved Immunity and Control of the Adaptive Immune Response

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Natural killer (NK) cells are critical for targeting and killing tumor, virus-infected and stressed cells as a member of the innate immune system. Recently, NK cells have also emerged as key regulators of adaptive immunity and have become a prominent therapeutic target for cancer immunotherapy and infection control. NK cells display a diverse array of phenotypes and function. Determining how NK cells develop and are regulated is critical for understanding their role in both innate and adaptive immunity. In this review we discuss current research approaches into NK cell adaptive immunity and how these cells are being harnessed for improving cancer and vaccination outcomes.

Keywords: natural killer (Nk) cell, adaptive immune cells, NK cell therapy, vaccine, innate and adaptive immune response

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NK CELL CELLS ARE INNATE IMMUNE KILLERS

Natural Killer (NK) cells are cytotoxic granular lymphoid cells that develop from a common progenitor of B and T cells (Kondo et al., 1997; Abel et al., 2018). NK cells have the innate ability to recognize both virally infected and tumor cells, play a key role in tumor clearing (Rosenau and Moon, 1961; Smith, 1966; Herberman et al., 1975; Kiessling et al., 1975; Yang et al., 2006), and the primary immunological response to viral infection (Biron et al., 1999; Vidal et al., 2011). When cytotoxic NK cells are activated, they release cytolytic granules and secrete inflammatory cytokines and chemokines that activate and recruit components of both the innate and adaptive immune response (Iannello and Raulet, 2013).

NK cell activation is governed by the ligand-receptor interactions of the activating and inhibitory receptors expressed on the NK cell surface (Tassi et al., 2006; Lanier, 2008; Bryceson et al., 2011). The balance of activating and inhibitory signals controls NK cell activation and function (MacFarlane and Campbell, 2006). NK cell activating receptors have well documented interactions with both viral (Alsheikhly et al., 1985; Mandelboim et al., 2001; Jarahian et al., 2009) and tumor derived ligands (Sivori et al., 1997; Vitale et al., 1998; Pende et al., 1999). NK cells also have killer cell immunoglobulin-like receptors (KIRs) that are vital to the normal function of NK cells and are critical for the education of NK cells. It is through these receptors that NK cells learn tolerance of self through HLA-I molecules, which serve as ligands to inhibitory KIRs (Ljunggren and Karre, 1990; Campbell and Purdy, 2011). Diversity of KIR genotypes among individuals that contribute to KIR-HLA interactions have implications for NK cell function and response against tumors and viruses.

NK CELLS WITH ADAPTIVE IMMUNE CELL PROPERTIES

Classically, NK cells are regarded as members of the innate immune system, but recent studies have elucidated that NK cells can display both adaptive and memory-like phenotypes. Antigen-specific NK cell memory was first described in T and B cell deficient mice displaying hapten-specific contact hypersensitivity (CHS) in skin cells after adoptive transfer of NK cells from a previously sensitized donor (O'Leary et al., 2006).

In addition to NK cell memory against haptens, it was discovered that murine NK cell receptor Ly49H showed specificity for MCMV-derived m157 expressed in mice (Daniels et al., 2001; Lee et al., 2001; Arase et al., 2002). This interaction between host Ly49H and virally-derived m157 elicits the clonal expansion of MCMV-activated NK cells, as well as the persistence of memory NK cells that possess increased responsiveness to m157 (Dokun et al., 2001; Bubic et al., 2004; Sun et al., 2009). This was an example of NK cell memory that was defined by specific NK cell receptor recognition of viral antigens. Additional adoptive transfer studies in mice have revealed that liver-resident NK cells have responded to several other pathogens including HSV-2 (Abdul-Careem et al., 2012), Vaccinia virus (Gillard et al., 2011) and Influenza A (IAV) (Li et al., 2017). Both the hapten and MCMV murine models demonstrated the specific recognition of foreign antigens by NK cells that contributed to a memory-like recall response but did not demonstrate if this occurred in humans and the extent NK cell memory contributed to virus control.

NK cell adaptive response can also be mounted by stress signals expressed by infected host cells (**Figure 1A**). In humans, hantavirus-infected endothelial cells have been shown to upregulate HLA-E, a ligand for NK cell activating receptor NKG2C, subsequently resulting in the expansion of NKG2C+ NK cells, and the persistence of this subset up to 2 months post infection (Bjorkstrom et al., 2011). Similarly, HCMV infection of peripheral blood cells and fibroblast cells elicits expansion of NKG2C+CD57+CD56dimCD16+ circulating NK cells in humans in acute infection models (Beziat et al., 2013; Newhook et al., 2017).

Simian-immunodeficiency virus (SIV) vaccination and infection models in rhesus macaques have provided evidence that hepatic and splenic NK cells had the capacity to specifically target and kill SIV Gag and Env-specific dendritic cells (DC), and that this killing was NKG2C-dependent (Reeves et al., 2015). Recently published data by Nikzad et al. demonstrated that human liver-resident NK cells in humanized BLT mice displayed antigen-specific killing *in vitro* against HIV Env-loaded DC's 14 days post vaccination with recombinant HIV Env (Nikzad et al., 2019). Moreover, they demonstrated that human NK cell memory is long-lived in humans. Individuals that had Varicella Zoster Virus (VSV) infection in their youth were injected with a VSV-STA vaccine and had a significantly higher percentage of degranulating NK cells localizing at the site of injection, compared to controls. Another study demonstrated NK cell memory in Hepatitis B virus infection and vaccination (Wijaya et al., 2020). These findings provide much-needed evidence that antigen-dependent memory NK cells may be induced in humans, and that NK cell memory might have the potential to persist decades after initial sensitization.

CYTOKINE-INDUCED MEMORY-LIKE NK CELLS

NK cells can undergo differentiation into memory-like effectors once exposed to various cytokines such as IL-12, IL-15, and IL-18 (**Figure 1A**). These cytokine-induced memory-like (CIML) NK cells display higher IFN-γ secretion upon re-challenge compared to their naïve counterparts, and has been demonstrated in both mice and humans (Cooper et al., 2009; Romee et al., 2012; Keppel et al., 2013; Berrien-Elliott et al., 2015). CIML NK cells may also be defined by up-regulation of CD25 (Leong et al., 2014), as well as complete demethylation of IFN-γ promoter regions and other epigenetic changes (Lee et al., 2015; Wiencke et al., 2016). Indeed, IFN-γ promoter region demethylation of NK cells is also observed in the expanding NKG2C+ NK cells of HCMV-infected individuals, independent of the presence cytokine treatment (Luetke-Eversloh et al., 2014; Schlums et al., 2015). This similarity might imply that CIML expansion and persistence might depend on HCMV infection and/or NKG2C+ expansion, and that CIML phenotypes can be evoked independent of cytokine treatment (Goodier et al., 2016). One key difference in HCMV-expanded NKG2C+ NK cells is that *in vitro* or vaccine-induced CIML NK cells have been associated with expansion of less differentiated NK cells. CIML NK cells have been a key player in recent developments in cancer immunotherapy and have shown enhanced killing against a variety of cancer cell lines *in vitro,* including leukemia and ovarian cancer (Romee et al., 2012, 2016; Uppendahl et al., 2019). More recently, Romee et al. demonstrated enhanced killing of leukemic targets after adoptive CIML transfer into patients with acute myeloid leukemia (AML) and have conducted a clinical trial evaluating the safety of ALT-803—an IL-15 super agonist complex that activates NK cell and CD8 T cell function—in patients with hematologic malignancies who had suffered a relapse post-Hematopoietic cell transplant (HCT) (Romee et al., 2016, 2018). Another ongoing clinical trial aims at evaluating the efficacy of adoptively transferred CIML NK cells in relapsed AML patients after HCT (NCT03068819). Future studies optimizing the *ex-vivo* generation of CIML NK cells for immunotherapy of cancer as well as determining if CIML NK cells can be generated *in vivo* through a vaccine, adjuvant, or other cytokine-stimulating molecule will be necessary to further advance this area of research in the clinic.

NK CELLS INFLUENCE ADAPTIVE IMMUNITY THROUGH REGULATION OF T AND B CELLS

NK cells and B cells have long been known to associate, given that NK cells mediate antibody-dependent cellular cytotoxicity

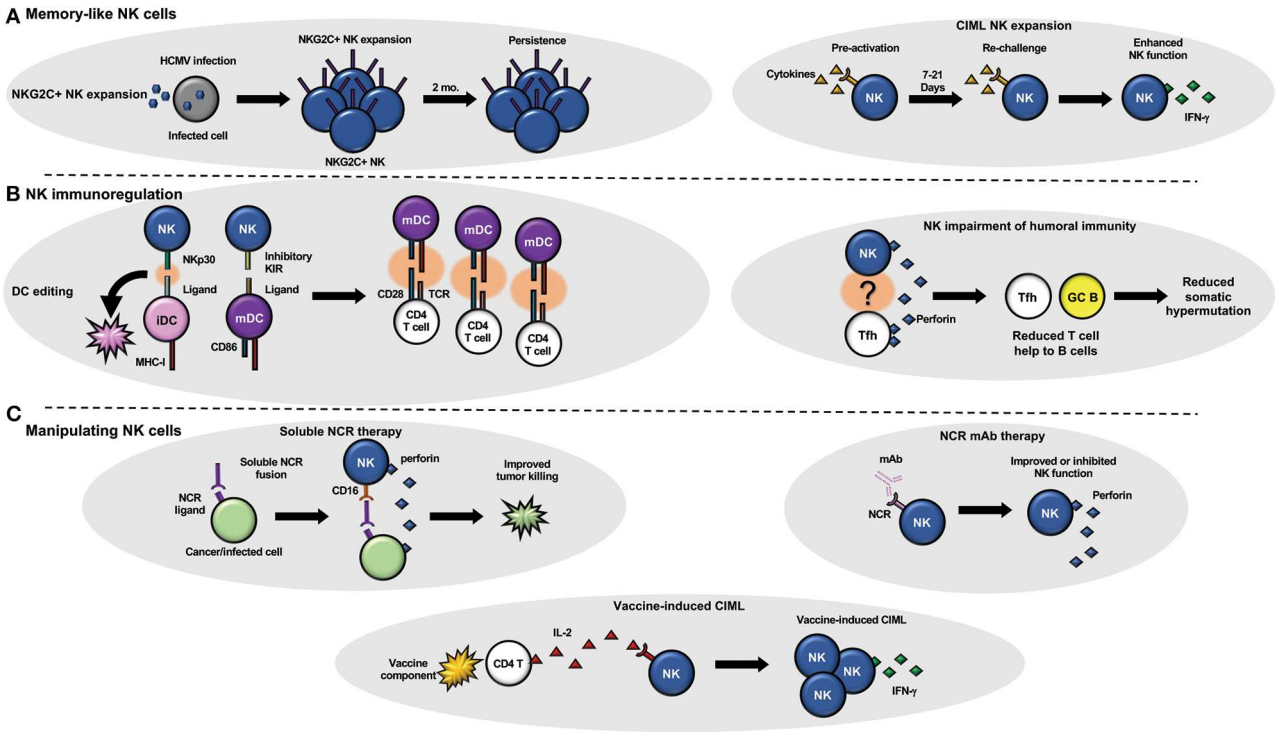

FIGURE 1 | NK cells and adaptive immunity. **(A)** NK cells can develop memory-like attributes in response to infection (left) or cytokine stimulation (right). **(B)** NK cells have been shown to regulate adaptive immunity by targeting dendritic cells (left) that can change the quality of the T cell response and T follicular helper cells (right) that can regulate B cell maturation (somatic hypermutation) and function. **(C)** Therapeutic manipulation of NK cell function using soluble NK cell receptors that target tumor or viral infected cells to improve NK cell targeting (top left) or monoclonal antibodies that block or stimulate NK cell receptors (top right) to modulate NK cell function are under current development. Vaccine components such as adjuvants (bottom) could also be utilized to generate CIML NK cells *in vivo* with a vaccine.

(ADCC) through the NK cell Fc receptor, CD16. Recent evidence suggests that NK cells impact B cell affinity maturation and immune function (**Figure 1B**). Recent reports by Rydyznski et al. have elucidated that murine NK cells impair humoral immunity through the inhibition of CD4 T follicular helper (Tfh) and germinal center (GC) B cell expansion and function (Rydyznski and Waggoner, 2015; Rydyznski et al., 2015, 2018). Using an NP-KLH (4-hydroxy-3-nitrophenylacetyl; keyhole limpet hemocyanin) conjugate model for immunization in mice, they demonstrated that NK cell-depleted mice, compared to control mice, had higher Tfh and GC B cell populations, greater expansion of splenic germinal centers, and an increase in the production of NP-specific antibodies that displayed higher affinities for NP following immunization. NK cell impairment of B cell affinity maturation in mice was shown to occur in a perforin-dependent manner, as perforin-deficient mice displayed a similar level of affinity maturation as NK cell depleted mice did (Rydyznski et al., 2018). Other studies have shown that NK cells directly activate B cell IgG and IgM production, as well as facilitate immunoglobulin class-switching and can control HIV-1 neutralizing antibody responses (Snapper et al., 1994; Gao et al., 2008; Bradley et al., 2018). Conversely, NK cells have also been shown to have inhibitory roles in B cell function. Poly:IC injection in mice inhibited IgM primary response, via NK cell activation (Abruzzo and Rowley, 1983). T-cell dependent (IL-2) NK cell activation has also been shown to have negative outcomes for antibody production after EBV and pokeweed mitogen stimulation (Rydyznski and Waggoner, 2015). In human NK cell-B cell co-culture experiments, NK cells have been shown to activate B cell antibody production via TNFα (Becker et al., 1990) and CD40-CD40 ligand interactions (Blanca et al., 2001).

Studies in humans and mice have revealed that NK cells indirectly influence the T cell repertoire via direct interaction with antigen presenting cells, most notably immature Dendritic Cells (iDC) and mature Dendritic Cells (mDC; **Figure 1B**). Human DC-NK cell cross-talk and subsequent activation of both cell types was first reported *in vitro*, where it was reported that DC-NK cell interaction enhances NK cell activation and DC maturation, with the former expressing IFN-γ, stimulating the latter to mature, secrete IL-12, and amplify expression of the co-stimulatory molecule CD86 (Gerosa et al., 2002). In subsequent studies, mDC-derived IL-12 was shown to enhance CD8 T cell responsiveness and activation (Mocikat et al., 2003; Adams et al., 2005). The tendency for NK cells to kill iDCs while sparing mDCs, termed "DC editing" is another example of indirect changes to T cell immunity modulated by NK cells (Morandi et al., 2012; Ferlazzo and Moretta, 2014). The elimination of iDCs is hypothesized to enhance T cell priming,

by decreasing competition between iDCs and mDCs which have the costimulatory molecules needed for T cell activation. In humans, this process is thought to be governed by ligand-receptor interactions of NKp30 ligands expressed on iDCs, and inhibitory KIR ligands expressed on mDCs (Ferlazzo et al., 2002).

During infection, NK cells target and kill infected host cells, which release antigen available for DC uptake. This enhancement of DC cross-presentation effectively improves cytotoxic T cell mediated immunity. After transfer of allogeneic B cells in mice, NK cell killing of the B cells resulted in apoptotic bodies taken up and presented by dendritic cells (Iyoda et al., 2002). NK cell killing of Ova-expressing splenocytes also resulted in release of antigen, leading to the enhancement of CD8 and CD4 T cell priming (Krebs et al., 2009). Activated murine NK cells are also capable of shaping T cell immunity directly. After activation, murine NK cells localize to the lymph nodes where they release IFN-γ, eliciting CD4+ T cell differentiation into the Th1 subtype (Martin-Fontecha et al., 2004).

NK CELL REGULATION OF T CELL IMMUNITY DURING VIRAL INFECTION

LCMV and MCMV infection studies in mice have produced variable results outlining the effect NK cells have on T cells during acute and chronic viral infection. Waggoner et al. demonstrated that NK cells targeted and killed CD4 T cells during LCMV infection in mice (Waggoner et al., 2011). However, other studies of LCMV infection have suggested that NK cells directly eliminated CD8 cells either through an NKG2D-dependent manner or another undefined mechanism during LCMV infection (Soderquest et al., 2011; Lang et al., 2012). Recently published data suggests that NK cells directly kill CD8 T cells during LCMV infection in mice, and that this killing is NCR-1 dependent (Pallmer et al., 2019). The presence of NK cells during LCMV infection in mice was reported to elicit T-cell exhaustion, and subsequently reduce both CD4 and CD8 T cell response to LCMV, and that NK cell depletion enhances T-cell mediated viral clearance (Cook and Whitmire, 2013). In chronic models of MCMV infection, TRAIL+ NK cells have been reported to target CD4 T cells in the salivary gland, which the authors suggest is to limit autoimmunity during chronic infection (Schuster et al., 2014). Other experiments showed that IL-10 secretion by NK cells during MCMV infection inhibited CD8 T cell response (Lee et al., 2009).

VACCINATING FOR MEMORY NK CELL GENERATION

Vaccines have historically relied on eliciting antigen-specific effector and memory B and T cells to protect against subsequent infection, but for challenging pathogens such as HIV-1 and TB, alternative strategies to boost immunity must be pursued.

CIML NK cell induction during vaccination has a clear advantage over antigen-specific NK cell memory, as it is not restricted to certain antigens. CIML NK cells have been proven to be elicited after immunization with several human vaccines, including TIV, YF-17D, and BCG (Marquardt et al., 2015; Goodier et al., 2016; Suliman et al., 2016; Darboe et al., 2017). IL-15 has been demonstrated to prime TIV-vaccinated human PBMC to produce innate myeloid cytokines, as well as generate CIML NK cells that have enhanced responsiveness to H3N2 influenza virus (Wagstaffe et al., 2019b). The persistence and functional significance of vaccine induced CIML NK cells during vaccination requires further investigation.

Although the recent findings of antigen-specific human NK cell memory are useful, there is a dearth of literature outlining how human NK cells mediate antigen-specific killing as well as how long human NK cell memory can persist *in vivo*. Findings on VZV-specific NK cell memory was limited by the fact that VZV-naïve individuals are rare, and thus were not available to be used as controls in the Nikzad et al. study. The HCMV-induced differentiation of CD56dimNKG2C+ into adaptive like NK cells was shown to occur via an epigenetic mechanism, however, it is not clear if all disease models that display NKG2C+ NK cell expansion go through the same epigenetic changes that HCMV infection elicits.

It is hypothesized that some models of vaccine-dependent, antigen-specific memory NK cells occur through genomic rearrangement, rather than the epigenetic mechanisms displayed in HCMV infection/host stress signal models. Several studies have elucidated the correlation between antigen-specific CD4 T cell derived IL-2 and improved NK cell response in a number of different vaccination models (**Figure 1C)** (Horowitz et al., 2012; Jost et al., 2014; Goodier et al., 2016). Thus, the mechanisms of adaptive NK cell memory generation must be studied on a pathogen-dependent basis, if they are to be implicated in vaccination.

ENHANCING CLASSICAL NK CELL EFFECTOR FUNCTIONS FOR BETTER VACCINATION OUTCOMES

A roadblock inhibiting vaccine-induced NK cell effector function is the limited understanding of how these processes occur in humans, and how these processes vary across different human vaccine models. To date, much of the work concerning NK cell effector function has centered around IAV and HIV models in mice and humans. Prophylactic and therapeutic vaccine trials need to investigate key NK cell effector functions—namely PAMP and myeloid cytokine-induced NK cell activation, DC editing, and NK cell ADCC induction.

Pathogen-associated molecular patterns (PAMPs) are often incorporated as adjuvants in vaccines (Miyaji et al., 2011). PAMP-induced NK cell activation has been correlated with overall vaccine immunogenicity (Feng et al., 2013; Martins et al., 2014). A recent report suggested that the presence of IFN-g derived from PAMP-induced activated human NK cells amplifies the pro-inflammatory cytokine profile of dendritic cells (Oth et al., 2018). Although these findings need to be further investigated in specific infection and vaccination models, they suggest that the presence of PAMPs in conjunction with IL-2, enhance DC editing, and thus could be a contributing factor to the enhanced

immunogenicity of PAMP-containing vaccines. Future efforts to determine which adjuvant or combinations of adjuvants that elicit superior NK cell function that results in improved protective adaptive immune responses should be considered for all vaccines to increase vaccine efficacy and durability.

Myeloid cell-derived cytokines (IL-12, IL-15, IL-18, IL-27) as well as T-cell derived IL-2 have all been documented to be involved with NK cell priming and activation. As described earlier, the IL-12, IL-15, IL-18 cytokine cocktail activated IFN-γ expression in NK cells during vaccination. A recent report has demonstrated that IL-27 promotes murine NK cell cytotoxicity and IFN-g production in an NKG2D-dependent manner during influenza infection (Kumar et al., 2019). Nanogram concentrations of IL-15 have been demonstrated to boost IL-12 production and boost human NK cell function after exposure to H3N2 *in vitro* (Wagstaffe et al., 2018). Many of these cytokines have been explored in terms of immunotherapy but have not been examined as extensively as adjuvants for vaccines against infectious disease. Influenza models would provide a convenient avenue to investigate the role that IL-27 plays in human models of influenza vaccination.

Improving NK cell ADCC via vaccination is also a key goal for Influenza, HIV-1, and other viruses (Hashimoto et al., 1983; Mielke et al., 2019). Recently, it was demonstrated that IL-15 is capable of improving ADCC-mediated killing against HIV-infected cells in seropositive donor plasma, and HVTN-100 vaccine trial (Fisher et al., 2019). In influenza-infected adults, ADCC antibodies specific to highly conserved viral proteins nucleoprotein (NP) and matrix 1 (M1) were found for both H1N1 and H5N2 strains of IAV (Vanderven et al., 2017). ADCC antibodies for M2, another highly conserved IAV protein, has been shown to elicit ADCC in mice, however human trials have not yet been conducted. Ebola-specific ADCC antibodies have been confirmed *in vitro* in human PBMCs and NK cell lines. Recently, it was reported that Ebola-specific ADCC is activated after various vaccination schedules of Adenovirus type 26.ZEBOV and modified vaccinia Ankara (MVA)–BN-Filo (Wagstaffe et al., 2019a).

Influenza and HIV remain persistent pathogens responsible for the deaths of millions every year. NK cell effector functions have been implicated in both disease models and should continue to be investigated. NK cells display direct recognition of influenza infected cells via interaction of NKp46 and hemagglutinin (HA). In mice, NK cells have been observed to localize to the lymph nodes during primary response to influenza vaccination and have ultimately been observed to regulate antibody production in an NK cell IFN-g and DC IL-6 dependent manner (Garcia et al., 2012; Chatziandreou et al., 2017; Farsakoglu et al., 2019). The exact mechanism behind the infection-induced IL-6 response remains unclear and should be investigated further in mice. The mechanisms by which NK cells regulate humoral immunity during influenza vaccination should be examined in mice and humans in order to investigate if an influenza vaccine with appropriate adjuvant combination can elicit a similar immune response as seen in the murine infection model. The positive effect that murine NK cells display during acute viral infection is interesting, given that chronic HIV infection models have suggested that functional NK cells play an inhibitory role in humoral immunity both in mice and humans—namely through inhibition of B cell maturation and decreased HIV-1 broadly neutralizing antibody production (Rydyznski et al., 2015, 2018; Bradley et al., 2018). In addition to cytokines and adjuvants, the use of soluble NK cell receptors and antibodies targeting activating or inhibitory NK cell receptors could be utilized to modulate NK cell function and influence the adaptive immune response during infection, autoimmunity and cancer (**Figure 1C**). The role that NK cells play in humoral immunity are dependent on the nature of an infection (acute vs. chronic) and the types of adaptive immune responses that are required to be protective.

CLOSING REMARKS

Here, we have summarized how NK cell phenotype and function can be manipulated to improve immunity to vaccines and cancer, and how NK cells can influence other arms of innate and adaptive immunity. Future efforts should attempt to elicit memory-like NK cell phenotypes, while enhancing innate NK cell effector functions. Limiting NK cell phenotypes that negatively impact the generation of protective immune responses must also be pursued. It should also be noted that future vaccination efforts should not seek to replace aspects of cell-mediated and humoral immunity with NK cell-mediated immunity but seek to modulate NK cell function in tandem with adaptive immunity.

AUTHOR CONTRIBUTIONS

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NK Cells Negatively Regulate CD8 T Cells to Promote Immune Exhaustion and Chronic *Toxoplasma gondii* Infection

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NK cells regulate CD4+ and CD8+ T cells in acute viral infection, vaccination, and the tumor microenvironment. NK cells also become exhausted in chronic activation settings. The mechanisms causing these ILC responses and their impact on adaptive immunity are unclear. CD8+ T cell exhaustion develops during chronic *Toxoplasma gondii* (*T. gondii*) infection resulting in parasite reactivation and death. How chronic *T. gondii* infection impacts the NK cell compartment is not known. We demonstrate that NK cells do not exhibit hallmarks of exhaustion. Their numbers are stable and they do not express high PD1 or LAG3. NK cell depletion with anti-NK1.1 is therapeutic and rescues chronic *T. gondii* infected mice from CD8+ T cell exhaustion dependent death, increases survival after lethal secondary challenge and alters cyst burdens in brain. Anti-NK1.1 treatment increased polyfunctional CD8+ T cell responses in spleen and brain and reduced CD8+ T cell apoptosis in spleen. Chronic *T. gondii* infection promotes the development of a modified NK cell compartment, which does not exhibit normal NK cell characteristics. NK cells are Ly49 and TRAIL negative and are enriched for expression of CD94/NKG2A and KLRG1. These NK cells are found in both spleen and brain. They do not produce IFNγ, are IL-10 negative, do not increase PDL1 expression, but do increase CD107a on their surface. Based on the NK cell receptor phenotype we observed NKp46 and CD94-NKG2A cognate ligands were measured. Activating NKp46 (NCR1-ligand) ligand increased and NKG2A ligand Qa-1b expression was reduced on CD8+ T cells. Blockade of NKp46 rescued the chronically infected mice from death and reduced the number of NKG2A+ cells. Immunization with a single dose non-persistent 100% protective *T. gondii* vaccination did not induce this cell population in the spleen, suggesting persistent infection is essential for their development. We hypothesize chronic *T. gondii* infection induces an NKp46 dependent modified NK cell population that reduces functional CD8+ T cells to promote persistent parasite infection in the brain. NK cell targeted therapies could enhance immunity in people with chronic infections, chronic inflammation and cancer.

Keywords: *Toxoplasma gondii*, NK cells, CD8 T cell exhaustion, chronic infection, ILC

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan that is the 3rd leading cause of foodborne illness in the U.S. (Mead et al., 1999) At least one-third of the human population is infected with this parasite and it is a major health concern for people who become immune compromised and in the developing fetus (Harms Pritchard et al., 2015; Gigley, 2016). Presently, there are no vaccines or drugs available to prevent or eliminate this infection and infection with this parasite is life long (Coppens, 2014; Radke et al., 2018). *T. gondii* infection induces a potent cell mediated response that is initiated by the production of IL-12 which helps activate CD8+ T cells to produce IFNγ (Suzuki and Remington, 1988; Suzuki et al., 1988; Gazzinelli et al., 1994a,b). CD8+ T cell IFNγ production is the major mediator of this infection. Despite induction of a robust Th1 response, the parasite is never cleared. The immunological reason why this infection is not cleared is still unknown.

In mouse models of chronic *T. gondii* infection the parasite can spontaneously reactivate causing the development of toxoplasmic encephalitis (TE) and death (Bhadra et al., 2011b). Parasite reactivation has been attributed to the development of immune exhaustion of parasite specific CD8+ T cells (Bhadra et al., 2011a,b, 2012; Hwang et al., 2016). The CD8+ T cells in mice harboring chronic *T. gondii* infection exhibit immune exhaustion characteristics similar to persistent viral infections (Wherry and Kurachi, 2015). Loss of activated CD8+ T cells resulting in a reduced functional cell population, expression of high levels of programmed death 1(PD1) and increased apoptosis of CD8+ T cells. This loss of functional CD8+ T cells results in parasite reactivation and death of the animals. Importantly, the exhausted CD8+ T cells can be rescued with anti-PDL1 therapy during chronic *T. gondii* infection and this also prevents parasite reactivation and death. The mechanisms underlying the development of CD8+ T cell exhaustion and dysfunction during chronic *T. gondii* infection are still unclear.

NK cells are innate lymphoid cells (ILCs) that provide early cytotoxicity and cytokine dependent protection during infections and cancer (Geiger and Sun, 2016). NK cells are important for control of acute *T. gondii* infection (Denkers et al., 1993; Johnson et al., 1993) and are activated early during parasite infection by IL-12 (Gazzinelli et al., 1993; Hunter et al., 1994). As a result of IL-12 signaling, NK cells produce high levels of IFNγ, which helps control the parasite prior to T cell activation. NK cells are more complex than previously thought and appear to not only be activated and work as a component of innate immunity during acute infections, but may also continue to work along side CD4+ and CD8+ T cells during the adaptive phase of immunity. NK cells have been shown to acquire memory-like features after exposure to haptens, during viral infections and after cytokine stimulation (O'Leary et al., 2006; Cooper et al., 2009; Sun et al., 2009; Paust et al., 2010). This highlights their ability to not simply fall into the background once adaptive immunity is established, but also to continue to play a role in immunity after acute infections are resolved. NK cells have also been shown to become exhausted (Gill et al., 2012; Sun et al., 2015; Alvarez et al., 2019; Zhang et al., 2019). This can occur in the tumor microenvironment, chronic stimulation and persistent HCV infection. In these different disease situations, NK cells become dysfunctional and as a result could contribute to the persistence of infections and reduced clearance of tumor cells. NK cells can also be negative regulators of the adaptive response during acute infections and cancer. Through several interactions including TRAIL, NKp46 and yet to be defined receptors, NK cells can lyse CD4+ and CD8+ T cells resulting in less effective adaptive responses thereby promoting pathogen and tumor persistence (Lang et al., 2012; Waggoner et al., 2012; Cook and Whitmire, 2013; Peppa et al., 2013; Crouse et al., 2014; Schuster et al., 2014). In addition, NK cells produce IL-10 during acute systemic infections including *T. gondii* infection dampening the activation of adaptive immune responses (Perona-Wright et al., 2009). Much of what is known about the development of these other non-protective NK cell responses is in the acute disease or infection setting and less in known about how NK cells behave during chronic infections long after acute infection is resolved.

Based upon the knowledge that CD8+ T cells become exhausted to promote *T. gondii* persistence, NK cells can remain active for long periods of time, NK cells have the potential to become exhausted and they can regulate development of adaptive immune responses we were interested to test how chronic *T. gondii* infection impacted the NK cells and how did NK cells impact the outcomes of chronic toxoplasmosis. Our results indicate that NK cells are still present during chronic *T. gondii* infection. They do not exhibit characteristics of immune exhaustion. They contribute to the loss of exhausted CD8+ T cells and their removal helps maintain control of chronic *T. gondii* infection. We also demonstrate that NK cells develop a unique phenotype that supports the hypothesis that NKp46 recognition of ligand and loss of NKG2A interaction with Qa-1b promotes the development of an NK cell population that negatively regulates CD8+ T cell function contributing to parasite reactivation and death. Our data highlight that NK cells could be therapeutic targets to enhance long-term immunity to chronic *T. gondii* infection.

MATERIALS AND METHODS

Mice

C57BL/6 (B6), B6.129S6-IL-10^{tm1Flv}/J (IL-10-GFP Tiger) mice were purchased from The Jackson Laboratory. All animals were housed under specific pathogen-free conditions at the University of Wyoming Animal Facility. This study was carried out in strict accordance following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The University of Wyoming Institutional Animal Care and Use Committee (IACUC) (PHS/NIH/OLAW assurance number: A3216-01) approved all animal protocols.

T. gondii Parasites and Infection

Tachyzoites of RH were cultured by serial passage in human fetal lung fibroblast (MRC5, ATCC) cell monolayers in complete DMEM (supplemented with 0.2 mM uracil for CPS strain). For mouse infections, parasites were purified by filtration through a

3.0-μm filter (Merck Millipore Ltd.) and washed with phosphate-buffered saline (PBS). Mice were infected intraperitoneally (i.p.) with 1×10^3 or 1×10^6 RH tachyzoites or 1×10^6 CPS tachyzoites. The brains of CBA mice 5 weeks after ME49 infection were used as a source of ME49 cysts. Mice were infected i.p. or i.g. (intragastrically) with 10 or 200 ME49 cysts.

NK Cell Depletion and NKp46 Blockade *in vivo*

To deplete NK cells, B6 mice were treated i.p. with 200 μg of anti-NK1.1 (PK136, Bio X Cell). To block NKp46 mice were treated i.p. with 50 μg non-depleting LEAF purified anti-NKp46 (29A1.4, Biolegend) (Narni-Mancinelli et al., 2012). Antibody treatments were started 5 weeks after infection with ME49 and continued every other day for 2 weeks for flow cytometry assays or until non-treated animal groups died from reactivation of *T. gondii*. No treatment animals were treated with 200 μl of 1 X PBS i.p.

Brain and Spleen T Cell Isolation and Stimulation

Single-cell suspensions of brain and spleen were prepared from mice. To harvest brain lymphocytes and assess their phenotype and function, mice were anesthetized and perfused with 20 ml of 0.9% saline with heparin as described (Donley et al., 2016). Brains were then homogenized in 1 X PBS using a dounce homogenizer. Brains were pelleted by centrifugation then homogenates were added to 30% percoll® and centrifuged at 2,000×g for 20 min at 15°C to collect lymphocytes from the pellet. Brain lymphocytes were then plated at $0.5–1.5 \times 10^6$ cells/well in complete Iscove's DMEM medium (10% FBS, Na Pyruvate, non-essential amino acids, penicillin, β-2 Mercaptoethanol) (Corning). 0.5×10^6 congenically marked CD45.1 splenocytes were also added to the brain lymphocyte wells as feeder cells for antigen restimulation. Spleens were crushed through 70 μm cell strainers (VWR) in 1 X PBS. Splenocytes were then treated with 3 ml of RBC lysis buffer for 3 min at 37°C to lyse erythrocytes, washed then resuspended in complete Iscoves DMEM. Spleen cells were plated at 1×10^6 cells per well. Brain and spleen cells were then pulsed with 20 μg/ml Toxoplasma lysate antigen (TLA) for 8 h and cultured at 37°C in 5% CO_2. After 8 h, 1X protein transport inhibitor cocktail (PTIC) containing Brefeldin A/Monensin (eBioscience, Thermo Fisher Scientific) with or without anti-CD107a (eBio1D4B, eBioscience, Thermo Fisher Scientific) was added to each well in complete Iscove's DMEM medium (Corning). After 4 h incubation at 37°C in 5% CO_2, cells were prepared for flow cytometry.

ILC Functional Assays

For ILC function assays, spleen cells were stimulated for 4 h with plate bound anti-NK1.1 in the presence of 1× protein transport inhibitor cocktail (PTIC) containing Brefeldin A/Monensin (eBioscience, Thermo Fisher Scientific) and anti-CD107a (eBio1D4B, eBioscience, Thermo Fisher Scientific) in complete Iscove's DMEM medium (Corning). Cells were incubated during stimulation at 37°C in 5% CO_2 for 4 h. Cells were then first surface stained then intracellularly stained to measure function. ILC phenotypes were measured directly *ex vivo*. Spleen cells were stained following procedures indicated below after fixable Live/Dead staining (Invitrogen).

Flow Cytometry

Single cell suspensions from brain or spleen were assayed for immune cell phenotype and functions. Phenotype assays were performed directly *ex vivo* after harvest. Function assays were performed after antigen pulse cells or stimulation. All flow cytometry staining was performed using the same procedure for all experiments. Cells were washed twice with PBS and stained for viability in PBS using Fixable Live/Dead Aqua (Invitrogen) for 30 min. After the cells were washed with PBS, surface staining was performed using antibodies diluted in stain wash buffer (2% fetal bovine serum in PBS and 2 mM EDTA) for 25 min on ice in the presence of 2.4G2 FcR blockade to reduce non-specific staining. For phenotype analysis cells were then fixed for 10 min using fixation/permeabilization solution (BD biosciences). For functional assays after fixable live/dead and surface staining, the cells were fixed and permeabilized for 1 h on ice in Fixation/Permeabilization solution (BD Bioscience), followed by intracellular staining in 1 X permeabilization wash buffer (BD Bioscience) with anti-IFNγ and anti-granzyme B (XMG1.2, NGBZ, eBioscience, Thermo Fisher Scientific) for 45 min. Antibodies used for surface staining were against: CD3 (17A2), CD49b (DX5), CD49a (HMα1), NKp46 (29A1.4), NK1.1(PK136), CD4 (RM4-5), CD8b (YTS156.7.7), KLRG1 (2F1/KLRG1), 2B4 (m2B4), Ly49I (YLI-90), Ly49H (3D10), CD94 (18d3), NKG2AB6 (16A11), LAG3 (C9B7W), PD1 (29F.1A12), PDL1(10F.9G2), CD107a (1D4B), CD45.1 (A20), CD45.2 (104). These antibodies were from Biolegend. Anti-Qa-1b (6A8.6F10.1A6) was from eBiosciences and anti-Ly49D (4E5) was from BD Biosciences. NKp46 (NCR1) ligand was stained using the soluble NKp46 receptor fused to human Fc (NCR1-hFc, RND systems). Bound soluble receptor was then detected using a secondary antibody anti-human IgG. To assess apoptosis, cells were also stained using Annexin V (Annexin V staining kit, Biolegend). The cells were resuspended in 1 X PBS and analyzed using Guava easyCyte 12HT flow cytometer (Millipore-SIGMA) and FlowJo software (Tree Star).

Parasite and Cyst Burdens

Cyst burdens were quantified using microscopy. Brains of mice infected with Type II strain ME49 were harvested and homogenized with a dounce homogenizer in 2 ml of 1 X PBS. Ten microliter of homogenized brain was placed onto a microscope slide and covered with a cover slip. Microscope slides were examined and cysts in the homogenate were counted. A minimum of 5 slides per mouse was counted.

Survival Studies

WT B6 mice were infected with 10 cysts i.g. of the type II parasite strain ME49. After 5 weeks of infection mice were treated or not i.p. with 200 μg anti-NK1.1 (PK136, BioXCell) or 50 μg LEAF purified anti-NKp46 (Biolegend). Mouse treatments were performed every other day until completion of experiments. Mice were monitored daily for morbidity and mortality. Mice were

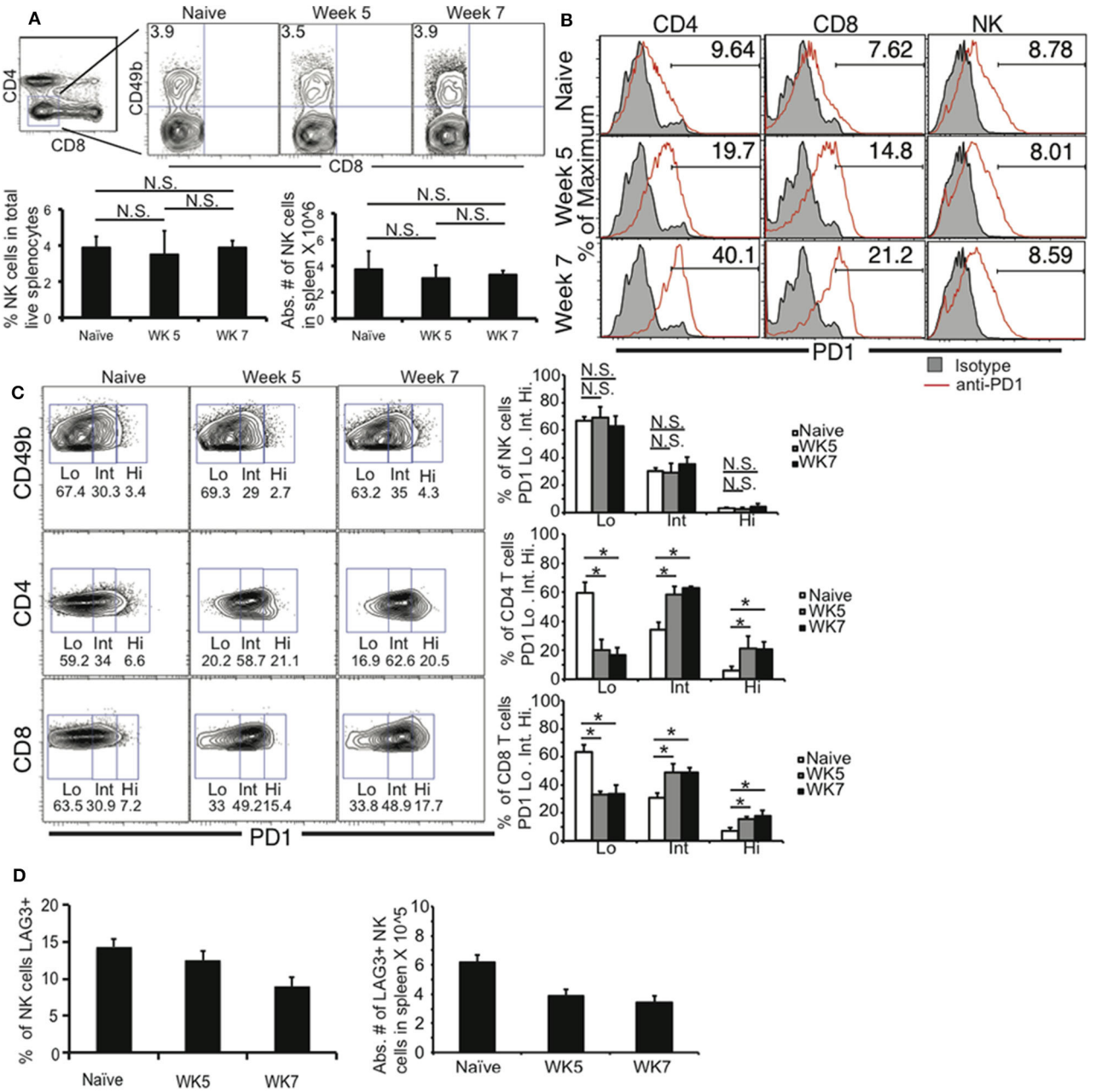

FIGURE 1 | NK cells do not exhibit immune exhaustion characteristics during chronic *T. gondii* infection. C57BL/6 mice were orally infected or not with 10 cysts of ME49 and 5 and 7 weeks after infection spleen cells were analyzed for **(A)** NK cell (Lineage-CD49b+) frequency and number. NK cells, CD4+ and CD8+ T cells were then analyzed for **(B)** PD1 MFI and **(C)** PD1+ frequency based on separating the populations into PD1 low (Lo), intermediate (Int), and high (Hi). NK cells were then assayed for **(D)** LAG3 expression. Raw flow data presented are representative and based on means from 3 independent experiments. All graphs are mean ± SD. Graphs present pooled data from 2 independent experiments. Significance is denoted by *with a $p \leq 0.05$, $n = 4$–5 mice per group.

evaluated on a 1–5 scale with 5 indicating highest morbidity. Mice reaching a level 5 score are not moving, severely hunched, eyes shut and not eating or drinking. Mice were sacrificed prior to death and after they reached level 5 clinical score for no more than 24 h. For survival experiments after rechallenge, ME49 infected animals were treated or not with anit-NK1.1 (PK136, BioXcell). After the 2nd dose of anti-NK1.1 mice were challenged with either a lethal dose 200 cysts of ME49 i.g. or 1,000 tachyzoites of the type I highly virulent strain RH i.p. ILC depletion was continued every other day as in other experiments. Control mice were uninfected naïve B6 mice only given the challenge infection (either ME49 or RH). Survival of chronically infected rechallenged mice were monitored and assessed on the 1–5 scale as described above.

Statistical Analysis

Statistical analysis was performed using Prism 7.0d (GraphPad) and Microsoft Excel 2011. Significant differences were calculated using either unpaired Student's *t*-test with Welch's correction, Mann-Whitney nonparametric test or analysis of variance (ANOVA). The log-rank (Mantel-Cox) test was used to evaluate survival rate. Data is presented in graphs as the mean± standard deviation (SD). Significance is denoted as follows: ns, not significant ($p > 0.05$) or significant with a maximum *p*-value of 0.05 or less.

RESULTS

NK Cell Exhaustion

Previous studies have demonstrated that during chronic *T. gondii* infection, CD4+ and CD8+ T cells develop immune exhaustion resulting in their dysfunction (Bhadra et al., 2011b, 2012; Hwang et al., 2016). This ultimately results in the death of B6 mice beginning in the late chronic stage of infection (∼WK 7 post infection) due to parasite reactivation. Parasite reactivation is detected by loss of cyst numbers in the brains and increased parasitemia in the blood. To further dissect the immune mechanisms contributing to T cell exhaustion during chronic *T. gondii* infection, we investigated the role of innate lymphoid cells and more specifically NK cells. NK cells can participate in immune responses long after the innate response has transitioned into the adaptive response (O'Leary et al., 2006; Sun et al., 2009). NK cells acquire characteristics of memory. NK cells can also develop characteristics of immune exhaustion in the tumor microenvironment (Gill et al., 2012). They traffic to tumor sites, have reduced numbers, effector function and upregulate PD1 expression on their surface. Based on the ability of NK cells to contribute to immunity after the innate response is over and their potential to develop immune exhaustion we determined whether NK cells were still in abundance during late chronic *T. gondii* infection and their immune exhaustion status. Mice were infected with 10 cysts of the Type II *T. gondii* strain ME49, known to induce T cell exhaustion during long-term infection. At week 5 and 7 post infection spleens were harvested and NK cell frequencies and numbers were measured using flow cytometry. Lineage negative (CD4–CD8–) cells were analyzed for CD49b+ cells. As shown in **Figure 1A**, the frequencies and absolute numbers of splenic NK cells (CD4–CD8–CD49b+) did not significantly decrease from week 5 to 7 post infection. As previously published week 7 is when CD4+ and CD8+ T cells decrease in both frequency and number (Bhadra et al., 2011b; Hwang et al., 2016). An increase in Programmed death 1 (PD1) on T cells is a hallmark of immune exhaustion. During late chronic *T. gondii* infection, both CD4+ and CD8+ T cells have been reported to increase their PD1 expression leading to loss of function of these T cells and parasite reactivation (Bhadra et al., 2011b; Hwang et al., 2016). To further assess whether NK cells exhibited characteristics of immune exhaustion during late chronic *T. gondii* infection we measured their PD1 expression. As shown in **Figure 1B**, the mean fluorescence intensity (MFI) of PD1 increased on both CD4+ and CD8+ T cells, however, NK cells did not increase their expression of PD1. In addition, the frequencies of CD4+ or CD8+ T cell PD1 high (PD1 Hi), PD1 intermediate (PD1 Int) both increased significantly at week 5 and 7 post infection (**Figure 1C**). The frequencies of PD1 Hi or Int did not change on NK cells over the course of infection. Another marker of exhaustion is lymphocyte activating gene 3 (LAG3) expression. LAG3 increases on CD4+ and CD8+ T cells during late chronic *T. gondii* infection (Hwang et al., 2016). We did not detect an increase in LAG3 expression (**Figure 1D**) on NK cells during chronic *T. gondii* infection. Based on the results splenic NK cells do not appear to decrease in number or express PD1 or LAG3 at high levels compared to CD4+ and CD8+ T cells during chronic *T. gondii* infection.

NK Cell Role in Chronic *T. gondii* Infection

Based on results of our studies, NK cells did not appear to develop characteristics of immune exhaustion raising the question about how might NK cells contribute to immune control of *T. gondii* during chronic infection. WT B6 mice typically succumb to spontaneous reactivation of the parasite in the CNS and die (Bhadra et al., 2011b). Parasite reactivation in the brain can be observed via reemergence of parasitemia and a gradual decline of cyst numbers in the brain. Interestingly, treatment with anti-PDL1 antibody rescues animals from death and slows down parasite reactivation resulting in higher cyst numbers in the CNS in a CD8+ T cell dependent manner (Bhadra et al., 2011a,b, 2012). To begin to address how NK cells are behaving during chronic *T. gondii* infection NK cells were depleted using anti-NK1.1 in mice starting at week 5 post infection. Mice were treated every other day until the experiment was terminated at 100 days post infection. Mice with NK cells began to succumb to the infection around week 7 (49 days) post infection (**Figure 2A**). Mice treated with anti-NK1.1 did not start to succumb to the infection until 80 days post infection. All mice with NK cells were dead by 80 days post infection whereas 50% of mice depleted of their NK cells were still alive at 100 days post infection (**Figure 2A**). We next measured whether cyst burdens in the brain were maintained better when NK cells were depleted because as previously published, cyst numbers decrease in the brain as the parasite reactivates due to immune exhaustion and rescuing exhausted CD8+ T cells with anti-PDL1 maintains higher cyst numbers in the brains (Bhadra et al., 2011a,b, 2012, 2013). As shown in **Figure 2B**, mouse brain cyst burdens were higher in mice that were depleted of NK cells than mice with NK cells. To test how NK cells were impacting secondary immune control of the parasite, B6 mice were infected with ME49 and at 5 weeks depleted of their NK cells or not with anti-NK1.1. 2 days after start of treatment, mice were challenged with a lethal dose of either 200 cysts of ME49 i.g. or 1,000 tachyzoites of RH i.p. and monitored for survival. As shown in **Figure 2C**, mice with their NK cells succumbed to challenge significantly earlier than mice with NK cells depleted. These results suggest that NK cells appear to have a negative affect on long-term immunity to chronic *T. gondii* infection modifying cyst levels in the brain and reducing the effectiveness of adaptive recall responses.

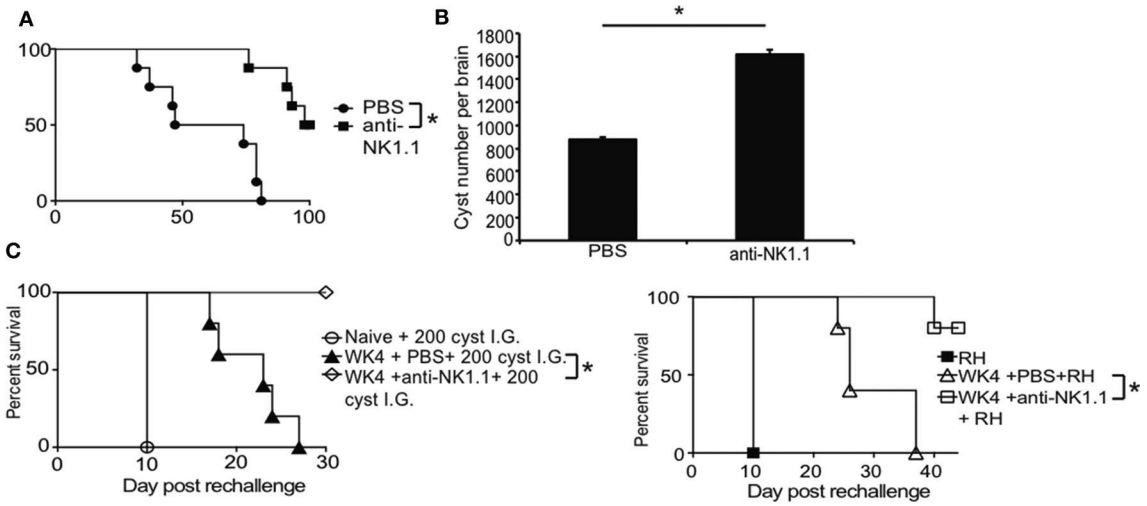

FIGURE 2 | NK cells promote immune exhaustion and parasite reactivation during chronic *T. gondii* infection. C57BL/6 mice were infected orally with 10 cysts of ME49 and infection outcome monitored. **(A)** Survival after 200 μg anti-NK1.1 or 200 μl 1 X PBS every 2nd day starting at week 5 p.i. Graph presents pooled data from 3 independent experiments, $n = 4$–5 mice per group **(B)** Brain cyst burden after anti-NK1.1 or 1 X PBS treatment starting at week 5 p.i. Graph presents data from 1 experiment repeated independently 2 times with $n = 5$ mice per group. **(C)** Survival of chronically infected animals after lethal secondary 200 ME49 cyst or 1,000 tachyzoite RH strain challenge with or without anti-NK1.1 treatment. Experiments were repeated independently 2 times with $n = 4$–5 mice per group. All graphs are mean ± SD. *denotes significance as $p \leq 0.05$.

NK Cell Impact on CD8+ T Cell Responses

Parasite reactivation and mouse death during chronic *T. gondii* infection occurs when parasite specific CD8+ T cells develop immune exhaustion (Bhadra et al., 2011b). Their exhaustion results in decreased frequency and number of polyfunctional (IFNγ+CD107a+, or IFNγ Granzyme B+) CD8+ T cells in the periphery and CNS. Since depletion of NK cells during chronic *T. gondii* resulted better survival we measured how this impacted polyfunctional CD8+ T cells in the spleen and brain. Infected mice were treated starting at week 5 post infection with anti-NK1.1 or 1 X PBS for 2 weeks as in previous experiments and spleen and brain cells were assayed for the frequency and absolute number of polyfunctional CD8+ T cells. As shown in **Figure 3A**, NK cell depletion of week 5 infected mice resulted in the maintenance of the frequency and absolute number of IFNγ+CD107a+ CD8+ T cells. In the brain, NK cell depletion starting at week 5 post infection did not significantly impact overall CD8+ T cell numbers, however, NK cell depletion resulted in a significant increase in the absolute numbers of IFNγ+ CD8+ T cells and in the frequency and absolute numbers of IFNγ+GrzB+ CD8+ T cells (**Figures 3C–E**). Several studies have demonstrated that during acute viral infections (MCMV, LCMV) infection, NK cells are negative regulators of priming of adaptive immune responses (Lang et al., 2012; Cook and Whitmire, 2013; Crouse et al., 2014; Schuster et al., 2014; Waggoner et al., 2014; Cook et al., 2015; Rydyznski et al., 2015). This negative regulation promotes viral persistence and immune exhaustion of the T cells. However, during acute *T. gondii* infection previous studies suggest that NK cells could be positive regulators of the priming of adaptive immune responses against the parasite (Combe et al., 2005; Goldszmid et al., 2007, 2012; Guan et al., 2007). Many of the earlier *T. gondii* studies used anti-asialo GM1 antibody to deplete NK cells without knowing that this antibody targets not only NK cells, but effector populations of CD8+ T cells (Ivanova, 2019). We tested whether NK cells provided a different function during acute *T. gondii* infection and promoted priming of CD8+ T cells as compared to NK cells in chronic *T. gondii* infection, which appear to inhibit CD8+ T cell function. B6 mice were treated with anti-NK1.1 1 or 1 X PBS alone a day prior to infection with ME49 strain of *T. gondii* and then infected with 10 cysts i.g. NK depleted mice were treated with anti-NK1.1 for 6 days and on day 7 all mice were harvested and their spleen CD8+ T cell functionality was measured. As shown in **Figures 3F,G**, CD8+ T cells were activated by day 7 post infection, however, NK cell depleted animals had significantly fewer activated CD8+ T cells (IFNγ+CD107a+ plus IFNγ+CD107a–) than mice that still have their NK cells. Thus, during acute infection NK cells are important for priming CD8+ T cells to protect against infection, but during chronic *T. gondii* infection, NK cells change their function and negatively regulate CD8+ T cells to promote parasite reactivation and mouse death. To further define why polyfunctional CD8+ T cells were reduced during chronic *T. gondii* infection in the presence of NK cells, we measured CD8+ T cell apoptosis using Annexin V staining and assessed CD8+ T cell PD1 expression in the presence or absence of NK cells. As presented in **Figure 4A**, CD8+ T cell apoptosis was significantly increased in chronically infected mice at week 5 and 7 post infection in the spleen. NK cell depletion significantly reduced the level of CD8+ T cell apoptosis in the spleen of chronically infected mice at week 7 post infection. NK cells also did not appear to impact the frequency of PD1+ CD8+ T cells or level of PD1 expression on CD8+ T cells in chronically infected

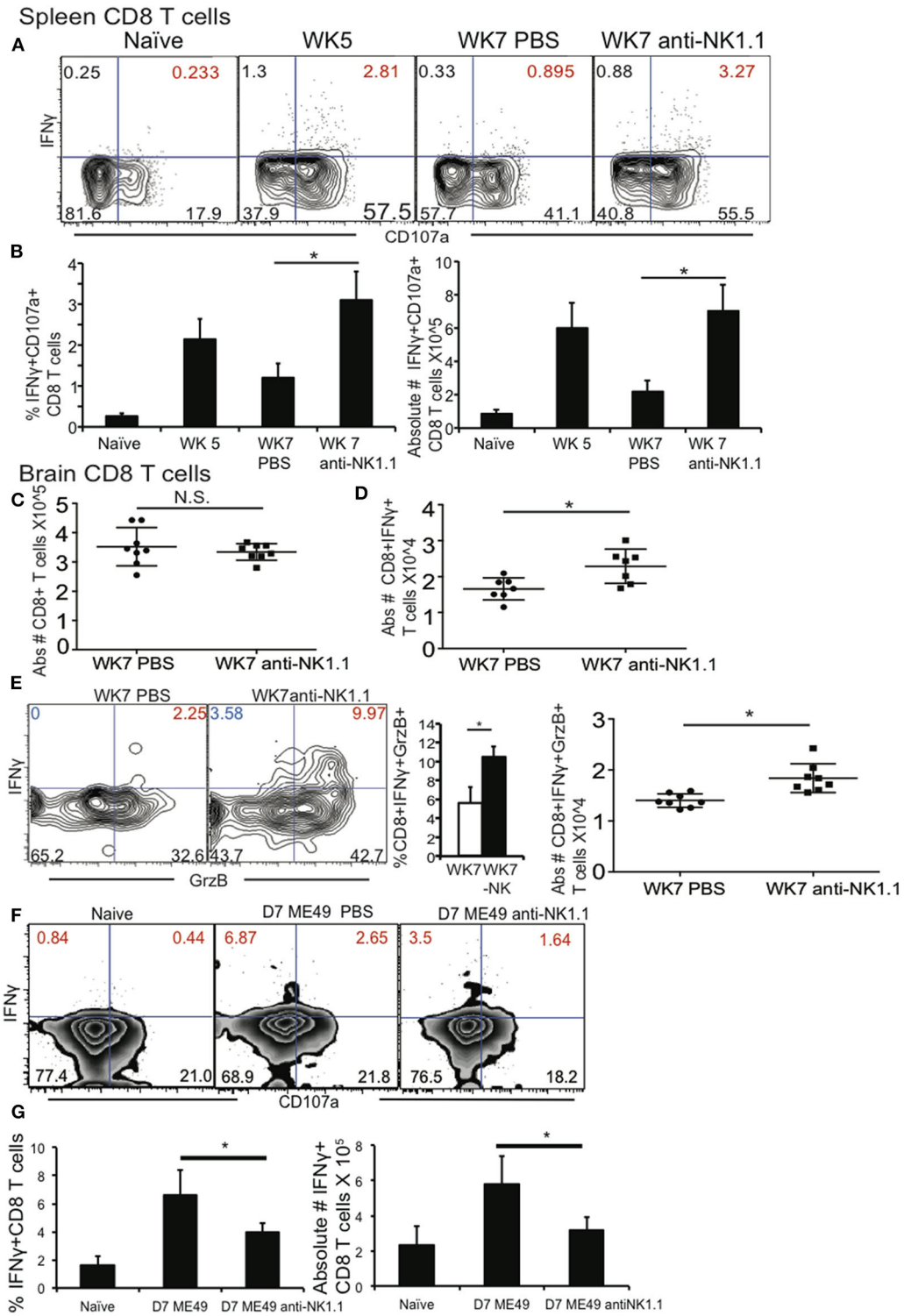

FIGURE 3 | NK cells reduce polyfunctional CD8+ T cells by increasing their apoptosis during chronic *T. gondii* infection. C57BL/6 mice were infected as described and 5 weeks after infection treated with 200 μg anti-NK1.1 or 200 μl 1 X PBS i.p. Brain and spleen cells were harvested and restimulated *ex vivo* with TLA. **(A)** Contour plots present spleen CD8+ T cells analyzed for IFNγ+ × CD107a+. **(B)** Graphs present frequency and absolute # of polyfunctional IFNγ+CD107a+CD8+T cells. Data presented is from 1 experiment repeated independently 2 times with $n = 5$ mice per group. **(C)** Graphs present pooled data from 2 independent experiments of absolute numbers of brain CD8+ T cells with or without anti-NK1.1 treatment starting at wk 5 post infection. **(D)** Graphs present pooled data

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FIGURE 3 | from 2 independent experiments of absolute numbers of brain IFNγ+ CD8+ T cells from animals treated with 200 μg anti-NK1.1 or 1 X PBS i.p. **(E)** Contour plots of brain IFNγ+GrzB+CD8+ T cells (red numbers are frequency) are presented. Graphs present the frequency and absolute number of brain polyfunctional (IFNγ+Grzb+) CD8+ T cells from 2 independent experiments with $n = 4$ mice per group. **(F,G)** B6 mice were treated or not with 200 μg of anti-NK1.1 starting at D-1 then infected with 10 cysts of ME49 strain i.g. Mice were treated every other day with anit-NK1.1. On day 7 post infection animals were sacrificed and spleen cells isolated then stimulated with TLA and stained for polyfunctionality (IFNγ+CD107a+). **(F)** Contour plots present CD8+ T cells stained for IFNγ+ × CD107a+ during acute *T. gondii* infection. **(G)** Graphs present pooled data from 2 experiments showing frequency (%) and absolute number of IFNγ+ CD8+ T cells in spleen. $N = 4$ mice per group. All graphs are mean ± SD. *denotes significance with a $p \leq 0.05$.

mice (**Figure 4B**). NK cells appear to contribute to CD8+ T cell exhaustion during chronic *T. gondii* infection possibly by increasing CD8+ T cell apoptosis, but not by increasing their PD1 expression.

NK Cell Phenotype During Chronic *T. gondii* Infection

Published studies indicate that NK cells use several mechanisms to regulate adaptive immune responses (Perona-Wright et al., 2009; Lang et al., 2012; Peppa et al., 2013; Crouse et al., 2014; Schuster et al., 2014; Xu et al., 2014; Crome et al., 2017; Kwong et al., 2017). Many of these mechanisms rely on the expression of specific NK cell receptors, which allow the NK cells to target specific adaptive immune cell populations and induce their apoptosis, lysis or suppression. NK cell receptors are also very important for normal protective NK cell functions including Ly49H, which recognize m157 of MCMV (Lanier, 2005; Sun et al., 2009). These specific interactions promote the enrichment of NK cell subpopulations expressing specific receptor combinations as was demonstrated for memory-like NK cells during MCMV infection. During acute *T. gondii* infection, we have previously published that there does not appear to be a dominant NK cell population activated. *T. gondii* infection may only induce cytokine dependent NK cell activation resulting in global activation of a large array of different IFNγ producing and protective NK cell subpopulations (Ivanova et al., 2016). In the studies presented here, NK cells appear to change their function to promote CD8+ T cell compartment dysfunction and alter levels of chronic infection. This suggests the NK cell compartment could be modified and a specific NK cell subpopulation develops to erode immunity to the parasite during chronic *T. gondii* infection. To begin to define what NK cell receptors might be involved in contributing to CD8+ T cell exhaustion during chronic *T. gondii* infection, we performed an exhaustive assessment of NK cell receptor expression during week 5 chronic *T. gondii* infection. As shown in **Figure 5A**, the NK cell compartment had significantly reduced frequencies of cells that expressed 2B4, Ly49H, Ly49D, and Ly49I. This was observed on lineage—CD49b+ cells in the spleen. We did not detect any differences in TRAIL expression (data not shown). We observed significant increases in the frequencies of NK cells (lin-CD49b+) that expressed KLRG1 and NKG2A (**Figure 5A**). The number of NK cells expressing KLRG1 also increased significantly during chronic *T. gondii* infection. The significant increase in KLRG1+ NK cells and NKG2A+ NK cells suggested these cells were being enriched within the NK cell compartment. A recent study suggests that CD49a+ ILC1 may develop from NK cells and exhibit this phenotype in the liver of mice infected with the vaccine strain of *T. gondii cps1-1* and a different limited cyst forming type II strain Prugniaud (Park et al., 2019). Therefore, to determine if this was also occurring in NK cells in the spleens after infection with the vaccine strain, we infected mice with *cps1-1* and 5 weeks later assayed the spleen cells for CD94+NKG2A+ NK cells. As shown in **Figure 5B**, *cps1-1* strain parasites did not induce the same increase in frequency of CD94+ NKG2A+ NK cells as did ME49. To further investigate whether ILC1 were enriched in the NK cell compartment, we measured the frequencies of ILC1 (CD49a+CD49b–) compared to NK cells (CD49a–CD49b+) in the CD3– and CD3– NKp46+ cell populations in the spleens of chronically infected mice. As shown in **Figure 5C**, NK cells (CD49b+CD49a–) comprised 22% of the CD3– population of cells and 60% of the lineage negative NKp46+ population of cells. ILC1 were present at a very low level. Therefore, in the spleen, NK cells appear to be the dominant population of ILC present and they are enriched for a specific receptor phenotype, which is Lin—CD49b+ CD49a–NKp46+ CD94+ NKG2A+KLRG1+.

NK Cell Function During Chronic *T. gondii* Infection

NK cells are the cytotoxic cells of the ILC lineage (Diefenbach et al., 2014; Eberl et al., 2015). NK cells are also capable of producing high levels of IFNγ upon activation. ILC1 are not cytotoxic and produce high levels of IFNγ upon activation. During acute *T. gondii* infection NK cells and ILC1 are known to produce IFNγ in an IL-12 dependent manner (Denkers et al., 1993; Gazzinelli et al., 1993; Hunter et al., 1994, 1995; Klose et al., 2014) We have recently demonstrated that after vaccination, NK cells respond a second time to help control challenge infection by producing IFNγ in an IL-12 and IL-23 dependent manner (Ivanova et al., 2019). We observe that NK cells during chronic *T. gondii* infection modify their role in immunity to the parasite and are not protective, but detrimental. They also express an altered receptor repertoire that suggests enrichment for a specific cell phenotype. Therefore, to begin to investigate how NK cells are negative regulators of CD8+ T cells during chronic *T. gondii* infection we first assayed their function. Mice were infected as above and starting at week 5 post infection we assessed NK cell (CD3–CD49b+ NKp46+) function (IFNγ × CD107a) by flow cytometry. As shown in **Figure 6A**, after *ex vivo* stimulation, naïve NK cells were capable of producing both IFNγ and expressing the surrogate cytotoxicity marker CD107a. However, after week 5 of infection (**Figure 6A**), NK cells produced very little IFNγ while significantly increasing their CD107a expression. This pattern of function was observed also at week 7 post infection. Increases in CD107a+ NK

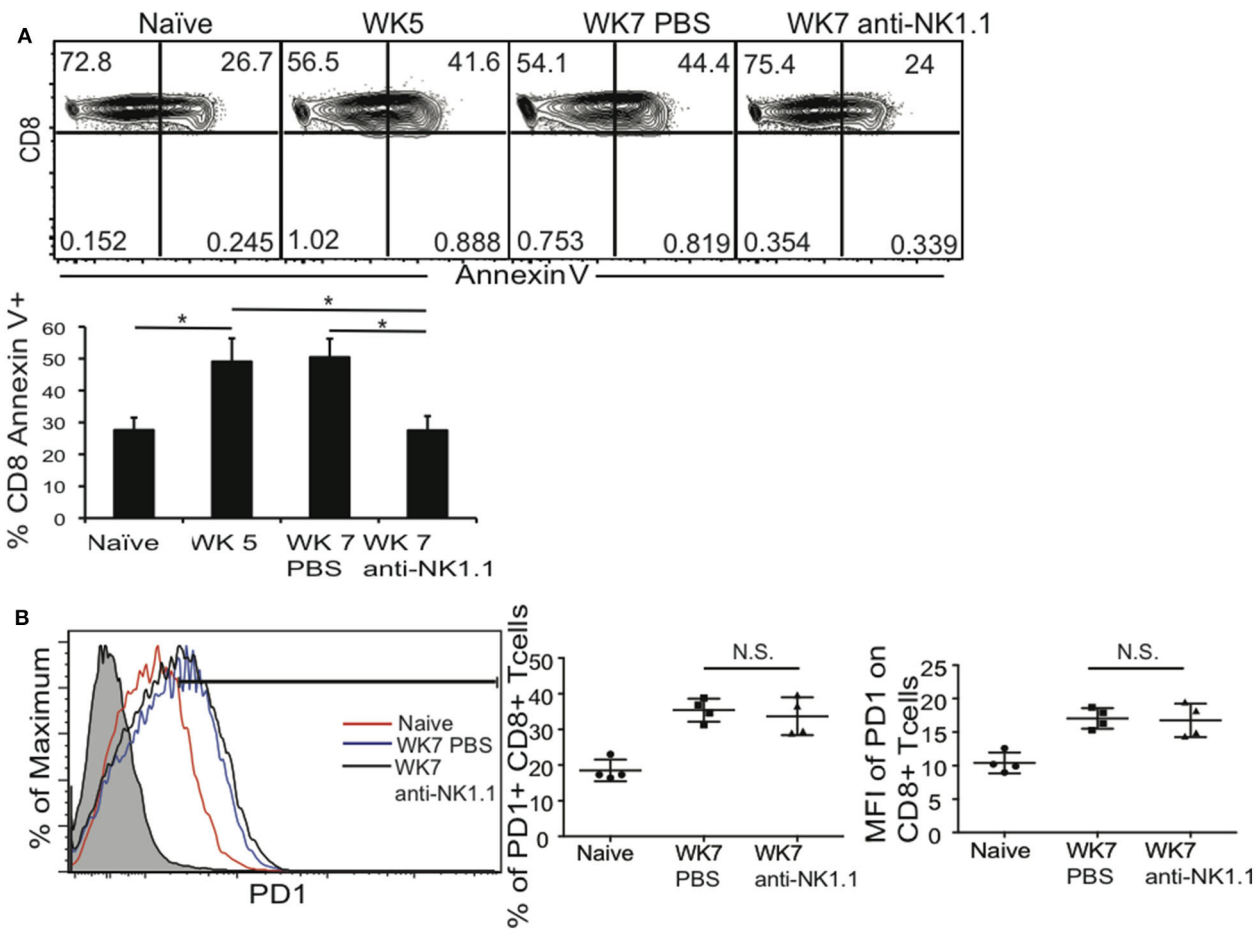

FIGURE 4 | NK cells increase CD8+ T cells apoptosis during chronic *T. gondii* infection. C57BL/6 mice were infected and some groups at week 5 after infection were treated with anti-NK1.1 or 1 X PBS i.p. as described. Week 5, week 7 non-treated and week 7 treated infected mice were sacrificed and spleen cells isolated for CD8+ T cell Annexin V staining and PD1 and compared to naïve animals. **(A)** Contour plots present frequency of Annexin V+ CD8+ T cells and graphs present mean ± SD. **(B)** Histogram presents PD1 expression on splenic CD8+ T cells from anti-NK1.1 treated or not treated mice. Graphs present frequency and MFI of PD1 on CD8+ T cells. Data presented are from 1 experiment repeated 2 independent times with $n = 4$ mice per group. Significance is denoted by *with a $p \leq 0.05$ non-significant results are denoted with N.S.

cells were observed in both frequency and absolute number. Interestingly, the frequency of IFNγ+ NK cells continued to decrease from weeks 5 to 7 post infection. The data shown in **Figure 6A** was generated *ex vivo* by using plate bound anti-NK1.1 crosslinking. We repeated *ex vivo* analysis using PMA/Ionomycin and still the NK cells did not produce IFNγ (data not shown). Interestingly, if these cells were ILC1, we would have expected them to produce IFNγ. We next measured whether the NK cells expressed PD-L1, the ligand for PD1. As shown in **Figure 6B**, splenic NK cells did not appear to increase their expression of PD-L1 as PD-L1 MFI was not significantly different between weeks 5 and 7 post infection. During acute systemic *T. gondii* infections, NK cells have been shown to produce IL-10 (Perona-Wright et al., 2009). Therefore, we obtained IL-10GFP TIGER reporter mice and infected them with 10 cysts of ME49 i.g. Comparing naïve to week 5 post infected mice (**Figure 6C**), we did not observe any IL-10 production by NK cells. A recent study demonstrated that NKp46+ ILC could contribute to the development of neurodegenerative disease by being in the CNS and promoting Th17 responses (Kwong et al., 2017). We next determined how chronic *T. gondii* infection impacted NK cells in the CNS. Mice were infected as previously described and at week 5 post infection, mice were perfused, brains dissected and immune cells isolated. Cells were analyzed for CD3–NKp46+ (**Figure 6D**) and CD3–CD49b+NK1.1+CD94+NKG2A+ (**Figure 6E**) populations. As shown in **Figure 6D**, we did not observe an increase in frequency of CD3– Nkp46+ NK cells in the CNS of *T. gondii* chronically infected mice at week 5 post infection. There appeared to be a decrease. However compared to naïve animals, we observed an increase in absolute number of CD3–CD49b+NK1.1+CD94+NKG2A+ NK cells in the brains of chronically infected mice, a similar phenotype to splenic NK cells. Our investigation of the function of the NK cells acting

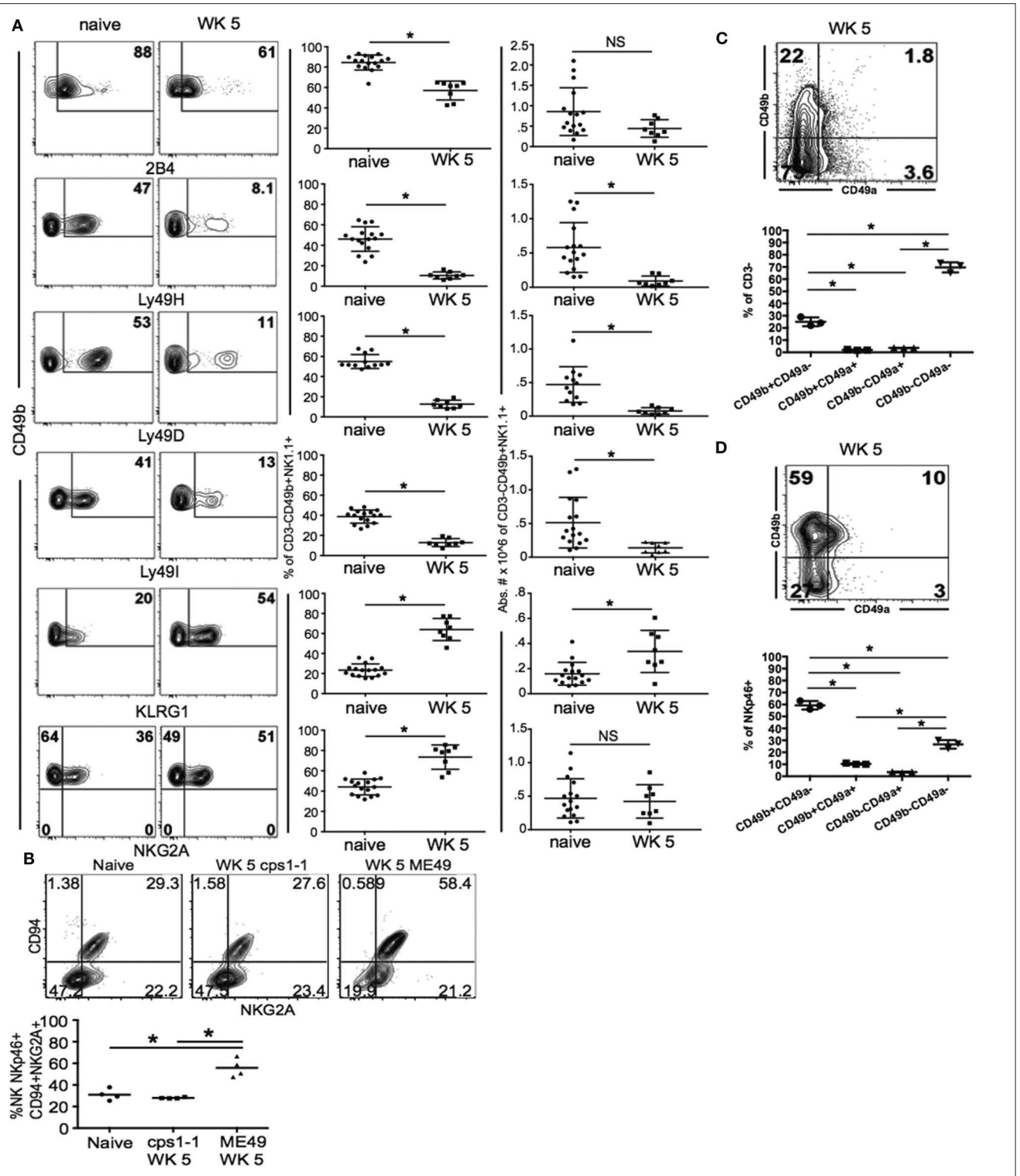

FIGURE 5 | NKp46+NKG2A+KLRG1+ NK cells are enriched in spleen during chronic *T. gondii* infection. C57BL/6 mice were orally infected with 10 cysts of ME49 and analyzed for the NK cell receptors 2B4, Ly49H, Ly49D, Ly49I, KLRG1, CD94, and NKG2A by flow cytometry. **(A)** Contour plots present the frequency of CD49b+ × receptor + cells comparing naïve animals to week 5 post infection. Graphs show pooled data from 3 experiments of frequency and absolute number of CD49b+ Receptor+ cells. **(B)** Mice were infected with either 1 × 10^6 tachyzoites of *csp1-1* i.p. or 10 cysts ME49 i.g. At week 5 post infection, lineage-CD49b+NKp46+ cells were analyzed for CD94 × NKG2A. Contour plots present data from one experiment showing frequency of CD94 × NKG2A cell populations. The graph presents

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FIGURE 5 | data from 1 experiment comparing the frequency of CD94+NKG2A+ cells between naïve, *cps1-1* and ME49 mice. **(C)** Mice were infected with 10 cysts of ME49 i.p. then spleen cells were analyzed at week 5 post infection for CD49a × CD49b to identify ILC1 compared to NK cells. Contour plot presents the frequency of CD49a × CD49b cells in the CD3– population. **(D)** Contour plot presents the frequency of CD49a × CD49b cells in the CD3–NKp46+ population. **(C,D)** Graphs present the frequency of CD49b+CD49a–, CD49b+CD49a+, CD49b–CD49a+, and CD49b–CD49a–. All experiments were repeated independently a minimum of 2 times with $n = 3$–4 per group. *denotes significance with a $p \leq 0.05$.

as negative regulators of immunity during chronic *T. gondii* infection suggests that NK cells have reduced IFNγ production, but may increase cytotoxicity. They do not produce IL-10 and similar to splenic NK cell phenotypes are enriched for CD94+ NKG2A+ population in the CNS of chronically infected mice.

NKp46 and NKG2A NK Cells During Chronic *T. gondii* Infection

The NK cell phenotype we observed Lin—CD49b+ CD49a– NKp46+ CD94+ NKG2A+KLRG1+ suggest that NKp46 and NKG2A may contribute NK cell negative regulation of the immune response to *T. gondii* in chronically infected mice. This is based on the concept of NK cell licensing (Kim et al., 2005). NK cell licensing determines the responsiveness of NK cells to self vs. non-self. A licensed NK cell expresses both activating and inhibitory receptors on its surface and as a result is tuned or permitted to respond when self is absent. An absence or reduction in self, usually reduced MHC expression can be detected on a target cell. At the same time, increases in non-self detected by elevated ligands binding to the activating receptor activate the NK cell. NKp46 is an activating receptor expressed on NK cells, ILC1 and some ILC3 (Eberl et al., 2015; Cortez and Colonna, 2016). The ligand NKp46 recognizes is not very well-described. Potential ligands for NKp46 vary in source and structure and to date may include Influenza virus HA, Sigma 1 protein of Reovirus and *Candida glabrata* proteins Epa 1, 6, and 7 (Mandelboim et al., 2001; Vitenshtein et al., 2016; Bar-On et al., 2017). NKp46 is a natural cytotoxicity receptor, also called NCR1 and is known once it engages its ligand (NCR1-ligand) to lyse target cells (Narni-Mancinelli et al., 2012). NKp46 can also promote the expansion and survival of NK cells similar to other activating receptors (Lee et al., 2009; Narni-Mancinelli et al., 2012). NKG2A is an inhibitory receptor that recognizes non-classical MHC Class I known as Qa-1b (Vance et al., 1998; Holderried et al., 2013). NKG2A prevents NK cell activation. Based on the licensing paradigm and our data we hypothesized that during chronic *T. gondii* infection, there was an increase in non-self (NCR1-ligand) while there was a decrease in self (Qa-1b) which in turn caused NK cells to negatively regulate CD8+ T cells resulting in parasite reactivation and death. To test this hypothesis spleens from chronically infected mice at weeks 5 and 7 post infection were isolated and the expression levels of NKp46-ligand and Qa-1b were measured on total splenocytes and CD8+ T cells and compared to naïve animals. NCR1-ligand was detected using soluble murine NCR1 (NKp46) fused to human Fc and Qa-1b using anti-Qa-1b antibody. In naïve mice total splenocytes were positive for Qa-1b and largely negative for ligands that were bound by NCR1 (**Figure 7A**, top and bottom panels, respectively). At week 5 post infection Qa-1b was significantly increased in expression and NCR1-ligand remained low compared to naïve mice. At week 7 post infection Qa-1b expression was decreased significantly compared to week 5 and naïve animals while NCR1-ligand was increased significantly (**Figure 7A**). As shown in **Figure 7B**, this pattern of Qa-1b and NCR1-ligand expression was similar when CD8+ T cells were gated and assessed. However, the changes in Qa-1b and NCR1-ligand did not appear to be greater on CD8+ T cells than total splenocytes. We performed preliminary assessments of whether the NK cells were actually more cytotoxic, but did not find any significant increase (data not shown), suggesting these interactions were promoting NK cell survival and maturation as measure by KLRG1 expression on the NK cells (**Figure 5A**). Overall the decrease in self (Qa-1b) and the increase in non-self (NCR1-ligand) support the concept that NK cell licensing was contributing to CD8+ T cell dysfunction in some way. Therefore, to test that these NK cells via a licensing process were contributing to immune dysfunction during chronic *T. gondii* infection, we infected animals as before and starting at week 5 post infection we treated or not mice with non-depleting anti-NKp46 blocking antibody (Narni-Mancinelli et al., 2012). This approach would not deplete NK cells, but simply block the interaction between NKp46 and the unknown ligand thus potentially decrease NK cell negative regulation of CD8+ T cells. As shown in **Figure 7C**, anti-NKp46 significantly prolonged the life of mice with chronic *T. gondii* infection compared to no treatment controls. We also wanted to test how blocking NKp46 could impact the frequency of NKG2A+ NK cells in chronically infected mice. As shown in **Figure 7D**, the frequency of NK1.1+ NKG2A+ cells was significantly decreased when NKp46 was blocked compared to 1 X PBS treated controls. These results suggest that modifications of self vs. non-self and NK cell recognition of these modifications via NKp46 and NKG2A receptors potentiate NK cell dependent negative regulation of CD8+ T cells responses during chronic *T. gondii* infection. The interaction of NK cells with NCR1 ligand may help promote the development of this population of cells. NK cells as a result contribute to immune exhaustion not early during infection, but later after chronic *T. gondii* infection is established.

DISCUSSION

The immune mechanisms regulating CD8+ T cell exhaustion resulting in reactivation of chronic *T. gondii* infections are poorly understood. In this study we sought to further explore these mechansisms and proposed that NK cells could contribute to this process. NK cells are innate immune cells and belong to a

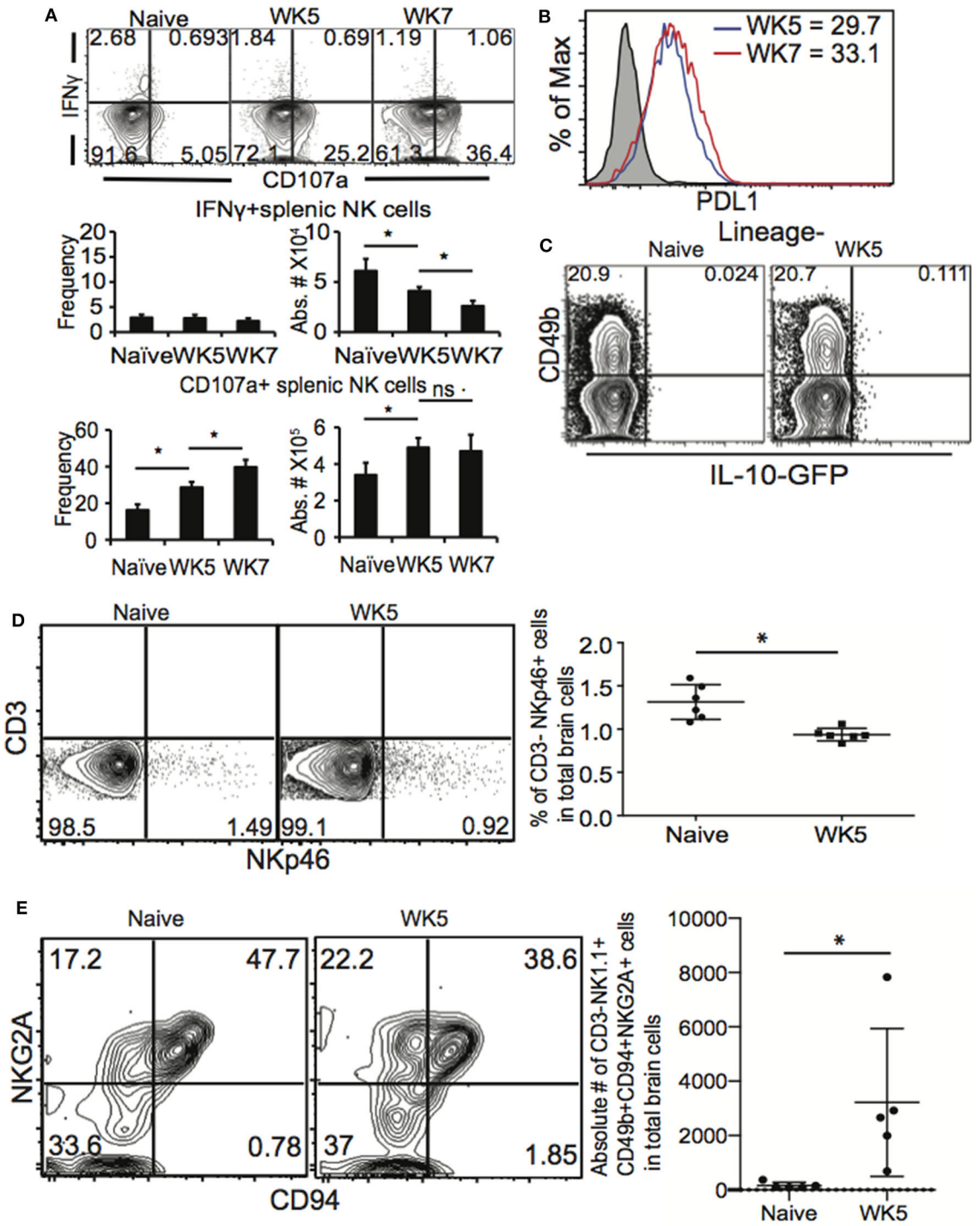

FIGURE 6 | NK cells have altered function during chronic *T. gondii* infection. C57BL/6 or IL-10 reporter TIGER-GFP mice were orally infected with 10 cysts of ME49 and 5 and/or 7 weeks after infection spleen cells analyzed for function. **(A)** Spleen cells were stimulated *ex vivo* with plate bound anti-NK1.1 then stained for NK cells (CD3–CD49b+ NKp46+) IFNγ and CD107a. Contour plots present frequency data gated on NK cells and compares IFNγ × CD107a. Graphs present the frequency and absolute number of IFNγ+ NK cells (top graphs) and CD107a+ NK cells (bottom graphs). Graphs present mean ± SD. **(B)** Splenic NK cells were assayed for PDL1 expression. Histogram presents the MFI of PD-L1 on NK cells from weeks 5 and 7 post infection mice. **(C)** Contour plots present the frequency of IL-10 GFP+ NK cells in naïve compared to week 5 post infection mice. **(D)** Brain cells were isolated and stained for lineage markers, CD49b and NKp46. Contour plots present the frequency of CD3–NKp46+ cells in the CNS. Graphs present the pooled data from 2 experiments of frequency of CD3–NKp46+ cells in the CNS with $n = 3$ mice per group. **(E)** Brain cells form naïve and WK5 infected mice were stained for CD3–CD49b+NK1.1+CD94+NKG2A+ cells. Contour plots present representative data from 1 experiment repeated 2 times with $n = 5$ mice per group showing CD3–CD49b+NK1.1+ cells analyzed for the frequency of CD94 and NKG2A+ cells. The graph presents absolute numbers of CD94+NKG2A+ cells in total NK cells from brains. Significance is denoted by *with a $p \leq 0.05$.

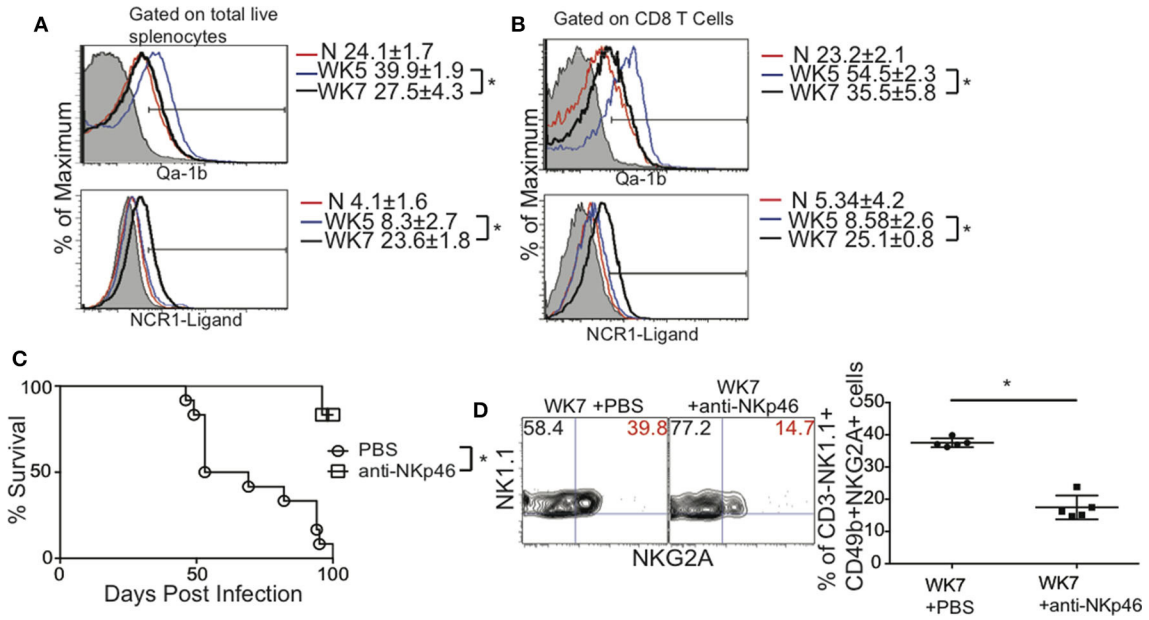

FIGURE 7 | Blockade of NKp46 rescues mice from death caused by CD8+ T cell exhaustion induced parasite reactivation. C57BL/6 mice were orally infected as above and total splenocytes were assayed for NKG2A ligand Qa-1b and NKp46 ligand using soluble NCR1 fused to human Ig Fc. **(A)** Histograms present the MFI ± SD of QA-1b (top) and NCR1-ligand (bottom) from total splenocytes. **(B)** Histograms present the MFI ± SD of QA-1b (top) and NCR1-ligand (bottom) from CD8+ T cells. **(C)** Mice were infected with 10 cysts of ME49 i.g. and treated with 50 μg of anti-NKp46 or 1 X PBS i.p. starting at week 5 p.i. Mice were treated every other day for the duration of this experiment. The survival graph presents pooled data from 3 independent experiments with $n = 4–5$ mice per group. The log-rank (Mantel-Cox) test was used to evaluate survival rates. **(D)** Mice were infected with 10 cysts of ME49 i.g. and treated with 50 μg of anti-NKp46 or 1 X PBS i.p. starting at week 5 p.i. Mice were treated every other day for the duration of this experiment and harvested 2 weeks after start of treatment. The frequency of CD3–NK1.1+CD49b+NKG2A+ NK cells was measured in the spleen. Contour plots and graph present frequency of NK1.1+NKG2A+ spleen cells from 1 experiment repeated independently twice with $n = 5$ mice per group. *denotes significance with $p \leq 0.05$.

growing family of immune cells known as innate lymphoid cells (ILCs) (Spits et al., 2013; Eberl et al., 2015). NK cells provide a first of defense against many pathogens via their ability to lyse tumor cells and infected cells and produce high levels of IFNγ. Although they have a primary role in innate immune protection, they can also contribute to long-term immunity. NK cells participate in memory responses by further differentiating and developing long life and more efficient recall responses (O'Leary et al., 2006; Cooper et al., 2009; Sun et al., 2009). During acute viral infections, systemic infections, and in the tumor microenvironment NK cells can dysregulate CD4+ and CD8+ T cell responses promoting pathogen and tumor persistence and immune exhaustion (Perona-Wright et al., 2009; Lang et al., 2012; Waggoner et al., 2012, 2014; Cook and Whitmire, 2013; Crouse et al., 2014; Schuster et al., 2014; Cook et al., 2015; Crome et al., 2017). In addition NK cells can become exhausted themselves in different tumors models and infection (Sun et al., 2015). Based on this published knowledge of the complexity of NK cell biology, we tested whether NK cells become exhausted during chronic *T. gondii* infection, how they impact long term immunity to the chronic stage of infection and the mechanisms involved. Our studies demonstrate that NK cells do not appear to become exhausted because their numbers are stable and they do not increase PD1 or LAG3 expression despite losing the ability to produce IFNγ. They appear to alter chronic infection load in brain and erode secondary immune responses in chronically infected animals. They may accomplish this by increasing CD8+ T cell apoptosis in the periphery and as a result decrease the number of protective CD8+ T cells in both spleen and brain. We did not measure CD8+ T cell apoptosis in brain where many antigen specific cells exist. However, since these cells are likely primed in secondary lymphoid organs what we observe in the spleen apoptosis may reflect what is occurring in other tissues. This will be clarified by future studies. NK cells have increased activation as indicated by high KLRG1 and CD107a expression. During chronic *T. gondii* infection NK cells develop a unique Lin-CD49b+CD49a–Ly49–NKp46+CD94+NKG2A+ phenotype in spleen and brain suggesting that these cells receive signals from altered self through NKp46 recognition of specific ligands and a reduction in Qa-1b. Indeed staining of total spleen and CD8+ T cells with soluble NCR1 and anti-Qa-1b indicate there is a significant change in altered self during chronic *T. gondii* infection. Our studies further support this hypothesis when we block NKp46 interaction and rescue chronically infected mice from death caused by CD8+ T cell exhaustion similarly to depletion of NK cells. This blockade also reduces the frequency of NKG2A+ NK cells in spleen. Overall we find that NK cells are essential for acute immune protection by helping to control the parasite with IFNγ and also by helping to prime CD8+ T cells. However, during chronic *T. gondii* infection NK cells develop a

response that contributes to CD8+ T cell dysfunction thereby promoting chronic infection in mice.

NK cells can develop immune exhaustion in the tumor microenvironment, after overstimulation and during HCV infection (Gill et al., 2012; Sun et al., 2015; Alvarez et al., 2019; Zhang et al., 2019). Our results suggest that NK cells are not becoming exhausted, but are developing into cells that negatively regulate the CD8+ T cell responses during chronic *T. gondii* infection. CD8+ T cells are known to develop immune exhaustion during chronic *T. gondii* infection (Bhadra et al., 2011b, 2012). This leads to the reactivation of encysted parasites in the CNS as indicated by reduced cyst numbers in brain and increased parasites in the peripheral tissues such as blood and spleen and ultimately results in death of B6 mice (Bhadra et al., 2011b; Hwang et al., 2016). We observe higher cyst numbers in the brains of NK cell depleted animals compared to non-depleted groups during chronic *T. gondii* infection. These data could suggest that NK cells are promoting the reactivation of chronic infection and that cysts are maintained better because there are higher numbers of functional CD8+ T cells in the brain. However, we have yet to thoroughly investigate whether there is an increase in parasite reactivation indicated by reemergence of parasitemia or life stage transformation from bradyzoite to tachyzoite in the brain. The higher cysts numbers we observe may be counterintuitive suggesting a potential different mechanism. The depletion of NK cells could increase the amount of parasite antigen due to lack of control of the parasite resulting in higher cysts numbers and CD8+ T cell responses. We have not ruled this out in this study and future investigation will be carried out to completely understand the mechanisms involved.

Exhausted CD8+ T cells have defective secondary recall responses to parasite challenge (Bhadra et al., 2011b). The maintenance of polyfunctional memory CD8+ T cells during chronic *T. gondii* infection wanes, likely resulting in loss of this long-term protective cell population (Bhadra et al., 2012). Other adaptive immune cells such as B cells may also contribute to long term immunity to the parasite and it is unclear at this time whether they also become exhausted (Kang et al., 2000; Sayles et al., 2000). This raised the question about how the presence of NK cells might contribute to the waning of long-term immunity to the parasite and how this might impact protection against a secondary challenge. Our results indicate that NK cell depleted animals were better able to survive a lethal secondary challenge with either Type II or Type I strain infections. One possible explanation for this result is that NK cells may negatively impact secondary memory CD8+ T cell responses, CD4+ T cell responses or memory B cell responses to the parasite. Another possibility to explain our results is that NK cells and/or ILC1, which are also targeted by our antibody depletion, could contribute to the death observed by increasing immune pathology associated with the secondary challenges (Klose et al., 2014). How NK cells are decreasing secondary responses are still unclear and this question will be addressed in future studies.

CD8+ T cell exhaustion during chronic *T. gondii* infection is marked by reduced CD8+ T cell numbers, decreased frequencies and numbers of IFNγ+CD8+ T cells in the spleen and brain, increased CD8+ T cell apoptosis and high expression of PD1 on the surface of CD8+ T cells. These are hallmarks of CD8+ T cell exhaustion in several infection and disease models (Wherry and Kurachi, 2015). Our results demonstrate that NK cells are present in the spleen and brain during chronic *T. gondii* infection, they do not have reduced numbers and do not express high levels of PD1 or LAG3 as compared to CD4+ and CD8+ T cells. When we investigated NK cell function (IFNγ and CD107a), we observed that although NK cells in chronic *T. gondii* infection lose the ability to produce IFNγ, they increase their CD107a expression indicating a gain of function. Moreover, NK cell depletion rescued mice from death during chronic infection. NK cell depletion helped restore CD8+ T cell function in spleen and brain of chronically infected animals and enhanced the survival of chronically infected mice after secondary parasite challenge. Our results suggest that one way NK cells may affect CD8+ T cell immunity to the parasite is by increasing their apoptosis. We only measured apoptosis in the spleen, whether NK cells also increase CD8+ T cell apoptosis in the brain is unclear. Although, NK cells may lose the ability to produce IFNγ, our results suggest that unlike tumor, overstimulation and persistent HCV infection (Gill et al., 2012; Sun et al., 2015; Alvarez et al., 2019; Zhang et al., 2019), they are also gaining function that negatively regulates the adaptive response to chronic *T. gondii* infection. The mechanism by which they are causing this negative regulation is unclear and will be important in future studies.

NK cells in the steady state express a stochastic array of activating and inhibitory receptors that help regulate their function (Pegram et al., 2011; Kruse et al., 2014; Sun, 2016). In mice this includes the Ly49 family of receptors (D-I), natural cytotoxicity receptors (NCRs), NKG2D, 2B4, and CD94/NKG2A. In the naïve state in B6 mice, these receptors are expressed on most NK cells in different combinations, but at relatively high frequencies. Our data demonstrates that NK cells during chronic *T. gondii* infection have altered expression of NK cell receptors. We observe a near complete loss of Ly49 D, H and I. At the same time we observed the maintenance of CD49b+ NKp46+ NK1.1+ cells. Within this population the frequency of CD94+NKG2A+ cells increased dramatically and this increase was only observed during persistent chronic *T. gondii* infection and not after infection with the non-persistent vaccine strain. There are many reasons why these NK cells develop such a different phenotype from conventional NK cells during chronic *T. gondii* infection. One reason could be due to the chronic inflammatory environment of the mice. In the absence of common gamma chain cytokines, NK cells can be developed via an IL-12 bypass pathway (Ohs et al., 2016). The NK cells that develop via this emergency NK cell lymphopoiesis pathways exhibit low level expression of Ly49 proteins and are enriched for CD94+NKG2A+ cells. A recent study has demonstrated that during *T. gondii* Prugniaud and attenuated *cps1-1* strain infection, NK cells are plastic and could differentiate into modified NK/ILC1 type cell that persist with memory-like features (Park et al., 2019). As we have previously published, in our hands NK cells do not persist during *T. gondii* infection (Ivanova et al., 2019). However, the NK/ILC1 like cells observed in the previous study exhibit a similar receptor phenotype to

those we observe in this study during chronic ME49 infection. The development of the NK/ILC1 like cell may be indirectly STAT4 dependent, but not through cell intrinsic IL-12 induced signaling. This could indicate the role of different continually expressed cytokines such as IFNγ, IL-6, or TGFβ during chronic *T. gondii* infection. The role of transcription factors Tbet and Eomes were also not clear in the previous study (Park et al., 2019). Whether the cells we observe and are calling NK cells are truly NK cells is therefor still an outstanding and complex question to be addressed. Given the level of ILC plasticity it is entirely possible that the cells we are studying are ILC1 or ILC3. We have not fully investigated transcription factor expression in these cells but are planning this for future studies. Therefore we hypothesize that the cells we observe are NK cells being generated de novo after chronic infection is established as a result of the chronic inflammatory environment. Our future studies are examining this possibility.

NK cells appear to have a licensed NK cell phenotype during chronic *T. gondii* infection because of the presence of both activating and inhibitory receptors on their surface (Kim et al., 2005). Thus, the licensing paradigm could also explain why this phenotype of NK cells develops during chronic *T. gondii* infection. In this situation, the chronic infection environment in Toxoplasmosis causes the activating receptor NKp46 to recognize a ligand expressed on target cells that potentiates the activation, survival and increased abundance of NKp46+ CD94+NKG2A+ NK cells. This is what occurs with other activating receptors including Ly49H after it recognizes m157 from MCMV (Lanier, 2005; Sun et al., 2009). As a result of Ly49H and m157 interaction, Ly49H+ NK cells are more abundant, have longer life and can respond more efficiently to secondary infection. While Ly49H interaction with MCMV m157 could directly activate all Ly49H positive NK cells regardless of inhibitory receptor expression, our data suggests that because of the higher frequency of CD94+NKG2A+ NK cells within the NKp46+ population, that loss of the inhibitory signal through NKG2A could also help promote the development of this NK cell population. What NKp46 could be recognizing is still a mystery during chronic *T. gondii* infection. The ligands for NKp46 vary in source and structure and to date may include Influenza virus HA, Sigma 1 protein of Reovirus and *Candida glabrata* proteins Epa 1, 6, and 7 (Mandelboim et al., 2001; Vitenshtein et al., 2016; Bar-On et al., 2017). Our data indicate that there is increased staining of spleen cells and CD8+ T cells with soluble NCR1. We also observed reduced Qa-1b expression. Moreover, blockade of NKp46 with a non-depleting anti-NKp46 antibody rescued mice to a similar level from death compared to NK cell depletion with anti-NK1.1. What protein modifications are occurring or genes that are being expressed to produce NCR1 ligand are unclear, however, the increase in binding of soluble NKp46 and anti-NKp46 blockade supports the hypothesis that the NKp46 signal is required for the development of this unique NK cell population during chronic *T. gondii* infection.

NKG2A is an inhibitory receptor expressed on NK cells and CD8+ T cells that recognizes the non-classical MHC protein Qa-1b, which presents the leader sequence of classical MHC Class I (Vance et al., 1998). Through NKG2A-Qa-1b interactions NK cell lysis of target cells and IFNγ production can be inhibited (Colmenero et al., 2007; Lu et al., 2007). Qa-1b expression by B cells during high dose LCMV infection can limit NK cells negative regulation of LCMV specific T cell responses (Xu et al., 2017). Blocking this interaction results in higher viral persistence. NKG2A interaction with Qa-1b can also limit the activation levels of virus specific CD8+ T cells in acute poxvirus infection resulting in reduced activation induced cell death and better antigen specific CD8+ T cell responses and control of virus (Fang et al., 2011; Rapaport et al., 2015). Interestingly, Qa-1b also is important for the development of protective non-conventional CD8+ T cell responses in the salivary gland of mice during acute MCMV infection in the absence of conventional CD8+ T cells (Anderson et al., 2019). Much of what is understood about NKG2A-Qa-1b interactions is in the context of acute infections. We present data that at WK5 post infection with *T. gondii,* Qa-1b is increased in expression. However, by WK7 post infection Qa-1b expression decreases. Along with increased NCR1-ligand expression at WK 7 post infection, the loss of Qa-1b expression could promote the negative regulation of CD8+ T cell responses by NK cells to promote chronic *T. gondii* infection. Therefore our data supports the hypothesis that during *T. gondii* infection the role of NKG2A-Qa-1b axis is to keep NK cells from eroding CD8+ T cell responses important for control of the parasite. Whether the loss of this interaction is important for the development of these NK cells during chronic *T. gondii* infection is still unclear. We also have not examined how NKG2A-Qa-1b interactions impact immunity during acute *T. gondii* infection. Other receptors could also impact the NK cell responses we observe during chronic *T. gondii* infection. These include DNAM1 since DNAM1 expression on NK cells can limit immature DC (Seth et al., 2009). NK cells with high DNAM1 expression also had low level expression of Ly49 markers in mice. The role of DNAM1 could be further explored in future studies.

Another important phenotype we observe is the increase in KLRG1+ NK cells in chronically infected mice. KLRG1 is an inhibitory receptor expressed more highly as NK cells mature (Tessmer et al., 2007; Geiger and Sun, 2016). NK cell maturation is activating receptor dependent. Recent studies investigating exhausted NK cells during chronic stimulation suggest that increased KLRG1 indicates NK cell exhaustion (Alvarez et al., 2019). In this study, NKG2D interaction with high levels of NKG2D ligands results in increased KLRG1 expression and loss of NKG2D expression on the cells and NK cell exhaustion. Therefore, another possible explanation of the development the phenotype of NK cells during chronic *T. gondii* infection could be that ligands for other receptors are highly upregulated during chronic *T. gondii* infection. This could then explain why we observe a loss of expression of Ly49 D, Ly49H, and Ly49I+ NK cells. However, we performed an exhaustive analysis of known murine NK cell receptor ligands and we did not detect any increase in their expression during chronic *T. gondii* infection (data not shown).

Our data demonstrates that NK cells present during chronic *T. gondii* infection alter their role in immunity and act as negative regulators of CD8+ T cells to promote reactivation

of the parasite. NK cells are the cytotoxic ILC (Cortez and Colonna, 2016). They also produce high levels of IFNγ and other cytokines after activation. NK cells are usually considered to be a first line of defense against many pathogens and tumors. However, many recent reports demonstrate that NK cells can also negatively regulate adaptive immune responses through several different mechanisms (Perona-Wright et al., 2009; Lang et al., 2012; Waggoner et al., 2012, 2014; Cook and Whitmire, 2013; Crouse et al., 2014, 2015; Schuster et al., 2014; Cook et al., 2015; Rydyznski et al., 2015; Crome et al., 2017; Kwong et al., 2017). These include the production of the immunosuppressive cytokine IL-10. NK cells are activated to produce IL-10 during acute stage systemic infections including *T. gondii*. NK cells can also induce apoptosis or kill CD4+ and/or CD8+ T cells during acute infections through TRAIL-TRAILR interactions, NKp46 dependent cytotoxicity and cytotoxicity through undefined receptor ligand pairs. NK cells can also kill tumor infiltrating lymphocytes (TILs) via an NKp46 dependent process. Another study recently published suggests that NK cells that become exhausted during persistent HCV infection lose their ability to produce IFNγ and as a result the CD8+ T cell effector population is unable to be maintained (Zhang et al., 2019). These studies suggest that NK cells can secrete immune suppressive cytokines to act systemically to suppress immunity, can act directly against T cells and kill them or because they are exhausted themselves they are unable to help maintain CD8+ T cell functions. During chronic *T. gondii* infection we observe that NK cells lose their ability to produce IFNγ while increasing the CD107a expression. Thus, while NK cells might lose one function during chronic *T. gondii* infection they appear to have a gain of function. We did attempt to measure whether NK cells from chronically infected mice were more cytotoxic, but we did not observe any increase (data not shown). Importantly CD107a is only a surrogate marker for NK cell cytotoxicity (Alter et al., 2004). CD107a can associate with other secretory vesicles and in particular can be surface expressed alongside MHC Class II on DCs (Alter et al., 2004; Michelet et al., 2015). Therefore, we believe that the increase in CD107a on NK cells during chronic *T. gondii* infection may indicate a different type of immune suppressive function. What that suppressive function might be is still unclear. We did not observe NK cells producing IL-10 during the chronic stage of parasite infection and they also did not increase their expression of PDL1, the ligand for PD1. PDL1 expression can promote exhaustion of CD8+ T cells during chronic *T. gondii* infection (Bhadra et al., 2011b). Therefore, based on our results we propose that NK cells are acquiring a different type of immune suppression than producing IL-10 or being cytotoxic. Another possibility is that sustained NK cell IFNγ is required to help maintain CD8+ T cell function during chronic *T. gondii* infection. NK cells are thought during acute *T. gondii* to help prime CD8+ T cell responses, especially in the absence of CD4+ T cell help (Combe et al., 2005). We confirmed the importance of NK cells for priming CD8+ T cells in this study. Based on these studies and our data along with data from the persistent HCV infection study, a lack of NK cell IFNγ could also not support CD8+ T cell function. However, our data show that NK cell depletion enhances CD8+ T cell function during chronic *T. gondii* infection making this less likely and that the negative regulation of CD8+ T cell responses by NK cells is via a different mechanism.

The results from our study open a question about where NK cells might negatively regulate CD8+ T cell responses during chronic *T. gondii* infection. We observe NK cells present in both spleen and in brain. Brain numbers are very low and we did not measure whether NK cells and CD8+ T cells came into contact in the spleen or brain during chronic *T. gondii* infection. Since ILCs can traffic to the CNS in EAE (Kwong et al., 2017) and cause pathology, one potential tissue where NK cells might decrease CD8+ T cell function is in the CNS. This has yet to be tested. Another possible location could be in secondary lymphoid organs where CD8+ T cells are receiving signals to traffic to the CNS. This will require extensive future studies to understand where NK cells are impacting CD8+ T cells during chronic *T. gondii* infection.

In this study we present data suggesting that during chronic *T. gondii* infection, NK cells are still present, do not appear exhausted based on cell number and PD1 or LAG3 expression. They negatively impact the mortality of chronically infected mice and NK cell depletion rescues animals during CD8+ T cell exhaustion (CD8+ T cell function is maintained and apoptosis reduced in the spleen). The NK cells develop a unique phenotype and are enriched for cells that are CD49b+ NKp46+ CD94+ NKG2A+ KLRG1+. The development of this population could be dependent upon activating receptor NKp46 recognition of a specific ligand while NKG2A interaction with Qa-1b is reduced. NK cells suppress the CD8+ T cell response by an as of yet identified mechanism that may be independent of cytotoxicity, IL-10 production or the expression of PDL1. Overall in chronic *T. gondii* infection, NK cells may contribute to CD8+ T cell exhaustion and persistence of the parasite and manipulating them to prevent the development of this response could improve health outcomes for individuals susceptible to parasite reactivation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Wyoming Institutional Animal Care and Use Committee (IACUC) (PHS/NIH/OLAW assurance number: A3216-01) approved all animal protocols.

AUTHOR CONTRIBUTIONS

RK and JG developed the scientific concept. RK, DI, SD, KF, GS, DR, RF, TM, JM, and JG carried out experiments, acquired and analyzed data, and helped generate figures for the manuscript. JG wrote the manuscript. ID consulted on the

manuscript. All authors contributed to the article and approved the submitted version.

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Harnessing Natural Killer Cell Innate and Adaptive Traits in HIV Infection

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Despite efficient virological suppression on antiretroviral therapy (ART), people living with HIV (PLWH), experience an increased burden of premature co-morbidities, such as cancer and end-organ disease. With remaining challenges in terms of access to therapy, adherence and potential long-term drug toxicity, improving their long-term healthcare outcome, including new strategies for HIV clearance, remains a global priority. There is, therefore, an ongoing need to better characterize and harness the immune response in order to develop new strategies and supplement current therapeutic approaches for a "functional" cure. Current efforts toward HIV eradication to enhance immune recognition and elimination of persistently infected cells have highlighted the need for an optimized "kill" approach. Natural killer (NK) cells play an important role in antiviral defense and by virtue of their innate and adaptive features hold great promise as a focus of "kill" efforts. Galvanized by advances in the cancer field, NK cell exploitation, represents a transformative approach to augment HIV therapeutic modalities, circumventing many of the limitations inherent to T cell approaches. In this review we will discuss recent advances in our understanding of the development of NK cell adaptive/memory responses in HIV infection and highlight new and exciting opportunities to exploit the beneficial attributes of NK cells for HIV immunotherapy.

Keywords: natural killer (NK) cells, human immunodeficiency virus (HIV), cytomegalovirus (CMV), adaptive NK cells, immunotherapy

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OVERVIEW OF THE FUNCTION OF NK CELLS AND ROLE IN HIV INFECTION

NK cells are multipotent innate effector cells that play pivotal roles in antiviral and tumor immunity (Vivier et al., 2008). They can rapidly eliminate virus-infected or transformed cells through contact dependent mechanisms and exocytosis of cytotoxic granules and/or via death receptor pathways that induce apoptosis (Vivier et al., 2008). Another important mechanism for the elimination of target cells by NK cells is antibody-dependent cell cytotoxicity (ADCC), mediated by the FcgRIIIA receptor (CD16), which binds the constant region (Fc) of immunoglobulin-opsonized cells. This interaction induces phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) domains of the high-affinity IgE receptor (FcεRIγ) and CD3ζ in NK cells, and initiates a signaling cascade that ultimately results in the killing of the antibody-coated cell (Bournazos et al., 2017). In addition to cytotoxic elimination of target cells, NK cells are potent producers of cytokines and chemokines with antiviral function (Lodoen and Lanier, 2006).

It is increasingly recognized that NK cells have an important immunoregulatory role with the ability to promote or suppress adaptive (i.e., T and B cells) and innate immune cells (i.e., dendritic cells) and influence the outcome of infection (Walzer et al., 2005; Waggoner et al., 2016). We have previously demonstrated the rheostat role of NK cells in human chronic viral infections, with the capacity to restrain antiviral immunity in chronic HBV infection (Peppa et al., 2013), and more recently the ability to regulate the development of broadly neutralizing antibodies (bNAbs) in HIV infection (Bradley et al., 2018).

NK cell activation is tightly regulated by the integration of signals from an array of germline-encoded inhibitory and activating receptors (Long et al., 2013). This dynamic balance ensures self-tolerance, whilst permitting robust responses against virally infected cells that have downregulated major histocompatibility complex class I (MHC I) molecules that ligate NK-expressed inhibitory receptors, such as killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A, and/or upregulated stress ligands or viral associated molecules recognized by activating receptors, such as NKG2D or natural cytotoxicity receptors (NCRs) (Lanier, 2008; Orr and Lanier, 2010). During NK cell development, the interaction between inhibitory receptors and self-MHC molecules is critical for promoting their education and fine tuning their level of responsiveness (Elliott and Yokoyama, 2011; Boudreau and Hsu, 2018).

NK cells precede adaptive immunity during the early stages of HIV infection, where a rapid expansion of cytotoxic $CD56^{dim}$ NK cells is observed prior to CD8 T cell expansion (Alter et al., 2007a). Evidence from immunogenetic, antiviral functional, and viral evolution/immune evasion studies further implicate NK cells as important contributors to immune control of HIV, linking specific KIR/HLA combinations with disease outcome and protective KIRs with enhanced NK cell function *in vitro* (Martin et al., 2002, 2007; Alter et al., 2007b; Shah et al., 2010). Further studies have indicated that the relative contribution of NK cells to control of viral replication is influenced by the degree of HIV-mediated changes to MHC class I expression and the strength of KIR/HLA interactions (Boudreau et al., 2016; Korner et al., 2017). Moreover, indirect NK cell-mediated ADCC is a potent means of control of HIV infection and has been associated with vaccine induced protective immunity and implicated in phenotypes of viral control and slower disease progression (Haynes et al., 2012; Wren et al., 2013; Kulkarni et al., 2017; Madhavi et al., 2017).

Whereas chronic HIV infection is well-documented to affect NK cell subset redistribution and functional ability (Mavilio et al., 2003; Fauci et al., 2005; Brunetta et al., 2010), these defects appear to be at least partially recovered following introduction of effective ART (Frias et al., 2015; Mikulak et al., 2017). More recently in treated HIV infection, phenotypic alterations in peripheral NK cells were not found to result in improved functional responses to HIV (Zhao et al., 2020). Thus, in ART-treated PLWH, targeting NK cell subsets to boost their range of antiviral properties and/or recover any residual dysfunction could improve control of HIV and restraint the development of detrimental co-morbidities.

With recent advances increasing our understanding of the anatomic control of NK cell development (Dogra et al., 2020) including potential for memory responses (O'Sullivan et al., 2015), the opportunities to direct and exploit these distinct features of NK cells to target HIV have grown. Here, we will consider current immunotherapeutic approaches to harness NK cells, highlighting the beneficial attributes of adaptive/memory NK cell subsets and potential advantage over their conventional counterparts.

NK CELL-BASED STRATEGIES FOR ELIMINATION OF HIV—LEARNING FROM THE CANCER FIELD

The success of NK cells in cancer immunotherapy is emerging as an exciting field in augmenting therapeutic approaches against chronic viral infections (Shimasaki et al., 2020). These are based on activating immunological mechanisms that would allow durable viral control by enhancing NK cell endogenous responses and/or generating new immune responses (**Figure 1**). An important consideration with such approaches continues to be a balance between promoting highly effective NK cell responses and abating any potential toxicity/bystander effects (**Table 1**).

RELEASE OF NK SUPPRESSION

The use of monoclonal antibodies (mAbs) that target the interaction between MHC class I and NK cell inhibitory receptors represents one strategy that is currently used to enhance NK cell anti-tumor activity (van Hall et al., 2019). In the setting of HIV, HLA-E interaction with its ligand NKG2A expressed on NK cells could serve as an evasion mechanism to escape NK-mediated responses. In particular, elevated levels of HLA-A expression where shown to result in enhanced expression of HLA-E and increased NKG2A-mediated inhibition and subsequent impairment of HIV control (Ramsuran et al., 2018). Therapeutic blockade of NKG2A/HLA-E interaction could, therefore, improve HIV control alone or in combination with other approaches. In addition, blocking inhibitory signals mediated through inhibitory KIRs by Lirilumab (IPH2102) augment NK mediated elimination of autologous HLA-C expressing tumor cells (Romagne et al., 2009). Even though it is widely accepted that HIV downregulates HLA-A/B and most primary HIV isolates can mediate downmodulation of HLA-C (Apps et al., 2016) unleashing NK cells from inhibition, the extent of HLA-C downregulation and any residual binding to inhibitory KIRs could influence NK cell function (Korner et al., 2017). Hence the use of inhibitory KIR blockade could augment NK cell antiviral potency in curative strategies. In addition, NKG2A and inhibitory KIR blockade could have an effect on CD8 T cells expressing these receptors and work in a complementary fashion to increase cytotoxic T cell (CTL) activity. However, these approaches raise questions about excessive negative NK cell immunoregulation, potential autoreactivity and bystander killing of activated uninfected T cells.

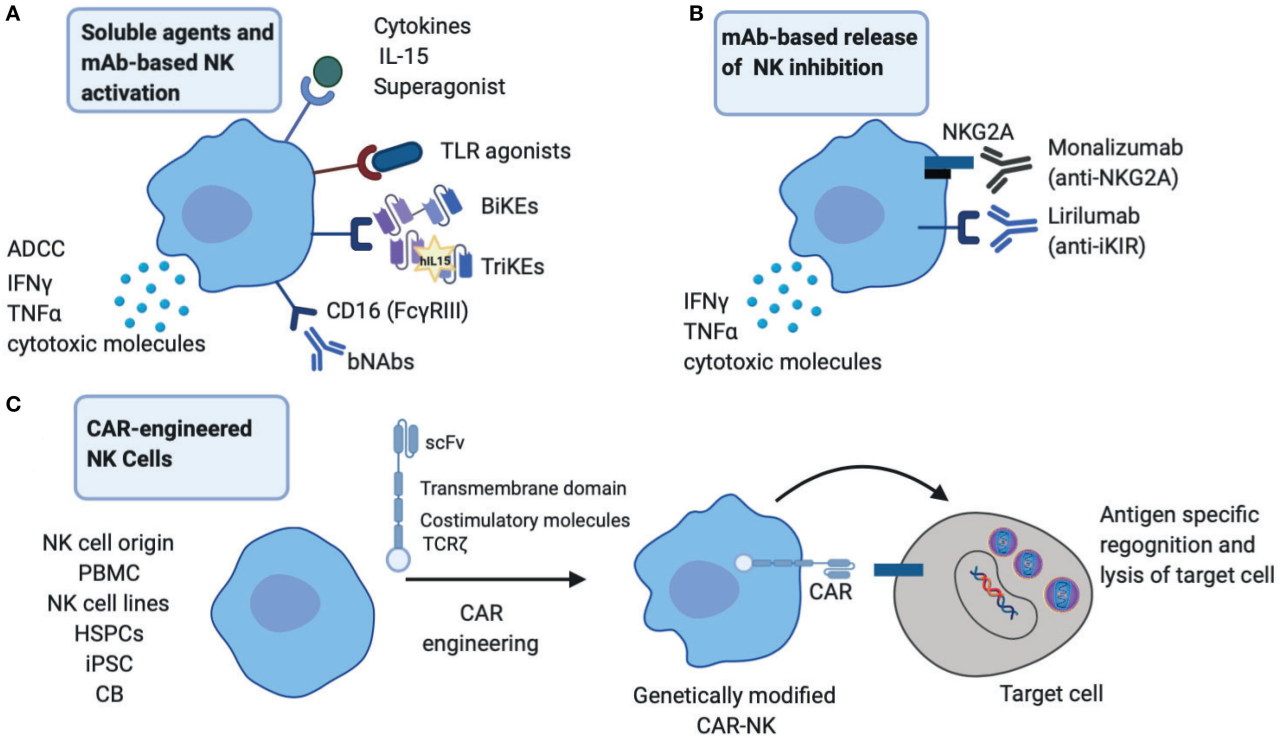

FIGURE 1 | Therapeutic startegies to harness NK cells in HIV infection. **(A)** NK cell activation strategies through broadly neutralizing antibodies (bNAbs), engineered proteins, Bi-specific or Tri-specific Killer engagers (BiKEs or TriKEs), soluble mediators such as cytokines and TLR agonists to boost NK effector functions including cytotoxicity and cytokine production. **(B)** Release of NK inhibition via engagement of monoclonal antibodies (mAb) directly against inhibitory receptors NKG2A and inhibitory Killer-cell immunoglobulin-like receptor (iKIRs). **(C)** CAR-engineered NK cells to target HIV infected cells. PBMC, peripheral blood mononuclear cells; HSPCs, hematopoietic stem/progenitor cells; iPSC, induced pluripotent stem cells; CB, cord blood.

REV-UP ENDOGENOUS NK CELL RESPONSES VIA BIKES/TRIKES AND BNABS

One exciting approach of improving NK cell functionality is through the use of the immunomodulators termed Bispecific and Trispecific Killer cell Engagers (BiKEs and TriKEs) (Tay et al., 2016). These are small molecules consisting of a single chain variable fragment (scFv) from the heavy and light variable chains of an antibody connected to one (BiKE) or two (TriKE) variable portions of antibodies specific to an antigen expressed on the surface of target cells. One of the components targets CD16 expressed on NK cells to induce direct killing via ADCC (Gleason et al., 2012; Vallera et al., 2016). Their use circumvents previous challenges associated with NK cell-based therapies including: (i) lack of specific targeting by NK cells and (ii) limitations in NK cell activation, survival and expansion *in vivo* which is overcome by the more superior TriKEs with IL-15 supplementation (Vallera et al., 2016). These approaches are already gaining ground as potent pharmacological interventions in cancer (NCT01221571) (Rothe et al., 2015; Sarhan et al., 2018a). BiKEs consisting of CD16A binding antibody domains fused through a linker to an engineered one-domain soluble human CD4 with high affinity to HIV-envelope glycoproteins have also been reported to show potential in HIV treatment/eradication (Li et al., 2017).

Following the rise of single cell cloning techniques, next-generation anti-HIV-1 broadly neutralizing antibodies (bNAbs) with greater potency/breadth and the ability to suppress viral replication and potential for Fc-mediated clearance of virus-infected cells have now entered the clinical arena (Halper-Stromberg and Nussenzweig, 2016) with promising results (Bar-On et al., 2018; Mendoza et al., 2018). The capacity of bNAbs to potentially induce NK-mediated ADCC following passive immunization (Lu et al., 2016) is of immense interests in current HIV cure attempts, where the *in vivo* effect of bNAbs on NK cells in humans remains to be determined (upcoming RIO trial).

TOLL-LIKE RECEPTOR (TLR) AGONISTS AND NK CELL ACTIVATION

TLR engagement with respective agonists/ligands coordinate some of the innate effector responses mediated by NK cells during viral infections. As such, TLR agonists have been utilized for the enhancement of the immunotherapeutic potential of NK cells especially in cancer immunotherapy, where they are being tested as adjuvants in clinical trials (Smith et al., 2018). In the

TABLE 1 | Selected trials and therapeutic approaches targeting natural killer (NK) cells in cancer and/or HIV1 immunotherapy and associated limitations of such approaches.

Therapeutic approaches	Type	Mode of action	Limitations and challenges	Trial registry identifier(s)*	References
Release of NK suppression	mAbs to NKG2A Monalizumab (previously IPH2201)	Blockade of NKG2A mediated inhibition of NK cells; synergizes with other checkpoint inhibitors or mAbs	Potential autoreactivity and off-target effects; optimal combination therapy	NCT02643550 NCT02921685 NCT02671435 NCT03822351 NCT03833440	Shimasaki et al., 2020
	mAbs to KIRs Lirilumab (IPH2102)	Blockade of inhibitory KIR mediated inhibition of NK cells	Potential autoreactivity and off-target effects; optimal combination therapy	NCT03532451 NCT01714739 NCT01687387 NCT01750580 NCT02252263 NCT02399917 NCT02481297	Ramsuran et al., 2018
Rev-up endogenous NK cell responses	BiKEs and TriKEs	Engage an activating receptor on NK cells (i.e., CD16), bridging it to a target cell; high efficacy; good safety profile	Complexity of design process; CD16 polymorphism; levels of CD16 expression on NK cells and CD16 cleavage	NCT01221571 NCT03192202 NCT03214666	Gleason et al., 2012; Rothe et al., 2015; Apps et al., 2016; Tay et al., 2016; Vallera et al., 2016; Sarhan et al., 2018a
	bNAbs	HIV neutralization; Fc mediated functions and ability to trigger NK cell-mediated ADCC	ADCC capacity of bNAbs; NK cell responsiveness; Fc receptor polymorphisms; possibility of generation of escape mutants	NCT02018510 NCT02825797	Halper-Stromberg and Nussenzweig, 2016; Li et al., 2017; Bar-On et al., 2018; Mendoza et al., 2018
Toll-Like receptor (TLR) agonists	TLR agonists	Enhance activation of components of adaptive and innate immunity, including NK cells	Potential off-target effects and toxicity	NCT02077868 NCT02200081 NCT02668770 NCT02858401 NCT03060447	Lu et al., 2016; Smith et al., 2018
Immunostimulatory cytokines +/- adoptive NK cell therapy	IL-2, IL12, IL-18, IL-15; IL-15 superagonists	*Ex vivo* cytokine NK cell expansion and activation; *In vivo* modulation and augmentation of NK cells responses	Systemic toxicity; optimal dosing required for expanded NK cells to prevent exhaustion; development of new compounds with improved pharmacokinetics	NCT01885897 NCT02191098 NCT04290546 NCT02890758 NCT03346499 NCT03899480	Romee et al., 2012; O'Sullivan et al., 2015; Huot et al., 2017
NK cell engineering	CAR-NK cell adoptive therapy; multiple cellular sources	Redirect NK cells against specific antigen to enhance lysis of target cells	Optimized CAR constructs to increase efficacy; remaining challenges to manufacturing and scaling up; potential toxicity	NCT02892695 NCT03056339 NCT03415100 NCT03941457	O'Sullivan et al., 2015; Romee et al., 2016; Oei et al., 2018; Angin et al., 2019

**Trial Registry Identifier(s) in cancer/or HIV immunotherapy.*

HIV cure field, studies in SHIV-infected non-human primates identified NK cell activation to be amongst the factors predictive of a delay in viral rebound in a vaccination regimen involving PGT121 bNAb infusion, a "kick" using TLR7 agonist (GS-9620), followed by antibody washout and treatment interruption (TI) (Borducchi et al., 2018). In this study, a proportion of PGT121 and GS-9620 treated animals remained undetectable for over 200 days following TI (Borducchi et al., 2018). These findings suggest a critical role of NK cells in strategies directed toward HIV eradication. Currently GS-9620 is being evaluated in two clinical trials, in HIV infected controllers (NCT03060447) and in individuals on suppressive ART (NCT02858401). These studies will provide valuable insights on safety, biological activity and impact on viral reservoirs.

IMMUNOSTIMULATORY CYTOKINES

The role of IL-15 cytokine in maintaining NK cell maturation, proliferation, homeostasis and antiviral immunity is well-established (Cooper et al., 2002). Levels of IL-15 correlate with NK cell antiviral function and natural control of SIV replication in lymph node B cell follicles in African green monkeys, a likely

viral reservoir (Huot et al., 2017). IL-15 is currently being tested in several clinical trials to improve NK cell function, persistence, and expansion (Guillerey et al., 2016). Remarkably, the IL-15 superagonist ALT-803 (also known as N-803) inhibited acute HIV infection through NK cell activation in humanized mice (Seay et al., 2015) and has been also shown to drive SIV-specific CD8 T cells to lymph nodes in macaques (Webb et al., 2018). ALT-803 is now being evaluated in a phase I clinical trial aiming to clear HIV-1 reservoirs (NCT02191098). The ability of IL-15 to enhance ADCC and augment NK cell mediated killing of HIV-infected target cells *ex vivo* (Garrido et al., 2018) could prove vital in the development of a functional cure for HIV. These findings, together with a recent report of IL-15 improving the efficacy of HIV-specific CD8 T cells from non-controllers (Angin et al., 2019), highlight the complementary effects of such an approach to simultaneously target and boost multiple arms of the immune response. IL-15 could synergize with other ongoing approaches that target NK cells. Along these lines, the safety and efficacy of IL-15 superagonists in combination with bNAbs to induce HIV control will be assessed in a future trial (NCT04340596).

(CAR)-ENGINEERED NK CELLS AND ADOPTIVE NK CELL TRANSFER

Chimeric antigen receptor (CAR)-engineered NK cells offer great promise as a new immunotherapeutic tool in the HIV field. The recent success of CAR NK cells derived from cord blood transduced with a retroviral vector, expressing the genes encoding anti-CD19, IL-15, and a safety switch (inducible caspase 9), in patients with refractory or relapsed CD19 positive cancers, represents a remarkable achievement in the field (Liu et al., 2020). Importantly the administration of CAR-NK cells was not associated with the development of cytokine release syndrome and toxicity seen with the use of CAR-T cells (Liu et al., 2020). With limited success of CAR-T cells in clinical trials to suppress HIV replication (Deeks et al., 2002), exploitation of NK cells from different sources that can be modified by the use of CAR constructs has several benefits. These include scalability, *in vivo* persistence and more favorable side effect profile. CAR-NK cells could, therefore, represent an attractive alternative to T cell approaches. In a humanized mouse model, anti-CD19 CAR-modified hematopoietic stem/progenitor cells (HSPCs) could differentiate into effector NK cells mediating an innate antiviral response and protection against HIV (Zhen et al., 2015). No doubt in the years to come we will see more exciting developments in this area.

In addition to genetic engineering of NK cells, adoptive cell transfer therapies using *ex vivo* expanded autologous or allogeneic NK cells or NK cell lines, such as NK-92, to treat human cancers (Shimasaki et al., 2020) could be considered as a therapeutic tool for HIV. The safety and tolerability of adoptive transfer of haploidentical NK cells and IL-2 (NCT03346499) or IL-15 superagonist N-803 (NCT03899480) in HIV infection along with any measurable impact on viral reservoirs are currently being evaluated.

THE CASE FOR ADAPTIVE NK CELLS IN HIV INFECTION

The recognition that NK cell subsets can expand and form long-lasting pools of memory-like cells in response to viral infection resembling the enhanced responsiveness previously regarded as a unique feature of adaptive T and B cell responses, represents a major advance in the field of NK cell research. Several types of adaptive NK cells have been described in humans, including in response to cytokines, antibody-mediated stimulation, vaccination and CMV-derived peptides (O'Sullivan et al., 2015; Reeves et al., 2015; Nikzad et al., 2019).

The best characterized adaptive NK cell subset in humans, expressing high levels of the activating receptor NKG2C, the activating counterpart of NKG2A that also binds to HLA-E (with lower affinity than NKG2A) (Guma et al., 2006; Beziat et al., 2013; Peppa et al., 2018) is driven by CMV infection. Recently a highly specific recognition of certain CMV-encoded HLA-E presented peptides was elegantly demonstrated to promote adaptive NK cell expansions (Hammer et al., 2018; Rolle et al., 2018). In addition, a rare CMV derived UL40 peptide that is identical to the HLA-E-binding peptide in the HLA-G signal sequence, was reported to trigger optimal NK stimulation and to enhance NK cell ADCC responses (Rolle et al., 2018). The NKG2C^{pos} NK cell population largely overlaps with the FcεRIγ adaptor protein-deficient subset that expands in response to antibody-opsonized targets or immune complexes (Hwang et al., 2012; Zhang et al., 2013). These CMV-reactive adaptive NK cells are delineated by a distinct epigenetic signature, similar to memory CD8 T cells, and changes in receptor expression, key transcription factors and signaling adaptor proteins (Guma et al., 2004; Luetke-Eversloh et al., 2014; Schlums et al., 2015). They are functionally specialized with a more selective recognition repertoire (oligoclonal pattern of self HLA-C KIRs) and reduced ability to respond to bystander activation/engagement of multiple activating receptors (Hammer and Romagnani, 2017). Instead they favor strong Fc receptor-dependent effector functions, especially IFN-γ production (Schlums et al., 2015).

CMV co-infection is near universal in HIV infected cohorts. We and others have shown the strong influence of CMV co-infection/reactivation in shaping the NK cell repertoire during chronic HIV infection. This leads to an accelerated differentiation and adaptive reconfiguration of the NK cell pool and a bias toward CD16 mediated NK cell effector functions (Zhou et al., 2015; Peppa et al., 2018). In particular during HIV/CMV co-infection, UL40 or HLA-G derived peptides may stabilize the expression of HLA-E and fine tune NK cell activation and antibody driven adaptive responses (Cubero et al., 2020); this blurs the dichotomous effect of the genetic polymorphism at position −21 of HLA-B on NK cell function described in CMV seronegative donors (Horowitz et al., 2016).

These expanded adaptive NK cells in HIV infected patients display enhanced responses to overlapping HIV envelope peptides (Zhou et al., 2015). Several reports also suggest that CMV-associated (NKG2C+) adaptive NK cells influence

the outcome of HIV infection and improve viral control (Thomas et al., 2012; Gondois-Rey et al., 2017; Ma et al., 2017). The presence of NK cells with mature/adaptive features (CD57+NKG2C+), during early HIV infection, is inversely correlated with HIV viral load and linked to lower viral set point, better early response to ART and better immunological outcome (Gondois-Rey et al., 2017; Ma et al., 2017). In contrast, NKG2C gene deletion is linked with higher HIV susceptibility and disease progression (Thomas et al., 2012).

In addition to the influence of CMV co-infection, a recent study demonstrated that the pro-inflammatory milieu in HIV infected patients drives the expansion of a defined NK subset with memory-like properties, characterized by $CD94^{+}CD56^{hi}$ and high expression of the transcription factor TCF7 (Wang et al., 2020). A combination of IL-12 and IL-15 was able to induce the generation of $CD94^{+}CD56^{hi}$ NK cells from $CD94^{-}CD56^{+}$ NK cells from HIV seronegative donors (Wang et al., 2020). These $CD94^{+}CD56^{hi}$ NK cells exhibited features in keeping with adaptive NK cells, including higher NKG2C expression, increased cytotoxicity and a more pronounced degranulation against HIV-infected CD4 T cells. The presence of $NKG2C^{+}TFC7^{+}CD56^{hi}$ NK cells correlated with HIV-induced inflammation and altered homeostasis of the gut resident and circulating innate lymphoid cells (ILCs) (Wang et al., 2020). The effect of CMV reactivation in the gut and influence on local NK cell populations was not addressed in this study but important to delineate given the pronounced contribution of CMV reactivation to loss of intestinal epithelial integrity and chronic inflammation in HIV, despite suppressive ART (Maidji et al., 2017).

Antigen-specific NK cells have been reported in rhesus macaques infected with SIV/SHIV (Reeves et al., 2015) and emerging evidence also supports the existence of human HIV-specific memory NK cells developing in response to vaccination, homing to the liver and expressing a $CXCR6+CD16^{lo}CD69+T\text{-}bet^{lo}Eomes^{hi}$ signature (Stegmann et al., 2016; Nikzad et al., 2019). Whether NK cell memory is restricted to resident populations (Paust et al., 2010; Nikzad et al., 2019) requires a deeper understanding of their relationship between blood and tissue and highlights the need for a closer examination of resident tissue populations in order to fully harness their therapeutic potential.

THE ADVANTAGE OF ADAPTIVE NK CELL-BASED IMMUNOTHERAPIES

Adaptive NK cells make a significant proportion of the peripheral NK cell pool in HIV infection (Zhou et al., 2015; Peppa et al., 2018). In addition to their longevity, and *in vivo* persistence they are endowed with enhanced capacity for ADCC (Schlums et al., 2015). Thus, by exploiting the specificities of monoclonal antibodies (mAbs), adaptive NK cells can exhibit increased reactivity against different immunogenetic epitopes and these attributes could be exploited in combination with HIV bNAbs already in clinical trials (Mendoza et al., 2018). Adaptive NK cells also exhibit a mature profile, specifically lack expression of NKG2A and preferentially express self-HLA KIRs, supporting the notion that adaptive NKG2C+ NK cells will have a superior ability to recognize "missing-self" on HIV-infected targets that have downregulated HLA-C (Apps et al., 2016). In addition, they are more resilient displaying elevated resistance to myeloid-derived suppressor cells (MDSC) and Treg suppression (Sarhan et al., 2016, 2018b) and exhibit enhanced bioenergetic profile, including enhanced mitochondrial oxidative phosphorylation (OxPhos) relative to their conventional NK counterparts (Cichocki et al., 2018). Adaptive NK cells also display a distinct activation threshold. For instance, while T cell immunoreceptor with Ig and ITIM domains (TIGIT) (co-inhibitory receptor) has been associated with NK cell exhaustion, TIGIT expression in HIV infection marks a mature NK cell subset with adaptive traits and enhanced responses to virus-infected cells (Vendrame et al., 2020). Taken together adaptive NK cell subsets have unique immunological features that are highly desirable, especially at the sites of immune engagement.

A concern with utilizing bulk NK cells for immunotherapy is their potential for unwanted immunoregulatory functions, such as regulation of T cell responses (Peppa et al., 2013) and B cell responses via elimination of CD4 follicular helper (Tfh) cells (Bradley et al., 2018). Adaptive NK cells are poised toward less immunoregulation exhibiting decreased degranulation against activated uninfected T cells (Schlums et al., 2015) and limited ability to restrain virus-specific T cells (Duhan et al., 2019). Exploiting the properties of adaptive NK cell subpopulations could, therefore, have the additional benefit of sparing activated bystander uninfected T cells and enhancing virus-specific responses against HIV.

Importantly adaptive NK cells have *in vitro* expansion capability with predictable selectivity as an alternative or combination strategy for a functional cure. Utilizing HLA-E expressing transfectants has been a successful strategy for obtaining robust proliferation of functional adaptive NK cells, with profound skewing toward a single self KIR, and enhanced NKG2C effector potential against allogeneic acute lymphoblastic leukemia primary blasts (Liu et al., 2017). The exquisite specificity of adaptive NK cells to HLA-E presented peptides (Hammer et al., 2018; Rolle et al., 2018), which has been shown to influence proliferation and ADCC responses (Rolle et al., 2018), opens up the possibility for further refinement of these approaches by tailoring the peptide ligand on HLA-E. Various combinations of specific cytokines have also been demonstrated to induce expansions of cytokine-induced memory like NK cells with increased responsiveness (Romee et al., 2012). In particular, IL-15 stimulated bulk NK cells from HIV infected donors on suppressive ART, were able to recognize and clear autologous latently HIV infected CD4 T cells following exposure to the potent latency reversal histone deacetylase inhibitor (HDACi), Vorinostat (Garrido et al., 2018). Such an approach could therefore be tailored to direct/boost specific adaptive NK cell subpopulations for functional cure strategies but so far has remained unexplored.

Indeed, adoptive transfer of cytokine-induced adaptive NK cells are being tested in phase I clinical trials in AML patients (Romee et al., 2016). CAR-transduced mature/adaptive NK cells exhibited enhanced effector responses relative to other conventional NK counterparts (Oei et al., 2018) and CD19-CAR memory-like NK cells exhibited antigen-specificity, higher anti-tumor responses, and a greater expansion compared with classical CAR NK cells (Berrien-Elliott et al., 2019).

FUTURE APPROACHES AND CONCLUDING REMARKS

The study of adaptive NK cell subpopulations and *ex vivo* expansion for clinical application represents an exciting new avenue for the development of novel therapeutic interventions in the field of HIV infection. These approaches can be combined with therapeutic antibodies improving their efficacy. In addition, the generation of memory NK cell represents a novel goal of new vaccination approaches incorporating targeted adjuvants or through enhancing presentation via HLA-E. Future innovative strategies for cure include manipulation of the metabolic machinery of immune cells and attempts to intrinsically rewire NK cells to improve their immunotherapeutic potential.

Despite the considerable amount of progress, additional work is required to fully unravel the unique properties of specialized and memory NK cells subsets, especially within key effector sites, along with their potential for functional exhaustion. This knowledge would be critical in order to leverage their distinct features and maximize their therapeutic use in chronic viral infections while offsetting any detrimental effects to adaptive immunity and the host.

AUTHOR CONTRIBUTIONS

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