Antiretroviral therapy partly reverses the systemic and mucosal distribution of NK cell subsets that is altered by SIV<sub>mac251</sub> infection of macaques

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**A B S T R A C T**

We characterized three subsets of NK cells in blood, and two subsets in mucosal tissues. SIV<sub>mac251</sub> infection increased total and CD16<sup>+</sup> NK cells in the blood. In the rectum, we observed a significant increase in total and NKG2A<sup>+</sup> NK cells during SIV infection. In contrast, the NKp44<sup>+</sup> subset significantly depleted in acute infection and continued to decline in frequency during chronic phase. During SIV infection, blood CD16<sup>+</sup> and mucosal NKG2A<sup>+</sup> subsets had increased cytotoxic potential. Intriguingly, the NKp44<sup>+</sup> NK cell subtype that likely mediates mucosal homeostasis via the production of cytokines, acquired cytotoxicity. Antiretroviral therapy significantly increased the frequency of mucosal NKG2A<sup>+</sup> NK cells and peripheral CD16<sup>+</sup> NK cells. However, it failed to restore the normal frequency of NKp44<sup>+</sup> NK cells in the rectum. Thus, SIV<sub>mac251</sub> infection causes changes in the distribution and function of NK cells and antiretroviral therapy during chronic infection only partially restores NK homeostasis and function.

**Introduction**

Understanding of the innate and adaptive immunological mechanisms that curtail HIV infection and dissemination will facilitate the development of more effective vaccines or therapies to combat HIV. The early phase of HIV infection is critical as the outcomes of the interaction between the virus and the immune system during this phase is likely to determine whether the virus is eliminated, locally controlled, or disseminates to the rest of the body (Naranbhai et al., 2013; Ansari et al., 2011). While it is clear that there is a rapid activation of innate immune responses after viral exposure (Chang and Altfeld, 2010; Haase, 2010) details on innate cell types and their role in the immune responses to HIV remains unclear.

NK cells are part of the innate immune system, which is the body’s initial defense against viral infections (Reeves et al., 2010a; Moretta et al., 2002). They kill virus-infected and neoplastic cells, via multiple mechanisms, including degranulation of cytotoxic granules and activation of death receptors (Cooper et al., 2001). NK cells also secrete a wide range of cytokines and chemokines that are pro-inflammatory or anti-inflammatory and are involved in regulating the adaptive immune responses (Farag and Caligiuri, 2006; Cooper et al., 2001). Increasing evidence supports an important role of NK cell subsets, in controlling HIV and SIV infection, both directly and indirectly (Ackerman et al., 2012; Fauci et al., 2005). Recent studies have demonstrated a significant association between slower HIV-1 disease progression and the presence of NK cell killer immunoglobulin-like receptor gene (KIR3DS1) with its ligand HLA-B alleles (Bw4-80lle) (Boulet et al., 2008; Martin et al., 2002). Furthermore, Alter et al. (2011)
showed HIV-I adaptation to NK cell mediated immune pressure. NK cells in conjunction with antibodies, can also kill target cells by antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated virus infection (ADCVI) (Chung et al., 2008). A recently concluded Phase III HIV vaccine trial (RV144), demonstrated 31% protection from infection, and an inverse correlation between the risk of HIV acquisition and ADCC (Bonsignori et al., 2012). Therefore, a better understanding of the mechanism of NK cells and HIV interactions may be important for the development of HIV vaccines.

Our lab and others have shown that SIV infection of Rhesus macaques is a valuable tool to mimic natural HIV infection in humans (Pegu et al., 2013; Morgan et al., 2008; Ansari et al., 2011). The majority of HIV infections are acquired through sexual transmission, thus HIV enters the body through the vaginal, rectal, or penile mucosa. In the initial phase, a small number of founder viruses may infect target cells in the mucosa and expand locally. Within few days however, the virus disseminates systemically and a persistent infection is established (Haase, 2005; Hladik and Hope, 2009; Yu and Vajdy, 2010). Recent reports suggest that the role of innate immune components, present at the mucosal site during this early phase of the infection, may determine the disease outcome (Borrow, 2011; Ansari et al., 2011).

Although, NK cell subsets have been described in human mucosal tissues, most of the studies on NK cell responses during HIV infection are limited to human peripheral blood (Sips et al., 2012; Alter et al., 2005). This is mainly due to practical difficulties in obtaining appropriate samples (vaginal or rectal mucosa) from humans. Recent transcriptional studies have shown a high degree of homology between Rhesus macaques and human NK cells (Hong et al., 2013). Thus, macaques are a powerful and relevant model to study how SIV/HIV infection changes NK cells frequency and function.

Accurate identification of NK cells subsets in the blood and tissues of nonhuman primates is complex and requires several surface markers (Pereira et al., 2008). Rhesus macaque peripheral blood NK cells are generally defined as CD3−CD20−CD80α−α and NKG2A+. NK cells can be further subdivided into three groups depending on the degree of CD56 and CD16 receptors expression (Reeves et al., 2010b; Hong et al., 2013; Ansari et al., 2011). The majority of naive Rhesus macaques blood NK cells are CD16+/CD56− (CD16 subset) and considered to be more cytotoxic, whereas the smaller CD16−/CD56+ (CD56 subset) subset produces more cytokines. In contrast, the CD16−CD56− (DN subset) may have both properties (Webster and Johnson, 2005; Pereira et al., 2008; Ansari et al., 2011).

Classifications of mucosal NK cell subsets are rather complicated. According to the recent classification, gut associated NK cells in human and mouse are grouped in the innate lymphoid cell group1 (ILC-group1) (Walker et al., 2013). However, two distinct NK cell subsets were recently defined in the mucosal tissues of the Rhesus macaque depending on NKP44 and NKG2A expression (Reeves et al., 2011). In naive animal’s NKG2A+ NK cells are more cytotoxic and NKP44+ NK cells produce cytokines. NKP44+ NK cells closely resemble the NK22 cells reported in human mucosal tissues (Cella et al., 2009) and they produce LIF, BAFF, and cytokines including IL7 and IL22 (Reeves et al., 2011). Thus, the NKP44+ subset may be important in maintaining gut mucosal integrity and regulating B-cell function.

Highly active anti-retroviral therapy (HAART) effectively suppresses HIV replication, restores CD4+ T-cell counts in blood, and reduces HIV morbidity. However, little is known about NK cell frequency and function in blood and tissues during HAART therapy. Indeed, only few studies addressed the effect of HAART on mucosal NK cell subsets (Costiniuk and Angel, 2012; Mela et al., 2007). Peripheral blood NK cells from patients treated with HAART showed durable activation of NK cells, and also reported a decrease in the expression of FcγRII11 receptor and reduced ADCC signaling (Lichtfuss et al., 2012; Leenensahy et al., 2010). Another study has shown a significant increase of NK cells in colonia lamina propria after HAART treatment in humans (Mela et al., 2007).

In this study, we characterized the NK cell subsets and their phenotypic and functional alterations during acute and chronic SIV infection in peripheral blood and rectal mucosa and also studied the changes of NK cell subsets after anti-retroviral therapy. During the acute phase of SIV infection, we observed a rapid increase of cytotoxic NK cells (CD16 NK cells) in the blood and NKG2A+ NK cells subsets in the gut. Mucosal NKP44+ cell subsets rapidly decreased in the acute phase and further decreased during the chronic phase. Mucosal NKG2A+ and NKP44+ NK cells altered their phenotypic and functional characteristics during SIV infection. This may lead to alteration of the gut homeostasis and subsequent chronic immune activation and disease progression. HAART treatment significantly increased CD16 NK cells in the blood and NKG2A+ NK cell subsets in the gut. However, mucosal NKP44+ NK cell subsets continue to decline, even after ART treatment in the chronically infected macaques.

Results
Phenotypic and functional characterization of NK cells subsets in the blood and mucosa of healthy un-infected macaques

Recent studies have defined macaque peripheral blood NK cell as CD3−CD80α− NKG2A+ (Total NK cells) (Xu et al., 2012; Reeves et al., 2011, 2010b). Three different NK cell subsets are identified based on the expression of surface receptors CD56 and CD16 (Fig. 1A). The predominant peripheral blood NK cell subsets are the CD16+CD56− (CD16 subset) and the CD56−CD16− (DN) cells, while the least frequent subset is the CD56−CD16+ (CD56 subset) (Fig. 1B). We characterized the activation/apoptotic potential, homing and cytokine production of each subset in the blood. The DN subset expresses the highest levels of the active form of caspase 3, suggesting a higher rate of turnover (Fig. 1C). The CD16 subset expresses the highest level of the CCR6, a gut homing marker (Ito et al., 2011) (Fig. 1D). The CD16 subset produced higher CD107a and low IFN-γ and TNF-α where as the CD56 subset produced higher IFN-γ and TNF-α when stimulated with 721.221 cells. This indicates that CD16 subset is potentially more cytotoxic while cytokine production may be a key feature of the CD56 NK subset in naive rhesus macaque peripheral blood (Fig. 1E).

Next, we studied the mucosal NK cell subsets in naïve macaques. Human mucosal NK cell subsets have been reclassified as an innate lymphoid cell Group1 (Walker et al., 2013). However, two different NK cell subpopulations have been recently reported at the mucosal tissues of rhesus macaques (Reeves et al., 2010b). Using a comparable gating strategy (Supplemental Fig. 1), we observed two distinct NK subpopulations; NKG2A+ NKP44− (NKG2A+ subset) and NKP44+ NKG2A− (NKP44+ subset) in the mucosal tissues (Fig. 2A). The NKG2A+ cells were the most frequent in mucosal tissues. Interestingly, the highest NKG2A+ frequency was observed in vaginal tissues followed by mesenteric lymph nodes, bronchoalveolar lavage (BAL), and rectal biopsies (Fig. 2A and B), whereas NKP44− NK cells were predominantly present in the rectal biopsies and mesenteric lymph nodes (Fig. 2A and C). We did not find NKP44− NK cells in peripheral blood, auxiliary, inguinal, or obturator lymph nodes in naïve Rhesus macaques (Fig. 2A). Most of the gut associated NKG2A+ NK cells expressed the classical NK cell marker CD56, and more than 50% of cells expressed CD16. However, NKP44+ cells expressed low amounts of CD56 and no CD16 in naïve animals (Fig. 2D and E). Although frequencies of NKP44+ NK cells were lower than
NKp44+ cells, they expressed higher levels of CCR6 than NKG2A+ cells (Fig. 2F). We did not observe a significant difference in proliferation or CCR5 expression between these subsets (data not shown). Since NK cells exhibit either cytotoxic, cytokine producing capabilities, or both, we stimulated mucosal NK cells with mitogens and measured intracellular cytokine production and expression of the degranulation marker CD107a. CD107a or LAMP-1 has been described as a functional marker for NK cells. CD107a on NK cells correlate with target cell lysis. Therefore, expression of CD107a can be use to estimate cytotoxic capability of NK cells (Alter et al., 2004). Following PMA and ionomycin stimulation, CD107a can be use to estimate cytotoxic capability of NK cells. Macaque's blood NK cell subsets were gated as CD3–CD20–CD8αα+ NKG2A+ cells. They were further delineated by expression of CD16 and CD56 receptors. (B). Relative distribution of different NK cell subsets CD16 (CD56–CD16+), CD56 (CD56+CD16) and DN (CD56–CD16–) in the peripheral blood. (C) Expression of Caspase-3 or (D) chemokine receptor CCR6 was measured on blood NK cell subsets. (E) Peripheral blood mononuclear cells were stimulated with 721.122 cells (E:T ratio at 5:1) for 12 h and measured CD107a expression, IFN-γ and TNF-α production on CD16, CD56 and DN NK cell subsets in naive macaques. The data represents seven naïve animals. Only significant P values are shown. Mann–Whitney test used to compare two groups and; 1way ANOVA used to compare multiple groups (*P < .05; **P < .01; ***P < .001). (ns: non significant).

Blood and gut NK cell subsets in Rhesus macaques during acute and chronic SIV infection

We studied the changes in the frequency of NK cell subsets in the blood and rectal mucosa during the acute and chronic phases of SIV infection. During the acute phase, total NK cells (CD3–CD20–CD8αα+ NKG2A+) in the blood were increased compared to naïve animals and continued to increase during the chronic phase (Fig. 3A). The CD16+ NK cell subset was increased significantly in acute infection and then began to decline during the chronic phase but remained higher than the pre-infection level (Fig. 3A). The CD56 subset showed a slight decrease, however it was not significant. Interestingly, the DN subset was significantly reduced during the acute phase, but gradually increased to pre infection level in the chronic phase (Fig. 3A). Of note, no correlation was observed between CD4 T cell count, viral load or frequency of C4 Ki67+ in the blood and CD16 NK cell subset (Supplemental Fig. 2) or any other peripheral NK cell subset (Data not shown).

We further examined the mucosal NK cell responses during acute and chronic SIV infection. Similar to the total peripheral blood NK cells, total NK cells (CD3–CD20–) in the gut also increased significantly post SIV infection and peaked during the acute phase. Further analysis of subsets showed that the NKG2A+ subset increased in acute SIV infection yet returned to the baseline levels in the chronic phase. Interestingly, NKP44+ cell subsets were severely depleted during acute infection and continued to decline in the chronic phase (Fig. 3B). Loss of NKP44+, cells that produce IL17 and potentially IL22, may lead to a loss of intestinal integrity causing microbial translocation and an increase in immune activation.

Chemokine receptor 6 (CCR6) expression on NK cells during SIV infection

CCR6 is the receptor for CCL20 ligand, which is primarily expressed in the gut. The CCR6/CCL20 combination plays an important role in gut immunity (Ito et al., 2011). CCR6 expression

Fig. 1. Phenotypical and functional characterization of NK cell subsets in peripheral blood of naïve Rhesus macaques. (A). Representative flow cytometric gating strategy to identify NK cell subsets in peripheral blood of naïve Rhesus macaques. Macaque's blood NK cell subsets were gated as CD3–CD20–CD8αα+ NKG2A+ cells. They were further delineated by expression of CD16 and CD56 receptors. (B). Relative distribution of different NK cell subsets CD16 (CD56–CD16+), CD56 (CD56+CD16) and DN (CD56–CD16–) in the peripheral blood. (C) Expression of Caspase-3 or (D) chemokine receptor CCR6 was measured on blood NK cell subsets. (E) Peripheral blood mononuclear cells were stimulated with 721.122 cells (E:T ratio at 5:1) for 12 h and measured CD107a expression, IFN-γ and TNF-α production on CD16, CD56 and DN NK cell subsets in naive macaques. The data represents seven naïve animals. Only significant P values are shown. Mann–Whitney test used to compare two groups and; 1way ANOVA used to compare multiple groups (*P < .05; **P < .01; ***P < .001). (ns: non significant).
Functional alteration of blood and gut NK cell subsets during acute and chronic SIV infection

NK cells have both effector and regulatory functions. A recent study on human gut mucosal NK cells showed two different NK cell populations present in the intraepithelial layer (IEL) and lamina propria (LP). Spontaneous controllers showed a stable IEL NK cell subset in rectal mucosa, vaginal tissues and mesenteric lymph nodes (MLN). Conversely, TNF-α and IL17 production was significantly decreased in the NKp44+ subset during acute SIV infection but increased during progression to the chronic phase. IFN-γ production was very low amounts of IL17 in naive animals and a further significant decrease is observed in SIV infection (Fig. 4D).

Interestingly, NKp44+ NK cell subsets demonstrated higher levels of CD107a production in the acute phase of the infection, which declined in the chronic phase but remained significantly higher than naive animals (Fig. 4A). Expression of CD107a by NKp44+ cells was surprising and suggests that the NKp44+ NK cell subset acquired cytotoxic capabilities when exposed to SIV (Fig. 4A). In addition, the NKp44+ subset demonstrated significantly increased production of Th1 cytokine IFN-γ during all stages of SIV infection (Fig. 4B). Conversely, TNF-α and IL17 production was significantly decreased in the NKp44+ subset, which could indicate a reduction in their ability to regulate mucosal homeostasis (Fig. 4C and D). NKp2A+ NK cells, the dominant NK cell subset in the gut, also had increased cytotoxic capabilities during acute SIV infection (Fig. 4A). Production of IFN-γ from NKp2A+ cells were significantly increased during the acute phase but reduced during progression to the chronic phase (Fig. 4C and D). TNF-α production was significantly reduced in NKp2A+ cells during acute SIV infection but increased during progression to the chronic phase (Fig. 4C). NKp2A+ cells produced very low amounts of IL17 in naive animals and a further significant decrease was observed in SIV infection (Fig. 4D).

Naive and infected blood NK cell subsets were analyzed for functional changes after stimulation with 721.221 cells, as described in “Materials and methods” section. We measured expression of the granzyme marker CD107a and production of IFN-γ, and TNF-α by different NK cell subsets. During the acute phase, CD107a expression was increased in all subsets. Interestingly, CD107a expression on the DN subset was significantly reduced in the chronic phase (Fig. 4E). IFN-γ production was elevated in CD16
subsets during the chronic phase (Fig. 4F) whereas; TNF-α production was transiently elevated during the acute phase in DN and CD56 subsets (Fig. 4G).

Incomplete restoration of NK cell subsets in blood and rectal mucosa of infected macaques treated with ART

ART therapy can lead to partial restoration of the peripheral and mucosal immune system. However, the effect of ART on the innate immune system, particularly NK cell subsets, has not been extensively studied. Therefore, in this study, seven chronically infected Rhesus macaques were treated with antiretroviral treatment for 11 weeks (Fig. 5A). ART significantly reduced plasma viral load (Fig. 5B), as well as SIV DNA in rectal biopsies (Fig. 5C). The effect of ART on NK cells in blood and rectal mucosa were studied by multi-parametric flow cytometry. The CD16 NK cell subset in the peripheral blood significantly increased after ART treatment. However, DN population (Fig. 5D) demonstrated a significantly lower frequency when compared to naive animals. In rectal mucosa, the NKG2A⁺ NK subset was significantly elevated after ART treatment (Fig. 5E). However, the Nkp44⁺ NK cell subset remained depleted despite ART treatment (Fig. 5E). No ART mediated functional changes were observed in any blood NK cell subsets stimulated with 721.221 cells (Fig. 5F–H).

Discussion

NK cells are a heterogeneous population that plays an important role in many viral infections and are involved in controlling SIV and HIV infection (Ansari et al., 2011; Naranbhai et al., 2013). They are part of the innate defense systems in the body and influence the development of adaptive immune responses. We assessed peripheral blood NK cells in macaques based on a previously reported gating strategy (Reeves et al., 2010b). We found that the CD16 subset is the most abundant NK cell population in the peripheral blood of naïve animals. This is consistent with findings in the peripheral blood of humans (Reeves et al., 2010b). Our results
also indicate a cytotoxic role for CD16 cells and a cytokine producing role for CD56 and DN subsets that express the highest levels of IFNγ and TNFα after in vitro stimulation.

The chemokine receptor CCR6 expressed by lymphocytes, including NK cells, binds to the chemokine ligand CCL20 and is an important receptor involved in regulating mucosal immunity.
Fig. 5. The effects of ART treatment on peripheral blood and gut NK cell subsets in SIV chronically infected Rhesus macaques. (A) Diagram representing ART treatment schedule. Chronically infected seven Rhesus macaques were treated with ART as described in "Material and methods" section. Samples were collected pre-ART, and 11 weeks post treatment from peripheral blood and gut tissues and the NK cell responses were evaluated. (B) Plasma viral load (SIV RNA) and (C) SIV viral load (SIV DNA) in rectal mucosa. The dot plots represent the changes of (D) blood NK cell subsets and (E) rectal mucosal NK cell subsets during pre- and post-ART treatment. Prior vaccinated animals are indicated in open squares or open triangles. As indicated in Supplemental Table 1, there were no differences in viral loads or CD4 counts between vaccinated and unvaccinated animals. Bar graphs represent the average and standard deviation for (F) expression of CD107a (G) and levels of IFN-γ and (H) TNF-α production by peripheral blood NK cell subsets following a 12 h stimulation with 721.122 cells at an E:T ratio of 5:1. Kruskal–Wallis tests were used for comparisons of each group. Only significant P values are shown (**P < .01; ***P < .001).
Interestingly, we observed that the frequency of NKG2A+ dominant NK cells in the gut and many other mucosal tissues, blood NK cells and exhibits cytotoxic properties and is the NKp44 subset (Reeves et al., 2011). The NKG2A+ NK cell subset is similar to blood NK cells and exhibits cytotoxic properties and is the dominant NK cells in the gut and many other mucosal tissues. Interestingly, we observed that the frequency of NKG2A+ NK cells was higher in vaginal tissues and in the lung than any other tissue. NKp44+ NK cells are cytokine producing cells and may represent the human NK22 counterpart. Other research also suggest that this subset is a part of innate lymphoid cell group (Xu et al., 2012).

The initial interactions of HIV/SIV with the immune system during the acute phase is critical to the outcome of the infection (Borrow, 2011). This phase determines whether the virus will be eliminated, or becomes established and the individual progresses to a chronic infection. We evaluated both peripheral blood and mucosal NK cell responses during the acute and chronic phases of the infection. Consistent with NK cell responses during acute HIV (Alter et al., 2005) and SIV infections, we also showed that CD16+, the cytotoxic NK cell subset in the blood, increased significantly during the acute phase and declined during the chronic phase of infection. CD16+ cells also showed significantly higher IFNγ production during the chronic phase. This early expansion of the CD16+ NK cell subset may be important for initial viral control. We also observed that the frequency of multifunctional DN NK cells in the blood is reduced during the acute phase with a slight increase during the chronic phase of infection. DN NK cells expressed higher levels of lymph node-homing marker CCR7 and CD62L (Reeves et al., 2011). Thus, initial reduction of DN cells in the blood may be due to trafficking to the lymph nodes during acute infection. The CD56 subset, which expressed CCR5, CCR6, CXCR3, and CXCR4 (Reeves et al., 2010a, b) decreased during chronic phase but did not show significant changes during both acute and chronic infection. However, TNFα production of this subset significantly reduced during the chronic phase. Interestingly, CCR6 expression in CD56 subset was significantly changed during the chronic phase of infection. This may suggest a possible recruitment of this NK cell subset to the mucosal tissues as infection progresses.

The frequency of cytotoxic mucosal NK cell subset (NKG2A+), was transiently increased during acute infection in the rectal mucosa, where the virus primarily replicates. The cells also significantly increased production of IFNγ during the acute phase of infection. This may improve the anti-viral status in the gut before the induction of adaptive immune cells. With progression to the chronic phase of infection, we observed a loss of frequency and a significant reduction of IFNγ production, which indicates the suppression of NK cell function during the chronic stages of the infection. However, the cytotoxic capability remained significantly increased. In addition, the NKp44+ NK cell subset was significantly reduced during the acute and chronic phase of infection. This subset also exhibited a significant reduction of IL17 and TNFα producing cells during disease progression. IL17, together with TNFα, has been shown to be an important cytokine in the host defense (Hartupee et al., 2007; Xu et al., 2012). Previous studies have shown that the IL17 producing cells were important for mucosal epithelial integrity during SIV and HIV infection (Brenchley et al., 2008; Hartigan-O’Connor et al., 2011; Reeves et al., 2011). Therefore, the loss of IL17 producing NKp44+ cells during infection in the gut may have a significant impact on gut homeostasis during SIV infection in macaques. Furthermore, NKp44+ cells significantly changed their functional capacity. The reduced IL17 production and TNFα production occurred in conjunction with a significant increase in the production of CD107a and IFNγ production during infection. Taken together, our study has shown that rapid changes occur both in the frequency and in the function of different NK cell subsets during the course of SIV infection.

HAART therapy restores CD4+ T-cell counts in blood and has reduced the incidence of AIDS in HIV-infected individuals. However, little is known about NK cell responses during HAART therapy. There are only a few reports about the mucosal NK cell subset during HAART therapy (Costiniuk and Angel, 2012; Mela et al., 2007). Recent reports from HAART treated patient's peripheral blood NK cells showed continued activation of NK cells and decreased expression of Fcγ receptor γ and reduced ADCC signaling. In this study, we showed that with ART treatment, the CD16 NK cell subset significantly increased in the blood. However, we did not observe restoration of functional capability of blood NK cells after ART. Interestingly, the frequency of the cytotoxic NKG2A+ NK cell subset in the rectal mucosa was also significantly elevated. Nevertheless, NKp44+ NK cell subsets continuously declined during ART. Thus, we observed a continued alteration of NK cell subsets in chronically infected macaques treated with ART. Further studies are needed to gain better understanding of the effect of antiretroviral therapy on HIV/SIV infections.

We have studied NK cell subsets in the peripheral blood and mucosal tissues during acute and chronic SIV infection and while we observed no significant associations with virus loads, we do observe significant alterations in NK cells during infection. During the chronic infection, NK cell subsets in both peripheral blood and mucosal tissues increased their cytotoxicity. Mucosal tissues, in particular, lost their cytokine producing NK cell subsets. Interestingly, the expression of chemokine receptor 6 (CCR6) and their trafficking patterns are changed during the infection. Furthermore, ART treatment in animals with chronic SIV infection demonstrated an up regulation of cytotoxic NK cell subsets in blood and rectal mucosa but ART failed to restore cytokine producing NKp44+ cells.

Materials and methods

**Animals and antiretroviral therapy**

We studied a total of 32 Indian Rhesus macaques. Twelve animals were vaccinated with ALVAC-SIV and/or HPV-SIV and gp120 and 20 animals were unvaccinated. All animals were challenged with repeated low doses of SIVmac251 either intra-rectally or intra-vaginally. Seven of the 32 animals were followed longitudinally during the acute and chronic phases. At 6 months post infection, these seven animals were treated daily with Raltegravir (100 mg/day/BID), Zerit (Stavudine) (1.2 mg/kg/BID) orally, PMPA (20 mg/kg/day), and Emtricitabine (FTC) (50 mg/kg/day) subcutaneously. The prior treatment of these macaques is summarized in Supplemental Table 1. Naïve samples were collected prior to vaccination and SIV infection. Post infection samples were obtained 2 weeks post infection (acute) and 21–41 weeks post infection (chronic). Vaccination did not reduce SIV viral burden or CD4 loss in the acute or chronic phase. All of the Rhesus macaques were housed and handled in accordance with the standards of the Association for the
Mononuclear cells from blood and tissues were isolated as described previously (Vargas-Inc et al., 2011; Reeves et al., 2011). For the phenotypic characterization, 2 x 10^6 cells were used, and flow cytometry staining was carried out for cell surface and intracellular molecules using standard protocols. NK cell functions were analyzed using 3 x 10^6 cells after stimulation with phorbol myristate acetate (50 ng/mL) and ionomycin (1 μg/mL) or 721,221 cells a MHC-devoid human cell line that are MICA/MICB-negative, and Golgi Plug™ (Brefeldin A) and Golgi Stop™ (Monensin) were added at final concentrations of 6 μg/mL. All samples were then cultured for 12 h at 37 °C in 5% CO2. Unstimulated (medium alone) samples were used as the negative controls. After incubation, cells were washed and stained for the surface and then fixed and permeabilized with a Cytofix/Cytoperm Kit™ (BD Biosciences), and stained for the specific intracellular molecules. The following anti-human fluorochrome-conjugated mAbs known to cross-react with rhesus macaques were used for the staining: V450 anti-IFN-γ (B27), PE-Cy7 anti-CD56 (NCAM 16.2), Alexa Fluor 700 anti-CD3 (SP34-2), Allophycocyanin anti-CD57 (H57-597) (all from BD Biosciences, San Jose, CA); PE-Cy5 anti-CD107a (eBioH4A3), Alexa 488 anti-IL17 (eBio64DEC17), eFluor 605NC anti-CD20 (2H7), and eFluor 605NC anti-CD8a (RPA-T8) (all from BD Biosciences, San Jose, CA). For intracellular molecules using standard protocols. NK cell functions were analyzed using 3 x 10^6 cells after stimulation with phorbol myristate acetate (50 ng/mL) and ionomycin (1 μg/mL) or 721,221 cells a MHC-devoid human cell line that are MICA/MICB-negative, and Golgi Plug™ (Brefeldin A) and Golgi Stop™ (Monensin) were added at final concentrations of 6 μg/mL. All samples were then cultured for 12 h at 37 °C in 5% CO2. Unstimulated (medium alone) samples were used as the negative controls. After incubation, cells were washed and stained for the surface and then fixed and permeabilized with a Cytofix/Cytoperm Kit™ (BD Biosciences), and stained for the specific intracellular molecules. The following anti-human fluorochrome-conjugated mAbs known to cross-react with rhesus macaques were used for the staining: V450 anti-IFN-γ (B27), PE-Cy7 anti-CD56 (NCAM 16.2), Alexa Fluor 700 anti-CD3 (SP34-2), Allophycocyanin anti-CD57 (H57-597) (all from BD Biosciences, San Jose, CA); PE-Cy5 anti-CD107a (eBioH4A3), Alexa 488 anti-IL17 (eBio64DEC17), eFluor 605NC anti-CD20 (2H7), and eFluor 605NC anti-CD8a (RPA-T8) (all from BD Biosciences, San Jose, CA). For intracellular molecules using standard protocols. NK cell functions were analyzed using 3 x 10^6 cells after stimulation with phorbol myristate acetate (50 ng/mL) and ionomycin (1 μg/mL) or 721,221 cells a MHC-devoid human cell line that are MICA/MICB-negative, and Golgi Plug™ (Brefeldin A) and Golgi Stop™ (Monensin) were added at final concentrations of 6 μg/mL. All samples were then cultured for 12 h at 37 °C in 5% CO2. Unstimulated (medium alone) samples were used as the negative controls. After incubation, cells were washed and stained for the surface and then fixed and permeabilized with a Cytofix/Cytoperm Kit™ (BD Biosciences), and stained for the specific intracellular molecules. The following anti-human fluorochrome-conjugated mAbs known to cross-react with rhesus macaques were used for the staining: V450 anti-IFN-γ (B27), PE-Cy7 anti-CD56 (NCAM 16.2), Alexa Fluor 700 anti-CD3 (SP34-2), Allophycocyanin anti-CD57 (H57-597) (all from BD Biosciences, San Jose, CA); PE-Cy5 anti-CD107a (eBioH4A3), Alexa 488 anti-IL17 (eBio64DEC17), eFluor 605NC anti-CD20 (2H7), and eFluor 605NC anti-CD8a (RPA-T8) (all from BD Biosciences, San Jose, CA). For intracellular molecules using standard protocols.


