Human natural killer cells confer protection against HIV-1 infection in humanized mice

Can M. Sungur, …, Wayne M. Yokoyama, Liang Shan


The role of natural killer (NK) cells against HIV-1 infections remains to be elucidated in vivo. While humanized mouse models potentially could be used to directly evaluate humans NK cell responses during HIV-1 infection, improved functional development of human NK cells in these hosts is needed. Here we report the humanized MISTRG-6-15 mouse model, in which NK cells were quick to expand and exhibit degranulation, cytotoxicity, and pro-inflammatory cytokine production in non-lymphoid organs upon HIV-1 infection, but had reduced functionality in lymphoid organs. Although HIV-1 infection induced functional impairment of NK cells, antiretroviral therapy reinvigorated NK cells in response to HIV-1 rebound after analytic treatment interruption. Moreover, a broadly neutralizing antibody PGT121 enhanced NK cell function in vivo, consistent with antibody-dependent cellular cytotoxicity. Monoclonal antibody depletion of NK cells resulted in higher viral loads in multiple non-lymphoid organs. Overall, our results in humanized MISTRG-6-15 mice demonstrated that NK cells provided direct anti-HIV-1 responses in vivo but were limited in their responses in lymphoid organs.
Human Natural Killer Cells Confer Protection Against HIV-1 Infection in Humanized Mice

Can M. Sungur¹, Qiankun Wang², Ayse N Ozanturk², Hongbo Gao², Aaron J Schmitz³, Marina Cella³, Wayne M. Yokoyama¹,⁴, *, Liang Shan²,⁴,*

1. Division of Rheumatology, Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA.
2. Division of Infectious Diseases, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA.
3. Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, USA.
4. The Andrew M. and Jane M. Bursky Center for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, Saint Louis, MO, USA.

* Correspondence:
Wayne M. Yokoyama
Mailing address: Campus Box 8045-0020-10, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO, 63110-1093.
Tel: 314-362-9075. Email: yokoyama@wustl.edu

Liang Shan:
Mailing address: Campus Box 8051, Washington University School of Medicine, 425 South Euclid Avenue, St. Louis, MO, 63110-1093.
Tel: 314-747-0050. Email: liang.shan@wustl.edu
Abstract

The role of natural killer (NK) cells against HIV-1 infections remains to be elucidated in vivo. While humanized mouse models potentially could be used to directly evaluate humans NK cell responses during HIV-1 infection, improved functional development of human NK cells in these hosts is needed. Here we report the humanized MISTRG-6-15 mouse model, in which NK cells were quick to expand and exhibit degranulation, cytotoxicity, and pro-inflammatory cytokine production in non-lymphoid organs upon HIV-1 infection, but had reduced functionality in lymphoid organs. Although HIV-1 infection induced functional impairment of NK cells, antiretroviral therapy reinvigorated NK cells in response to HIV-1 rebound after analytic treatment interruption. Moreover, a broadly neutralizing antibody PGT121 enhanced NK cell function in vivo, consistent with antibody-dependent cellular cytotoxicity. Monoclonal antibody depletion of NK cells resulted in higher viral loads in multiple non-lymphoid organs. Overall, our results in humanized MISTRG-6-15 mice demonstrated that NK cells provided direct anti-HIV-1 responses in vivo but were limited in their responses in lymphoid organs.

Brief Summary

Natural killer (NK) cell responses to HIV-1 infections in vivo were studied in humanized mice. NK cell depletion enhanced infection, indicating they control HIV-1 infection.
**Introduction**

NK cells recognize ligands presented on the surface of HIV-1-infected cells, including infection-induced self-proteins (1, 2), and possibly the viral envelop protein (3), and Fc regions of viral-specific antibodies (4, 5), as well as MHC class I molecules (6, 7), that engage either activating or inhibitory NK receptors, respectively. Genetic and epidemiological studies strongly support an active role for NK cells in HIV-1 infection. Interestingly, the combined genotype of certain germline-encoded killer immunoglobulin-like receptors (KIRs), generally selectively expressed on NK cells, and their HLA ligands is associated with slower HIV-1 disease progression (8, 9) or protection against HIV-1 acquisition (10-12), and KIR-dependent NK cell activities appear to directly mediate immune pressure that leads to HIV-1 evolution in infected individuals (13). In addition, antibody-dependent cellular cytotoxicity (ADCC), potentially mediated by NK cells, is associated with HIV-1 control (14-17) and is linked to vaccine-induced protective immunity against HIV infection (18-20). NK cells can inhibit HIV replication but these studies have been limited to *in vitro* and *ex vivo* studies on NK cell from peripheral blood samples (21-25). Thus, the role of NK cells in direct *in vivo* control of HIV-1 infections has not been elucidated.

In mouse experimental models, NK cells have disparate effector functions across tissues and tissue-resident NK cells acquire unique functions different from blood NK cells for viral containment (26, 27). In humans, evidence for discrete stages of differentiation and functional development of blood and tissue NK cells has also been reported. While most blood NK cells are mature (CD56dimCD16+) with strong capacity to lyse target cells, the immature and non-cytolytic CD56brightCD16− NK cells are the predominant subset in secondary lymphoid tissues (28-30), the major sites for HIV-1 infection in untreated individuals (31-33) and the most important anatomical
reservoirs for latent HIV-1 in people on suppressive antiretroviral therapy (34). However, there is a relative scarcity of studies assessing the pleiotropic functions of tissue NK cells during HIV-1 infection because of the lack of easy access to tissue samples. Therefore, it remains unknown how NK cells influence HIV-1 infection in vivo despite strong evidence from epidemiological and in vitro studies.

Generation of human immune system mice (humanized mice) can be achieved in various strains of immunodeficient mice transplanted with human hematopoietic stem and progenitor cells (HSPCs). Humanized mice are useful tools to study HIV-1 infection and immune responses (35) but human NK cells reconstituted in immunodeficient mice are numerically and functionally deficient, secrete lower levels of IFN-gamma and do not respond to NK cell-susceptible targets (36, 37). This may be potentially due to the lack of cross reactivity between murine IL-15 and human IL-15 receptors since IL-15 is essential for the development and survival of NK cells and human IL-15 injection can rescue differentiation of human NK cells (38, 39). However, IL-15 supplementation is only temporary and the appropriate distribution of IL-15-expressing cells critical for development and maintenance of NK cells with circulating or tissue-resident identities (40) cannot be achieved through cytokine injection or transgenic expression. Both hematopoietic and non-hematopoietic cells provide IL-15 and IL-15 receptor alpha (IL-15Rα) and both can drive NK cell differentiation with hematopoietic-derived IL-15Rα being more important to the late maturation process (41). MISTRG mice with knock-in expression of human M-CSF, IL-3/GM-CSF, SIRPα, and THPO that together support efficient development of human myeloid cells, the hematopoietic source of IL-15, showed improved development of human NK cells in tissues (42). Since human IL-15 production in the MISTRG model is solely dependent on human myeloid cells,
experimental conditions that destroy the myeloid cell compartment such as clodronate or HIV-1 infection lead to rapid loss of NK cells (42, 43). On the other hand, a mouse with human IL-15 and signal regulatory protein alpha (SIRPA) knock-in, named SRG-15, can provide human IL-15 from the non-hematopoietic compartment comprising murine stromal cells and epithelial cells since it does not support efficient human myelopoiesis (40). SRG-15 mice promote improved human NK cell maturation in the bone marrow and periphery, which relies on the suboptimal cross-reactivity between human IL-15 and murine IL-15RA (44). To further improve NK cell development, we generated MISTRG mice with humanized IL-6 and IL-15, termed MISTRG-6-15, which produce human IL-15 from both hematopoietic and non-hematopoietic cell compartments, more physiologically relevant than the MISTRG and SRG-15 models. Since IL-6 stimulates hematopoietic progenitor cells and myeloid differentiation in mice (45), IL-6 humanization partially blocks murine hematopoiesis to better support overall reconstitution of multiple lineages of human immune cells.

Here we characterized human NK cell development and their functional dynamics during acute and chronic HIV-1 infection, or post antiretroviral therapy (ART) in humanized MISTRG-6-15 mice. More importantly, we showed that NK cell depletion led to increased HIV-1 replication and accelerated disease progression in vivo. Thus, we provide direct evidence that human NK cells can control HIV-1 infection in vivo.
Results

Functional development of human NK cells in humanized MISTRG-6-15 mice

We first compared reconstitution and functionality of human NK cells in reconstituted MISTRG-6-15 and the commonly used NSG mice. MISTRG-6-15 mice had significantly more circulating NK cells and monocytes compared to NSG mice engrafted with the same cord blood sample (Fig. 1A and B). In addition, tissue NK cells (defined by CD56 and not NKp46 to reduce inclusion of group 1 and 3 innate lymphoid cells) were also significantly higher in MISTRG-6-15 mice (Fig. 1C and Fig. S1). Furthermore, NK cells in all tissues of the MISTRG-6-15 mice produced more cytokines (Fig. 1D-G) and had stronger cytolytic capacity (Fig. S2A) than those in NSG mice. Concanamycin A was used to confirm that the killing of K562 cells was perforin-dependent (Fig. S2B). Thus, human NK cells show improved reconstitution in both number and function in MISTRG-6-15 mice as compared to NSG mice.

Human NK cell functions vary widely across tissues, particularly with respect to lymphoid NK cells. Indeed, the frequency of NK cells in lymph nodes (LNs) of MISTRG-6-15 mice was low and vast majority of LN NK cells (>90%) were functionally immature (Fig. 1G) as compared to the spleen, liver, and lung (Fig. 1D-F). To further compare functions of lymphoid NK cells from humans and MISTRG-6-15 mice, we purified NK cells from human tonsil and blood samples. Similar to previous studies (28-30), we found that the frequency of NK cells expressing CD16, granzyme B (GZMB), or perforin was significantly higher in blood compared to tonsil (Fig. 2A-C), and tonsil NK cells could not lyse K562 cells (Fig. 2D) or autologous CD4+ T cells infected by HIV-1 (Fig. 2E). The predominance of immature NK cells in lymphoid tissues is likely due to their expression of the homing receptor CCR7, which was virtually absent in mature CD16+ NK cells (Fig. S3). To evaluate the ADCC activity, we co-cultured blood or tonsil NK cells with
autologous CD4+ T cells infected with a recombinant HIV-1 reporter virus (HIVivo-HA) (Fig. S4). The humanized IgG1 anti-HA antibody can bind to HIVivo-HA infected cells but had no neutralizing activity (46). Blood NK cells were able to lyse more than 40% of the infected autologous blood CD4+ T cells, which was Fc-dependent, as evidenced by the complete loss of cell lysis when treated with a mutant anti-HA antibody carrying mutations (GRLR) that abrogated binding to activating Fc receptors. In contrast, tonsil NK cells exhibited minimal cytolysis of the autologous tonsil CD4+ T cells regardless of treatment, similar to no antibody controls. Similar to the disparate tissue distribution patterns in humans, the vast majority of NK cells in blood and non-lymphoid tissues of MISTRG-6-15 mice exhibited mature phenotypes with CD16 expression (Fig. 2F and S5). By contrast, the dominant presence of CD16- immature NK cells was observed in LNs of MISTRG-6-15 mice (Fig. 2F) and these NK cells produced very low levels of effector molecules (Fig. 2G) and were unable to kill K562 cells (Fig. 2H). Overall, the functional development of human NK cells in MISTRG-6-15 mice with tissue-specific disparities comparable to humans allowed us to compare tissue-specific NK cell responses to HIV-1 infection.

**Dynamics of NK cell responses in MISTRG-6-15 mice during acute and chronic HIV-1 infection**

We infected MISTRG-6-15 with HIV-1BaL to characterize viral infection. Plasma HIV-1 RNA was readily detectable as early as 7-8 days post infection and peaked at around 3 weeks (Fig. 3A). Viral replication was paralleled by CD4+ T-cell depletion in blood and tissues (Fig. 3B and C). Viral infection was detected by cell-associated HIV-1 RNA (cavRNA) quantification in various tissues of infected mice, showing clear viral dissemination (Fig. S6A). Levels of CD16 expression and functions of NK cells in blood, lymphoid and non-lymphoid tissue were enhanced during acute
infection, albeit the enhancement in lymphoid tissues was less robust (Fig. 3D and E, and S6B). CD8+ T cells exhibited similar functional patterns with reduced degranulation and pro-inflammatory cytokine production in the lymph nodes (Fig. S7). Previous studies showed that several immune checkpoint receptors (ICRs) were up-regulated in NK cells in the setting of cancer or chronic viral infection and these receptors negatively regulated NK cell cytotoxicity (47-50). Similarly, we found rapid increases of KLRG1, LAG-3, PD-1, and TIGIT expression in blood and tissue NK cells within two weeks post HIV-1 infection (Fig. 3F and S6C). ICR upregulation in CD8+ T cells was also observed in the same groups of mice 21 days after HIV-1 infection (Fig. S8). Although NK cells were more functionally active during acute infection, upregulation of ICRs might lead to functional impairment. To further evaluate the possibility of functional impairment in NK cells during the course of HIV infection (Fig. 4A), we monitored the survival, proliferation, and functionality of NK cells from day 0 to day 168 post infection. Rapid expansion of NK cells was seen in all organs (Fig. 4B). A slow decline in NK numbers was seen between day 42 and 168. Correspondingly, NK cells proliferated early during the course of infection in the organs and returned to baseline when infection was sustained beyond 100 days (Fig. 4C). After ex vivo stimulation with PMA/ionomycin, NK cells at early stage of infection showed increased potency for degranulation and IFNγ and GZMB production, which then declined during the chronic phase (Fig. 4D-F). Notably, without ex vivo stimulation, the baseline functions of NK cells from acutely and chronically infected mice were similar and were higher than those from uninfected mice (Fig. S9), suggesting continuous NK cell activation. Taken together, these results suggest that HIV-1 results in chronic activation and functional impairment of NK cells in all tissues.
Liver NK cells had the highest percentage degranulation and cytokine production when compared with NK cells in other organs, but NK cells in all organs showed increased functionality after HIV infection that persisted throughout the studied span of disease. Additionally, the liver NK cells had the least decline in functionality throughout the course of infection when compared with the other organs. These data suggest that liver NK cells retained their functionality throughout the course of infection possibly through establishment of immunological memory in the liver (3, 51).

**ART prevents the progressive loss of NK cell functionality**

In people living with HIV-1, functional impairment of NK cells due to chronic HIV-1 infection can be partially restored by ART (52-54). In the MISTRG6-15 mouse model, the expansion and functional activation of NK cells occurred within the first 10 days of infection, whereas NK cell numbers and functions began to decline on day 50, and almost returned to baseline after day 100 (Fig. 4). Notably, the levels of inhibitory receptors including LAG3, PD-1, and TIGIT in NK cells increased during acute infection (Fig. 3F), suggesting that early ART initiation may better improve NK cell functional restoration. To test this hypothesis, we studied mice that were initially infected for 4 weeks and then compared NK cells in mice on ART for 8 weeks to those from untreated mice (Fig 5A). The levels of inhibitory receptors including LAG-3, PD-1, and TIGIT all increased on NK cells after infection and were partially reduced in mice receiving ART (Fig 5B-D). Next, we performed analytic treatment interruption (ATI) to determine whether NK cell functions were preserved by ART. Mice under ART had undetectable viral load, and virus rebounded in all animals within 4 weeks after ATI (Fig 5E). We found that the number of NK cells in various tissues increased following ATI (Fig 5F). In addition, NK cell functions including cytokine production and cytotoxicity were also increased after ATI (Fig 5G-K). Notably, LN NK cells
responded to initial infection (Fig 3) but did not respond to viral rebound after ATI, suggesting that additional approaches might be needed for the functional restoration of LN NK cells. Nonetheless, for non-lymphoid NK cells, these results suggest that ART prevented their functional impairment, including loss of proliferation capacity and degranulation seen in chronically infected mice (Fig 4); and ART reinvigorated NK cells in response to HIV-1 rebound after ATI.

**NK cells suppress HIV-1 infection in vivo**

Previous studies showed that transfusion of human blood NK cells suppressed HIV infection in humanized mice (55, 56). While our studies here show NK cells responded to HIV-1 infection as well as viral rebound after ATI, it is unclear whether endogenous NK cells can directly impact HIV-1 replication in vivo. To address this, we first isolated NK cells from various organs of infected mice and co-cultured them with HIV-infected donor-matched CD4+ T cells for 4 hours. NK cells from all of the organs did exhibit degranulation and killed HIV-1-infected target cells, but liver NK cells showed the most pronounced response (Fig. 6A and B). Next, the in vivo control of HIV-1 by NK cells was studied by depleting NK cells with a monoclonal antibody against NKp46 that has been shown to deplete human NK cells in humanized mice (57). Here depletion of NK cells was successful in all studied organs (Fig. 6C and D). On day 14 post HIV-1 infection, except for LNs, CD4:CD8 ratios were significantly lower upon NK cell depletion as compared to non-NK cell depleted mice (Fig. 6E). No apparent CD4 depletion was observed in LNs even in control mice that is likely due to the small number of CD4+ T cells expressing CCR5 (Fig. S10).

Next, we collected blood and tissues to measure plasma HIV-1 RNA and cell-associated HIV-1 RNA, respectively. NK depletion led to an increase in plasma HIV-1 RNA by 5-10 fold (Fig. 6F). Depletion of NK cells also caused increased cell-associated viral RNA in tissues, especially in the
liver (Fig. 6G), which was consistent with the robust NK cell response in the liver. These results strongly suggest that NK cells directly suppress HIV-1 replication \textit{in vivo}.

**Antibody treatment improves NK cell functionality**

Administration of broadly neutralizing antibodies (bNab) into viremic individuals enhances viral-specific T cell responses (58-62) and may also modulate NK cell functions through Fc-dependent mechanisms. To address the potential role of bNab therapy may have on NK responses, we used an HIV neutralizing antibody PGT121 (63) to understand the Fc-dependent NK cell functions. (Although IgM$^+$ or IgD$^+$ human B cells develop properly in most humanized mice, a scarce amount of hypermutated, class-switched IgG antibodies are produced, mainly due to the lack of germinal center response (64), necessitating a passive antibody treatment approach to study ADCC by NK cells.) Moreover we also used PGT12 with GRLR mutations that block antibody binding to Fc receptors (46). PGT121 or PGT121$_{\text{GRLR}}$ were injected into infected mice, and viral loads in the plasma were reduced by both antibodies (Fig 7A and B). In mice treated with PGT121, the spleen and lung had greater reduction of cell-associated HIV-1 RNA and numbers of infected cells than the PGT121$_{\text{GRLR}}$ group (Fig 7C and D), indicating Fc-dependent clearance of HIV-1-infected cells. No reduction was seen in the LNs, likely due to the lack of functionally mature NK cells in LNs. Surprisingly, the number of HIVp24$^+$ cells in the liver was reduced by PGT121, whereas liver HIV-1 RNA was unchanged. It is possible that HIV-1 RNA signals were detected from kupffer cells that engulfed HIV-1-infected T cells upon antibody treatment. Since the GRLR mutation abolishes both ADCC (Fig. S4) and ADCP (46), it is possible that macrophages and NK cells both contributed to the clearance of HIV-1-infected cells mediated by PGT121 interacting with the intact Fc fragment. Next, we aimed to evaluate the influence of antibody therapy on NK cells. NK
cell functions such as degranulation and GzmB production were selectively enhanced by PGT121 in the spleen, livers, and lungs, and slightly in the lymph nodes. By contrast, the PGT121GRLR did not have any effect on NK cell functionality (Fig. 7E). These results support antibody therapies improving NK cell cytolytic activity in an Fc-dependent manner.
Discussion

Despite strong epidemiological evidence from large-scale cross-sectional studies, there is still a nascent understanding of the roles of NK cells in HIV-1 infection in humans. The lack of clarity is likely due to several factors: the dependence of NK cell function on the associated HLA genotype, the functional diversity not fully captured by examination of circulating NK cells, and finally the inadequacy of animal models. The MISTRG-6-15 mouse model recapitulates the human immune system beyond prior humanized mouse models. Of note, the NK population is robust and more functional than NSG mice and better mirrors human NK responses to immunological challenges in the various organs. More importantly, the various organs also showed dramatically different responses and functionality of the NK cells especially when comparing lymphoid versus non-lymphoid tissues. While NK cells from lymphoid tissues from human donors had reduced functionality when compared to blood, human NK cells in MISTRG-6-15 mice had similar properties. Thus, human NK cells in reconstituted MISTRG-6-15 mice more closely resembled NK cells in humans.

Here we further showed, in humanized MISTRG-6-15 model infected with HIV-1, the depletion of NK cells by monoclonal antibody resulted in higher levels of viral replication and accelerated loss of CD4+ T cells. For the first time, we found the direct evidence for control of HIV-1 infection by NK cells *in vivo*. Furthermore, we found no change in disease progression in the lymph nodes after NK depletion, consistent with the reduced functionality of human NK cells in lymphoid tissue. Thus, this mouse model allows us to study NK responses to HIV-1, especially in different organs, such as lymphoid versus non-lymphoid tissues.
The humanized MISTRG-6-15 mice also allowed investigation of how bNab therapy modulates NK cell functions, as related to antibody effector functions. In HIV-1-infected mice, NK cell functionality was improved with PGT121 administration in all organs, but was least improved in the lymph nodes. These results highlight the potential difficulty in eliminating the remaining infected cells in lymphoid tissues by bNabs, perhaps due to the lack of functional effector cells.

During acute HIV-1 infection in humanized MISTRG-6-15 mice, NK cells rapidly expanded and had greater percentages of cells degranulating and producing inflammatory cytokines in non-lymphoid tissues, while those in lymphoid tissues were immature or dysfunctional throughout the course of infection, consistent with results from humans and studies of non-human primates infected with simian immunodeficiency virus (65). This highlights a key concern of HIV-1 persistence in lymphoid tissue (34, 66, 67), as overall the NK cells appear to be less cytotoxic and pro-inflammatory. In chronically infected mice, the functions of NK cells declined in various organs tested, which was accompanied by increases in potential NK exhaustion markers in all of the organs studied that may allude to overall dampened response over time to HIV-1 by these cells. The dampened response was partially reduced after ART, which resulted in the return of functional NK cells after treatment interruption. Notably, NK cells in the lymph nodes not being strongly affected by ART suggests either NK cells were overall minimally responding to HIV-1 in the lymphoid tissue initially or there remains continuous immunological stimulation due to the persistence of HIV-1-infected cells in the lymphoid tissues despite ART (68).

By better understanding the role of NK cells during the course of HIV-1 infection in different organs, more targeted therapies and approaches to treatment can be pursued. Therefore going
forward, it will be important to use this humanized mouse model to dissect the molecular and cellular mechanism for NK cell-mediated HIV-1 suppression and to develop new strategies to enhance NK cell functions, especially in the lymphoid tissues.
Methods

Mouse strains

The generation of knock-in mice encoding human M-CSF, IL3/GM-CSF, SIRPA, THPO, IL6, and IL15 in a 129xBALB/c (N3) genetic background (42, 44) was performed using Velocigene technology by Regeneron Pharmaceuticals. Mice were bred to a Rag2\(^{-/-}\) Il2rg\(^{-/-}\) background with homozygous knockin to generate two mouse colonies including MCSF\(^{h/h}\) GMCSF\(^{h/h}\) IL3\(^{h/h}\) THPO\(^{h/h}\) IL6\(^{h/h}\) Rag2\(^{-/-}\) Il2rg\(^{-/-}\) and MCSF\(^{h/h}\) GMCSF\(^{h/h}\) IL3\(^{h/h}\) SIRPA\(^{h/h}\) THPO\(^{h/h}\) IL6\(^{h/h}\) IL15\(^{h/h}\) Rag2\(^{-/-}\) Il2rg\(^{-/-}\). To produce mice for engraftment with human cord blood CD34\(^+\) cells, the two colonies were crossed to generate MCSF\(^{h/h}\) IL3\(^{h/h}\) GMCSF\(^{h/h}\) SIRPA\(^{h/m}\) THPO\(^{h/h}\) IL6\(^{h/h}\) IL15\(^{h/m}\) Rag2\(^{-/-}\) Il2rg\(^{-/-}\) mice, labeled MISTRG-6-15. Human SIRPA and IL15 loci were used as heterozygotes for engraftment throughout the study. Nonobese diabetic (NOD) severe combined immunodeficient (Scid) Il2rg\(^{null}\) (NSG) mice were obtained from The Jackson Laboratory. Full characterization of the IL-6 knock-in mouse will be described in further detail in subsequent publication.

Human samples

De-identified human cord blood samples were collected at the St. Louis Cord blood bank. Anonymous peripheral blood samples were acquired from the Mississippi Valley Regional Blood Center as waste cellular products. Human tonsils were collected from elective tonsillectomies from Children’s Hospital in Saint Louis, which were provided as surgical waste, with no identifiers attached.

Plasmids, monoclonal antibodies and viruses
The anti-HA and anti-HA-GRLR IgG1 heavy chain and light chain expressing plasmids as well as the HIVo-HA viral plasmid were obtained from Michel Nussenzweig laboratory at The Rockefeller University (46). Codon optimized PGT121 heavy chain and light chain expressing plasmids were obtained from Dennis Burton laboratory at The Scripps Research Institute. GRLR mutations were introduced to the PGT121 heavy chain plasmid. PGT121 and anti-HA antibodies were produced by transfecting the FreeStyle™ 293-F cells. Replication-competent HIV_{Bal} viruses were prepared from PHA-stimulated CD8-depleted healthy donor PBMCs. The HIV-1 reporter virus NL4-3-ΔEnv-EGFP (AIDs reagent program #11100) pseudotyped with VSVG envelop was prepared from transient transfection of plasmid DNA into 293T cells. HIVo-HA virus was also prepared from transient transfection of 293T cells. Concentrated viral stocks were prepared using Lenti-X™ Concentrator (TaKaRa #631232).

In vitro ADCC Assay

De-identified frozen tonsillar and blood mononuclear cells were used for the functional analyses. To obtain purified human NK cells from blood and tonsil mononuclear cells, CD3/CD19 depletion was performed before NK cell purification using EasySep™ Human NK Cell Isolation Kit (Stemcell tech. #17955). Human CD4^{+} T cells were isolated using MojoSort™ Human CD4 T Cell Isolation Kit (Biolegend #480010). Purified CD4^{+} T cells were co-stimulated with plate-bound CD3 (Biolegend #300465) and soluble CD28 (Biolegend #302943) antibodies with the presence of 20ng/mL IL-2 (Biolegend #589106) for 3 days prior to viral infection. Activated CD4^{+} cells were infected with HIVo-HA virus by spin inoculation at 1,200g for 2 hours at 30°C and incubated at 37°C in medium supplemented with 20ng/mL IL-2. 4 days post infection, infected tonsillar or blood CD4^{+} T cells were co-cultured with their autologous NK cells in a 1:1 effector
to target ratio with anti-HA or anti-HA_{GRLR} (1\mu g/ml) for 4 hours. Target cells lysis was determined by the percentage of lysed infected cells (CD3^{+}CD8^{+}HA^{+}7-AAD^{+}) in the total infected cells (CD3^{+}CD8^{+}HA^{+}).

**Generation of humanized mice**

Human CD34^{+} cells were isolated from cord blood using EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Stemcell tech. #17896) and were cryopreserved in Iscove’s Modified Dulbecco’s Medium containing 7.5% DMSO. For MISTRG-6-15 mice, 1-3 days old newborn mice were humanized through injection of 1.5-3x10^{4} cord blood CD34^{+} cells intrahepatically. For NSG mice, 1-3 days old newborn mice preconditioned with sublethal irradiation (80cGy) followed by intrahepatic injection of 1x10^{5} cord blood CD34^{+} cells. Reconstitution of human CD45^{+} cells in blood was determined 9-10 weeks post engraftment. Mice were grouped after checking for blood engraftment to ensure that animals from different treatment groups or time points had similar levels of human T cells, NK cells, and macrophages. In each treatment group and at any time point, both male and female mice were used. Mice were randomly sorted into different treatment groups and time points. Identical cord blood donor was used when possible for experiments, but variation in donors between experiments exists.

**HIV-1 infection and treatment of humanized mice**

9-10 weeks post engraftment, the MISTRG-6-15 mice were infected with HIV-1_{BAL} (10ng p24) by retro-orbital injection. Uninfected mice received 100\mu l PBS by retro-orbital injection. To quantify HIV-1 infection in tissues by flow cytometry, intracellular HIV-p24 staining (Beckman Coulter #KC57-RD1) was performed using the Cytofix/CytopermTM kit (BD #554714). To
quantify plasma HIV-1 RNA, blood samples were collected by retro-orbital or submandibular bleeding. Plasma viral RNA was extracted by Quick-RNA Viral Kits (Zymo Research #R1035) before reverse transcription using SuperScript™ III Reverse Transcriptase (Thermo Fisher #18080085). Quantification of tissue HIV-1 RNA was described previously (69). Briefly, after single cell suspensions of tissues are obtained as described below, a fixed portion of each tissue was used for RNA extraction by Direct-zol RNA Kits (Zymo Research #R2071), then reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). The HIV-1 gag-based qPCR assays using ten-fold serial dilutions of HIV-1 genomic DNA as standard (70) were used to quantify plasma and tissue HIV-1 RNA. Total HIV-1 copy numbers are then obtained by multiplying by the proportion of the total sample used for analysis.

To suppress HIV-1 replication in mice, antiretrovirals were added into mouse food as previously described (71). Briefly, final concentrations of drugs in mouse food are 1g/kg tenofovir disoproxil fumarate, 1g/kg Emtricitabine, and 2g/kg raltegravir. To depletion human NK cells in vivo, mouse anti-human NKp46 antibodies (clone B-L46) (cell sciences #CDM306B) were administered by intraperitoneal injection on day 0 and day 7 post infection. Each mice received 50μg antibody per injection. Control mice received 100 μl PBS by intraperitoneal injection. For bNab treatment, PGT121 or PGT121GRLR was administered by retroorbital injection. Each mice received 250μg antibody per injection. Control mice received 100μl PBS by retro-orbital injection.

Functional analysis of NK cells and CD8+ T cells from humanized mice

Spleens, livers, and submandibular, axillary, and thoracic lymph nodes were excised from mice and filtered through 100 μm strainer using the back end of a syringe. Strainer was washed with tissue buffer (PBS+FBS). Samples were centrifuged followed by resuspension of pellet in tissue
buffer. For livers, cells were resuspended in 8 ml of tissue buffer and 5 ml of 100% percoll and then centrifuged for 20 minutes at 2000 rpm. Lungs were excised and cut into small pieces by scissors. They were then incubated at 37°C with digestion buffer contained collagenase and DNase I for 45 minutes. Samples were then filtered through 100 μm strainer using the back end of a syringe. Strainer was washed with tissue buffer (PBS+FBS). Samples were centrifuged followed by resuspension of pellet in 10 ml of 40% percoll. Samples were centrifuged for 20 minutes at 2000 rpm. After single cell suspensions from all tissues were obtained, cells were utilized for three purposes including ICR staining, intracellular cytokine staining, and target cell lysis. ICR staining was performed using freshly isolated cells. For intracellular cytokine production, cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) and stained with anti-CD107a antibody for 4 hours. Brefeldin A and monensin (golgiplug) was used for the final hour of stimulation. Cells were then washed and stained with CD56, CD3, CD8, NKp44, and CD16. Cytofix/Cytoperm kit (BD #554714) was then utilized and intracellular staining was performed with IFNγ, TNFα and GZMB (Figure S11). Purified NK cells were used to assess target cell lysis.

To purify NK cells from mice, single cell suspensions were obtained from specified organs as outlined above followed by elimination of CD45+ mouse cells with EasySep™ Mouse CD45 Positive Selection Kit (Stemcell tech. #18945) followed by NK cell isolation using EasySep™ Human NK Cell Isolation Kit (Stemcell tech. #17955). Purified NK cells were co-incubated with CFSE-labeled (Thermo Fisher #C34554) K562 cells (ATCC, CCL-243), effector to target ratios of 1:2, for 4 hours and cytotoxicity was assayed by 7-AAD uptake. Background spontaneous K562 blast death (no effector control wells) was subtracted to yield percentage specific killing. For experiments investigating perforin mediated killing, concanamycin A (Sigma #C9705) was utilized at 100 nM with cells incubated for 120 minutes prior to killing assay. To determine lysis
of HIV-1-infected target cells, NK cells and CD4\(^+\) T cells were purified separately from mice engrafted with CD34\(^+\) cells from the same cord blood donor. Purified CD4\(^+\) T cells were infected with the HIV-1 reporter virus NL4-3-ΔEnv-EGFP for three days. Infected cells were then co-cultured with freshly isolated NK cells from various tissues in a 1:1 effector to target ratio for 4 hours and cytotoxicity was assayed by 7-AAD uptake in GFP\(^+\) cells.

*Flow Cytometry analysis*

Flow cytometry was performed using BD LSRFortessa or BD Canto II and data were analyzed by Flowjo software. The following antibodies used for surface and intracellular staining were purchased from Biolegend mCD45 (Clone #30-F11), hCD45 (Clone #HI30), hCD3 (Clone #HIT3a), hCD4 (Clone #OKT4), hCD8 (Clone #HIT8a, #RPA-T8), hCD14 (Clone #M5E2), hNKp46 (Clone #9E2), hCD56 (Clone #HCD56), hCD16 (Clone #3G8); from eBioscience hCD3 (Clone #UCHT1), hCD16 (Clone #CB16); from Beckman Coulter anti-HIV-1 p24 (Clone #KC57-RD1) and from NIH AIDS Reagent Program anti-HIV-1 p24 (Clone #KC57); from BD Biosciences hCD56 (Clone #NCAM16.2); from BD Pharmingen hCD3 (Clone #SK7), hCD107a (Clone #H4A3), hIFN\(\gamma\) (Clone #4S.B3), hTNF\(\alpha\) (Clone #mAB11) and 7AAD (#51-2359KC); from Invitrogen hGranzymeB (Clone #GB12).

*Statistics*

Data shown are mean values with error bars denoting SEM. In figures comparing two groups \(P\)-values were calculated using unpaired, two-tailed t-test. When more than 2 groups were compared one-way ANOVA was utilized with Tukey multi-comparison post-test. In figures comparing multiple groups with multiple outcomes or organs, \(P\)-values were calculated using
one-way ANOVA with Tukey multi-comparison post-test, or two-way ANOVA with Sidak’s multiple comparison test or Tukey multi-comparison post-test. Analysis was performed with GraphPad Prism 8 (Graphpad Software). A $P$-value less than 0.05 was consider significant.

Study Approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine, approval #20-0224. Human cord blood and tonsil samples were classified as surgical waste with no identifiers attached and did not require further approval for usage.
Author Contributions

C.S., W.M.Y., and L.S. designed the study and wrote the manuscript; C.S., Q.W., A.N.O., and L.S. performed in vitro and animal experiments and analyzed the data; H.G. and A.J.S. performed antibody production; M.C. contributed to the analysis of NK cell functions in tonsils.

Acknowledgements

We thank Regeneron Pharmaceuticals and the Richard Flavell laboratory at Yale University for generating the human cytokine knock-in mice. We thank Michel Nussenzweig for providing the HIVivoHA viral plasmid and HA antibody plasmids. We thank Dennis Burton for providing PGT121 antibody plasmids. This work was supported by NIH grants R00AI125065, R21AI143413, R01AI155162, UM1AI164568, T32CA009547, and 2T32AR007279, and the Bursky Center for Human Immunology and Immunotherapy Programs, and Barnes-Jewish Hospital Foundation.

Competing interests

The authors declare no competing financial interests.
References


**Figure Legends**

**Fig. 1. Improved human NK cell reconstitution and functionality in humanized MISTG-6-15 versus NSG mice.**

A, Representative flow cytometry plots of NKp46<sup>+</sup> and CD14<sup>+</sup> cells in blood between MISTRG-6-15 and NSG mice. B, Percentage of CD3<sup>+</sup>, CD14<sup>+</sup>, and NKp46<sup>+</sup> cells in blood. Cells were gated on human CD45<sup>+</sup> population. C, Percentage of CD3<sup>−</sup>CD56<sup>+</sup> NK cells (defined by CD56 and not NKp46 to reduce inclusion of group 1 and 3 innate lymphoid cells) in spleen, liver, lung, and LN. Cells were gated on human CD45<sup>+</sup> population. D-F, Percentage of CD107α<sup>+</sup>, GZMB<sup>+</sup>, IFNγ<sup>+</sup>, and TNFα<sup>+</sup> NK cells in spleen (D), liver (E), lung (F), and LN (G) after 4 hours of ex vivo stimulation with PMA/ionomycin. Error bars show mean values with SEM. 8 mice per group. *P*-values were calculated using two-way ANOVA with Sidak’s multiple comparison test. ** denotes *p*-value <0.01, *** denotes *p*-value <0.001, and **** denotes *p*-value <0.0001.
Fig. 2. Lymphoid tissue NK cells isolated from healthy human donors are functionally defective against HIV-1 infected cells. A-E, NK cells from human blood and tonsil were gated on CD3−CD56+ population. A, Representative flow cytometry plots of CD56 and CD16 expression of human blood and tonsil NK cells. B, Percentage of CD16+ NK cells in blood (n=13) and tonsil (n=5). C, Percentage of Perforin+ or GZMB+ NK cells in blood and tonsil after 4hrs of PMA/ionomycin stimulation. D, E, Percentage of killing of K562 (D) and HIV-1 infected CD4+ T cells (E) after co-culture for 4 hours at 1:1 effector-to-target ratio. Symbols represent biologically independent samples isolated from blood (n=5) and tonsil (n=3) from healthy donors. F-H, in humanized MISTRG-6-15 mice, percentage of (F) CD16+ NK cells (n=5), (G) GZMB+ NK cells (n=3), and (H) K562 killing (n=5) by NK cells in the blood, lung, liver, spleen, and LN after ex vivo culture for 4 hours with K562 targets at 1:1 E:T. Error bars show mean values with SEM. In B-E, P-values were calculated using unpaired, two-tailed t-test. In F-H, P-values were calculated using one-way ANOVA with Tukey multi-comparison post-test. ** denotes p-value <0.01, *** denotes p-value <0.001, and **** denotes p-value <0.0001.
Fig. 3. Human NK cells respond to HIV-1 infection in humanized MISTRG-6-15 mice.

MISTRG-6-15 mice were infected with HIV-1\textsubscript{BAL}. A, Longitudinal viral load measurement (n=8). Lines connect data from the same mice. For analysis in B-F, blood and tissue samples were collected on day 21 post infection. B-C, Percent CD4\textsuperscript{+} of total T cells in blood (B) (n=15) or tissues (C) (n=6) of uninfected and infected MISTRG-6-15 mice. Cells were gated on human CD45\textsuperscript{+}CD3\textsuperscript{+} population. D. Percentage of blood and tissue NK cells positive for CD16. E, Percentage of tissue NK cells positive for CD107\text{a}, GZMB, IFN\textgamma, and TNF\textalpha after ex vivo stimulation with PMA/ionomycin for 4 hrs. In D and E, 5 mice were used per group. F, Percentage of tissue NK cells positive for KLRG1, LAG3, PD-1, TIGIT, and TIM3. In D and E, 5 mice were used per group. In F, 4 mice were used per group. Error bars show mean values with SEM. In B and C, \textit{P} values were calculated using unpaired, two-tailed \textit{t}-test. In E and F, \textit{P}-values were calculated using Two-way ANOVA with Sidak’s multiple comparison test. ** denotes \textit{p}-value <0.01, *** denotes \textit{p}-value <0.001, and **** denotes \textit{p}-value <0.0001.
Fig. 4. NK cell expansion and increased functionality during the course of HIV-1 infection wanes with time. MISTRG-6-15 mice were infected with HIV-1BAL. On day 0, 10, 21, 42, 56, 112, and 168 post infection, 4-6 mice at each time point were euthanized for blood and tissue collection. A, Plasma HIV-1 RNA levels. Red line illustrates the average. B, Number of human NK cells (huCD45+CD3-CD56+) throughout the course of infection in spleen, liver, lung, and LN. C-F, Ki67, CD107a, IFNγ, and GZMB expression by NK cells in blood, spleen, liver, lung, and LN. NK cells purified from tissues were stimulated with PMA/ionomycin ex vivo for 4 hours before flow cytometry analysis. Error bars show mean values with SEM.
**Fig. 5.** ART restores NK cell responses during HIV-1 infection. MISTRG-6-15 mice were infected with HIV-1BAL. A, Infection and treatment scheme for the uninfected, untreated, and ART groups. Copies of plasma HIV-1 RNA were measured by RT-qPCR for the untreated and ART groups. B-D, Percentage of tissue NK cells positive for KLRG1, LAG3, PD-1, and TIGIT. E, viral load measurement after ATI. Lines connect data from the same mice. F, Number of CD3⁺CD56⁺ NK cells in spleen, liver, lung, and LN 4 weeks after ATI. G-K, Percentage of CD107a⁺, GZMB⁺, IFNγ⁺, and TNFα⁺ NK cells in indicated tissues. NK cells purified from tissues were stimulated with PMA/ionomycin ex vivo for 4 hours before flow cytometry analysis. Error bars show mean values with SEM. In B-K, 4 mice were used for each time point. In B-D and F-K, P-values were calculated using two-way ANOVA with Sidak’s multiple comparison test. * denotes p-value <0.01, ** denotes p-value <0.01, *** denotes p-value <0.001, and **** denotes p-value <0.0001.
Fig. 6. NK cells exhibit direct anti-HIV-1 control in vivo. MISTRG-6-15 mice were infected with HIV-1BAL. A-B, NK cell degranulation and target cell killing. CD4⁺ T cells were purified from uninfected mice and then infected with HIV-1 reporter virus NL4-3-ΔEnv-EGFP. Autologous NK cells were purified from indicated tissues from infected mice and then co-cultured with infected CD4⁺ T cells for 4 hours at indicated effector-to-target ratio. (A) NK cell degranulation and (B) live/dead staining of HIV-1-infected target cells (GFP⁺) was determined by flow cytometry. Cells were purified from three mice. C-D, NK depletion by αNKp46 antibodies. Percentage of CD3⁻CD56⁺ NK cells in mouse tissues with or without αNKp46 antibody treatment. E, CD4:CD8 ratio with or without αNKp46 antibody treatment. F-G, Copies of plasma HIV-1 RNA (F) and copies of cell-associated viral RNA (cavRNA) in tissues (G) with or without αNKp46 antibody treatment. Error bars show mean values with SEM. 5 mice were used in the isotype treated group and 4 in the αNKp46 treated group. In B, P-values were calculated using one-way ANOVA with Tukey multi-comparison post-test. In D, E, and G, P-values were calculated using two-way ANOVA with Sidak’s multiple comparison test. In F, P-value was calculated using unpaired, two-tailed t-test. ** denotes p-value <0.01, *** denotes p-value <0.001, and **** denotes p-value <0.0001.
Fig. 7. PGT121 antibody mediated viral suppression enhances NK cell functionality.

MISTRG-6-15 mice were infected with HIV-1BAL. Two doses (20mg/kg) of PGT121 (n=9) or PGT121<sub>GRLR</sub> (n=5), or PBS (n=10) were administered on day 14 and 17 post infection. Blood samples were collected on day 14 and 20. Tissues were collected on day 20. A, Plasma viral load before and after antibody treatment. Lines connect individual mice. P-values were calculated using two-way ANOVA with Sidak’s multiple comparison test. B, Plasma viral load fold change from day 14 to day 20. P-values were calculated using one-way ANOVA with Tukey multi-comparison post-test. C, Copies of cell-associated HIV-1 RNA in spleen, lung, and LN. D, Percentage p24<sup>+</sup> of CD3<sup>+</sup>CD8<sup>-</sup> cells in spleen, lung, and LN. E, Expression of CD107a or GZMB in NK cells in the spleen, lung, and LN. NK cells purified from tissues were stimulated with PMA/ionomycin ex vivo for 4 hours before flow cytometry analysis. PGT121 (n=3), PGT121<sub>GRLR</sub> (n=4), and PBS (n=3). Error bars show mean values with SEM. In C-E, P-values for each tissue were calculated using two-way ANOVA with Tukey multi-comparison post-test. * denotes p-value <0.05, ** denotes p-value <0.01, *** denotes p-value <0.001, and **** denotes p-value <0.0001.
Figure 1

A. Flow cytometry analysis of NSG and MISTRG-6-15 cells.

B. Percentage of huCD45+ cells among CD3+, CD14+, and NKp46+ populations.

C. Percentage of NK cells of huCD45+ cells in different organs: Spleen, Liver, Lung, LN.

D. Spleen: % Positive for CD107a, GZMB, IFNγ, TNFα in NSG and MISTRG-6-15.

E. Liver: % Positive for CD107a, GZMB, IFNγ, TNFα in NSG and MISTRG-6-15.

F. Lung: % Positive for CD107a, GZMB, IFNγ, TNFα in NSG and MISTRG-6-15.

G. LN: % Positive for CD107a, GZMB, IFNγ, TNFα in NSG and MISTRG-6-15.
Figure 4

A  Plasma HIV-1 RNA (copies/ml blood)

B  Number of CD3CD56+ cells

C  %Ki67+ of CD3CD56+

D  %CD107a+ of CD3CD56+

E  %IFNγ+ of CD3CD56+

F  %GZMB+ of CD3CD56+

Days post infection