

UCSF

UC San Francisco Previously Published Works

Title

Altered distribution of mucosal NK cells during HIV infection

Permalink

<https://escholarship.org/uc/item/6hq6b5sx>

Journal

Mucosal Immunology, 5(1)

ISSN

1933-0219

Authors

Sips, Magdalena
Sciaranghella, Gaia
Diefenbach, Thomas
et al.

Publication Date

2012

DOI

10.1038/mi.2011.40

Peer reviewed



HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2013 August 12.

Published in final edited form as:

Mucosal Immunol. 2012 January ; 5(1): 30–40. doi:10.1038/mi.2011.40.

Altered distribution of mucosal NK cells during HIV infection

Magdalena Sips¹, Gaia Sciaranghella¹, Thomas Diefenbach¹, Anne-Sophie Dugast¹, Christoph T. Berger¹, Qingquan Liu¹, Douglas Kwon¹, Musie Ghebremichael¹, Jacob D. Estes², Mary Carrington^{1,3}, Jeffrey N. Martin⁴, Steven G. Deeks⁴, Peter W. Hunt⁴, and Galit Alter¹

¹Ragon Institute of Massachusetts General Hospital, Harvard University and Massachusetts Institute of Technology (former known as Partners AIDS Research Center of Massachusetts General Hospital) Boston, MA, USA

²AIDS and Cancer Virus Program, Frederick Inc., National Cancer Institute, Frederick, Maryland, USA

³Cancer and Inflammation Program, Laboratory of Experimental Immunology, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA

⁴University of California, San Francisco, Positive Health Program, San Francisco, 94110, USA

Abstract

The human gut mucosa is a major site of HIV infection and infection-associated pathogenesis. Increasing evidence shows that natural killer (NK) cells play an important role in control of HIV infection but the mechanism(s) by which they mediate antiviral activity in the gut is unclear. Here we show two distinct subsets of NK cells exist in the gut, one localized to intraepithelial spaces (IEL) and the other to the lamina propria (LP). The frequency of both subsets of NK cells was reduced in chronic infection, whereas IEL NK cells remained stable in spontaneous controllers with protective KIR/HLA genotypes. Both IEL and LP NK cells were significantly expanded in immunologic non-responsive (INR) patients, who incompletely recovered CD4⁺ T cells on HAART. These data suggest that both IEL and LP NK cells may expand in the gut in an effort to compensate for compromised CD4⁺ T cell recovery, but that only IEL NK cells may be involved in providing durable control of HIV in the gut,

Introduction

Over the course of past decade, our understanding of HIV-pathogenesis has changed considerably, as we have begun to appreciate the role of the gastrointestinal (GI) tract in HIV-associated immunopathology^{1–3}. Several lines of evidence suggest that HIV preferentially infects activated memory CD4⁺ T cells⁴, and therefore the gut, which harbors

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use:http://www.nature.com/authors/editorial_policies/license.html#terms

Corresponding Author: Galit Alter, 149 13th Street, Room 6618b, Charlestown, MA, 02129, galter@partners.org, (phone) 617.724.0546, (fax) 617.726.5411.

Disclosure:

No conflicts of interest.

a large concentration of activated memory CD4+ T cells, provides fertile ground for rapid HIV infection and dissemination. As early as the first few weeks of HIV infection, significant physical and immunological changes occur in the gut, including a massive accumulation of CD8+ T lymphocytes^{2,5,6} and a massive depletion of CD4+ T cells⁷. These dramatic changes in cell frequencies in the gut are accompanied by increased gut permeability resulting in microbial translocation, leading to elevated levels of microbial products in the blood such as lipopolysaccharide (LPS) that has been shown to contribute to the induction of immune activation⁸.

Despite the early burst of HIV replication in acute infection, in both blood and the gut, acute viral replication is brought down to a viral set-point that persists throughout chronic infection⁹. Given that HIV replicates robustly in the GI tract, it is likely that immune responses must work aggressively during acute infection in the gut to contain the ongoing dramatic viral replication. It is therefore plausible that these GI antiviral responses may represent the most robust control over the virus, and measurements of the immune response in the peripheral blood may not necessarily reflect responses in the gut.

Interestingly, epidemiologic and functional evidence suggest that NK cells play an important role in controlling AIDS progression¹⁰⁻¹². Highly functional clonal populations of NK cells expand rapidly during acute HIV infection, prior to the induction of adaptive immune responses^{10,11} and particular killer immunoglobulin-like receptors (KIR), KIR3DS1 and KIR3DL1, that interact with a subclass of HLA-B alleles, called HLA-Bw4-80I, are associated with slower HIV-1 disease progression^{13,14}. Thus increasing evidence points to an important role for NK cells in early and durable antiviral control; however, the mechanism(s) and location of their antiviral activity remains unknown.

NK cells are large granular lymphocytes that play a major role in elimination of both tumors and virally infected cells without the need for antigen sensitization¹⁵. Several lines of evidence suggest that dramatic changes occur within the NK cell compartment during HIV infection, including phenotypic and functional changes¹⁶⁻¹⁹ that potentially contribute to the failure to control infection and progression to AIDS^{20,21}. However, NK cells in individuals expressing protective KIR/HLA genotypes expand rapidly in acute infection and exhibit strong antiviral activity against HIV *in vitro*¹⁴. Furthermore, recent studies from Vieillard et al. demonstrated that spontaneous HIV controllers exhibit robust NK cell responses²². Yet, given that the majority of HIV viral replication occurs in the gut, it is essential to define whether gut mucosal NK cells contribute to antiviral control at this vulnerable site of infection in these unique individuals that control HIV infection in the absence of therapy.

Early studies suggest that NK cells reside in the intra-epithelial space of the GI tract and in fact, NK cells have been identified as intra-epithelial lymphocytes (IEL) in various species of animals²³⁻²⁶ and humans²⁷. In humans, gut NK cells, similar to uterine NK (uNK) cells, express high levels of CD56, KIR, and CD16²⁸, produce proinflammatory cytokines (IFN- γ and TNF- α), and kill MHC negative target cells (K562 cells) in the presence of IL-2, IL-12, or IL-15²⁹. Furthermore, gut NK cells have been shown to be crucially involved in controlling murine enteric coronaviral infections³⁰. More recent data have documented a new subset of NK cells or lymphoid tissue inducers, the NK-22 cells, that secrete IL-22,

which is required for the maintenance of epithelium integrity^{31,32}. Yet little is known about the distribution of NK cells in the human gut, and how these change during viral infections.

Here we sought to define whether durable control of HIV infection was associated with the preferential recruitment of NK cell populations to the gut, by analyzing rectosigmoid mucosal biopsies collected from patients at various stages of HIV infection. In our studies, we identified two distinct populations of gut mucosal-resident NK cells, one present in the IEL (IEL NK) and the other in the lamina propria (LP NK). Interestingly, we observed changes in IEL and LP NK cell frequencies in HIV infection. Unexpectedly, increased frequencies of both IEL and LP NK cells were only observed in subjects with incomplete peripheral blood CD4+ T cell recovery (CD4<350 cells/ul) despite long-term antiretroviral therapy (ART)-mediated viral suppression. However importantly, spontaneous controllers expressing protective KIR/HLA genotypes had higher IEL NK cell numbers than those with non-protective genotypes, suggesting that IEL NK cells may contribute to durable control of HIV in these individuals. These data suggest that two subsets of NK cells exist in the human gastrointestinal tract, one in the IEL and one in the LP, one of which may be involved in durable control of HIV infection, and both that may expand at this vulnerable site in response to incomplete immune recovery following virologic suppression.

Results

GI tract CD3^{neg}NKp46^{pos} NK cells are localized within the intraepithelial space and lamina propria

Epidemiologic data suggest that NK cells may play a critical role in controlling HIV-1 infection¹³, however the majority of studies have focused on NK cells in the blood, whereas the virus replicates most profusely in the gut³. While several reports suggest that NK cells can be detected in gut mucosa in animals^{23–26,33–35}, less is known about NK cells in human gut. Thus to define the frequency of NK cells in the gut, as well as their distribution and localization, immunohistochemistry (IHC) was used to visualize and quantify these cells. Traditionally, NK cells are defined as CD3^{neg}CD56^{pos/neg}CD16^{pos/neg}, however this combination of markers is difficult to use in immunohistochemical staining, due to the fact that CD56 also stains neural tissue that is abundant in the GI tract. In contrast, specific family of receptors, the natural cytotoxicity receptors (NCRs), are thought to be exclusively expressed on NK cells³⁶. Thus, NKp46, an NCR expressed on nearly all NK cells, was selected. Two NKp46 antibodies were used in the study to detect NK cells in human colon: polyclonal goat anti-human (GAH) NKp46 and monoclonal mouse anti-human (MAH) NKp46. Unexpectedly, these two antibodies labeled two distinct cell populations in the gut. The GAH NKp46 exclusively labeled cells located in intraepithelial spaces (IEL NK) (Fig. 1A, 1C), whereas MAH NKp46 labeled lamina propria cells (LP NK) (Fig. 1B, 1D). LP NK cells were located in basolateral part of mucosa and within gut submucosa. Neither of these antibodies labeled cells within lymphoid aggregates, suggesting that NK cells may be excluded from inductive sites in the gut (data not shown). Additionally, a subset of IEL NK cells (Fig. 2A), but not LP NK cells (Fig. 2B), co-expressed CD57, a marker of terminal differentiation, present on NK cells and T cells³⁷, suggesting that these two NK cell subsets represent phenotypically, and potentially functionally distinct cell types. Thus two

populations of NK cells were identified in healthy human gut, located in distinct regions of the tissues, potentially reflecting unique functional characteristics.

Confirmation of NKp46 antibody specificity

Having identified two populations of NK cells in the gut stained with different NKp46 antibodies, we next sought to confirm that these IHC antibodies labeled NK cells in other tissues as well. We investigated whether these antibodies recognized NK cells in uterine tissue, known to be abundant in NK cells³⁸. Abundant levels of NKp46⁺ cells were identified in healthy endometrial tissues with both GAH and MAH NKp46 antibodies (Fig. 3A, 3B), suggesting that these antibodies did in fact recognize NK cells at other mucosal sites.

To further confirm that NKp46 antibodies labeled CD3^{neg} cells, we performed a double staining by IHC and multi color IF to simultaneously label CD3^{pos} and NKp46^{pos} cells. Neither of the NKp46^{pos} cell subsets stained positively with the CD3 antibody confirming that neither the IEL nor LP NK cells belong to the T cell lineage (Fig. 1C, 1D, 2A, 2B).

To ultimately validate that these NKp46 antibodies labeled NK cells specifically, both NKp46 antibodies were tested by flow cytometry and compared to the standard BD NKp46 antibody (clone 9E2) widely used to stain peripheral blood NK cells. Both the GAH and MAH NKp46 stained similar frequencies of CD3^{neg}CD56^{pos/neg}CD16^{pos/neg} blood NK cells as did the traditional BD antibody (Fig. 4A). Moreover, these antibodies did not label other cell subsets (data not shown). To further confirm the specificity of these antibodies for NK cells, NK cells were also costained with a range of additional NK cell markers, including NKp30, NKG2A, KIR and NKG2D (Fig. 4A), demonstrating that both GAH and MAH NKp46 antibodies labeled similar frequencies of NK cells bearing these other phenotypic markers.

Finally, to confirm that the GAH and MAH NKp46 antibodies stain mucosal NK cells, freshly collected-enzymatically digested gut tissue was stained with the two antibodies and frequencies of NK cells were compared with the traditional BD antibody NK cell frequencies. All three NKp46 antibodies labeled CD45^{pos}CD3^{neg} lymphocytes isolated from the gut (Fig. 4B), that also coexpressed CD56 and CD16 (data not shown). However, interestingly, the three antibodies labeled different frequencies and intensities of CD56^{pos} NK cells, in such a way that the MAH labeled more CD56^{bright} NK cells than the GAH NKp46 antibody, suggesting some differences in NK cell subset specificity in the gut. Furthermore, all three antibodies stained CD45^{pos}CD3^{neg}NKp46^{pos} cells that also co-expressed NKG2A, confirming that the stained mucosal cells belong to the NK cell lineage (Fig. 4B). Thus overall, these data suggest that both NKp46 antibodies, used to detect NK cells in the gut, are highly specific for NK cells.

No alteration in the IEL:LP NK cell balance during HIV infection

Given the fact that the two NKp46 antibodies labeled two distinct NK cell populations in the gut, we next sought to determine if HIV infection is associated with differential recruitment of either of these NK cell subsets to this vulnerable site, where they may play a role in antiviral control. Slides from colon biopsies were collected from 65 subjects, including 15

HIV negative individuals and 50 HIV positive patients with differential control of HIV infection: 20 untreated controllers (11 elite controllers: <75 RNA copies/ml and 9 viremic controllers: 75–2000 RNA copies/ml), 9 chronic untreated patients (<2000 RNA copies/ml) and 21 treated patients (11 with optimal CD4+ T cells recovery and 10 with suboptimal recovery: all <75 RNA copies/ml). Slides were stained with the GAH and MAH NKp46 antibodies and the frequencies of IEL and LP NK cells were counted manually in whole tissue scans. Patients were grouped into HIV negative controls and HIV-infected individuals for initial studies.

In healthy controls IEL NK cells were more frequent than LP NK cells (Fig. 5A). Despite the presence of both of these subsets of NK cells in the gut, there was no relationship between the frequency of each subset in the gut of healthy individuals (Fig. 5C), suggesting that they may be recruited independently to these sites. Interestingly, while the ratio of IEL:LP NK cells was preserved in HIV infection (Fig. 5B), a positive correlation was observed between these two NK cell populations in HIV+ subjects (Fig. 5D), suggesting that in HIV infection these cells may be synergistically recruited to the gut, to contribute to immune control. These data suggest that under homeostatic conditions, the frequency of the IEL and LP NK cells is unrelated, but during infection the recruitment/expansion of these cells in the gut may occur in a coordinated manner.

Expansion of NK cells in immunologically non-reconstituted patients after HAART

Given the alteration in the relationship between IEL and LP NK cells in HIV infection, we were intrigued to next define whether these cells were specifically expanding/accumulating in specific HIV-infected patient populations. We hypothesized that both of these NK cell subsets would expand preferentially in the controller group, potentially contributing to durable control of HIV infection, whereas we speculated that the frequency of these cells would be depressed in chronically infected individuals off therapy. As expected, we observed a trend towards a decrease in the frequency of both IEL and LP NK cells in chronic untreated patients with high viral loads (VL) as compared to HIV- subjects (Fig. 6A, 6B). However, we observed a remarkably heterogeneous distribution of NK cells in controllers, marked by a wide distribution of both IEL and LP NK cell frequencies, with some individuals exhibiting some of the highest levels of both subsets of NK cells in their guts, whereas the vast majority exhibited some of the lowest frequencies of these cells. No difference was observed in either IEL or LP NK cell frequencies among elite compared to viremic controllers (Fig. 6C). Conversely, the most dramatic expansion of both IEL and LP NK cells was observed in the treated patient group that exhibited incomplete or suboptimal CD4+ T cell recovery despite long-term anti-retroviral therapy, (immunological non-responders (INR)) (ART INR:CHRO UNTX $P < 0.05$ for both IEL and LP NK cells, Fig. 6A, 6B). LP NK cells in immunologic non-responders were also significantly more frequent compared to LP NK cells in spontaneous controllers (ART INR:CONTRL $P < 0.05$). Interestingly, this expansion of IEL and LP NK cells in the INR patients was related (Fig. 6D), and appeared to drive the IEL/LP NK cell correlation observed in Fig. 5D. These data suggest for the first time that NK cell distribution in the gut of controllers is quite heterogeneous, NK cell frequencies decline in chronic HIV infection, but that both IEL and LP NK cells expand in the GI tract of incompletely reconstituted HIV+ subjects on ART

suggesting that NK cells may accumulate in the absence of viral replication in this vulnerable mucosal tissue in response to incomplete immunologic reconstitution, to potentially provide a compensatory mechanism for the compromised immunity.

Positive correlation of gut IEL NK cells and CD4 T cells in periphery in chronic infection

Given that the absolute number of circulating CD4+ T cells and plasma viral set point serve as markers of disease progression, we were next interested in defining whether the frequencies of either of the two populations of NK cells in the gut of HIV-infected patients were related to changes with either of these 2 clinical parameters. We therefore compared the frequency of NK cells to the VL, CD4 and CD8 count in the peripheral circulation. We found no correlation between VL and NK cells in any of the patient populations, potentially suggesting that the peripheral level of viremia may not drive these changes in NK cell frequencies, but that perhaps local viral replication could have an impact. However, a positive correlation between IEL NK cells and peripheral blood CD4+ T cells ($R^2=0.3322$, $P=0.0498$) and a negative correlation between IEL NK and peripheral blood CD8+ T cells ($R^2=0.4071$, $P=0.0256$) was observed in untreated, chronically infected patients (Fig. 6D). These data show that the inversion of the CD4:CD8 ratio, associated with inflammation, may potentially reflect a dampening of the NK cell response in the setting of an increase in CD8+ T cell numbers, as has been previously shown in acute HIV infection¹¹.

Higher frequency of IEL NK cells in spontaneous controllers with protective KIR/HLA genotypes

Previous epidemiologic studies have shown that particular KIR/HLA compound genotypes are associated with spontaneous control of HIV^{13,39}. Given the wide variation in NK cell frequencies in spontaneous controllers (Fig. 6A and 6B), unrelated to the degree of antiviral control (Fig. 6C), we next sought to determine whether these protective KIR/HLA genotypes may account for differences in NK cell frequencies in the gut in spontaneous controllers. Thus, ECs and VCs were genotyped for KIR and HLA, and were grouped into those with protective genotypes (KIR3DS1 or KIR3DL1 *001, *002, *008, *015 and *009 in combination with HLA-Bw4 alleles with an isoleucine at position 80) and those without protective genotypes (the rest). Interestingly, there was a trend towards significantly higher frequencies of IEL NK cells detected in controllers with protective KIR/HLA compound genotypes ($p=0.0549$) but not in LP NK cells (Fig. 7). These data support a potential role for a persistent expansion of protective IEL NK cell populations in the gut mucosa in spontaneous controllers that may directly contribute to durable control of viral replication at this vulnerable site beyond acute infection.

Discussion

Based on our recent understanding of the role of the gut as the primary site of HIV viral replication, we sought to define whether NK cells, a subset of innate immune cells, may accumulate preferentially at this site in individuals that progress more slowly to disease. In our study we identified two populations of NK cells in the GI tract, one that resides in intraepithelial space of the crypts (IEL NK) and one present in the lamina propria (LP NK) of the colon tissue (Fig. 1). Furthermore, we observed a remarkable heterogeneity in the

distribution of IEL and LP NK cells in spontaneous controllers, where some of these patients expressed the highest frequencies of NK cells in the gut, but the majority exhibited reduced frequencies of these innate immune cells, similar to the levels observed in chronic HIV infection. Moreover, we observed that the frequency of both IEL and LP NK cells were expanded in HAART treated patients that did not fully reconstitute their CD4+ T cell numbers (Fig. 6A, 6B). Additionally, IEL NK cells were preserved in spontaneous controllers with protective KIR/HLA genotypes compared to controllers with non-protective KIR/HLA genetic backgrounds. These data suggest for the first time that IEL NK cells may contribute to persistent durable control of viral replication in the gut in controllers with protective KIR/HLA compound genotypes, and that both IEL and LP NK cells expand at this vulnerable site in individuals that are incompletely able to recover from the assault of the virus, potentially in an effort to compensate for the persisting compromised environment within this barrier.

It is incompletely understood what contributes to the lack of immunologic reconstitution. Some recent data show that collagen deposition and disruption of the lymphatic tissue architecture contribute to persistent CD4+ T cell depletion prior to treatment, and the extent of these changes affects the efficiency of immune reconstitution after the initiation of therapy⁴¹. Such observations have been made at first in lymph nodes (LN)⁴¹ and subsequently in gut-associated lymphatic tissue (GALT)⁴², the latter suffering from a more pronounced loss in CD4+ T cell numbers^{3,43-45}. Furthermore, immunological non-responders exhibit particular unique clinical characteristics including fibrosis in LNs and gut tissue, increased persistent immune activation, and elevated markers of chronic inflammation despite undetectable VLs⁴⁶. Thus despite the direct-antiretroviral effects on reducing viral replication, these individuals still exhibit some markers of chronic disease. In addition to these markers of continued disease pathology, we report here that these individuals also exhibit a remarkable expansion of NK cells in the gut (Fig. 6A, 6B). We speculate that incomplete immunological reconstitution causes some additional inflammatory stimuli in the gut, potentially due to some persistent low level viral replication, resulting in the persistent recruitment of innate immune cells to help provide some level of protection in the absence of CD4+ T cells. Thus mucosal NK cells may expand in an effort to compensate for the compromised gut mucosal immunity. Interestingly, this NK cell expansion is not observed in chronic untreated patients, despite the fact that they exhibit reduced CD4+ T cell numbers as well, however, these individuals also exhibit high levels of viral replication and persistent immune activation, due to microbial translocation⁸, through accessory cells⁴⁰. This hyper-activation, however, may drive direct NK cell apoptosis⁴⁷, thereby preventing the observed increase of these cytolytic effector cells within tissues.

Epidemiologic work strongly suggests that NK cells may be directly involved in antiviral control of HIV^{10,13,14}. We therefore speculated that these innate effectors may expand preferentially in the gut of individuals that exhibit durable control of HIV, including the elite and viremic controllers. Overall controllers exhibited a heterogeneous distribution of both IEL and LP NK cells in mucosal sections (Fig. 6A and B), that did not differ in ECs or VCs (Fig. 6C), and were similar in overall numbers from that seen in chronically infected individuals. However, given the strong epidemiologic data suggesting that specific

KIR/HLA combinations contribute to spontaneous control of HIV infection, we examined IEL and LP NK cell distributions among controllers with protective and non-protective KIR/HLA genotypes. Interestingly, we observed a trend towards a elevated IEL, but not LP, NK cells in controllers with protective KIR/HLA genotypes, suggesting for the first time that particular NK cell populations may persist in these unique individuals at this vulnerable site, aimed at maintaining long-term control of HIV infection. The specific preservation of IEL, and not LP, NK cells may reflect functional differences between these subsets of lymphocytes. Previous work has suggested that lymphocytes able to traffic in the IEL exhibit more cytolytic functional properties than those found in the LP⁴⁸. This may suggest that the elevated IEL NK cells in controllers with protective KIR/HLA genotypes may confer an enhanced capacity to rapidly deploy the cytolytic antiviral activity of NK cells, should the cell encounter a potential viral burst that have been suggested to occur in some controllers⁴⁹. However, additional in depth functional studies are required to begin to dissect the functional and phenotypic properties of NK cells in these two compartments of the tissues, and particularly those that may expand in spontaneous control in the setting of protective KIR/HLA genotypes.

Early studies from animal models have shown IEL lymphocytes to have higher cytolytic activity than LP⁴⁸ or Peyer's patches lymphocytes³⁵, however the specific distribution and functional properties of NK cells in the gut have been poorly explored. A population of cells, called NK-22, was reported to reside in the lamina propria of tonsils, lacking classical markers (including NKp46) of NK cells, but expressing NKp44 and secreting IL-22 required for the maintenance of epithelium integrity³¹. Yet, several lines of evidence now suggest that NK-22 cells belong to a subset of lymphoid-tissue inducer (LTi) cells, rather than conventional NK cells³². However, here we report the presence of two NK cell subsets, distinct from NK-22 as they express NKp46, that are distributed in distinct effector compartments of the gut. Based on their location within mucosa, we speculate that IEL NK cells may be more cytolytic and may serve as the first line of defense, just beneath the epithelial border, whereas LP NK cells, located in basolateral surface of gut mucosa and in submucosa may constitute an immunoregulatory type of NK subset, similar to that seen in the uterine tissues. These two NK cell subsets exhibit distinct phenotypes what supports this potential functional difference, as only IEL NK cells co-express the terminal differentiation marker CD57 (Fig. 2) that is expressed on more cytolytic blood NK cells³⁷. The fact that these two NK cell populations were identified by two antibodies targeting the same antigen mounted in different species, suggests that the NKp46 receptor may exhibit conformational changes depending on the activation status of NK cells. A similar scenario has been observed for the CD43 molecule, where two distinct antibodies recognize differentially glycosylated isoforms of the receptor, reflecting distinct activation states of the lymphocytes on which they are expressed⁵⁰. Given that NKp46 is highly glycosylated⁵¹, we speculate that these two antibodies may recognize NKp46 antigens with unique glycosylation patterns or conformational changes on IEL and LP NK cells, providing a useful mechanism by which to identify mucosal NK cell subpopulations.

In summary, our data demonstrate that two distinct populations of NK cells reside in the gut and may represent two innate effector cell subsets with unique functional properties related to their tissue localization. We show that the frequency of both subsets of NK cells change

following HIV infection, with an overall loss of both subsets in chronic infection, but with a significant expansion in immunologic non-responders. Furthermore, we show that the protective KIR/HLA genotypes are associated with a preservation of IEL NK cells in spontaneous controllers. These data suggest that while chronic HIV infection is associated with reduced frequencies of these innate effector cells in the gut, these cells respond robustly and expand in the setting of incomplete immune reconstitution potentially aimed at compensating for incomplete immunological recovery in patients on HAART and in spontaneous controllers with protective KIR/HLA genotypes what may provide durable protection from infection following acute infection.

Materials and methods

Subjects

Formalin-fixed-paraffin-embedded (FFPE) tissue slides from rectosigmoid mucosal biopsies from a total of 65 individuals from the SCOPE cohort were used in this study, among which 50 were HIV-infected patients and 15 were uninfected healthy controls. HIV-infected samples included 20 untreated HIV spontaneous controllers, that spontaneously control HIV viral replication in the absence of therapy (11 'Elite Controllers' with viral load (VL)<75 copies/ml and 9 'Viremic Controllers' with VL 75–2000 copies/ml); 9 untreated non-controllers ('Chronic untreated'), that remained off-therapy during infection with VL>2000 copies/ml (most with VL>10000 copies/ml); 21 treated patients on antiretroviral therapy with VL<75 copies/ml (11 'Immunologic responders' (IRs) with optimal CD4 recovery>500 cells/mm³ of blood; and 10 'Immunologic non-responders' (INRs) with suboptimal CD4+ T cell recovery <350 cells/mm³). IRs and INRs maintained ART-mediated viral suppression for a median of 8.2 years and 4.4 years, respectively. Detectable episodes of viremia <500 copies/ml were allowed during these intervals if preceded and followed by undetectable values. In addition to having lower CD4+ T cell counts at the time of the rectal biopsy (<350 cells/mm³ vs. >500 cells/mm³), INRs also had a lower median rate of CD4+ T cell recovery in the two years prior to the rectal biopsy (+11.9, IQR: +8.2 to +15.2 CD4+ T cells/mm³ per year) than the IRs (+24.8, IQR: +18.3 to +36.7 CD4+ T cells/mm³ per year, P=0.002 for between group comparison). Laboratory personnel were blinded to HIV disease status until measurements were completed.

KIR/HLA genotyping

We performed genotyping of the *HLA* locus following the PCR-SSOP (sequence-specific oligonucleotide probing) typing protocol recommended by the 13th International Histocompatibility Workshop (<http://www.ihwg.org/components/ssopr.htm>). For *KIR3DL1/S1* subtyping, polymorphic exons 3, 4, 5, 7, 8 and 9 were selectively amplified in four PCR reactions using locus-specific primers (a separate PCR for each of exons 3, 4 and 5, and a fourth PCR for exons 7, 8 and 9 combined). The PCR products were blotted on nylon membranes and hybridized with a panel of 56 sequence-specific oligonucleotide (SSO) probes designed to detect unique sequence motifs of known *KIR3DL1* alleles. *KIR3DL1* alleles were assigned by the reaction patterns of the SSO probes based on the known *KIR3DL1* sequences, as previously described³⁹. Ambiguous SSO typing results were resolved by sequencing analysis.

Immunohistochemistry (IHC)

FFPE slides were de-paraffinized and hydrated. Antigen retrieval was performed in Deckloaking Chamber (Biocare medical), inducing temperature of 125°C and pressure of 25 psi. Endogenous peroxidase activity was blocked with buffered hydrogen peroxide (Biocare medical) for 5 mins at room temperature (RT). Unspecific binding of antibodies (Abs) was blocked using purified casein and other proteins (Background Sniper, Biocare medical) for 10 mins in RT and donkey serum (Sigma) for 15 mins. Slides were incubated with the following primary Ab for 1h at RT: mouse monoclonal anti-human NKp46 (R&D), goat polyclonal anti-human NKp46 (R&D), rabbit polyclonal anti-human CD3 (DAKO), mouse monoclonal anti-human CD57 (DAKO). The following control Abs were used: mouse IgG2B Isotype Control (R&D), goat control Ab (R&D), mouse IgM Isotype Control (DAKO). EnVision+Systems-HRP (anti-mouse and anti-rabbit) or rabbit anti-goat-HRP Ab (DAKO) were used for 45 min at RT as a secondary Ab and visualization was performed with a diaminobenzidine (DAB+) substrate chromogen (DAKO). For double staining procedure, denaturing solution, AP conjugated secondary Abs and Vulcan Fast Red chromogen (Biocare medical) were used for the detection of the second antigen. Sections were counterstained with hematoxylin, dehydrated and mounted in mounting medium.

Immunofluorescence (IF)

De-paraffinization, hydration and antigen retrieval steps were performed as described in IHC section. Prior to incubation with the primary Ab, slides were immersed in 0.3% Sudan Black B in 70% ethanol for 2hrs at RT in order to mask autofluorescence present in tissues. Slides were blocked and incubated with primary or control Abs as described in IHC section. The following secondary Abs were used for detection of cells: donkey anti-mouse AlexaFluor 555 or A488 (Invitrogen), donkey anti-goat AlexaFluor A488 (Invitrogen), and donkey anti-rabbit AlexaFluor A647 (Invitrogen). Nuclei were stained with Hoechst 33258 (Invitrogen). Slides were mounted with Gold ProLong antifade reagent (Invitrogen).

Microscopy

Slides were scanned with Zeiss MIRAX MIDI automated slide scanner in brightfield mode for IHC staining or fluorescence mode for IF staining. High quality, high magnification images were taken using Zeiss LSM510 laser scanning confocal microscope.

Image analysis

Cells were counted manually from 12 bit TIFF images acquired from whole tissue scans. The tissue area measurements were performed using ImageJ software (NIH, Bethesda, MD) and obtained frequencies of cells were presented as number of cells/mm² of tissue.

Flow cytometry

Flow cytometric evaluation of NKp46 antibody staining was performed on peripheral blood mononuclear cells (PBMC) and freshly isolated lymphocytes from enzymatically digested gastrointestinal tissues. At least 10⁶ PBMCs or gut lymphocytes were stained with anti-CD3-PacificBlue, anti-CD16-APC-Cy7, anti-CD56-PE-Cy7 (BD Biosciences, San Jose, CA USA), anti-CD158a/anti-CD158b/anti-NKB1-FITC, anti-NKG2D-PE (BD Biosciences),

anti-NKG2A-Alexa Fluor 647 (Beckman Coulter, Fullerton, CA USA) and one of 3 NKp46 antibodies followed by a secondary conjugated to Alexa Fluor 488 or Alexa Fluor 647. Cells were then washed and fixed by 4% paraformaldehyde, prior to acquisition on a BD LSRII. The expression of NKp46 was quantified on CD3^{neg}CD56^{pos/neg}CD16^{pos/neg} lymphocytes using FlowJo, (Tree Star Inc., Ashland, OR USA). Additionally, CD45-Pe-Cy5.5 (BD Biosciences) and LIVE/DEAD stain (Invitrogen) were added for tissue lymphocyte staining and the expression of NKp46 was quantified on live, CD45^{pos}CD3^{neg}CD56^{pos/neg}CD16^{pos/neg} lymphocytes.

Statistics

Statistical analyses for comparison of IEL and LP NK cells in healthy and infected groups were performed using a Mann Whitney U test. Comparison of protective and non-protective KIR/HLA compound genotypes in spontaneous controller group was performed using a Mann Whitney U test. Spearman rank correlation coefficient was used to examine the relationships between IEL and LP NK cell frequencies. Statistical comparisons of NK cell frequencies among patient groups were made using Kruskal-Wallis test. P-values of pairwise comparisons were adjusted by using Dunn's method. Reported p-values are two sided and values <0.05 were considered significant.

Acknowledgements

We would like to thank Dr. Joseph Misdraji for his expertise on our staining data, Dr. Georg Lauer and Michelle Tomlinson for their help with healthy control tissue collection.

References

1. Brenchley JM, Douek DC. The mucosal barrier and immune activation in HIV pathogenesis. *Curr Opin HIV AIDS*. 2008; 3:356–361. [PubMed: 19372990]
2. Brenchley JM, Douek DC. HIV infection and the gastrointestinal immune system. *Mucosal Immunol*. 2008; 1:23–30. [PubMed: 19079157]
3. Brenchley JM, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med*. 2004; 200:749–759. [PubMed: 15365096]
4. Douek DC, et al. HIV preferentially infects HIV-specific CD4+ T cells. *Nature*. 2002; 417:95–98. [PubMed: 11986671]
5. Clayton F, et al. Rectal mucosal pathology varies with human immunodeficiency virus antigen content and disease stage. *Gastroenterology*. 1992; 103:919–933. [PubMed: 1499943]
6. Rodgers VD, Fassett R, Kagnoff MF. Abnormalities in intestinal mucosal T cells in homosexual populations including those with the lymphadenopathy syndrome and acquired immunodeficiency syndrome. *Gastroenterology*. 1986; 90:552–558. [PubMed: 2935443]
7. Mattapallil JJ, et al. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature*. 2005; 434:1093–1097. [PubMed: 15793563]
8. Brenchley JM, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006; 12:1365–1371. [PubMed: 17115046]
9. Pantaleo G, Graziosi C, Fauci AS. Virologic and immunologic events in primary HIV infection. *Springer Seminars in Immunopathology*. 1997; 18:257–266. [PubMed: 9089948]
10. Alter G, Altfeld M. NK cells in HIV-1 infection: evidence for their role in the control of HIV-1 infection. *J Intern Med*. 2009; 265:29–42. [PubMed: 19093958]
11. Alter G, et al. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J Infect Dis*. 2007; 195:1452–1460. [PubMed: 17436225]

12. Berger CT, Alter G. Natural killer cells in spontaneous control of HIV infection. *Curr Opin HIV AIDS*. 6:208–213. [PubMed: 21399495]
13. Martin MP, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002; 31:429–434. [PubMed: 12134147]
14. Alter G, et al. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med*. 2007; 204:3027–3036. [PubMed: 18025129]
15. French AR, Yokoyama WM. Natural killer cells and viral infections. *Curr Opin Immunol*. 2003; 15:45–51. [PubMed: 12495732]
16. Collins KL, Baltimore D. HIV's evasion of the cellular immune response. *Immunol Rev*. 1999; 168:65–74. [PubMed: 10399065]
17. De Maria A, et al. The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol*. 2003; 33:2410–2418. [PubMed: 12938217]
18. Mavilio D, et al. Characterization of CD56-/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals. *Proc Natl Acad Sci U S A*. 2005; 102:2886–2891. [PubMed: 15699323]
19. Fogli M, et al. Significant NK cell activation associated with decreased cytolytic function in peripheral blood of HIV-1-infected patients. *Eur J Immunol*. 2004; 34:2313–2321. [PubMed: 15259029]
20. Cai Q, Huang XL, Rappocciolo G, Rinaldo CR Jr. Natural killer cell responses in homosexual men with early HIV infection. *J Acquir Immune Defic Syndr*. 1990; 3:669–676. [PubMed: 2141073]
21. Poli G, et al. Natural killer cells in intravenous drug abusers with lymphadenopathy syndrome. *Clin Exp Immunol*. 1985; 62:128–135. [PubMed: 2415279]
22. Vieillard V, Fausther-Bovendo H, Samri A, Debre P. Specific phenotypic and functional features of natural killer cells from HIV-infected long-term nonprogressors and HIV controllers. *J Acquir Immune Defic Syndr*. 53:564–573. [PubMed: 20147841]
23. Wilson AD, Stokes CR, Bourne FJ. Morphology and functional characteristics of isolated porcine intraepithelial lymphocytes. *Immunology*. 1986; 59:109–113. [PubMed: 2428733]
24. Chai JY, Lillehoj HS. Isolation and functional characterization of chicken intestinal intra-epithelial lymphocytes showing natural killer cell activity against tumour target cells. *Immunology*. 1988; 63:111–117. [PubMed: 3338816]
25. Flexman JP, Shellam GR, Mayrhofer G. Natural cytotoxicity, responsiveness to interferon and morphology of intra-epithelial lymphocytes from the small intestine of the rat. *Immunology*. 1983; 48:733–741. [PubMed: 6187670]
26. Tagliabue A, Befus AD, Clark DA, Bienenstock J. Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. *J Exp Med*. 1982; 155:1785–1796. [PubMed: 7077224]
27. Chiba M, Bartnik W, ReMine SG, Thayer WR, Shorter RG. Human colonic intraepithelial and lamina propria lymphocytes: cytotoxicity in vitro and the potential effects of the isolation method on their functional properties. *Gut*. 1981; 22:177–186. [PubMed: 6262195]
28. Eiras P, et al. Intestinal intraepithelial lymphocytes contain a CD3- CD7+ subset expressing natural killer markers and a singular pattern of adhesion molecules. *Scand J Immunol*. 2000; 52:1–6. [PubMed: 10886777]
29. Leon F, et al. Human small-intestinal epithelium contains functional natural killer lymphocytes. *Gastroenterology*. 2003; 125:345–356. [PubMed: 12891535]
30. Carman PS, et al. Intraepithelial leukocytes contain a unique subpopulation of NK-like cytotoxic cells active in the defense of gut epithelium to enteric murine coronavirus. *J Immunol*. 1986; 136:1548–1553. [PubMed: 3005395]
31. Cella M, et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*. 2009; 457:722–725. [PubMed: 18978771]
32. Crellin NK, Trifari S, Kaplan CD, Cupedo T, Spits H. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. *J Exp Med*. 207:281–290. [PubMed: 20142432]

33. Arnaud-Battandier F, Bundy BM, O'Neill M, Bienenstock J, Nelson DL. Cytotoxic activities of gut mucosal lymphoid cells in guinea pigs. *J Immunol.* 1978; 121:1059–1065. [PubMed: 690435]
34. Nauss KM, Pavlina TM, Kumar V, Newberne PM. Functional characteristics of lymphocytes isolated from the rat large intestine. Response to T-cell mitogens and natural killer cell activity. *Gastroenterology.* 1984; 86:468–475. [PubMed: 6607187]
35. Tagliabue A, Luini W, Soldateschi D, Boraschi D. Natural killer activity of gut mucosal lymphoid cells in mice. *Eur J Immunol.* 1981; 11:919–922. [PubMed: 7327195]
36. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? *Nat Immunol.* 2002; 3:6–8. [PubMed: 11753399]
37. Lopez-Verges S, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood.* 116:3865–3874. [PubMed: 20733159]
38. Hamperl H, Hellweg G. Granular endometrial stroma cells. *Obstet Gynecol.* 1958; 11:379–387. [PubMed: 13517743]
39. Martin MP, et al. Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet.* 2007; 39:733–740. [PubMed: 17496894]
40. Alter G, et al. Single-stranded RNA derived from HIV-1 serves as a potent activator of NK cells. *J Immunol.* 2007; 178:7658–7666. [PubMed: 17548602]
41. Schacker TW, et al. Collagen deposition in HIV-1 infected lymphatic tissues and T cell homeostasis. *J Clin Invest.* 2002; 110:1133–1139. [PubMed: 12393849]
42. Estes J, et al. Collagen deposition limits immune reconstitution in the gut. *J Infect Dis.* 2008; 198:456–464. [PubMed: 18598193]
43. Veazey RS, et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science.* 1998; 280:427–431. [PubMed: 9545219]
44. Guadalupe M, et al. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol.* 2003; 77:11708–11717. [PubMed: 14557656]
45. Mehandru S, et al. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med.* 2004; 200:761–770. [PubMed: 15365095]
46. Estes JD, et al. Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor beta 1-positive regulatory T cells and begins in early infection. *J Infect Dis.* 2007; 195:551–561. [PubMed: 17230415]
47. Poggi A, Zocchi MR. HIV-1 Tat triggers TGF-beta production and NK cell apoptosis that is prevented by pertussis toxin B. *Clin Dev Immunol.* 2006; 13:369–372. [PubMed: 17162379]
48. Parrott DM, et al. Analysis of the effector functions of different populations of mucosal lymphocytes. *Ann N Y Acad Sci.* 1983; 409:307–320. [PubMed: 6223566]
49. Hunt PW, et al. Relationship between T cell activation and CD4+ T cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. *J Infect Dis.* 2008; 197:126–133. [PubMed: 18171295]
50. Santamaria M, Lopez-Beltran A, Toro M, Pena J, Molina IJ. Specific monoclonal antibodies against leukocyte-restricted cell surface molecule CD43 react with nonhematopoietic tumor cells. *Cancer Res.* 1996; 56:3526–3529. [PubMed: 8758921]
51. Mendelson M, et al. NKp46 O-glycan sequences that are involved in the interaction with hemagglutinin type 1 of influenza virus. *J Virol.* 2010; 84:3789–3797. [PubMed: 20147410]

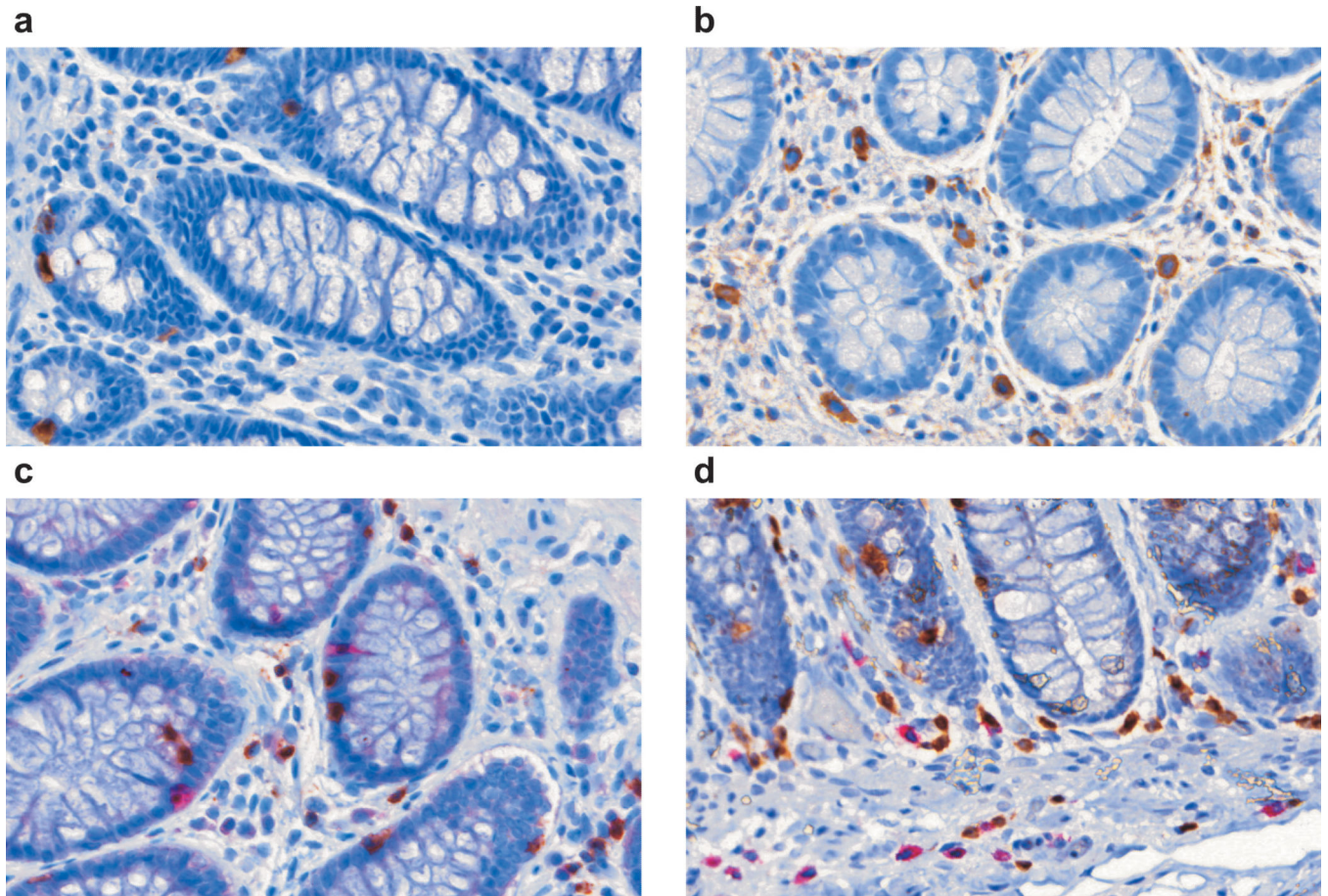


FIG.1. Two distinct subsets of gut NK cells exist in different regions of the human colon
Immunohistochemical (IHC) staining depicts IEL NK cells stained in brown with GAH NKp46 antibody (a) and LP NK cells stained in brown with MAH NKp46 antibody (b). To confirm that NKp46+ cells were not T cells, double staining IHC was performed with CD3 in brown and GAH NKp46 (c) and MAH NKp46 (d) in red. All images are displayed at 400x magnification.

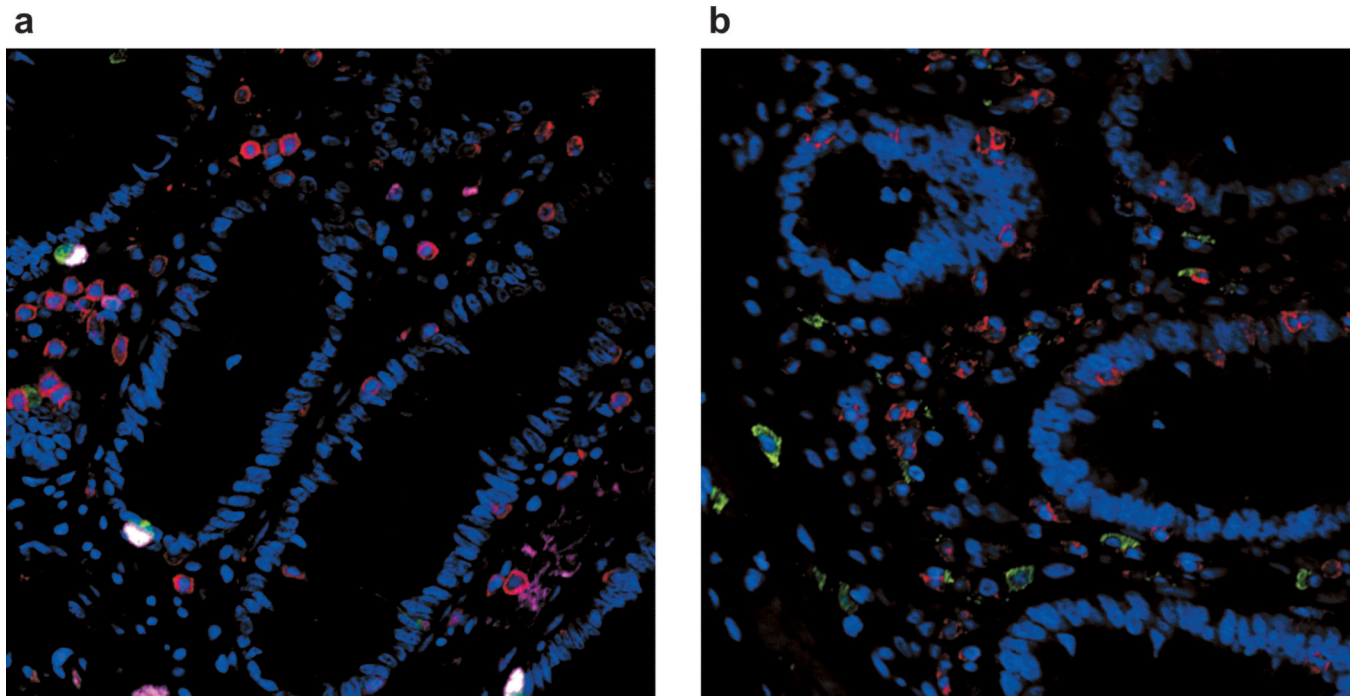


FIG.2. IEL, but not LP, NK cells express CD57

Multicolor confocal immunofluorescence images show co-expression of CD57 (white), a marker of differentiated NK cells, on a subset of IEL NK cells (a) defined as CD3-negative (red) and NKp46-positive (green). LP NK cells (b) showed no such co-expression of CD57. Both images are displayed at 630x magnification.

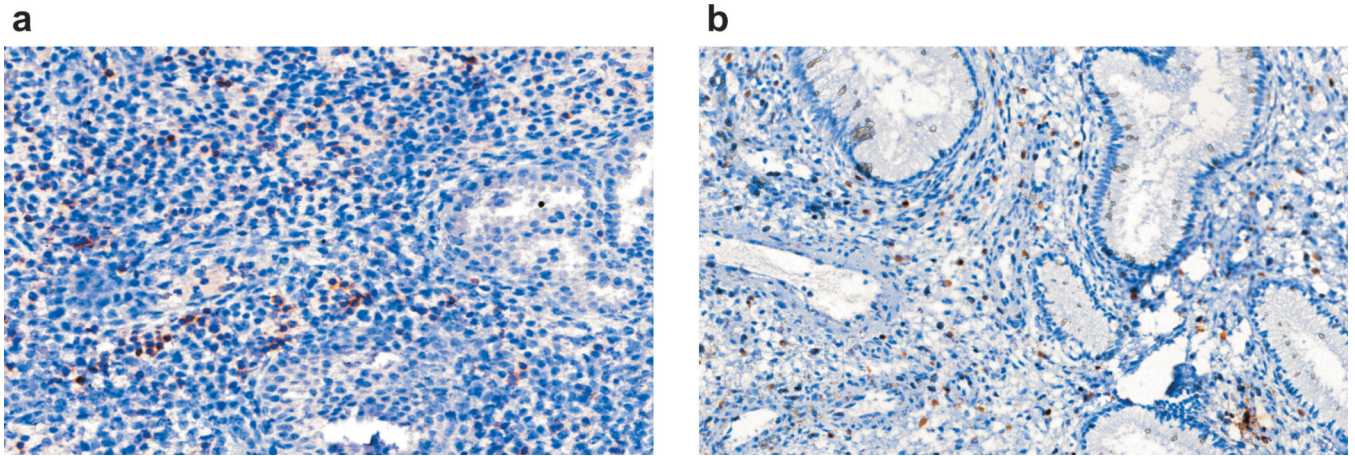
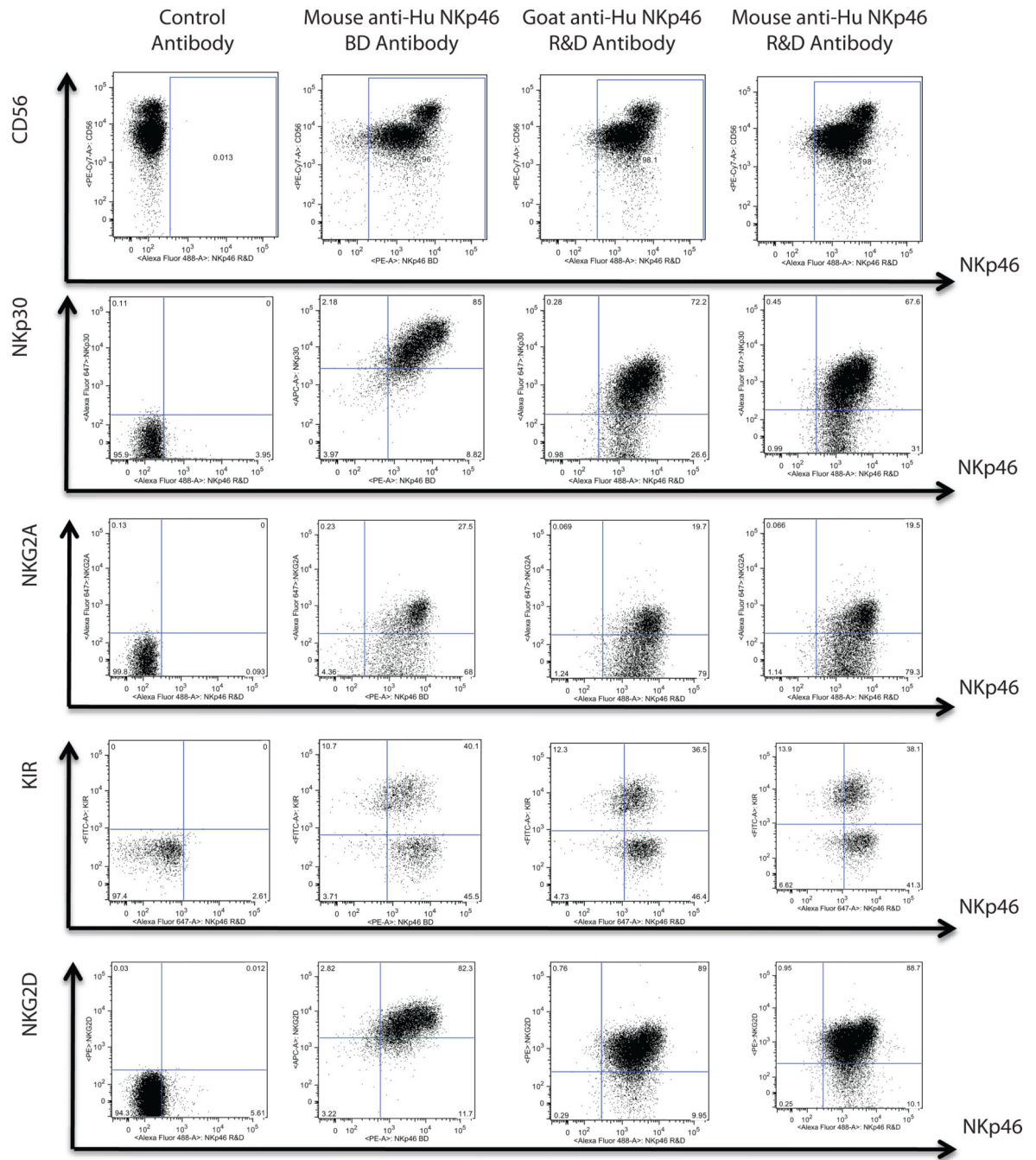


FIG.3. NKp46 antibodies also stain NK cells in the uterus

Both the GAH (a) and MAH (b) NKp46 antibodies labeled NK cells (brown) in the endometrial section. All images are displayed at 200x magnification.

a



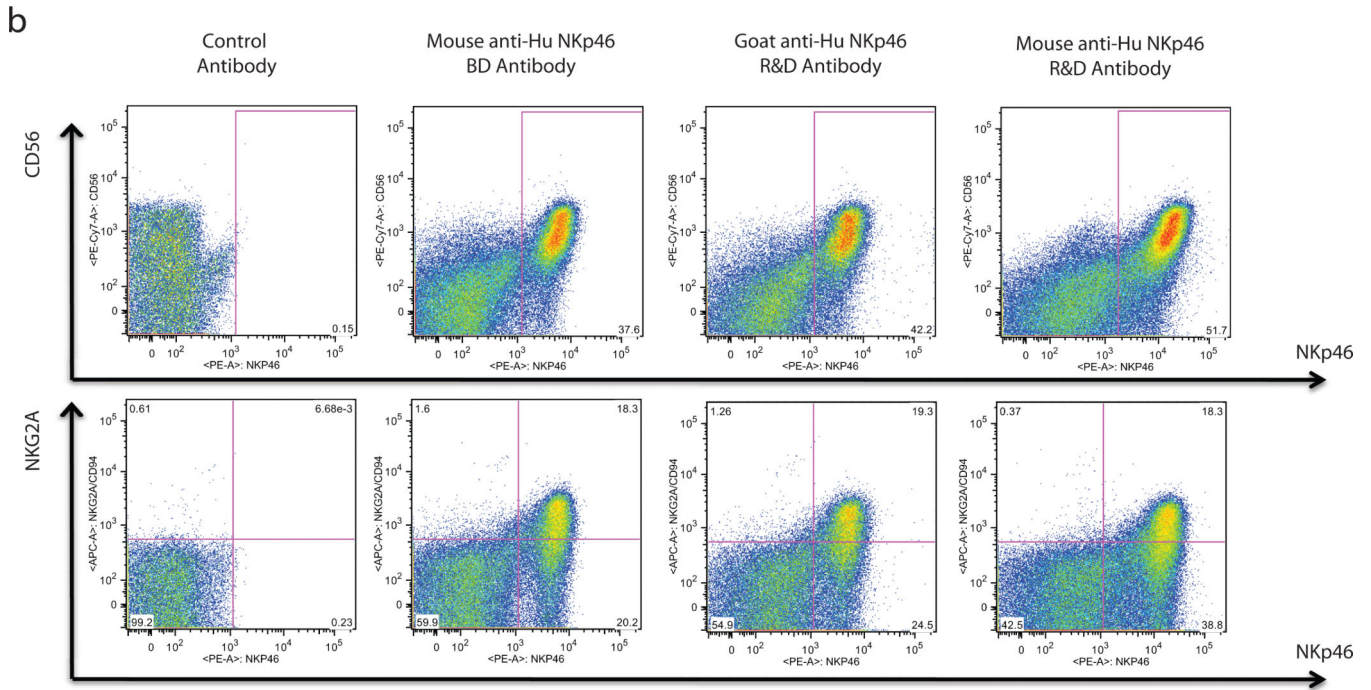


FIG.4. Flow cytometric confirmation of NKp46 antibody specificity for NK cells in the peripheral blood

To confirm that the 2 NKp46 antibodies specifically stained NK cells, the specificity of the antibodies was verified by flow cytometry using a panel of NK cell specific antibodies. The overall frequency of CD3⁻CD16^{+/-}CD56^{+/-} NK cells stained with the traditional BD NKp46, the GAH NKp46, and the MAH NKp46 were similar. Additional NK cell markers, including NKp30, NKG2A, KIR, NKG2D were expressed at equivalent levels on blood NK cells labeled with the 3 different NKp46 antibodies (a). Furthermore, lymphocytes isolated from the gut tissue were stained with the traditional BD NKp46, the GAH NKp46 and the MAH NKp46 to confirm that these antibodies label CD45^{pos}CD3^{neg} NK cells in mucosal samples (b).

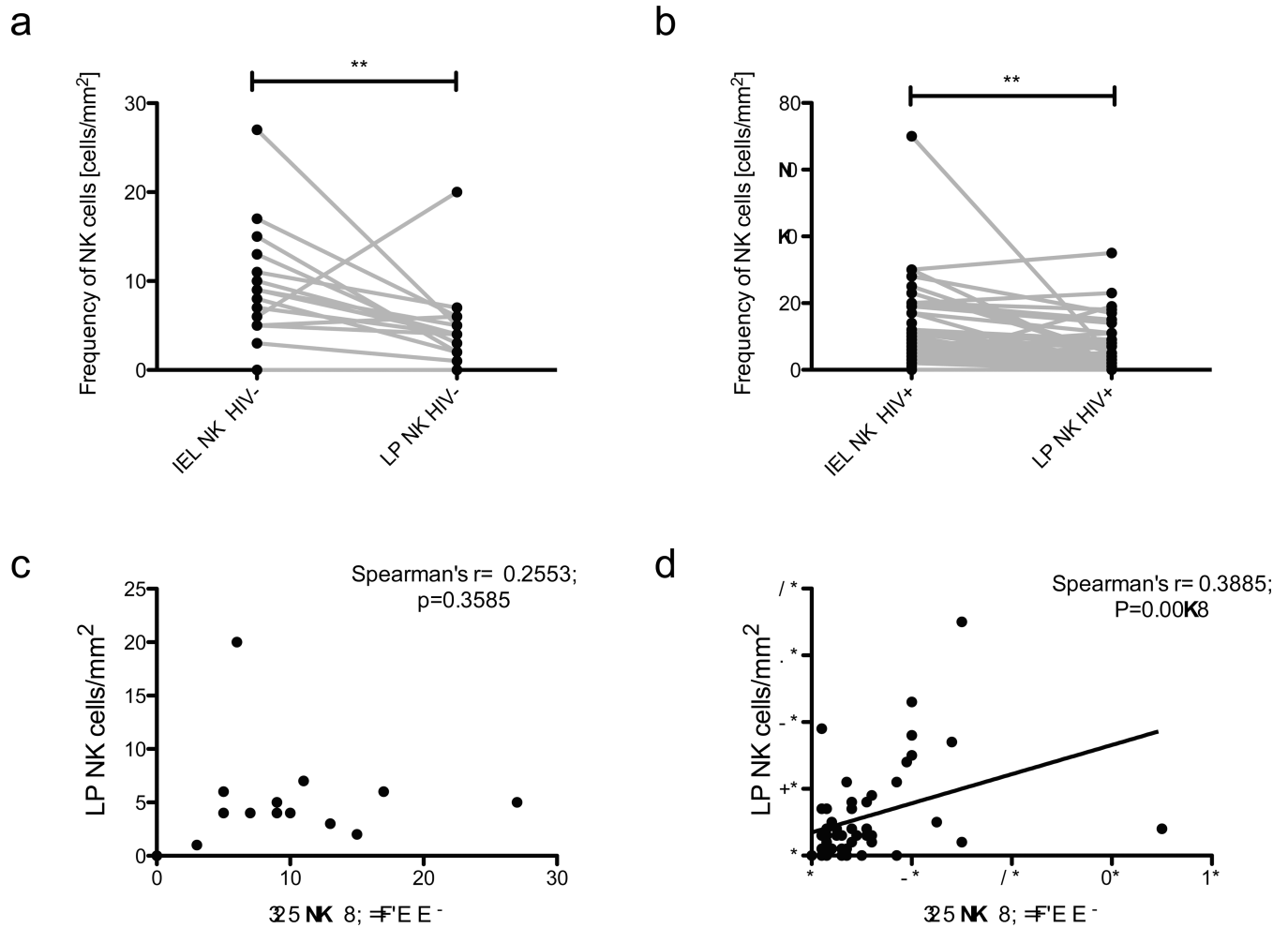


FIG.5. IEL NK cells are more abundant than LP NK cells in both healthy and HIV-infected populations

The total frequency of IEL and LP NK cells were counted per mm² of colon tissue in healthy individuals, demonstrating that IEL NK cells are more abundant in the gut mucosa compared to LP NK cells ** $P < 0.01$ (a). Similarly, IEL NK cells exceeded the frequency of LP NK cells in the colon tissue of HIV-infected individuals ** $P < 0.01$ (b). Furthermore, the relationship between IEL and LP NK cells was explored, demonstrating no visible correlation in cell frequencies in healthy individuals (c), but a significant correlation in HIV + patients (d).

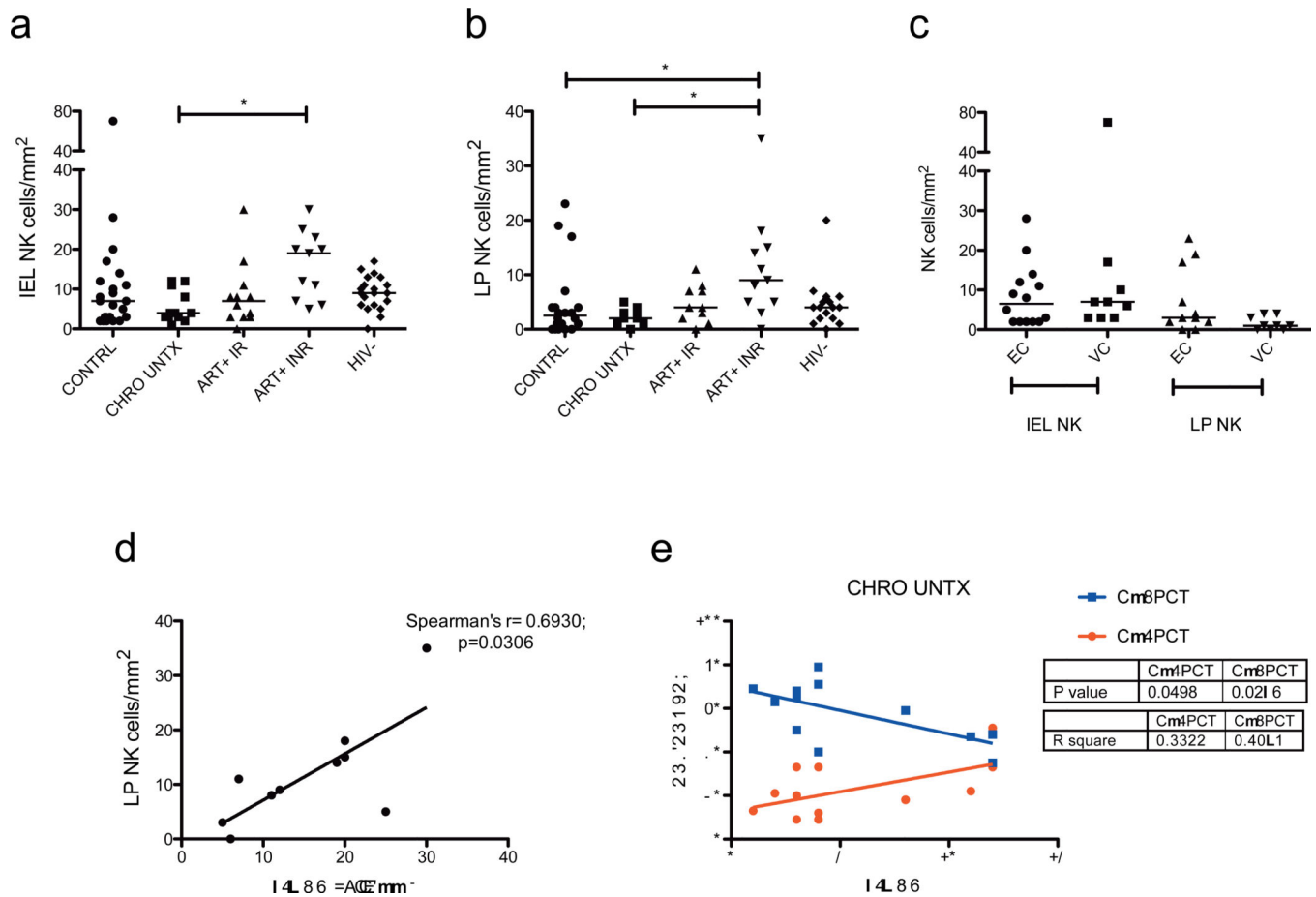


FIG.6. Frequency changes in IEL and LP NK cells in HIV+ patients with differential control of infection and healthy controls

The frequency of IEL (a) and LP (b) NK cells was compared among the different HIV+ patient populations, including spontaneous controllers (CONTROL-), chronic untreated patients (CHRO UNTX-), treated chronically infected patients that exhibited immunologic reconstitution following HAART (ART+ IR-), treated chronically infected patients that were immunologic non-responders (ART+ INR-), and healthy controls (HIV NEG-) * $P < 0.05$.

These data demonstrate the expansion of both IEL and LP NK cells in ART-INR patients. Furthermore, the frequency of IEL and LP NK cells were also compared among elite (EC) and viremic (VC) controllers (c). IEL and LP NK cells frequencies are correlated in the INR group (d). The relationship between the frequency of IEL NK cells and the percent CD4+ T cells (CD4 PCT) and the percent CD8+ T cells (CD8 PCT) in the periphery is illustrated in panel (e).

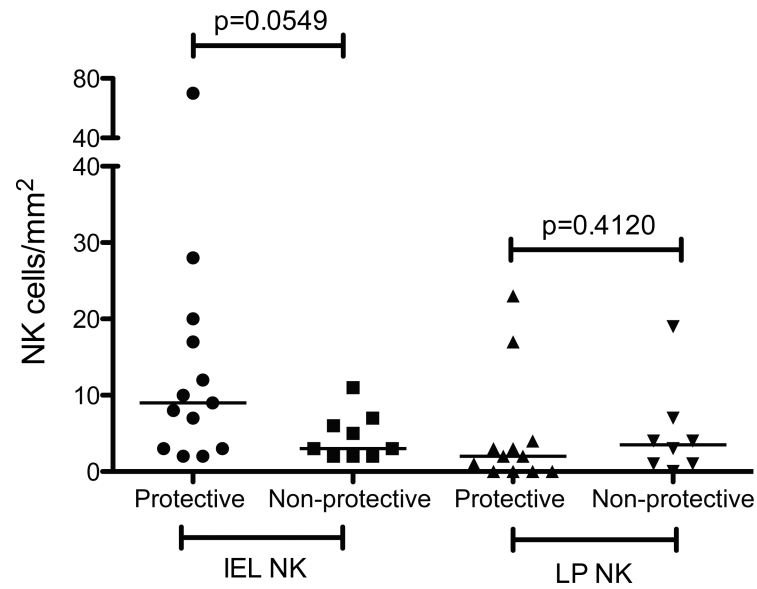


FIG.7. Impact of KIR/HLA compound genotypes on the frequency of gut mucosal NK cells
 The difference in numbers of IEL NK cells and LP NK cells was compared between controllers expressing protective KIR/HLA alleles (KIR3DS1-Bw4-80I or KIR3DL1*h-Bw4-80I) with those not expressing protective KIR/HLA alleles.