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INFLAMMATORY COLONIC INNATE LYMPHOID CELLS ARE INCREASED DURING UNTREATED HIV-1 INFECTION AND ASSOCIATED WITH MARKERS OF GUT DYSBIOSIS AND MUCOSAL IMMUNE ACTIVATION

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Abstract

BACKGROUND—HIV-1 infection is associated with intestinal inflammation, changes in the enteric microbiota (dysbiosis) and intestinal epithelial cell (IEC) damage. NKp44^{+} innate lymphoid cells (ILCs) play an important role in epithelial barrier maintenance via the production of IL-22, but also display functional plasticity and can produce inflammatory cytokines (e.g. IFNγ) in response to cytokine milieu and stimulatory signals. The objective of this pilot study was to enumerate frequencies of IL-22 and IFNγ-expressing colonic NKp44^{+} ILCs during untreated, chronic HIV-1 infection.

SETTING—A cross-sectional study was performed to compare numbers of cytokine-expressing ILCs in colonic biopsies of untreated, chronic HIV-1 infected (n=22) and uninfected (n=10) study participants. Associations between cytokine[+] ILC and previously established measures of virological, immunological and microbiome indices were analyzed.

METHODS—Multi-color flow cytometry was used to measure the absolute number of colonic CD3^{−}NKp44[±]CD56[±] ILCs expressing IL-22 or IFNγ following *in vitro* mitogenic stimulation.

RESULTS—Numbers of colonic NKp44^{+} ILCs that expressed IFNγ were significantly higher in HIV-1 infected versus uninfected persons and positively correlated with relative abundances of

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dysbiotic bacterial species in the Xanthomoadaceae and Prevotellaceae bacterial families and with colonic mDC and T cell activation.

CONCLUSION—Higher numbers of inflammatory colonic ILCs during untreated chronic HIV-1 infection that associated with dysbiosis and colonic mDC and T cell activation suggest that inflammatory ILCs may contribute to gut mucosal inflammation and epithelial barrier breakdown, important features of HIV-1 mucosal pathogenesis.

Keywords

HIV-1; innate lymphoid cells; dysbiosis; microbial translocation; inflammation

INTRODUCTION

Disruption of intestinal homeostasis, intestinal epithelial cell (IEC) damage and changes in the microbiota (dysbiosis)[1–5] are associated with HIV-1 infection. IEC damage leads to translocation of dysbiotic microbes[6] and microbial translocation is linked to tissue and systemic immune activation and predicts disease progression in untreated HIV-1 infected persons[7–10]. Understanding the mechanisms that drive IEC damage and microbial translocation will be critical to controlling both gut and systemic inflammation during HIV-1 infection.

Maintenance of intestinal barrier integrity and homeostasis relies in part on the presence of IL-22[11,12]. Depletion of gut T helper 22 (Th22) and Th17 cells, subsets of which co-produce IL-22, is considered to be a major contributor to HIV-1 pathogenesis[1,13–15]. A pivotal publication by Cella *et.al.*, described non-T cells capable of producing IL-22 in human mucosa-associated lymphoid tissues[16]. These non-T cells identified by the expression of an NK cell activation receptor NKp44[17] and the classic NK cell marker CD56, were termed NK22 cells. NK22 cells are now considered members of the recently identified family of Group 3 innate lymphoid cells (ILC3s), which play important and diverse roles in mucosal immunity[18–20]. In untreated HIV-infected individuals, frequencies of colonic IL-22-expressing non-T-cells that included NKp44+ cells were increased early in infection[13]. Further, frequencies of NKp44+ IL-22-producing cells positively correlated with an intact intestinal barrier in long-term ART-treated HIV infected persons[21]. Thus, NKp44+ ILCs may also play a critical role in regulating epithelial barrier health during HIV infection.

Multiple studies have suggested that ILCs, including ILC3s, display a degree of functional plasticity and can produce inflammatory cytokines (*e.g.* TNFα, IFNγ) in response to the local cytokine milieu (*e.g.* IL-2, IL-23, IL-12p70, IL-1β, IL-18) and/or exposure to stimulatory signals (*e.g.* ligation of NKp44)[22–25]. We have previously demonstrated that colonic myeloid dendritic cells (mDCs) produce IL-23 and IL-1β in response to exogenous exposure to mucosa-associated colonic commensal bacteria that are increased in relative abundance in untreated HIV-1 infected persons[26]. Moreover, HIV-1 induces the expression of NKp44L on CD4 T cells both *in vitro* and *in vivo*[27–29]. These observations raise the possibility that a microenvironment exists in the gut of HIV-1-infected persons that is conducive to IL-22-producing ILCs switching into inflammatory ILCs. Indeed, colonic NKp44+ ILCs demonstrated an altered phenotype by producing IFNγ rather than IL-17/

IL-22 during pathogenic SIV infection[30,31]. Therefore, we evaluated the frequencies of IL-22-, IL-17- and IFNγ-producing colonic NKp44-expressing ILCs in the setting of untreated chronic HIV-1 infection.

METHODS

Study participants

Untreated, chronically HIV-1-infected adults and HIV-1-seronegative (uninfected) controls were enrolled in this cross-sectional study at the University of Colorado Anschutz Medical Campus. Inclusion and exclusion criteria are extensively detailed in previous publications[26,32,33]. All study participants voluntarily gave written, informed consent. This study was approved by the Colorado Multiple Institutional Review Board (COMIRB).

Surface and intracellular flow cytometry staining assays, acquisition and analysis

The collection, processing and storage of colon biopsies are detailed elsewhere[26,32,33]. Colonic cells were cultured in RPMI (Invitrogen) + 10% human AB serum (Gemini Bioproducts) + 1% penicillin/streptomycin/L-glutamine (Invitrogen) and stimulated with PMA (250ng/ml; Sigma-Aldrich) and ionomycin (1μg/ml; Sigma-Aldrich) and 0.1% Brefeldin A (Golgi Plug, BD Biosciences) for 16hrs. Cells were collected and frequencies of NKp44$^+$CD56$^-$, NKp44$^+$CD56$^+$ and NKp44$^-$CD56$^+$ cells and cytokine-expressing NKp44$^+$CD56$^-$, NKp44$^+$CD56$^+$ and NKp44$^-$ CD56$^+$ cells were determined using standard multi-color intracellular cytokine flow cytometry protocols[26,32–36]. Cells were stained with viability dye (Aqua, Invitrogen), CD45 (clone: 2D1; PerCp-Cy5.5, eBioscience), CD3 (UCHT1; PE Texas Red, Beckman Coulter), NKp44 (P44-8; APC, Biolegend), CD56 (B159; PE-Cy5, BD Biosciences), IL-22 (22URTI; PE, eBioscience), IL-17 (N49-653; V^{450}, BD Biosciences) and IFNγ (B27; AF700, BD Biosciences). All flow cytometry data was acquired on an LSRII Flow Cytometer (BD Biosciences) and analyzed using BD FACSDiva software version 6.1.2 (BD Biosciences)[36] (Figure S1 Supplemental Digital Content). Evaluation of cytokine-expressing ILCs was only performed when there were at least 25 NKp44$^+$CD56$^-$, NKp44$^+$CD56$^+$ and NKp44$^-$CD56$^+$ events.

Enumeration of immunological, virological, and microbial parameters

Measurements of plasma IL-6, C-reactive protein, TNFα, IFNγ, IL-10, soluble CD14, lipoteichoic acid, lipopolysaccharide and intestinal fatty acid binding protein, colonic mucosa levels of HIV-1 RNA, frequencies of colonic mDC, plasmacytoid DC (pDC), CD4 and CD8 T cells, measurements of mDC activation/maturation (CD40/CD83) and T cell activation (CD38$^+$HLA-DR$^+$) and frequencies of IFNγ, IL-22 and IL-17-expressing Th cells have been previously published[26,32,33]. Laboratory and analytic methods used to profile the intestinal microbiomes were as described[26,32,33,35].

Statistical analysis

Non-parametric statistics were performed with no adjustments for multiple comparisons due to the exploratory nature of this study. Analyses were performed using GraphPad Prism Version 6 for Windows (GraphPad Software, San Diego, CA). A p-value <0.05 was considered significant.

RESULTS

Absolute numbers of colonic ILCs and cytokine-expressing colonic ILCs were determined in a subset of untreated HIV-1 infected (N=22) and uninfected (N=10) study participants from a previously detailed clinical study[26,32,33]. Study participant characteristics are detailed Table S1, Supplemental Digital Content.

Absolute numbers of colonic ILCs

There were no significant differences in the absolute number of colonic NKp44$^+$CD56$^-$ and NKp44$^+$CD56$^+$ ILCs between uninfected and HIV-1-infected subjects (Figure S2 Supplemental Digital Content). Conversely, HIV-1 infected individuals had significantly fewer NKp44$^-$CD56$^+$ cells compared to uninfected controls.

Cytokine profiles of colonic ILCs in healthy individuals

We first compared absolute numbers of IL-22, IL-17, and IFNγ-expressing CD3$^-$ILCs in healthy uninfected persons (Figure 1A). Greater numbers of NKp44$^+$CD56$^-$ ILCs expressed IL-22 compared with IFNγ or IL-17, although this latter comparison did not reach statistical significance. Similar numbers of NKp44$^+$CD56$^+$ expressed IL-22 or IFNγ, whereas fewer were capable of producing IL-17. Very few NKp44$^+$CD56$^+$ ILCs co-expressed IL-22 and IFNγ (data not shown), suggesting distinct cytokine-producing populations. The number of NKp44$^-$CD56$^+$ cells that expressed IFNγ was significantly greater than IL-22$^+$ or IL-17$^+$ expressing cells.

Altered ILC cytokine profiles in HIV-infection

In HIV-1 infected individuals, comparable numbers of NKp44$^+$CD56$^-$ ILCs expressed IL-22 or IFNγ (Figure 1B) with few NKp44$^+$CD56$^-$ ILCs expressing both IL-22 and IFNγ (data not shown). Numbers of IL-22$^+$ and IFNγ$^+$ NKp44$^+$CD56$^-$ ILCs were significantly greater than IL-17- expressing NKp44$^+$CD56$^-$ ILCs. Cytokine profiles of the remaining ILC populations in HIV-1 infected persons were generally reflective of those observed in uninfected persons, with similar numbers NKp44$^+$CD56$^+$ ILCs producing either IL-22 or IFNγ and with NKp44$^-$CD56$^+$ ILCs primarily producing IFNγ (Figure 1B).

We next compared the absolute number of cytokine-producing ILCs between uninfected and HIV-1 infected persons. IFNγ-producing NKp44$^+$CD56$^-$ and NKp44$^+$CD56$^+$ ILCs were significantly increased in number in HIV-1 infected relative to uninfected persons (Figures 2A, 2C). Absolute numbers of IFNγ$^+$NKp44$^-$CD56$^+$ ILCs were not statistically different between the two cohorts (data not shown). Numbers of IL-22-expressing NKp44$^+$CD56$^-$ or NKp44$^+$CD56$^+$ ILC populations were not significantly different between HIV-1 infected and uninfected study participants (Figure 2E). Although numbers of IL-22-expressing NKp44$^-$CD56$^+$ cells were low compared to IFNg$^+$NKp44$^-$CD56$^+$ cells, significantly fewer NKp44$^-$CD56$^+$ cells expressing IL-22 were observed in HIV-1 infected persons (Figure 2E). No statistical differences between uninfected and HIV-1 infected individuals in the absolute numbers of IL-17-expressing ILC populations were noted (data not shown).

Associations of IFNγ-expressing colonic ILCs with dysbiotic microbes and markers of immune activation in HIV-1 infection

Given the finding of increased IFNγ[+] ILCs in HIV-infected persons, we next addressed associations of these ILC populations with previously reported measures of clinical, virological, immunological and microbiome indices for the HIV-1 infected cohort[26,32,33] (Table S2 Supplemental Digital Content). Absolute numbers of colonic IFNγ^{+}NKp44^{+}CD56^{−} ILCs significantly correlated with the relative abundance of Xanthomonadaceae and Prevotellaceae families, with the *Prevotella* genus (Figure 2B), and with the individual species *P. copri* and *P. stercorea* (R=0.68, P=0.01; R=0.63, P=0.02 respectively; data not shown). Numbers of IFNγ^{+}NKp44^{+}CD56^{−} ILCs inversely correlated with the percentages of CD83^{+}CD1c^{+} mDC (R=− 0.51, P=0.03) and positively correlated with numbers of IFNγ-producing CD4 T cells (R=0.53, P=0.01) (data not shown). IFNγ^{+} NKp44^{+}CD56^{+} ILC numbers were not significantly associated with dysbiotic microbes but instead positively correlated with colon CD1c^{+} mDC activation levels and with absolute numbers of activated colon CD4 and CD8 T cells in HIV-1 infected individuals (Figure 2D).

DISCUSSION

This study highlights that untreated, chronic HIV-1 infection is associated with higher numbers of colonic NKp44^{+} ILCs that express IFNγ. The difference in the number of these cells expressing IFNγ is unlikely related to an increase in the number of NKp44^{+} ILCs given that no significant difference in the overall number of each NKp44^{+} ILC subsets was observed between the two cohorts. IFNγ is an inflammatory cytokine that increases intestinal epithelial barrier permeability primarily via alterations in tight junction protein expression and thereby enhances bacterial transcytosis[37–39]. Accordingly, the presence of these inflammatory ILCs likely contributes to epithelial barrier breakdown and the resultant microbial translocation.

We, and others, have demonstrated that alterations in intestinal mucosa-associated bacterial communities during HIV-1 infection are associated with indicators of mucosal HIV-1 pathogenesis (reviewed in[40]). In our study, major findings from the microbiome analysis included higher relative abundance of mucosa-associated bacteria belonging to the Proteobacteria phylum (including Xanthomonadaceae) and of *Prevotella spp.* in HIV-1 infected persons[26,33]. Increased relative abundance of *Prevotella spp.* in HIV-1 infected persons associated with increased colonic mDC and T cell activation[26,33]. Other HIV-associated mucosal abnormalities included decreased percentages of colonic mDC expressing CD83[26], a molecule reported to play a role in intestinal immune regulation[41]. In this current study, numbers of IFNγ-expressing NKp44^{+} ILCs positively correlated with relative abundances of bacterial species in the Xanthomoadaceae and Prevotellaceae families, with colonic mDC and T cell activation and inversely associated with the fraction of colonic mDCs expressing CD83. We have previously shown that enteric bacterial species found in high abundance in HIV-1 infected persons (e.g. *Prevotella*), are capable of inducing IL-23 and IL-1β from colonic mDC *in vitro*[26], cytokines that contribute to the induction of inflammatory cytokines such as IFNγ and TNFα by ILCs[25,42]. Thus, we hypothesize that in the setting of HIV-1 infection, the shift in phenotype of primarily IL-22-producing ILCs to

IFNγ- producing ILCs is linked to an intricate relationship between translocating bacteria, colonic mDC, and other signals within the inflammatory environment (e.g., NKp44L).

In contrast to the dramatic alterations in numbers of IFNγ-expressing NKp44^{+} ILCs associated with HIV-1 infection, absolute numbers of IL-22-expressing NKp44^{+} cells were similar in both HIV-1 infected and uninfected individuals, an observation in keeping with a number of other studies investigating gut ILCs during HIV-1 infection[13,21,43,44]. Conversely, one study which identified colonic tissue ILCs as CD3^{−}IL-22^{+} using immunohistological techniques, found decreased numbers of these cells in untreated HIV-1 infected versus uninfected individuals[45]. In contrast to HIV-1 infection, both absolute frequencies of ILCs and frequencies of IL-22/IL-17- producing ILCs were significantly reduced during early and chronic SIV infection[30,31,46–48]. Of note, we observed a decrease in frequencies of NKp44^{−}CD56^{+} cells that expressed IL-22, likely related to the overall decrease in absolute numbers of colonic NKp44^{−}CD56^{+} cells as a whole. However, NKp44^{−}CD56^{+} cells do not typically produce IL-22, so the impact of an overall decrease in this small population in untreated, chronic HIV-1 infected persons may be minor in the context of maintenance of IL-22^{+}NKp44^{+} ILCs.

We acknowledge a number of limitations to this pilot study. The study was not powered to address alterations in numbers of colonic cytokine-producing ILCs in the setting of HIV-1 infection. The two study groups were not matched for sexual practice, which has recently been reported to impact the intestinal microbiome independent of HIV-1 infection[49,50] and may drive mucosal immune cell activation and inflammation[50] and therefore be a contributing factor to our current observations. Enumeration of the specific ILC subsets was based on criteria used to identify NK22 cells[16]. Since that time, the field of ILC biology has greatly expanded and a more rigorous and specific identification paradigm for the various ILC subsets (i.e., NK, ILC1s, ILC2, ILC3s) is being utilized[19,20,42,51,52]. Our ability to determine the composition of the specific ILC subsets (NK v ILC1 v ILC3) within the cytokine-producing populations based on this more recent nomenclature is limited here and future studies will be needed to incorporate these most recent definitions.

Despite these limitations, the observations that higher frequencies of IFNγ-producing ILCs, particularly in the NKp44^{+}CD56^{−} ILC population which typically do not produce IFNγ in the absence of HIV-1 infection, underscores the role these innate immune cells may play in HIV-1-associated mucosal inflammation and pathogenesis. Additional studies both *in vivo* and *in vitro* will be required to determine the extent to which the increased numbers are a result of a switch from IL-22 to IFNγ production versus expansion of the IFNγ-producing population versus an influx of inflammatory cells into the mucosal tissue. In summary, higher numbers of inflammatory colonic ILCs during untreated chronic HIV-1 infection associate with dysbiosis and gut mDC and T cell activation suggesting a critical interplay between gut ILCs, the microbiome and local immune responses that should be further explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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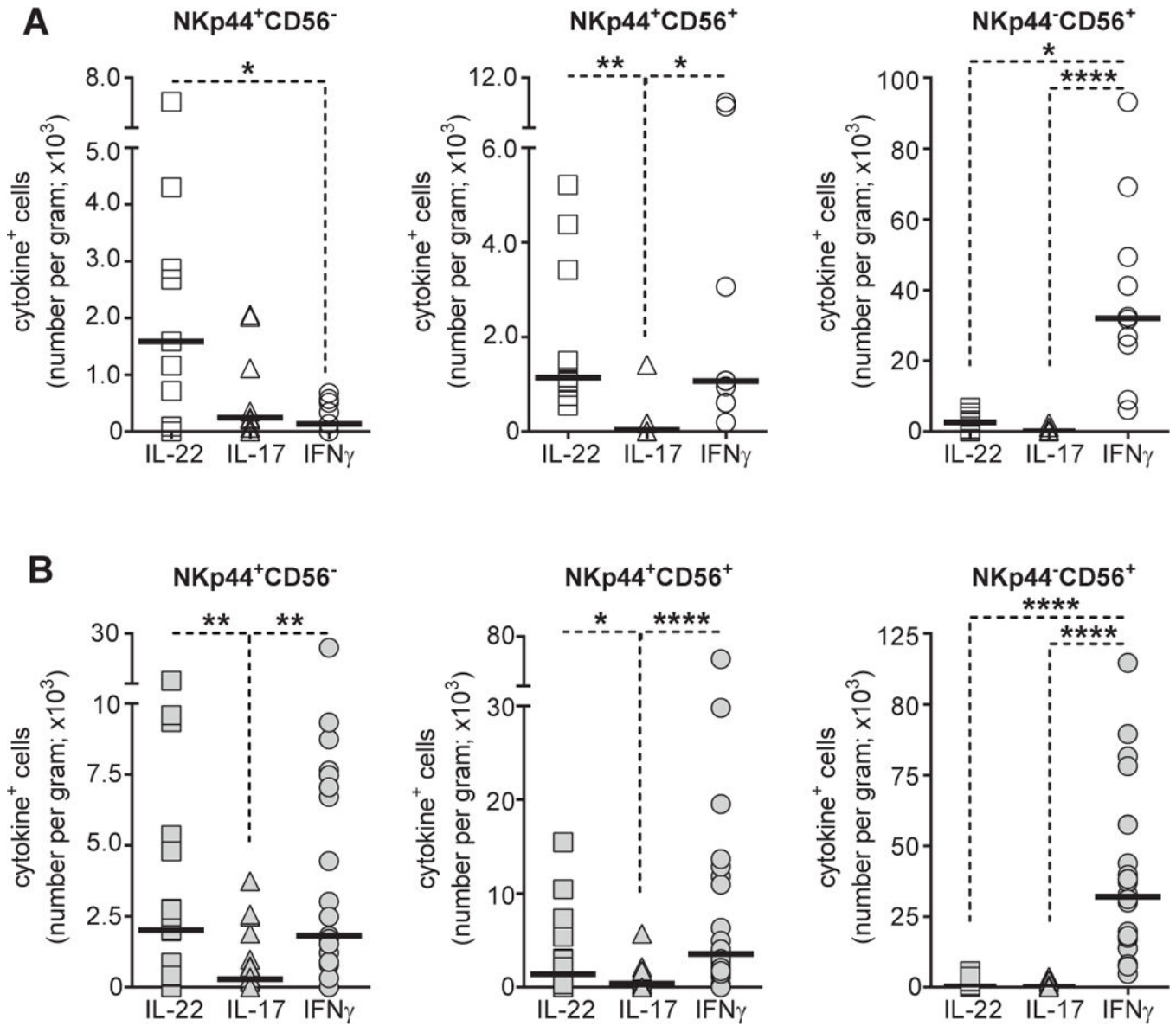
Figure 1.
Cytokine profiles of colonic ILC subsets. A, Absolute numbers of cytokine^{+} NKp44^{+}CD56^{−} (N=9), NKp44^{+}CD56^{+} (N=9) and NKp44^{−}CD56^{+} (N=10) colonic ILCs in uninfected study participants. B, Absolute numbers of cytokine^{+} NKp44^{+}CD56^{−} (N=21), NKp44^{+}CD56^{+} (N=22) and NKp44^{−}CD56^{+} (N=22) colonic ILCs in HIV-1 infected study participants. Lines indicate the median value. Statistical analysis was performed using Dunn's multiple comparisons test. *P<0.05; **P<0.01; ****P<0.0001.
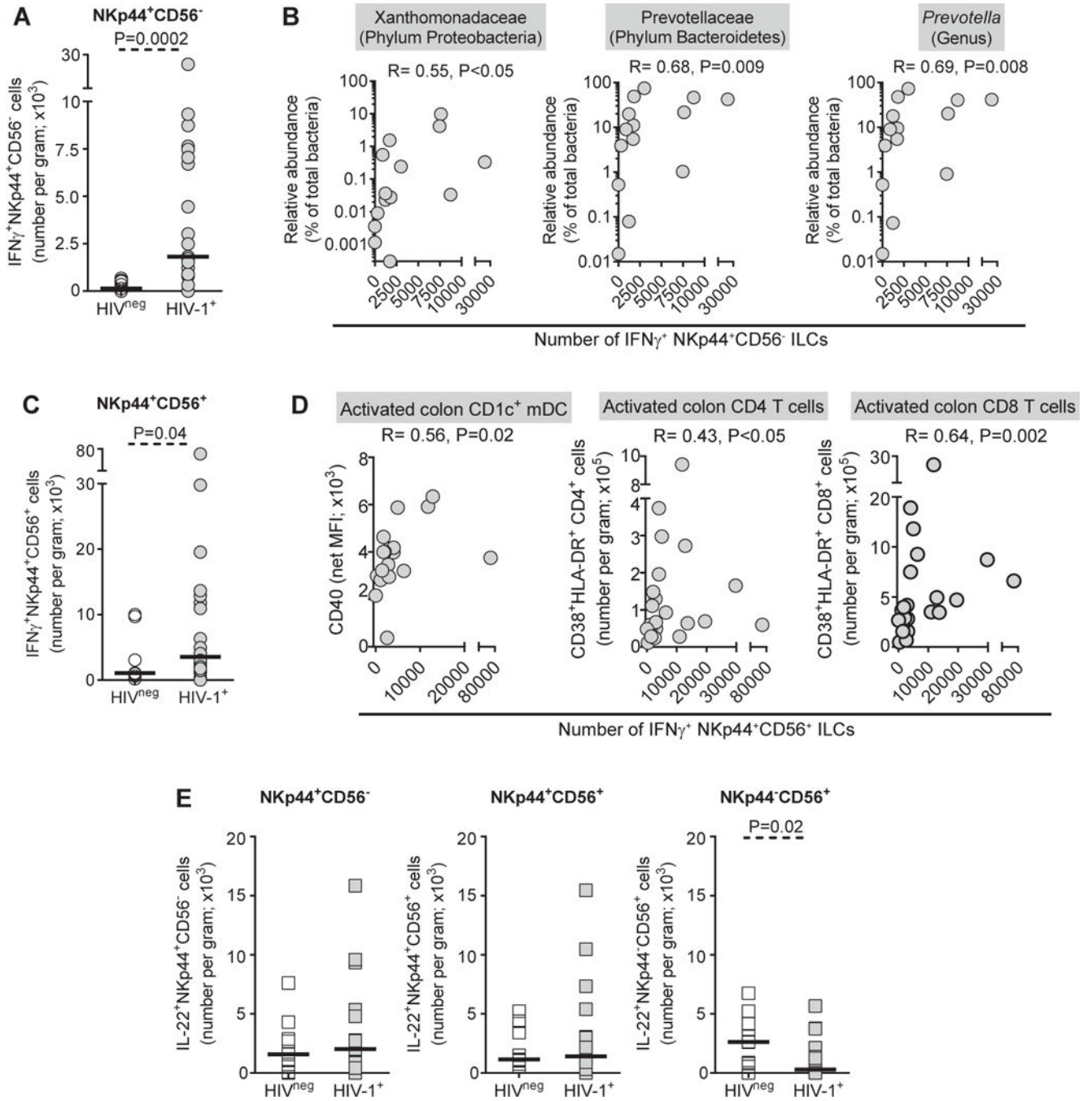
Figure 2.
Absolute numbers of IFNγ-expressing NKp44+ ILCs are higher in HIV-1 infection and associate with microbial dysbiosis and colonic immune activation. A, Absolute numbers of IFNγ- expressing NKp44+CD56− ILCs in uninfected (HIVneg; N=9) and HIV-1-infected (HIV-1+; N=21) study participants expressed as the total number of IFNγ+ NKp44+CD56− ILC per gram of tissue. B, Correlations between the number per gram of IFNγ-expressing NKp44+CD56− ILCs and relative abundances of colonic mucosa-associated microbiota in HIV-1 infected study participants (N=14). C, Absolute number of IFNγ-expressing

NKp44$^+$CD56$^+$ ILCs in HIV^{neg} (N=9) and HIV-1$^+$ (N=22) study participants expressed as the total number of IFNγ$^+$ NKp44$^+$CD56$^-$ ILC per gram of tissue. D, Correlations between the absolute number of IFNγ-expressing NKp44$^+$CD56$^+$ ILCs and CD40 expression levels on colonic CD1c$^+$ mDC (N=18) and numbers of colonic CD38$^+$HLA-DR$^+$ CD4 and CD8 T cells (N=22). E, Absolute numbers of IL-22-expressing NKp44$^+$CD56$^-$, NKp44$^+$CD56$^+$, NKp44$^-$CD56$^+$ ILCs measured in uninfected (HIV^{neg}; NKp44$^+$CD56$^-$ N=9; NKp44$^+$CD56$^+$ N=9; NKp44$^-$CD56$^+$ N=10) and HIV-1-infected (HIV-1$^+$; NKp44$^+$CD56$^-$ N=21; NKp44$^+$CD56$^+$ N=22; NKp44$^-$CD56$^+$ N=22) study participants expressed as the total number of IL-22$^+$ ILC subsets per gram of tissue. Lines represent median values. Statistical analysis to compare between independent groups were made using the Mann–Whitney test and correlations between variables were assessed using the Spearman test.