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Bacteroides are associated with GALT iNKT cell function and reduction of microbial translocation in HIV-1 infection

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Invariant natural killer T (iNKT) cells are innate-like T cells that respond to lipid antigens presented by CD1d. These immunoregulatory cells have the capacity for rapid cytokine release after antigen recognition and are essential for the activation of multiple arms of the immune response. HIV-1 infection is associated with iNKT cell depletion in the peripheral blood; however, their role in the gastrointestinal-associated lymphoid tissue (GALT) is less well studied. Our results show that iNKT cells are found at a higher frequency in GALT compared with blood, particularly in HIV-1 elite controllers. The capacity of iNKT cells to produce interleukin-4 (IL-4) and IL-10 in the GALT was associated with less immune activation and lower markers of microbial translocation, whereas regulatory T cell frequency showed positive associations with immune activation. We hypothesized that the composition of the microbiota would influence iNKT cell frequency and function. We found positive associations between the abundance of several *Bacteroides* species and iNKT cell frequency and their capacity to produce IL-4 in the GALT but not in the blood. Overall, our results are consistent with the hypothesis that GALT iNKT cells, influenced by certain bacterial species, may have a key role in regulating immune activation in HIV-1 infection.

INTRODUCTION

HIV-1 infection leads to the development of chronic inflammation that persists even in antiretroviral therapy (ART)-treated individuals with undetectable viral loads.[1,2] This inflammation is associated with non-HIV comorbidities, including cardiovascular disease, neurologic disorders, cancers, and an overall increased mortality. It has become apparent that immune activation is a better predictor of HIV-1 disease progression than either peripheral blood $CD4^+$ T-cell count or viral load,[3] highlighting the importance of chronic immune activation. However, distinct pathways of immune activation (innate vs. adaptive) appear to have differential prognostic capacity, depending on the cohorts.[4] Importantly, while ART significantly diminishes immune activation (particularly if initiated early after infection[5]), the levels do not normalize to those of uninfected individuals. Invariant natural killer T (iNKT) cells are innate-like T cells that respond to lipid antigens presented on CD1d, an major histocompatibility complex class I-like molecule expressed on antigen-presenting cells (APCs).[6] iNKT cells are characterized by their expression of the semi-invariant T-cell receptor chain Vα24-Jα18 preferentially paired to a Vβ11 chain. Upon stimulation, iNKT cells are capable of rapid production of a vast array of cytokines and chemokines and are instrumental in orchestrating innate and adaptive immune response.[7] iNKT cells can recruit and modulate other immune cells, including natural killer (NK) cells, dendritic cells (DCs), and conventional $CD4^+$ and $CD8^+$ T cells.[8] Depending on the type of specific interactions between

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iNKT cells and DCs, the cytokines secreted by activated iNKT cells may either activate or suppress adaptive immune responses.

Mouse studies have shown that the symbiotic microbiota can impact the maturation and function of iNKT cells in the mucosa.[9,10] A sphingolipid produced by the human commensal *Bacteroides fragilis* has been shown to bind CD1d and modulate iNKT cells.[11] When compared with specific pathogen-free mice, germ-free mice have a greater frequency of iNKT cells in intestinal lamina propria and epithelium, but these cells express lower levels of activation and produce less cytokines in response to stimulation.[12] Therefore, the gut microbiome influences the post-thymic maturation of iNKT cells, and intestinal bacterial reconstitution is a potential strategy for correcting systemic iNKT hyporesponsiveness in individuals with an altered microbial landscape. Dysbiosis of gut microbiota, particularly depletion of Bacteroidia members (including *B. fragilis*), has recently been described in the context of untreated HIV-1 infection, and is associated with markers of systemic immune activation and chronic inflammation.[13]

Mouse studies have revealed a role for iNKT cells in the control of viral infections, but their involvement in viral immunity in humans is less well characterized.[14,15] Previous studies have shown that iNKT cells in the peripheral blood are selectively and rapidly depleted in early HIV-1 infection[16] and in models of Simian immunodeficiency virus (SIV)-infected non-human primates.[17] Some studies reported reduced iNKT proliferation and cytokine secretion (interferon-γ (IFNγ), tumor necrosis factor (TNF), and IL-4 in response to α-Gal-Cer/IL-2/PMA (phorbol 12-myristate 13-acetate) stimulation in HIV-1 infection, with variable restoration of function on antiretroviral therapy (ART).[18–20] The role of iNKT cells in HIV-1 progression, whether defined by viral replication or immune activation, is unclear. Furthermore, HIV-1 has evolved to escape direct recognition of infected cells by iNKT cells (Paquin-Proulx *et al*, unpublished observation). Given their ability to produce IL-10 and activate regulatory T cells (Tregs), iNKT cells have the potential to help control pathologic T-cell activation.[21] In the present study, we investigated the role of peripheral blood and gut iNKT cells in controlling immune activation in HIV-1 infection and the consequences of gut microbial dysbiosis on iNKT frequency and function.

RESULTS

iNKT cells are reduced in the blood but not in the GALT during HIV-1 infection

A total of 23 HIV-1-infected subjects and 10 healthy controls were enrolled in the study and paired blood and gut-associated lymphoid tissue (GALT) samples were obtained (**Table 1**). Thirteen of the HIV-infected subjects were on ART at the time of sampling, and 3 of the 10 untreated patients met the definition of viremic controllers (viral load below 200 copies per ml). Mononuclear cells were isolated from the samples and flow cytometry was performed. Staining with Vα24, together with PBS57-CD1d tetramer, was used to identify iNKT cells (**Figure 1a**). iNKT cells were increased in the GALT compared with the blood across all subjects (**Supplementary Figure 1A** online). As reported previously, iNKT cells were found at a reduced frequency in the blood of viremic and ART-treated HIV subjects compared with healthy controls (**Figure 1b**).[22–24] There was a trend for increased iNKT frequency in the ART-treated group compared with the viremic group ($P = 0.07$). Surprisingly, no change in iNKT cell frequency was observed in the GALT of viremic and ART-treated HIV-infected individuals compared with healthy controls (**Figure 1c**). HIV controllers appeared to have preserved iNKT cell frequency in the blood and higher frequency in the GALT compared with all other groups. Next, we investigated the distribution of the CD4+ subset of iNKT cells as this population has been shown to be preferentially depleted in the peripheral blood during HIV infection.[22,23] No significant differences were observed between HIV-infected individuals and controls (**Figure 1d**) and between the viremic and ART-treated groups (**Supplementary Figure 1B,C**) both in the blood and in GALT. However, HIV-infected subjects had a significantly increased proportion of CD4+ iNKT cells in GALT compared with the blood (**Figure 1d**). Our results confirm the loss of iNKT cell in the blood of HIV-infected individuals that has been reported by several studies before and suggest for the first time that iNKT cells may be preserved in the GALT in these patients.

iNKT cells in the GALT of HIV-infected individuals have a Th2 cytokine profile

Previous studies demonstrated that cytokine production by iNKT in the blood of HIV-infected subjects is impaired.[19] However, cytokine production by iNKT cells in the GALT may be more relevant during HIV-1 disease progression. To assess

Table 1 Subjects demographics

	Gender	Age (years)	CD4 count	Viral load	Duration of infection (years)	Time on ART (total years)
Healthy ($n = 10$)	9M, 1F	32.5 (23–59)	828 (538–1,173)			
HIV (n = *23)*						
Viremic ($n = 7$)	5M, 1M to F, 1F	46 (31–61)	458 (257–887)	13,187 (1,102–305,178)	6.0 (0–23)	0 (0–0.97)
ART ($n = 13$)	12M, 1F	54 (32–66)	616 (374–1,023)	Undetectable	24.5 (1–34)	7.9 (0.9–19.6)
Controllers ($n = 3$)	2M, 1F	46 (25–50)	573 (393–900)	Undetectable (undetectable—141)	15(3–27)	0 (0–0.56)

Abbreviations: ART, antiretroviral therapy; F, female; M, male.

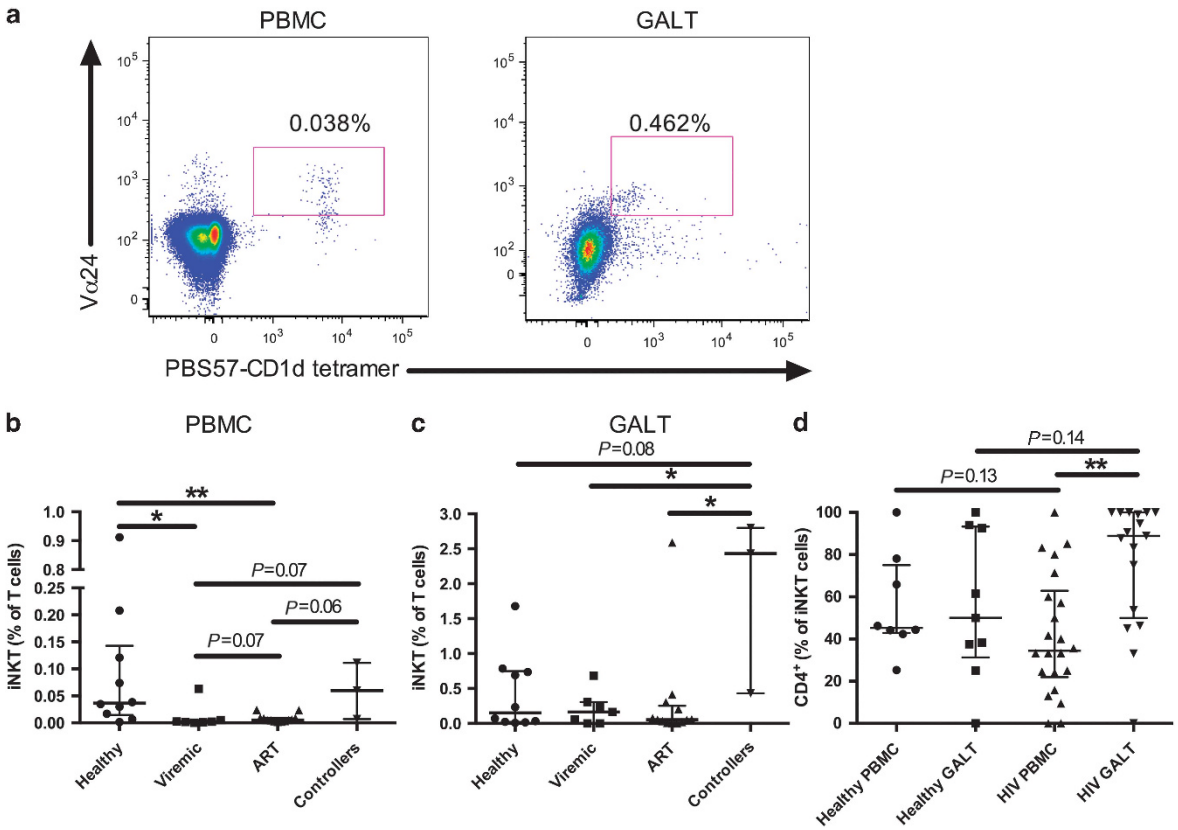

Figure 1 Frequency of invariant natural killer T (iNKT) cells in the blood and gastrointestinal-associated lymphoid tissue (GALT). (**a**) Representative gating strategy, iNKT cells were identified based on Vα24 and PBS57-CD1d tetramer-positive stainings. Frequency of iNKT cells in the (**b**) blood and in the (**c**) GALT for healthy controls ($n=10$), viremic ($n=7$), antiretroviral therapy (ART)-treated ($n=13$), and viremic controllers HIV-infected subjects ($n=3$). (**d**) Frequency of iNKT cells expressing CD4 in the blood and GALT of healthy controls (blood, $n=8$ and GALT, $n=9$) and HIV-infected subjects (blood, $n=22$ and GALT, $n=17$). $^{*}P<0.05$ and $^{**}P<0.01$. PBMC, peripheral blood mononuclear cell.

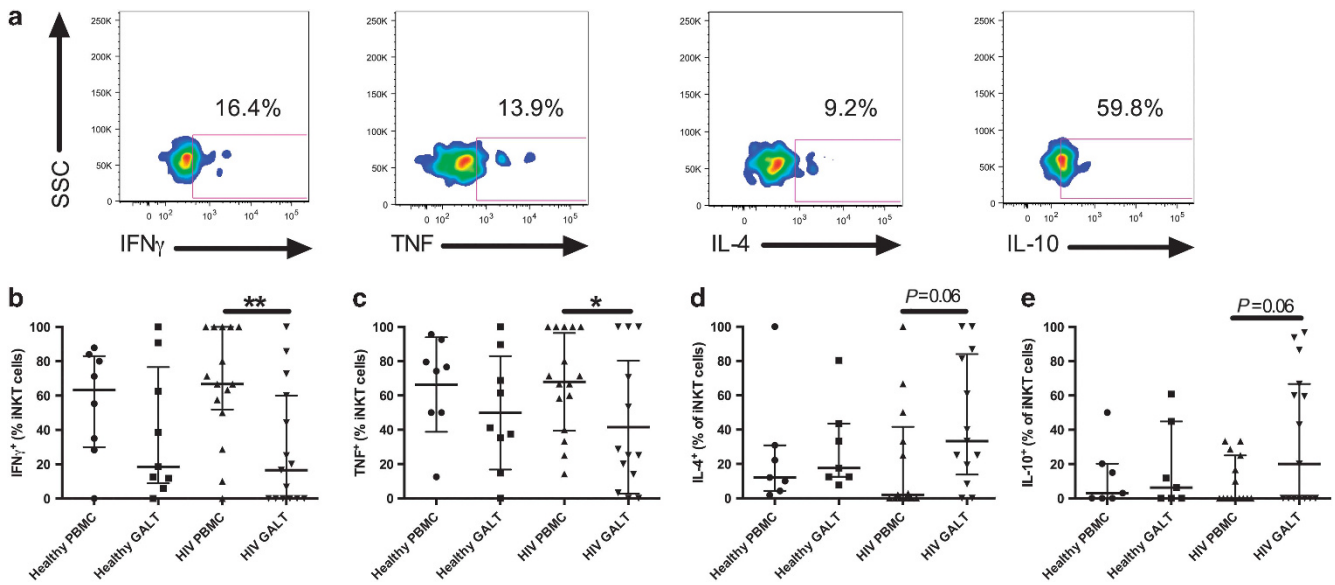

Figure 2 Cytokine production by invariant natural killer T (iNKT) cells in the blood and gastrointestinal-associated lymphoid tissue (GALT). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin before intracellular staining for cytokines. (**a**) The gates were set using unstimulated controls, representative staining for interferon-γ (IFNγ), tumor necrosis factor (TNF), interleukin-4 (IL-4) and IL-10. Percentage of iNKT cells producing (**b**) IFNγ (controls: blood, $n=8$ and GALT, $n=9$ and HIV-infected subjects: blood, $n=16$ and GALT, $n=15$), (**c**) TNF (controls: blood, $n=8$ and GALT, $n=9$ and HIV-infected subjects: blood, $n=16$ and GALT, $n=13$), (**d**) IL-4 (controls: blood and GALT, $n=7$ and HIV-infected subjects: blood and GALT, $n=13$) and (**e**) IL-10 (controls: blood and GALT, $n=7$ and HIV-infected subjects: blood and GALT, $n=15$). $^{*}P<0.05$ and $^{**}P<0.01$. PBMC, peripheral blood mononuclear cell; SSC, side scatter.

the functional potential of iNKT cells, peripheral blood mononuclear cell (PBMC) and rectal mononuclear cells were stimulated with PMA and ionomycin and the production of IFNγ, TNF, IL-4, and IL-10 was evaluated by flow cytometry (**Figure 2a**). No differences were observed in cytokine production by iNKT cells in the blood and GALT of HIV- individuals compared with healthy controls. The majority of iNKT cells in the blood of healthy controls produced IFNγ and TNF, while the frequencies of IL-4- and IL-10-producing iNKT were low (**Figure 2b–e**). However, we observed a trend for a lower frequency of IFNγ + and TNF + iNKT cells in the GALT of healthy individuals compared with the blood. This difference was even more marked in the HIV-infected subjects. The percentage of iNKT in the GALT-producing IL-4 and IL-10 varied greatly, ranging from undetectable to 100%. There was a trend for higher IL-4 + ($P = 0.06$) and IL-10 + ($P = 0.06$) iNKT cells in the GALT compared with the blood for HIV-infected individuals only. Our results suggest that a greater proportion of GALT iNKT cells in HIV subjects have a Th2 cytokine profile compared with the blood.

Production of IL-4 and IL-10 by iNKT is associated with lower immune activation in the blood of HIV-infected subjects

Next, we evaluated immune activation of $CD4^+$ and $CD8^+$ T cells in the blood and in the GALT by measuring coexpression of CD38 and HLA-DR. In healthy controls, we found a trend towards increased activation of $CD4^+$ T cells in the GALT compared with the blood (**Figures 3a**, $P = 0.08$). Significantly higher levels of CD38 and HLA-DR coexpression were found

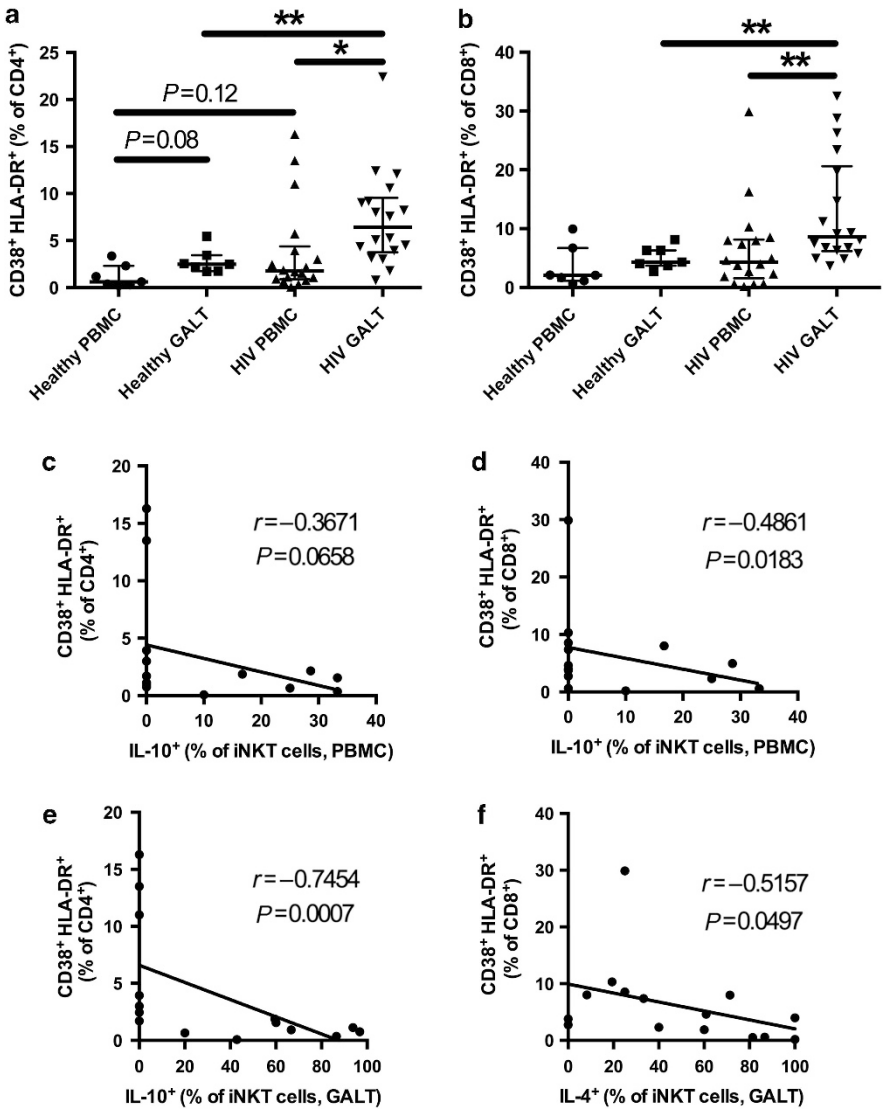

Figure 3 $CD4^+$ and $CD8^+$ T-cell activation in the blood and gastrointestinal-associated lymphoid tissue (GALT) and associations with invariant natural killer T (iNKT) cell cytokine production. Coexpression of CD38 and HLA-DR on (**a**) $CD4^+$ T cells and (**b**) $CD8^+$ T cells in the blood and GALT of controls ($n = 7$) and HIV-infected subjects ($n = 18$). Associations between interleukin-10-positive ($IL\text{-}10^+$) iNKT cells in the (**c**) blood and $CD38^+HLA\text{-}DR^+$ $CD4^+$ and (**d**) $CD8^+$ T cells of HIV-infected subjects. Associations between $IL\text{-}10^+$ and $IL\text{-}4^+$ iNKT cells in the GALT of (**e**) HIV-infected subjects and $CD38^+HLA\text{-}DR^+$ $CD4^+$ and (**f**) $CD8^+$ T cells, respectively. *$P < 0.05$ and **$P < 0.01$. PBMC, peripheral blood mononuclear cell.

on $CD4^+$ and $CD8^+$ T cells in the GALT of HIV-infected individuals when compared with paired blood samples (**Figure 3a,b**). A nonsignificant trend for greater cellular activation in the blood was observed when comparing HIV-infected subjects to controls. However, HIV-infected individuals had significantly increased activation of both $CD4^+$ and $CD8^+$ T cells in the GALT compared with healthy controls. Single expression of CD38 was also analyzed (**Supplementary Figure 3A,B**). iNKT cells are believed to have regulatory functions, which are in part mediated by their capacity to produce cytokines.[7] Therefore, we investigated if there was any association between cytokine production by iNKT cells and immune activation in HIV-infected subjects. We found no associations between immune activation in the GALT and iNKT cell cytokine production (data not shown). However, IL-10 production by iNKT cells in the blood was associated with lower $CD4^+$ and $CD8^+$ T-cell activation in the blood (**Figure 3c,d**). Furthermore, IL-10 and IL-4 production by GALT iNKT cells were, respectively, associated with lower $CD4^+$ and $CD8^+$ T-cell immune activation in the blood (**Figure 3e,f**). HIV-infected subjects were then grouped according to the capacity of iNKT cells to produce IL-10 and the levels of immune activation were compared. Subjects with GALT iNKT cells producing IL-10 had significantly lower frequencies of activated peripheral $CD4^+$ T cells (**Supplementary Figure 2A**) and subjects with blood iNKT cells producing IL-10 showed a trend for lower levels of activated peripheral $CD4^+$ and $CD8^+$ T cells (**Supplementary Figure 2B, C**). In addition, we analyzed CD38 single expression and its associations with iNKT cell cytokine production. CD38 expression was inversely associated with IL-4 and IL-10 production by GALT iNKT cells and IL-10 production by blood iNKT cells (**Supplementary Figure 3**). These results suggest a role for GALT iNKT cells in dampening the pathological peripheral immune activation in HIV-1 infection.

In addition to iNKT cells, Tregs are also known to have an important role in modulating immune activation.[25] Therefore, we analyzed Tregs (defined as $CD3^+$, $CD4^+$, $CD25^+$, and $Foxp3^+$) frequencies in peripheral blood and GALT of HIV-infected individuals. Similar frequencies of Tregs across groups were observed in the blood and in the GALT (**Supplementary Figure 4A**) and no difference in the frequency of Tregs in the blood compared with the GALT was observed for controls and HIV-infected individuals. We observed a positive association between Treg frequency in the GALT and $CD4^+$ and $CD8^+$ T-cell activation in the GALT of HIV-infected individuals (**Supplementary Figure 4B,C**). No associations were found between Treg and iNKT cell frequency or function (data not shown).

Cytokine production by GALT iNKT cells is associated with lower microbial translocation

Damage to the integrity of the gut epithelial barrier by HIV-1 infection has been reported to lead to the presence of microbial products in the circulation, often referred to as microbial translocation (MT). MT has been associated with the pathologic immune activation that is characteristic of HIV-1 disease.[26] The kynurenine (Kyn) pathway of tryptophan (Trp) catabolism has been demonstrated to be dysregulated in HIV-1 infection, leading to an elevated Kyn/Trp ratio in the blood.[27] This dysregulation is reported to be associated with changes in the composition of the microbiome of HIV-infected individuals and with established markers of disease progression such as IL-6[13]. We postulated that the immunoregulatory activity of GALT iNKT cells may limit disturbance to the gut barrier and therefore lower immune activation by reducing MT. For this purpose, we measured the concentration of soluble CD14 (sCD14) and LPS-binding protein (LBP) in the plasma as they have been shown to be indirect markers of MT.[26,28] We also measured intestinal fatty acid-binding protein 2 (, a marker of gut damage[29]), IL-6, and the Kyn/Trp ratio. As expected, HIV-infected subjects had significantly elevated levels of sCD14, LBP, and intestinal fatty acid-binding protein 2 (**Figure 4a–c**), suggesting gut epithelial barrier dysfunction and MT. The Kyn/Trp ratio was also elevated in HIV-infected individuals (**Figure 4d**) as well as the levels of IL-6 (**Supplementary Figure 5**). We then looked for relationships between all of the above parameters and iNKT frequency or function. The capacity of peripheral iNKT cells to produce IL-4 was positively associated with plasma levels of sCD14 and there was a trend for an inverse association between the capacity of intestinal iNKT cells to produce IL-10 and the levels of sCD14 (**Figure 4e,f**). iNKT cell production of IL-4 and TNF-α in the GALT showed negative associations with the levels of LBP (**Figure 4g,h**). Finally, IL-4 production by peripheral iNKT cells was associated with elevated Kyn/Trp ratios (**Figure 4i**). Additionally, HIV-infected subjects were grouped according to capacity of iNKT cells to produce IL-4 or IL-10. We observed that individuals in the group with higher production of IL-4 had lower levels of sCD14 (**Supplementary Figure 6**). Taken together, our results show that a higher capacity to produce cytokines by iNKT in the GALT is associated with lower markers of MT, suggesting a role for GALT iNKT cells in modulating this pathological process in HIV-1 infection.

Bacteroides are associated with iNKT frequency and IL-4 production in the GALT

The composition of the bacterial gut microbiota of untreated HIV-infected subjects is distinct from that of healthy individuals, with ART-treated patients having an intermediate change in the microbiome profile.[30] One of the genera significantly depleted in HIV-infected individuals is *Bacteroides*. We confirmed that *Bacteroides* were reduced in ART-treated HIV-infected subjects in our study (**Figure 5a**). Next, we performed an unbiased analysis of all gut-resident operational taxonomic units (OTUs) abundances compared with GALT iNKT cell frequency in ART-treated subjects and found that negative correlations existed between GALT iNKT cell frequency and OTUs in the *Prevotella* genus (Benjamini–Hochberg Q–value <0.15; **Supplementary file**), a genus that has been shown to be increased in abundance in HIV-infected subject gut microbiomes and associated with elevated mucosal immune

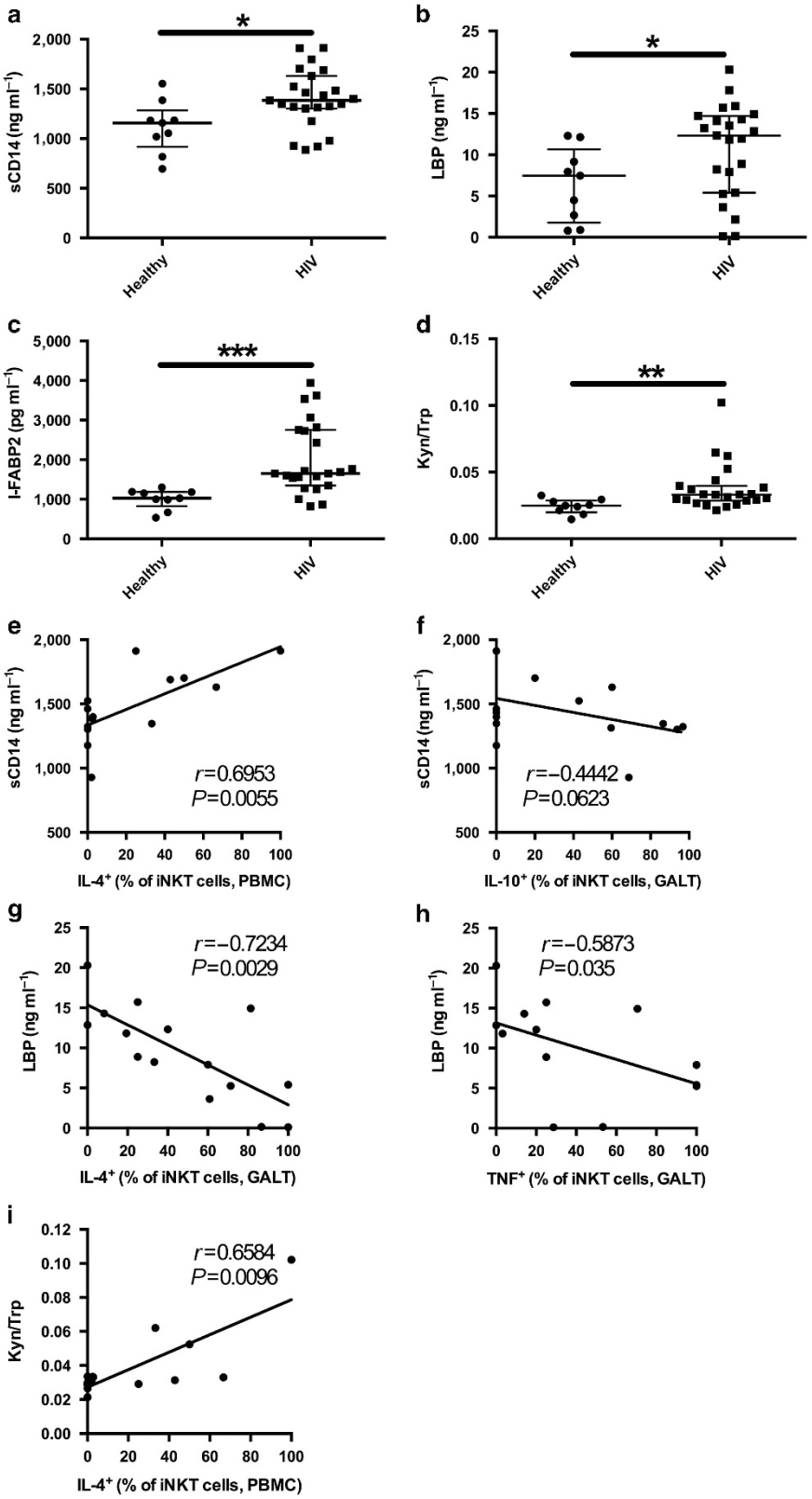

Figure 4 Markers of microbial translocation and associations with invariant natural killer T (iNKT) cell cytokine production. The levels of (**a**) sCD14, (**b**) LPS-binding protein (LBP), (**c**) intestinal fatty acid-binding protein 2 (I-FABP2), and (**d**) kynurenine/tryptophan (Kyn/Trp) ratio were determined in the serum of healthy controls ($n = 9$) and HIV-infected subjects ($n = 23$). Associations between the levels of sCD14 in HIV-infected subjects and IL4$^+$ iNKT cells in the (**e**) blood and IL10$^+$ iNKT cells in the (**f**) gastrointestinal-associated lymphoid tissue (GALT). Associations between LBP levels in the serum of HIV-infected subjects and (**g**) IL4$^+$ and (**h**) TNF$^+$ iNKT cells in the GALT. (**i**) Association between Kyn/Trp ratio in HIV-infected subjects and IL4$^+$ iNKT cells in the blood. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. PBMC, peripheral blood mononuclear cell.

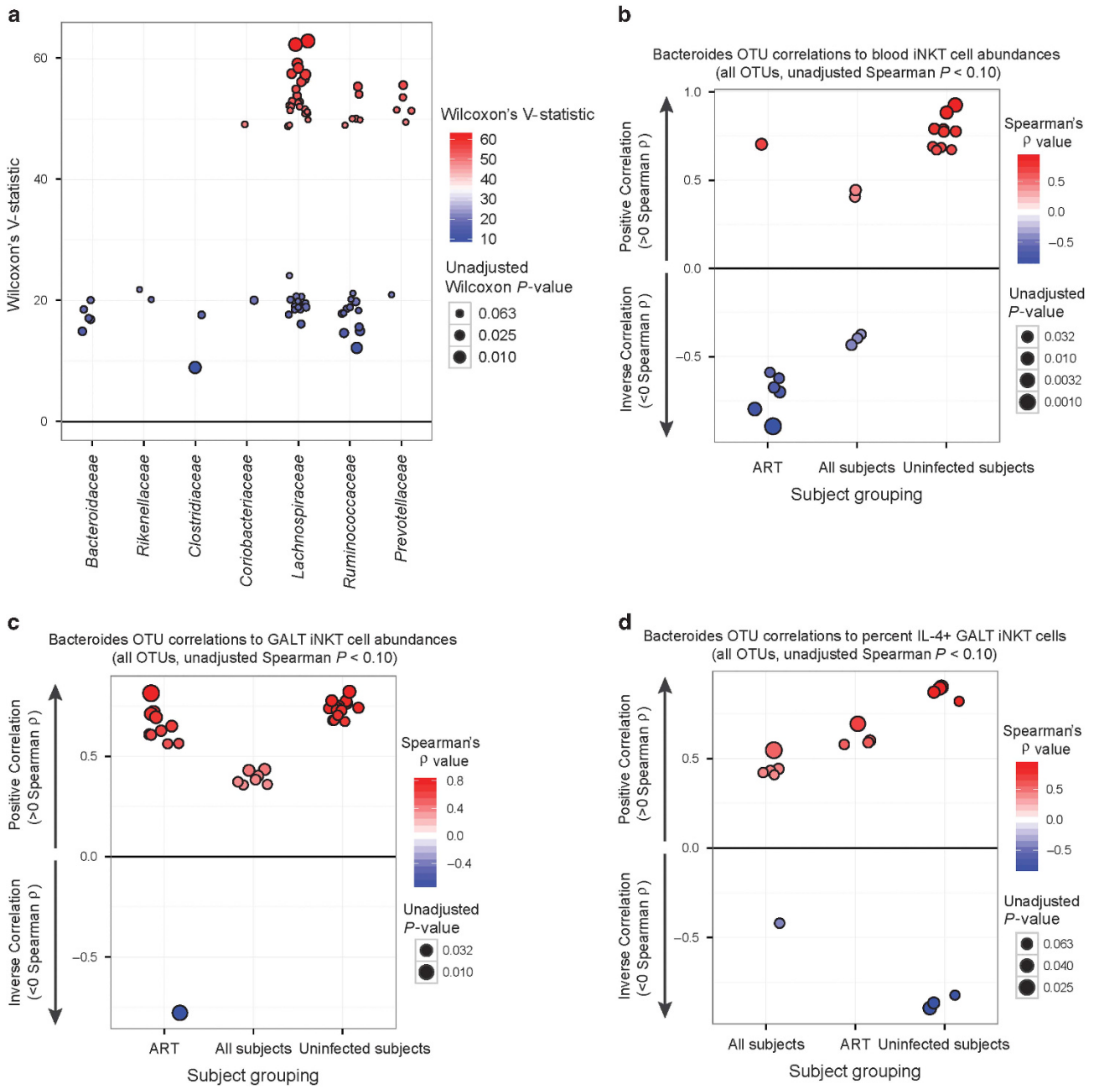

Figure 5 Change in microbiota in HIV-infected individuals and associations with invariant natural killer T (iNKT) frequency and function. Wilcoxon's rank-sum tests were performed comparing gut mucosal operational taxonomic unit (OTU) abundances between HIV-infected subjects undergoing antiretroviral (ART) and uninfected subjects. OTUs with $P<0.15$ are shown including taxonomic families to which each OTU belongs. (**a**) Wilcoxon's V-statistics (y axis) provide nonparametric enrichment/depletion information, and unadjusted *P*-values are depicted by point sizes. (**b**) Abundance of all detected OTUs within the *Bacteroides* genus were compared with blood iNKT cell percent abundances for three subject groups (ART HIV-infected, uninfected, and all subjects combined). (**b**) Spearman's ρ values depict directionality of correlation, and all OTUs with $P<0.10$ shown. (**c**) Abundance of all *Bacteroides* OTUs were compared with gastrointestinal-associated lymphoid tissue (GALT) iNKT cell percent abundances for the same subject groups ($P<0.10$ shown). Spearman's correlations were performed comparing all *Bacteroides* OTU relative abundances and proportions of IL-4$^+$ GALT iNKT cells following phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation ($P<0.10$ shown) (**d**).

activation.[31] Given that *B. fragilis* expresses a glycolipid that can activate human iNKT cells,[11] we investigated whether the abundance of OTUs within the *Bacteroides* genus was associated with frequencies of iNKT cells in both peripheral blood and GALT within ART-treated HIV-infected subjects, all study subjects combined, and uninfected subjects only (**Figure 5b,c** and **Supplementary file**). Fewer OTUs reached $P<0.10$ for comparisons to peripheral blood as opposed to GALT iNKT frequencies, and no trends were consistent across all subject groupings for comparisons to peripheral blood. However, when comparing OTU abundances to GALT iNKT frequencies, consistent positive associations were found between several *Bacteroides* OTUs and iNKT frequencies across subject groups. Finally, we looked for associations between *Bacteroides* OTUs and

cytokine production by iNKT cells. We found a positive association between specific *Bacteroides* OTUs and the capacity of GALT iNKT cells to produce IL-4 (**Figure 5d** and **Supplementary file**), although these observations exhibited Benjamini–Hochberg false discovery rate Q values > 0.70. These results suggest that loss of the *Bacteroides* genus in HIV-infected individuals could influence both the frequency and function of iNKT cells in the gut.

DISCUSSION

Several studies have examined the frequency and function of peripheral blood iNKT cells in HIV-1 infection but limited information is available for the GALT, an important target in HIV-1 pathogenesis. We found that iNKT cells were depleted in the blood but not in the GALT of HIV-infected subjects and that GALT iNKT cells consisted of an increased proportion of the $CD4^+$ subset. This is in contrast to a previous study that reported that $CD4^+$ iNKT cells are lost in the gut of HIV-1-infected individuals.[32] The discrepancy between our results and those of Ibarrondo *et al.*[32] could be explained by important differences in the HIV-1 cohorts and the methods used to identify iNKT cells. The majority of our HIV-1-infected individuals had an undetectable viral load (ART-suppressed or controllers) and we used a CD1d tetramer to identify iNKT cells while the cohort of Ibarrondo *et al.*[32] consisted exclusively of untreated subjects, and iNKT cells were identified using antibodies against the invariant T-cell receptor. Altogether, this would suggest that viral suppression prevents the depletion of the $CD4^+$ subset of iNKT cells in the GALT. Although based on a small number of subjects, our results suggest that HIV-1 elite controllers maintain normal iNKT cell frequency in the blood and have high levels of iNKT cells in the GALT. Further studies enrolling more controllers will be required to confirm the validity of these results.

$CD4^+$ iNKT are known to produce more Th2 cytokines than the $CD4^-$ subsets.[33–35] Therefore, given our finding of higher percentages of $CD4^+$ iNKT in the gut as compared with blood in HIV-infected subjects, it is not surprising that we observed more production of IL-4 and IL-10, two Th2 cytokines, and less IFNγ and TNFα by iNKT cells in the GALT compared with the blood. Our results suggest that iNKT cells can regulate the pathologic chronic immune activation in HIV-1 infection by their production of IL-4 and IL-10 in the GALT as well as IL-10 in the blood. We could not detect iNKT cell production of IL-10 by a significant proportion of HIV-infected individuals, suggesting that they could be separated in two groups based on the capacity of iNKT cells to produce IL-10. However, this could be due to the detection limit of the assay. Further studies with more subjects in each group are required to resolve this matter. Our findings are consistent with the model proposed by Rout *et al.*[36] who have shown that sooty mangabeys, natural hosts of SIV that do not progress to AIDS, exhibit a preservation of iNKT cell frequency and function after SIV infection. They have thus suggested that iNKT cell dysfunction has a role in AIDS pathogenesis.[36] MT has been proposed as a major component driving immune activation in HIV-infected individuals. We found that production of IL-4, IL-10, and TNF by iNKT cells in the GALT, but not in the blood, were associated with lower MT as measured by sCD14 and LBP, suggesting that iNKT cell production of IL-10 in the blood could have a localized effect on immune activation while production of the same cytokine in the GALT could have a more generalized effect by reducing MT. In contrast, the frequency of Tregs in the GALT was positively associated with $CD4^+$ and $CD8^+$ T-cell activation in the GALT. We saw no association between Tregs and markers of MT, suggesting a specific role for iNKT cells. How production of IL-4, IL-10, and TNF by GALT iNKT cells contributes to reduced MT remains to be determined. It is a likely possibility that the ability of iNKT cells to interact with antigen-presenting cells to shape the adaptive immune response may be involved. Moreover, mice lacking IL-10 have been shown to have increased gut permeability caused by an excessive Th1 response against enteric bacteria.[37] On the other hand, the positive associations between IL-4 production by peripheral iNKT and Kyn/Trp ratio and sCD14 might represent in part their response to MT.

To our knowledge, this is the first study looking at the influence of the microbiome on iNKT cell frequency and function in humans. A study performed in mice showed that a sphingolipid derived from *B. fragilis* can inhibit iNKT cell activation and that *B. fragilis* colonization reduces the frequency of iNKT cells in the colon but not in other organs or in the blood.[10] A different group reported that *B. fragilis* produced a sphingolipid that can activate iNKT cells.[11] In our hands, multiple *Bacteroides* OTUs exhibited positive associations with GALT iNKT cells and only one OTU presented a negative association in HIV-infected subjects (with the later association not being present in uninfected subjects). This would suggest that *Bacteroides* produce an antigen that can activate and expand iNKT cells in the GALT. The reported greater abundance of *Bacteroides* in HIV elite controllers compared with viremic patients[38] could therefore contribute to the higher frequency of iNKT in the GALT of elite controllers in our study. Mouse studies have shown that animals kept in germ-free conditions have lower IFNγ, TNF, and IL-4 production by iNKT cells following stimulation with α-Gal-Cer,[12] but the bacteria responsible for the functional maturation of iNKT cells were not identified. In this study, we have found a positive association between *Bacteroides* and IL-4 production by GALT iNKT cells. While fetal human iNKT cells have been reported to mature and acquire function in the small intestine before colonization by the normal microbiota,[39] our results are consistent with the hypothesis that in adults the normal microbiota may provide signals that support GALT iNKT cell frequency and functionality. These findings would be strengthened by further study in larger human cohorts. $V\alpha24^-$ cells specifically binding to α-Gal-Cer-loaded CD1d tetramer have been detected in PBMC following *in vitro* expansion.[40] However, the limited amount of GALT material obtained in our study did not allow us to study this population of NKT cells.

Based on our results, we propose a model where iNKT cells in the GALT have an important role in limiting MT and chronic pathologic immune activation in HIV-1 infection. This role of GALT iNKT is influenced by the composition of the gut

microbiota, with loss of the *Bacteroides* genus in HIV-infected individuals, possibly affecting both iNKT frequency and function. This suggests that strategies boosting GALT iNKT cells could reduce the MT and persistent immune activation that are important factors in the morbidity caused by HIV-1.

METHODS

Study subjects. PBMCs and GALT samples were obtained from participants in the San Francisco-based HIV-1-infected SCOPE (Study on Cognition and Prognosis in the Elderly) cohort. Samples from HIV-1-seronegative controls were obtained from healthy volunteers. The study was approved by the local Institutional Review Board (University of California San Francisco Committee on Human Research), and individuals gave written informed consent. Samples were obtained from the following numbers and categories of HIV-1-infected individuals: 3 untreated virologic "controllers" (viral load, <200 HIV-1 copies per ml), 13 HAART-suppressed patients (viral load, <50 copies per ml), and 7 untreated "virologic noncontrollers" (viral load, $>1{,}000$ copies per ml). All had $CD4^+$ T-cell counts of >250 cells per mm^3. See **Table 1** for baseline subject characteristics.

Blood and GALT samples. Five milliliters of blood was collected in BD Vacutainer EDTA-coated tubes for PBMC and plasma isolation purposes. After centrifuging at 400 *g* for 10 min without braking, the plasma layer was removed and frozen at $-80\,^{\circ}C$ for enzyme-linked immunosorbent assay quantification. The cellular fraction from the first spin was used to isolate PBMC by centrifugation over a Ficoll-Paque (GE Healthcare, Uppsala, Sweden) layer at 800 *g* for 25 min without braking. The PBMC layer was then removed and washed two times in RPMI with L-glutamine, penicillin/streptomycin, HEPES, and 10% fetal bovine serum (referred now on as R-10) at 400 *g* for 10 min with braking. GALT from rectosigmoid biopsy specimens were placed on a shaking incubator at 37 °C with a digestion mix of RPMI with + L-glutamine, HEPES, penicillin/streptomycin, and collagenase type II (0.25 mg ml^{-1}) (Sigma-Aldrich, St Louis, MO). After one digestion of 30 min, the sample was strained through a 70 μM cell strainer and washed through with cold R-10. Undigested biopsies were transferred into the collagenase digestion mix for repeat digestion of 30 min. Strained and digested biopsies were washed in R-10 and spun down at 700 *g* for 6 min at 4 °C to isolate the rectal mononuclear cell. PBMCs and rectal mononuclear cells were then resuspended and counted using Guava Viacount (Millipore, Brillerica, MA) on the Accuri C6 (BD Biosciences, San Jose, CA).

Flow cytometry and mAbs. For surface staining, cells were stained with surface markers for 30 min on ice and washed two times with FACS buffer (phosphate-buffered saline with 2% fetal bovine serum and 2 mM EDTA buffer). Later, cells were fixed and permeabilized with Fix/Perm buffer (BD Biosciences) for 20 min on ice, washed two times with BD Perm/Wash, and stained with the intracellular antibodies for 60 min on ice. For PoxP3 staining, cells were fixed and permeabilized with Fix/Perm Buffer (eBiosciences, San Diego, CA) for 60 min on ice, washed two times with Perm/Wash buffer (eBiosciences), and stained with intracellular antibodies for 60 min on ice. Subsequently, the cells were washed two times with the respective Perm/Wash buffer and kept in 2% paraformaldehyde. Antibodies used: PBS57-CD1d tet antigen-presenting cell (kindly donated by NIH Tetramer Resource Facility, Emory University Vaccine Center, Atlanta, GA), Vα24 FITC, and CD3 ECD were from Beckman Coulter (Fullerton, CA), CD4 Qdot655, CD8 Qdot 605, and the viability marker AmCyan were from Life Technologies (Carlsbad CA), CD25 APC, CD38 PE, HLA-DR PerCP, IFNγ V450, TNF Alexa700, IL-10 PE, and IL-4 PE-Cy7 were all from BD Bioscience. Data were acquired on a BD LSRFortessa instrument (BD Biosciences) and analyzed using FlowJo Version 9.8.5 software (TreeStar, Ashland, OR).

Enzyme-linked immunosorbent assay. IL-6, sCD14, LBP, and FABP-2 levels were detected in plasma isolated from peripheral blood with a commercially available Enzyme-Linked Immunosorbent Assay (all from R&D Systems, Minneapolis, MN) and performed according to the standard protocol. For sCD14 and IL-6, commercially available quality controls (R&D Systems) were also performed to ensure accurate detection of the kits.

Microbiome sample processing and analysis. DNA was extracted from gut biopsy samples using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Each DNA sample was PCR amplified in triplicate using primer pairs that targeted the V4 hypervariable region of the 16S rRNA gene, contained a unique barcode sequence to enable demultiplexing of pooled samples, and contained an adapter sequence that enables the amplicon to bind to the MiSeq flow cell. Successful amplicons were pooled in approximately equal molar concentrations and sequenced on the Illumina MiSeq platform. Paired sequencing reads were quality filtered and demultiplexed using the QIIME[41] software package. Briefly, assembled sequencing read pairs were binned into OTUs (OTUs using a 97% similarity to the Greengenes database) and reads that either did not cluster to the Greengenes database or that were chimeric were removed from subsequent analyses. OTUs that had a cumulative read count across all samples of $<0.001\%$ of the total reads were removed from downstream analysis. Sample read numbers were rarefied to the read number of the lowest sample after processing (94,780) resulting in a rarefied OTU table. OTU abundances were compared between uninfected and ART-treated HIV-infected subjects using a custom R script in conjunction with the "exactRankTests" package. Spearman's correlations were performed using the "Hmisc" R package using a custom script. Data visualizations were performed using the R package "ggplot2".

Trp metabolism. Liquid chromatography–tandem mass spectrometry was used to assess Kyn and Trp levels as described previously.[42]

Statistical analysis. All statistical analysis was performed using Graph Pad Prism version 6.0f for Mac OSX (GraphPad Software, La Jolla, CA). Groups were compared using the Mann–Whitney test, paired blood and GALT samples were compared using Wilcoxon's marched-pairs signed-rank test. Associations between groups were determined by Spearman's rank correlation. P values <0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS

D.P.P. analyzed data and wrote the manuscript; C.C. performed experiments and helped write the manuscript; I.V.-C. analyzed microbiome data, helped write the manuscript, and helped obtain grant funding; D.F. performed microbiome profiling; L.L. helped with flow cytometry panels; Y.H. performed kynurenine/tryptophan assays; M.S. performed GALT biopsies; S.V.L. oversaw microbiome analyses; P.W.H. and D.F.N. gave input on overall study design,

analysis, and helped write the manuscript; D.S. designed and obtained grant funding for the study, performed experiments and helped write the manuscript.

DISCLOSURE

The authors declared no conflict of interest.

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