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Gut Dendritic Cell Activation Links an Altered Colonic Microbiome to Mucosal and Systemic T Cell Activation in Untreated HIV-1 infection

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Abstract

HIV-1-associated disruption of intestinal homeostasis is a major factor contributing to chronic immune activation and inflammation. Dendritic cells (DCs) are crucial in maintaining intestinal homeostasis, but the impact of HIV-1 infection on intestinal DC number and function has not been extensively studied. We compared the frequency and activation/maturation status of colonic myeloid DC (mDC) subsets (CD1c⁺ and CD1c^{neg}) and plasmacytoid DCs in untreated HIV-1-infected subjects with uninfected controls. Colonic mDCs in HIV-1-infected subjects had increased CD40 but decreased CD83 expression, and CD40 expression on CD1c⁺ mDCs positively correlated with mucosal HIV-1 viral load, with mucosal and systemic cytokine production, and with frequencies of activated colon and blood T cells. Percent of CD83⁺CD1c⁺ mDCs negatively correlated with frequencies of IFN- γ -producing colon CD4⁺ and CD8⁺ T cells. CD40 expression on CD1c⁺ mDCs positively associated with abundance of high prevalence mucosal *Prevotella copri* and *P. stercorea*, but negatively associated with a number of low

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DISCLOSURE

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prevalence mucosal species including *Ruminococcus bromii*. CD1c⁺ mDC cytokine production was greater in response to *in vitro* stimulation with *Prevotella* species relative to *R. bromii*. These findings suggest that during HIV infection, colonic mDCs become activated upon exposure to mucosal pathobiont bacteria leading to mucosal and systemic immune activation.

Keywords

Human intestinal dendritic cells; HIV-1 pathogenesis; microbiome; mucosal immunology

INTRODUCTION

A hallmark of HIV-1 disease is a gradual decline in peripheral blood CD4 T cells associated with chronic immune activation, defined by increased levels of pro-inflammatory cytokines, innate and adaptive immune cell activation, and soluble markers of inflammation.¹ T cell activation, and in particular CD8⁺ T cell activation, has been shown to be a strong predictor of disease progression²⁻⁴. In the era of combination anti-retroviral therapy (cART), low levels of T cell activation and inflammation persist in many individuals despite controlled viral replication and have been linked to poor immune reconstitution and adverse clinical outcomes.¹ Thus, understanding the mechanisms that drive chronic immune activation and the attendant inflammation in the setting of HIV-1 infection is important in order to develop therapeutic approaches to prevent inflammation-associated morbidity and mortality.

Although multiple factors likely contribute to chronic immune activation during HIV-1 infection, microbial translocation (MT) – the movement of bacteria or bacteria products from the gut lumen into the lamina propria (LP) and systemic circulation – has recently been implicated as a major driving force.⁵ Plasma bacterial lipopolysaccharides (LPS) levels have been associated with systemic T cell activation, and LPS levels in the first years of chronic HIV-1 infection were found to predict HIV-1 disease progression.^{6,7} In addition to LPS, other indicators of systemic MT such as sCD14, intestinal fatty acid binding protein (iFABP) and zonulin have also been associated with disease progression in untreated, and with mortality in treated, HIV-1-infected subjects.^{8,9}

Increased MT occurs as a result of HIV-1-associated immunological and structural damage to the gastrointestinal (GI) tract. Within days of infection, irrespective of the route of transmission, HIV-1 replication results in the severe and rapid depletion of intestinal memory CD4 T cells including preferential depletion of T helper (Th)17 and Th22 cells, T cell subsets involved in normal mucosal defense and epithelial barrier maintenance.¹⁰ In addition, increased activated CD8⁺ T cell frequencies,¹¹⁻¹³ increased pro-inflammatory cytokines,¹⁴ and alterations in the composition of microbial communities have been observed in the GI tract of HIV-1-infected subjects.^{10,15} We recently identified an altered colonic mucosal microbiome in untreated, HIV-infected subjects that was associated with plasma LPS levels and mucosal and systemic T cell activation.¹⁶ Furthermore, these altered microbial communities were associated with increased expression of the activation marker, CD40, on intestinal myeloid dendritic cells (mDCs).

Intestinal DCs sample luminal microbes and their products and are critical in mediating the delicate balance between immunogenic and tolerogenic intestinal immune responses,¹⁷ yet few studies have directly addressed the contribution of intestinal DCs to HIV-1-associated mucosal pathogenesis. We previously identified a subset of resident mDCs present in the LP of normal small and large bowel that were capable of producing pro-inflammatory cytokines (including IL-23) in response to *in vitro* stimulation with a viral Toll-like Receptor (TLR) ligand that mimicked innate signaling by HIV-1.¹⁸ Moreover, levels of pro-inflammatory IL-23 were synergistically increased when mDC were stimulated by a combination of bacterial and viral TLR ligands, suggesting that during HIV-1 infection, concurrent exposure to both virus and translocating enteric bacteria and bacterial products could result in enhanced production of pro-inflammatory cytokines by intestinal mDCs *in vivo*. Further, we showed that exposure to certain commensal bacteria enhanced HIV-1 infection of intestinal CD4 T cells *in vitro*, and this process was dependent upon the presence of mDCs.¹⁹ Based on these findings and the likelihood that LP DCs would be exposed to translocating mucosa-associated bacteria, we hypothesized that intestinal DCs would play a critical role in mediating viral and bacterial signals during HIV-1 infection *in vivo*.

RESULTS

CD40 expression is increased and CD83 expression decreased on colonic mDCs in HIV-1-infected subjects

Twenty-four HIV-1-infected individuals and 14 age- and sex-matched HIV-1 uninfected controls were enrolled into a cross-sectional study from whom rectosigmoid biopsies, peripheral blood, and stool samples were collected. Based on study entry criteria, HIV-1-infected subjects were ART-treatment naïve or had not been on treatment for more than 7 days in the preceding 6 months. Of the 24 HIV-1-infected subjects, 5 subjects reported in a study questionnaire that they had taken ART at some point during their course of HIV-1 infection. Of these, 3 had stopped ART at least 8 years (range 8–14yrs) prior to the study, 1 subject stopped 3 years prior, and the remaining subject stopped 13 months prior to inclusion in our study. Additional exclusion criteria are detailed in Supplementary Materials and Methods. Subject characteristics are provided in Table 1.

In initial studies, two phenotypically distinct colonic LP mDC subsets were identified, both of which expressed HLA-DR and CD11c but were delineated by expression of CD1c, (Supplementary Results, Supplementary Figure 1). Similar frequencies of both CD1c⁺ and CD1c^{neg} mDCs were observed in uninfected and HIV-1-infected subjects when enumerated as either a percent of viable, CD45⁺ cells (Supplementary Table S1) or as an absolute number of DCs per gram of mucosal tissue (Figure 1a). Histological techniques were also utilized to enumerate CD11c⁺ DCs and HAM56⁺ tissue macrophages in colonic tissue sections obtained from a subset of HIV-1-infected (n=6) and uninfected (n=6) subjects. A similar number of CD11c⁺ DCs were enumerated in both cohorts (HIV-1-infected: median 12.1 CD11c⁺ cells/mm², range 3.8–19.1; uninfected controls: 12.4 CD11c⁺ cells/mm², 7.2–23.7; p=0.75), but a higher number of HAM56⁺ cells per mm² of tissue were found in HIV-1-infected subjects (15.7 HAM56⁺ cells/mm², 8.7–20.3) compared to uninfected controls (3.9 HAM56⁺ cells/mm², 2.3–13.0; p=0.02). Increased frequencies of macrophages

have also recently been reported in the duodenal mucosa of treatment naïve HIV-1-infected subjects.²⁰

Colon CD303⁺ pDCs, normally found at very low frequencies,¹⁸ were next assessed for frequency and activation status. We did not observe any statistical difference in the frequencies of colonic pDCs (Figure 1a, Supplementary Table S1) although a trend towards higher numbers of pDCs in HIV-1-infected subjects (median: 9354 pDC/gram, 1835–59658) compared to uninfected subjects (4243, 2104–14155; p=0.09) was noted.

Colonic CD1c⁺ mDC and CD1c^{neg} mDC activation based on CD40 expression was significantly higher in HIV-1-infected subjects compared to uninfected controls (Figure 1b). Conversely, CD40 expression on pDC was not statistically different between the two subject cohorts (Figure 1b). However, the absolute number of CD40⁺ pDCs was statistically greater in HIV-1-infected subjects (9047 CD40⁺ pDC/gram, 561–56192; n=21) compared to uninfected subjects (4380, 2212–11500; n=21; p<0.05). CD1c⁺ mDC activation levels significantly correlated with the number of CD40⁺ colonic pDCs (r=0.61, p=0.007; n=18). The percent of CD1c⁺ mDCs, CD1c^{neg} mDCs and CD303⁺ pDCs expressing the DC maturation marker CD83 were all lower in HIV-1-infected subjects (Figure 1c).

Colonic CD1c⁺ mDC activation is associated with mucosal HIV-1 viral load

CD40 expression on CD1c⁺ mDCs positively associated with mucosal HIV-1 viral load whereas CD1c^{neg} mDC CD40 expression did not (Figure 2a). Unlike our previous observations of activated blood DCs,²¹ CD40 expression on colon CD1c⁺ mDCs and CD1c^{neg} mDCs did not correlate with either plasma viral load (Figure 2b) or with peripheral CD4 count (CD1c⁺ mDCs: r=-0.12, p=0.60; CD1c^{neg} mDCs: r=-0.28, p=0.25). Although pDCs are known to be directly activated by HIV-1,²² no direct associations were observed between the number of CD40⁺ pDCs and either mucosal (r=0.16, p=0.50) or plasma viral load (r=0.03, p=0.88), or with peripheral CD4 count (r=0.09, p=0.69).

Colonic and systemic T cell activation correlate with colonic mDC activation

Activated colon CD4⁺ and CD8⁺ T cell frequencies were increased in HIV-1-infected subjects (Figure 3a, b, Supplementary Table S2), and CD40 expression levels on CD1c⁺ mDCs strongly associated with the number of activated colonic CD4⁺ and CD8⁺ T cells. Similar, but weaker associations were noted between CD1c^{neg} mDC activation and activated colonic T cells (Figure 3a, b).

A larger infiltrate of mononuclear cells was measured by histology in the colonic LP of HIV-1-infected subjects relative to control subjects, and CD40 expression levels on both CD1c⁺ and CD1c^{neg} mDCs were positively associated with the degree of mononuclear cell infiltration (Figure 3c).

Significantly higher percentages of activated blood CD4 and CD8 T cells were found in HIV-1-infected subjects relative to uninfected controls, as expected (Figure 4a, b, Supplementary Table S2). Blood T cell activation frequencies were positively associated with activation levels of CD1c⁺ but not of CD1c^{neg} mDCs (Figure 4a,b).

Significant decreases in the frequencies of colonic Th1, Th17 and Th22 cells in conjunction with increased frequencies of IFN- γ ⁺ CD8 T cells were observed in HIV-1-infected subjects compared to controls (Supplementary Table S3). However, no significant associations were found between levels of mDC activation and frequencies of cytokine-producing CD4 or CD8 T cells (data not shown).

Colonic mDC activation is associated with mucosal and plasma cytokine production

CD40 expression on CD1c⁺ mDC strongly associated with levels of a number of inflammatory mucosal cytokines including IL-23, IL-1 β , IL-6 and TNF- α as well as with IL-10 levels in HIV-1-infected subjects (Table 2). Moreover, CD1c⁺ mDC activation positively associated with both mucosal IFN- γ and IL-17 production. Weaker but significant associations were observed between CD1c^{neg} mDC activation levels and mucosal levels of IL-23 and IFN- γ .

Plasma IL-6 levels were significantly increased in HIV-1-infected subjects (1.43pg/ml, 0.43–5.09; n=24) compared to controls (0.70pg/ml, 0.19–2.16; n=14; p=0.002); however, no significant correlation between mDC activation and IL-6 levels was observed in HIV-1-infected subjects (r=0.08, p=0.76). Plasma levels of other cytokines were evaluated in a subset of HIV-1-infected subjects (n=18). TNF- α levels strongly associated with plasma HIV-1 viral load (r=0.62, p=0.006) and with plasma IL-6 (r=0.64, p=0.004) and sCD14 levels (r=0.79, p=0.001). IL-10 levels also associated with plasma HIV-1 viral load (r=0.69, p=0.001) and with sCD14 (r=0.73, p=0.0007). TNF- α and IL-10 levels strongly correlated with each other (r=0.87, p<0.0001). CD1c⁺ mDC activation was positively associated with plasma levels of TNF- α (r=0.63, p=0.02; n=14) and IL-10 (r=0.76, p=0.002; n=14), whereas CD1c^{neg} mDC activation was associated with plasma IFN- γ (r=0.59, p=0.03; n=14) and weakly with IL-10 (r=0.54, p=0.05; n=14).

Colonic mDCs are identified in association with tissue LPS to a greater extent than LTA

Levels of gram-negative bacterial LPS in the plasma of HIV-1-infected subjects were increased relative to controls (Figure 5a). In agreement with the early studies,⁶ plasma LPS levels in HIV-1-infected subjects correlated with blood CD4 (r=0.62, p=0.002) and CD8 (r=0.41, p=0.058) T cell activation. Moreover, plasma LPS levels significantly associated with levels of mucosal IL-1 β (r=0.58, p=0.02), IL-6 (r=0.54, p=0.03) and weakly with mucosal TNF- α (r=0.47, p=0.058). Increased plasma levels of the gram-positive cell wall component lipoteichoic acid (LTA) were also observed in HIV-1-infected subjects compared to controls (Figure 5a). However, LTA levels correlated only with blood CD4 T cell activation (r=0.47, p=0.03). No significant associations between these indicators of systemic MT and activated colonic CD1c⁺ mDCs (LTA (n=16): r=-0.16, p=0.55; LPS (n=17): r=0.22, p=0.39) or CD1c^{neg} mDCs (LTA: r=-0.31, p=0.23; LPS: r=-0.03, p=0.91) were observed in HIV-1-infected subjects.

Tissue LTA and LPS levels were both higher in HIV-1-infected subjects compared to control subjects, but only LTA levels reached statistical significance (Figure 5b). In HIV-1-infected subjects, no significant associations were found between tissue LTA or LPS levels and

CD1c⁺ mDC (LTA (n=14): $r=-0.14$, $p=0.63$; LPS (n=14): $r=-0.09$, $p=0.76$) or CD1c^{neg} mDC (LTA: $r=0.21$, $p=0.47$; LPS: $r=0.15$, $p=0.61$) activation.

In HIV-1-infected subjects, a greater fraction of CD11c⁺ mDCs and HAM56⁺ macrophages were associated with LPS than with LTA, although this did not reach statistical significance for macrophages (Figure 5c, d). Similar trends were observed in uninfected subjects (Figure 5e, f). Both LTA and LPS were more frequently associated with macrophages than with mDCs. When the numbers of LTA⁺ or LPS⁺ mDCs were compared between HIV-1-infected and uninfected subjects, no significant differences were observed ($p=0.46$, $p=0.90$ respectively) (Figure 5e). More LTA⁺ macrophages were observed in the LP of HIV-1-infected subjects than in uninfected subjects, whereas the number of LPS⁺ macrophages was similar between the two cohorts (Figure 5f).

Percent of CD83-expressing colonic mDCs is negatively associated with IFN- γ -producing colonic T cells

In HIV-1-infected subjects, the percent of CD83⁺ CD1c⁺ mDCs negatively correlated with the number of IFN- γ -producing colonic CD4 and CD8 T cells, whereas the percent of CD83⁺ CD1c^{neg} mDCs negatively associated only with IFN- γ ⁺ CD4 T cells (Figure 6a, b). A significant correlation between the percent of CD83⁺ pDCs and the percent of IFN- γ -producing CD8⁺ T cells ($r=0.53$, $p=0.02$; $n=20$) was noted; however, no association with the absolute number of IFN- γ ⁺ CD8⁺ T cells or with any other immunological or virological parameters were observed.

Abundances of altered mucosal bacterial species are associated with colonic mDC activation

We previously evaluated mucosal and fecal microbiomes to the genus level in a subset of study subjects¹⁶ and have now identified 21 mucosa-associated bacterial species, based on 99% identity to sequences in the SILVA database²³ that are significantly over (6) or under (15) represented in HIV-1-infected subjects, termed “HIV-altered mucosal bacteria” (HAMB) species (Supplementary Table S4). Similar to results in our previous study,¹⁶ greater abundance of the two Proteobacteria spp. was noted only in the mucosa, whereas *Prevotella* species abundance was significantly greater in both mucosa and stool of HIV-1-infected subjects compared to controls.

Levels of CD40 on CD1c⁺ mDCs trended ($p<0.1$) toward positive associations with high abundance *Prevotella copri* and *P. stercorea* and toward negative associations with low abundance *Bacteroides acidifaciens*, *Blautia schinikii* and *Ruminococcus bromii* (Table 3). CD1c^{neg} mDC activation also trended towards a negative association with *R. bromii*, but no clear associations with any other HAMB species were noted (Table 3).

High abundance HAMB species induce greater cytokine⁺ CD1c⁺ mDC frequencies *in vitro* compared to low abundance HAMB species

Production of IL-23, IL-1 β and IL-10 by CD1c⁺ mDC following stimulation of total LP mononuclear cells (LPMC) with *P. copri* and *P. stercorea* (high abundance HAMB) and *R.*

bromii (low abundance HAMB) was assessed. These cytokines and HAMB were specifically chosen based on their *in vivo* associations with CD1c⁺ activation.

Exposure to each of the HAMB induced significant frequencies of IL-23-, IL-1 β - and IL-10-producing CD1c⁺ mDCs, indicating that all three HAMB species activate colonic CD1c⁺ mDCs *in vitro* to some degree (Supplementary Table S5). *P. copri* and *P. stercorea* induced a higher percentage of IL-23⁺ CD1c⁺ mDCs compared to *R. bromii*, with this reaching statistical significance for *P. stercorea* (Figure 7a). *P. copri* induced the highest fraction of IL-1 β ⁺ CD1c⁺ mDCs, and this difference was highly significant ($p < 0.01$) when compared to *R. bromii* (Figure 7b).

P. copri induced the highest percentage of IL-10⁺ CD1c⁺ mDCs, which was, on average, 3.3x and 7.7x that induced by *P. stercorea* and *R. bromii*, respectively, and reached statistical significance ($p < 0.01$) relative to *R. bromii* (Figure 7c). In response to *P. copri* stimulation, a small fraction of IL-10⁺ CD1c⁺ mDC co-produced IL-23 (mean 8.2% \pm 3.9% (SEM)), whereas a higher percentage of IL-10⁺ CD1c⁺ mDC co-produced IL-1 β ⁺ (62% \pm 6.1%), suggesting that IL-23-producing CD1c⁺ mDC are a separate population of cells to those producing IL-10.

DISCUSSION

To our knowledge, this is the first study to address whether colonic mDC phenotype and function is altered in chronic untreated HIV-1 infection. In agreement with a number of previous studies in pathogenic SIV infection,²⁴⁻²⁷ no significant differences were observed in the frequency of colon CD1c⁺ or CD1c^{neg} mDCs in HIV-1-infected subjects compared to uninfected controls. However, HIV-1 infection induced an activated, but dysregulated intestinal mDC phenotype characterized by increased levels of CD40 expression and decreased CD83 expression, similar to what we previously described for lymph node DCs during HIV infection.²¹

We show for the first time that specific *Prevotella* species, increased in the stool and colonic mucosa of HIV-1-infected subjects, correlated in abundance with colonic mDC activation levels *in vivo* and also had the capability of inducing strong pro-inflammatory cytokine production by colonic mDCs *in vitro*. These findings expand upon our previous observations that in HIV-1-infected subjects, mucosal abundance of *Prevotella* spp. (genus level) was associated both with CD1c⁺ mDC activation and with colon CD4 and CD8 T cell activation.¹⁶ Taken together, these results suggest that increased abundance of these pathobiont bacterial species in the intestinal mucosa may contribute to HIV-associated mucosal inflammation and immune activation, supporting previous studies that demonstrated the ‘pathogenic potential’ of *Prevotella* species in periodontal disease,²⁸ ulcerative colitis,²⁹ and arthritis.³⁰

Although typically indicative of “maturation”, the precise role for DC expression of CD83 in directing immune responses is not well understood. Down-regulation of membrane-bound CD83 by RNA interference^{31,32} or by viruses such as HCMV³³ and HSV-1³⁴ on human blood DCs resulted in decreased T cell stimulatory capacity. However, fewer CD83⁺ cells

were detected in the inflamed areas of colonic and ileal Crohn's disease samples compared to control and uninfamed areas,³⁵ suggesting that in the intestinal mucosa, CD83 may have regulatory effects. This concept of CD83-mediated mucosal regulation is further supported by our observation that in HIV-1-infected subjects, frequencies of colonic CD83⁺ mDCs were inversely associated with IFN- γ -producing colonic T cells. However, further studies are warranted to determine the mechanistic relationship between CD83-expressing mucosal mDC and IFN- γ -producing T cells and to evaluate if this is an mDC-mediated process or, conversely, IFN- γ -producing T cells play a role in modulating intestinal mDC activation during HIV-1 infection.

A potential 'central role' for activated colonic mDC in HIV-associated pathogenesis is further highlighted by our observations that CD40 expression levels on CD1c⁺ mDCs positively correlated with colonic CD4 and CD8 T cell activation. Further, CD1c⁺ mDC activation also associated with blood CD4 and CD8 T cells activation, thereby linking colon mDC activation to a marker of HIV-1 disease progression.^{2,3} Moreover, activated CD1c⁺ mDCs in HIV-1-infected subjects was associated with numerous mucosal cytokines, including IL-23 and IL-1 β . Within the mucosa, increased levels of IL-23 and IL-1 β have been implicated in intestinal inflammation mediated, in part, through the promotion of T cell-associated IFN- γ and IL-17 production.^{36,37} In our study, levels of CD40 expression on colonic mDCs were also associated with mucosal levels of IFN- γ and IL-17, suggesting an intricate relationship between mDC activation, mucosal T cell activation, and cytokine-production in the setting of HIV-1 infection. These *in vivo* observations expand on our previous *in vitro* study that demonstrated a requirement for LP mDCs in the *in vitro* expansion and enhanced infection of Th1 and Th17 cells in response to exposure to commensal bacteria and HIV-1.¹⁹ Although we did not see direct correlations between mDC activation levels and absolute Th1 or Th17 frequencies, this finding may be due to the fact that these mucosal Th subsets are depleted early in the course of HIV infection³⁸ and thus absolute Th cell numbers might not be expected to reflect ongoing mucosal inflammation during chronic disease.

Intriguingly, HIV-1-associated colonic mDC activation levels positively associated with mucosal and systemic IL-10 production, a cytokine with well described immuno-regulatory functions.³⁹ Increased levels of IL-10, in conjunction with increased levels of pro-inflammatory cytokines, have been reported in both acute and chronic HIV-1 infection.^{40,41} Systemically administered IL-10 stimulated the production of IFN- γ during human endotoxemia,⁴² suggesting that IL-10 can have pro-inflammatory effects, especially concurrent with exposure to microbial products. IL-10 regulates production of IL-23 by human blood mDCs in response to commensal bacteria *in vitro*⁴³ and a similar negative feedback mechanism to compensate for increased production of pro-inflammatory IL-23 by intestinal mDCs in response to translocating commensal bacteria may be at play in the colon of HIV-1-infected subjects. Indeed, we observed production of IL-10 by CD1c⁺ mDC in response to *in vitro* exposure to HAMB, and these DCs were a different population to those producing IL-23. Although our current *in vivo* and *in vitro* observations suggest a role for intestinal mDCs in IL-10 production, the exact nature of the immune-regulatory versus pro-inflammatory effects of IL-10 in the setting of HIV-1 infection requires further investigation.

Estes *et al.* utilized quantitative image analysis to directly demonstrate translocation of LPS and *E. coli* in the colon of chronically SIV-infected rhesus macaques, and increased levels of MT were due, in part, to ineffective phagocytosis by intestinal macrophages.⁴⁴ To our knowledge, no studies have quantitated levels of microbial products in the human colonic LP during chronic untreated HIV-1 infection nor evaluated the co-localization of microbial products with resident LP mDCs and macrophages. In our study, HIV-1-infected subjects had heightened tissue levels of both LTA and LPS, although neither appeared to directly correlate with mucosal mDC activation. These results suggest that both gram-negative and gram-positive bacteria and their products are translocating even though only gram-negative bacteria were increased in abundance in the mucosa. A greater fraction of LTA and LPS was associated with macrophages than with mDCs, in keeping with the reported robust phagocytic ability of tissue macrophages.⁴⁵ Despite an increase in LTA and LPS tissue levels and in the number of LP macrophages in HIV-1-infected subjects, we only observed an increase in the number of LTA⁺ LP macrophages in this cohort, suggesting a defect in macrophage function in the context of bacterial uptake of LPS or gram-negative bacteria in chronic HIV-1 infection. Indeed, the ability of LP macrophages and mDCs to limit MT in the LP of both LTA and LPS must still be somewhat ineffective given the increased levels of these bacterial products in the plasma of HIV-1-infected subjects.

In a previous study, stimulation of LPMC with a synthetic TLR7/8 ligand that mimics HIV-1 ssRNA, in conjunction with a gram-negative bacterial TLR4 ligand (LPS), resulted in a synergistic increase in IL-23 production by intestinal mDC *in vitro*.¹⁸ In this clinical study, levels of CD1c⁺ mDC activation correlated with mucosal HIV-1 viral load, suggesting that HIV-1 itself may play a role in intestinal mDC activation. Moreover, in both uninfected and HIV-1-infected subjects, the fraction of mDCs found in association with LPS was higher than that with LTA, suggesting an increased likelihood of gram-negative bacteria directly activating colonic mDC. This finding is in keeping with our observations that gram-negative *Prevotella* species abundance correlated with colonic mDC activation *in vivo*, and that *Prevotella* species induced higher frequencies of cytokine-expressing mDCs *in vitro* than did gram-positive *R. bromii*. These observations raise the possibility that HIV-1 and translocating gram-negative bacteria act in concert to induce intestinal mDC activation *in vivo* and thereby potentiate mucosal inflammation. Further studies will be needed to understand whether HIV-1-associated activation is mediated by direct HIV-1/mDC interactions or in a bystander fashion via effects of HIV-1 on colonic pDCs⁴⁶ or other cells, as well as to identify the exact viral and bacterial determinants responsible for intestinal DC activation and cytokine production.

As part of this study, we also investigated the impact of HIV-1 infection on colonic pDC frequency and activation state. A trend towards increased frequencies of intestinal pDCs, a significant increase in numbers of CD40⁺ pDCs, and a decrease in percentages of CD83-expressing pDCs were observed in HIV-1-infected subjects compared to uninfected controls. Further, numbers of activated pDCs were directly associated with levels of CD1c⁺ mDC activation, suggesting that common factors might be driving activation in both DC subsets. These observations are in keeping with recent studies demonstrating increased pDC frequencies in the ileum of chronically HIV-infected subjects⁴⁷ as well as increased pDC frequencies with poly-functional cytokine phenotypes in pathogenic SIV infection

models^{25,26,48}. Unlike mDCs, pDCs are rarely found in intestinal tissue under steady state conditions,^{18,22} thus accumulation of activated pDCs during chronic HIV-1 infection is likely due to increased migration from the blood to the colon.^{25,26,47}

In conclusion, we propose a model whereby colonic mDCs drive mucosal immune activation and inflammation during chronic untreated HIV-1 infection (Figure 8). Increased translocation of gram-negative *Prevotella* into the LP synergizes with HIV to induce intestinal mDC activation. Activated mDC subsequently stimulate bacteria-specific CD4 T cells^{19,49} through cell-cell contact and production of inflammatory cytokines (IL-23, IL-1 β), with additional non-specific T cell activation potentially occurring via CD83-mediated loss of mucosal T cell regulation. This process leads to expansion of Th1, Th17 and Th22 as well as inflammatory IFN- γ -producing CD8 T cells. Activated Th1/17/22 cells are targets for viral replication¹⁹ which ultimately results in their infection and depletion.⁴⁹ Increased mucosal inflammation, a loss of DC-mediated regulation, and a lack of “protective” Th17 and Th22 cells further contribute to epithelial barrier breakdown and MT, thereby potentiating a vicious cycle that ultimately leads to systemic immune activation and its attendant comorbidities. The clinical implications of our *in vitro* findings are currently speculative and additional *in vivo* clinical studies that block microbial translocation^{50,52} and those that alter the microbiome composition and bacteria-associated metabolic pathways are required to provide further evidence that interactions between the microbiome and mDC contribute to intestinal inflammation during HIV-1 infection. These types of studies would also be invaluable in furthering our understanding of the factors contributing to HIV-1-associated mucosal pathogenesis by determining if the microbiome is altered due to ongoing mDC-mediated mucosal inflammation or if the increased abundances of pathobionts induce mDC activation, or if both processes are involved.

MATERIALS AND METHODS

Study participants and study design

Twenty-four HIV-1-infected adult subjects and 14 HIV-1-seronegative (uninfected) adult control subjects were enrolled in this cross-sectional study at the University of Colorado Anschutz Medical Campus. Efforts were made to enroll control subjects who were matched for age and sex to the HIV-1-infected subjects. Clinical characteristics for study subjects are detailed in Table 1. Based on study entry criteria, HIV-1-infected subjects were cART-naïve or had not been on treatment for greater than 7 days in the preceding 6 months. Exclusion criteria are extensively described in a previous publication¹⁶ and detailed in Supplementary Materials. All subjects voluntarily gave written, informed consent. This study was approved by the Colorado Multiple Institutional Review Board (COMIRB) at the University of Colorado Anschutz Medical Campus.

Collection, storage and processing of clinical samples

Collection, storage and processing of rectal swabs, colon biopsies and peripheral blood mononuclear cells (PBMC) are detailed in Supplementary Materials.

Enumeration of colonic mucosal HIV-1 viral load

Colonic mucosal HIV-1 viral load was determined as previously described.¹⁶ To account for variation in the number of CD4⁺ T cells in different samples, HIV RNA copy numbers were normalized per CD4 T cell within each biopsy.

Determination of mucosa-associated bacterial species

Laboratory and analytic methods used to profile the intestinal microbiomes of study participants were described previously.¹⁶ Species-level taxonomic classification of 16S rRNA sequence datasets was obtained via BLAST⁵³ of subject sequences against a database built from Silva²³ bacterial sequences marked as type strains, cultivars, or genomes. A species name was assigned when a sequence overlapped the Silva database sequence by at least 95% sequence length with at least 99% sequence identity and the taxonomy of the database hit matched the taxonomy returned by SINA⁵⁴ as determined previously.¹⁶

Plasma LTA, LPS and sCD14 measurements

Serum LTA levels were assessed using a custom ELISA.¹⁶ LPS levels were measured in EDTA plasma samples using the Limulus Amebocyte Lysate (LAL) assay (Lonza, Switzerland) following the manufacturer's protocol as previously detailed.¹⁶ sCD14 levels were measured in heparin plasma using a commercially available ELISA (R&D Systems, Minneapolis, MN).¹⁶

Mucosal and plasma cytokine measurements

A Custom Q-plex Array (Quansys Biosciences, Logan UT) was used to measure mucosal cytokine levels in culture supernatants and measurement of plasma cytokine levels were performed using the Human Cytokine High Sensitivity Screen as detailed in Supplementary Materials. Levels of plasma IL-6 were evaluated in EDTA plasma samples using a commercially available ELISA (R&D Systems).¹⁶

Histological staining and analysis of colonic biopsies

Assessment of microbial product levels, CD11c⁺ mDCs, and HAM56⁺ macrophages in colonic LP is detailed extensively in Supplementary Materials. Using Zeiss Zen Software (Jena, Germany), the total area and the area that stained with LTA/LPS was calculated within the LP. To analyze whether microbial products preferentially associated with mDCs or macrophages the total number of mDCs and macrophages that either did or did not associate with microbial products (LTA/LPS) were enumerated per square millimeter of LP using Image J Software (NIH Bethesda, MD)

Assessment of mononuclear infiltration is detailed in Supplementary Materials. Evaluation was performed by a gastrointestinal pathologist who was blinded to the HIV-1 status of each patient. The degree of mononuclear infiltration was quantified on a scale of 0 = Not present, Minimal = 0.5, Mild = 1, Moderate = 2, and Severe = 3.

In vitro stimulations

In vitro mitogenic stimulation of single-cell colon biopsy preparations: Evaluation of frequencies of colonic CD4 T cells capable of producing IFN- γ (Th1), IL-17 (Th17) or IL-22 (Th22) and frequencies of IFN- γ -producing CD8 T cells from isolated colon cells following mitogenic stimulation are detailed in Supplementary Materials. *In vitro exposure of LPMC to commensal bacteria:* Cytokine responses by LP CD1c⁺ mDCs to *P. copri*, *P. stercorea* and *R. bromii* were assessed utilizing an *ex vivo* tissue culture model consisting of isolated colon LPMC from normal tissue^{18,19,49,55} and is detailed in Supplementary Materials.

Commensal bacteria stocks

Expansion of *P. copri* (DSM# 18205, DSMZ, Braunschweig, Germany), *P. stercorea* (DSM# 18206) and *R. bromii* (ATCC# 27255, ATCC Manassas, VA) was performed at 37°C under anaerobic conditions per manufacturer's protocols as described in Supplementary Materials.

Surface and intracellular flow cytometry staining assays, acquisition and analysis

DC and T cell frequencies, activation and cytokine production from colon cells isolated from biopsies and from PBMC: Multi-color flow cytometry protocols to evaluate colon and blood DC and T cell frequencies and activation status and to determine T cell cytokine frequencies are detailed in Supplementary Materials. For enumeration of colonic DC and T cell frequencies, the percentage of DCs or T cells within viable, CD45⁺ cells was converted to an absolute number per gram based on the frequency within viable, CD45⁺ cells, initial cell counts and biopsy weights. Similarly, the percent of activated colon CD4 and CD8 T cells as well as the percent of cytokine⁺ CD4 and CD8 T cells were also converted to a total number per gram of mucosal tissue.

Phenotypic and functional characterization of mDC subsets in LPMC from normal colon tissue: Multi-color flow cytometry staining protocols used to characterize LP CD1c⁺ mDCs and CD1c^{neg} mDCs and enumerate cytokine⁺ CD1c⁺ mDCs following *in vitro* stimulation of LPMC in normal colon tissue are detailed in Supplementary Materials.

Flow cytometry acquisition: All flow cytometry data were acquired on an LSRII Flow Cytometer (BD Biosciences). Routine quality control using the Cytometer Setup & Tracking feature within the BD FACSDiva software version 6.1.2 (BD Biosciences) was performed daily as previously detailed¹⁹.

Statistical analysis

Non-parametric statistics were performed with no adjustments for multiple comparisons due to the exploratory nature of this study. Analysis and graphing were performed using GraphPad Prism Version 6 for Windows (GraphPad Software, San Diego, CA). Comparisons between independent groups were made using the Mann-Whitney test and the Friedman test with a multiple Dunn comparison test for matched-paired comparisons across multiple groups. To determine the differences between groups of matched paired data, the Wilcoxon matched-pairs signed-rank test was performed. Correlations between variables were assessed

using the Spearman test. Fisher exact tests and Chi-squared tests were used for comparisons of categorical data. A p value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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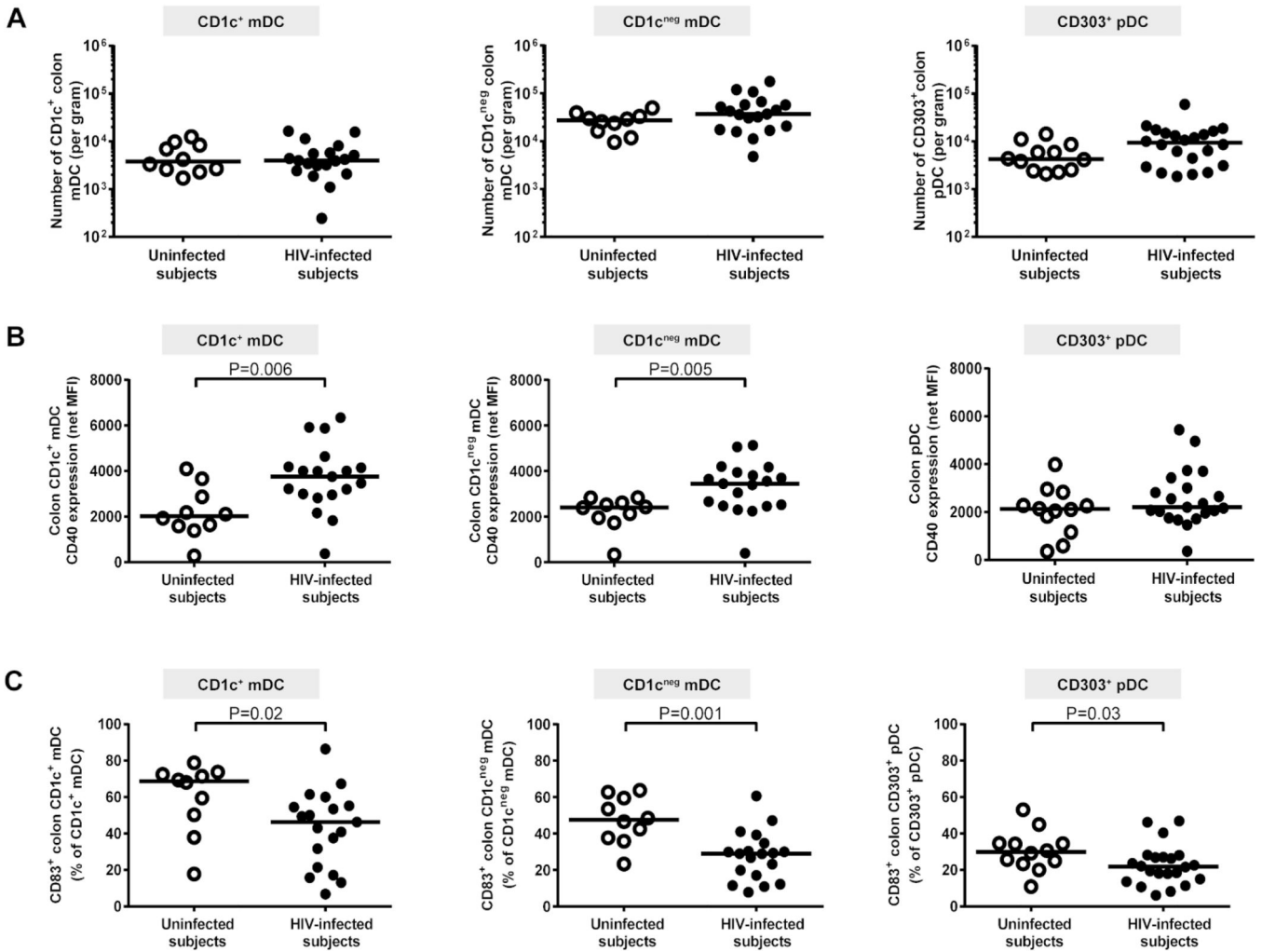


Figure 1. Colon dendritic cells (DCs) from HIV-1-infected subjects have an altered activation profile

Multi-color flow cytometry techniques were used to determine frequencies and activation/maturation states of colon CD1c⁺ myeloid DCs (mDCs), CD1c^{neg} mDCs and CD303⁺ plasmacytoid DCs (pDC) in uninfected (open circles) and HIV-1-infected (HIV-infected; closed circles) subjects. (A) Frequencies of CD1c⁺ mDCs, CD1c^{neg} mDCs (uninfected n=10; HIV-infected n=19) and CD303⁺ pDCs (uninfected n=12, HIV-infected n=22) were evaluated as a percent of viable, CD45⁺ leucocytes and converted into a total number of DC per gram of tissue. (B) CD40 expression levels (Mean Fluorescence Intensity; MFI) and (C) percent of CD83⁺ DCs were assessed on CD1c⁺ mDCs, CD1c^{neg} mDCs (uninfected n=10; HIV-infected n=19) and CD303⁺ pDCs (uninfected n=12, HIV-infected n=22). Appropriate isotype controls were removed to control for background staining (net). Lines represent median values and statistical analysis was performed using the Mann-Whitney test.

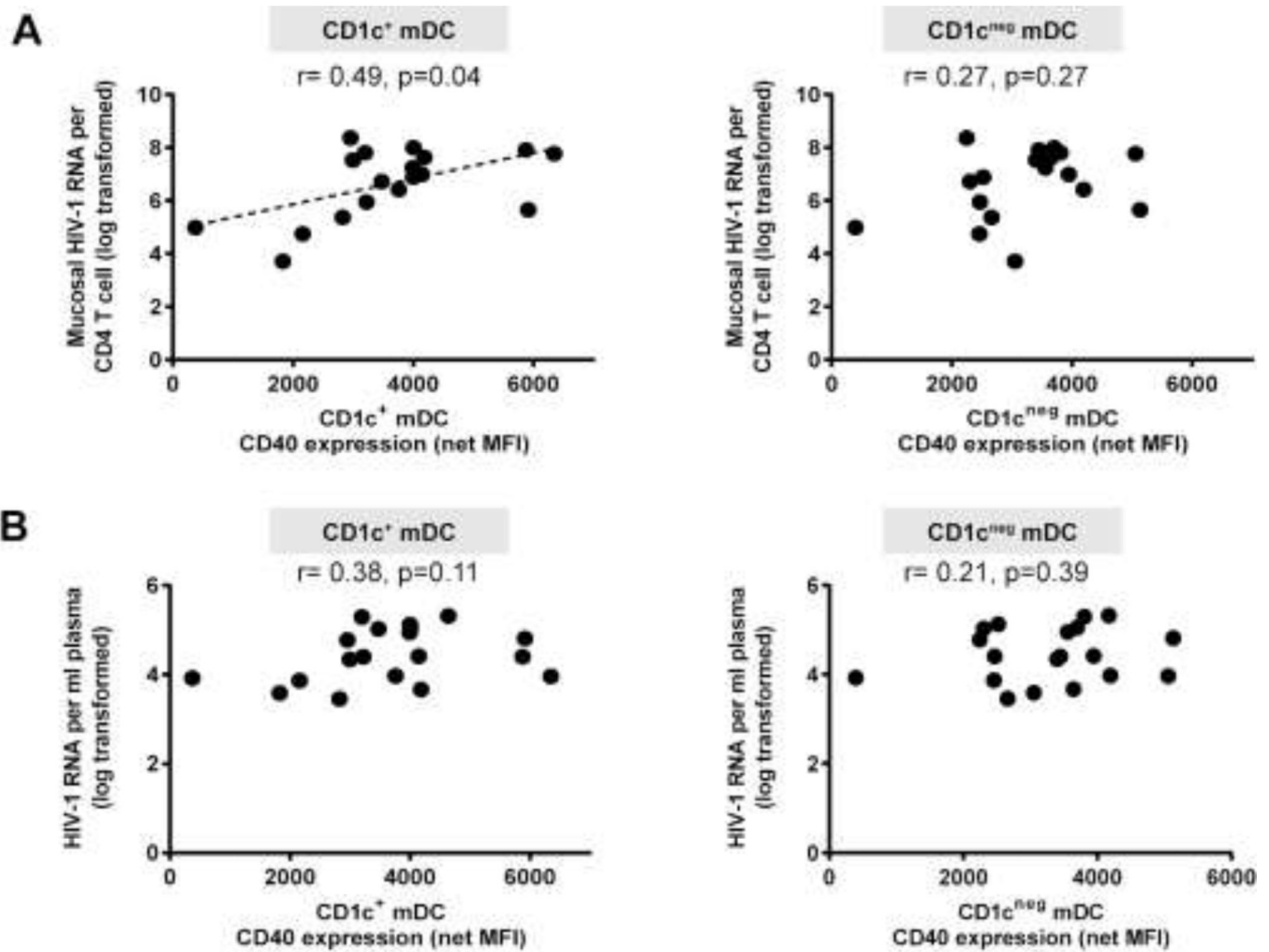


Figure 2. Activated colon CD1c⁺ myeloid dendritic cells (mDCs) correlate with mucosal HIV-1 viral load

Correlations between CD40 expression levels (mean fluorescence intensity; MFI) on CD1c⁺ and CD1c^{neg} mDCs (shown with background isotype values removed; net MFI) with (A) mucosal HIV-1 viral load (n=18) and (B) plasma HIV-1 viral load (n=19). Statistical analysis was performed using the Spearman test. Dotted line is a visual representation of the significant association.

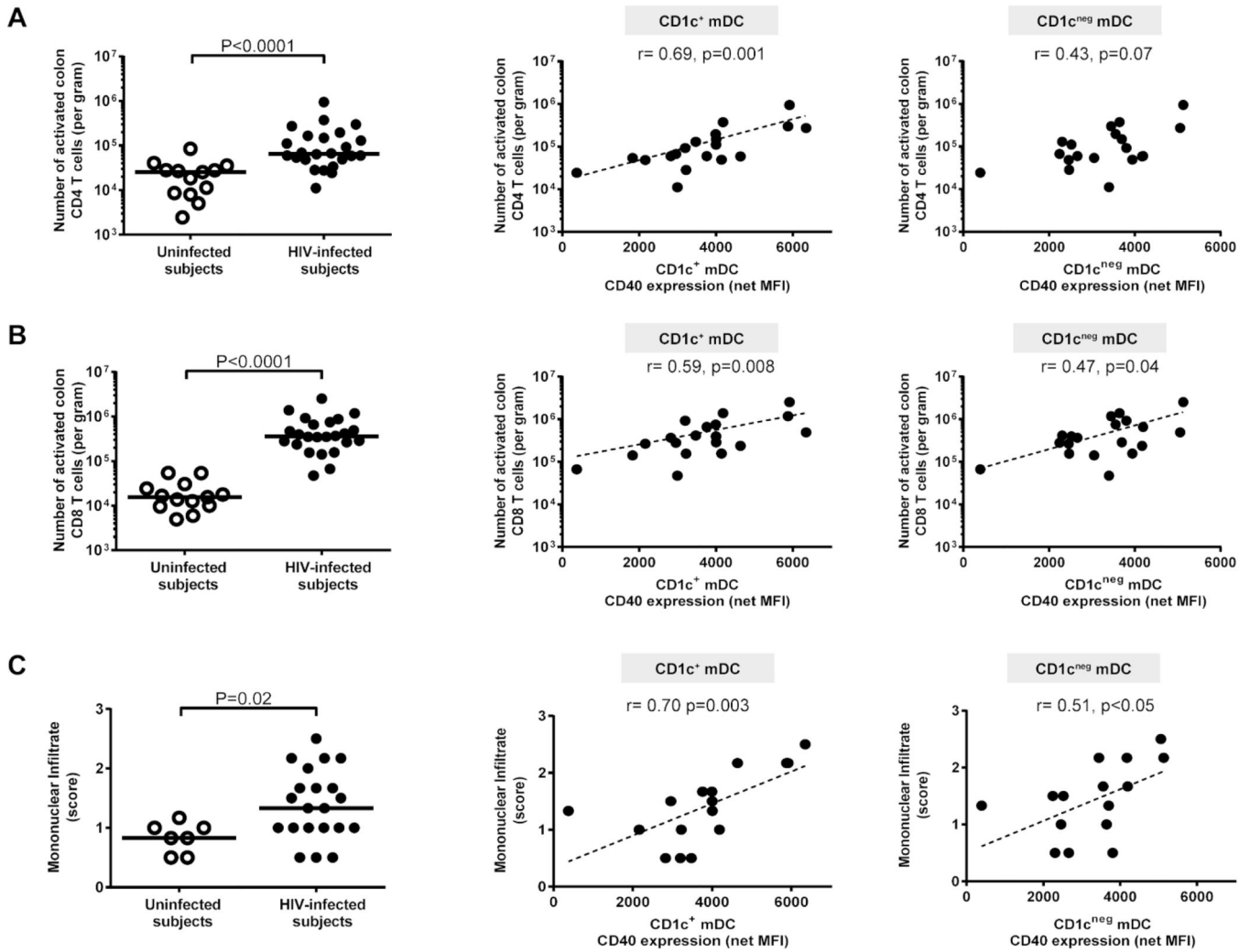


Figure 3. Activated colon CD1c⁺ myeloid dendritic cells (mDCs) correlate with mucosal T cell activation and mononuclear infiltration

Multi-color flow cytometry techniques were used to determine frequencies of activated (percent CD38⁺ HLA-DR⁺) colonic mucosal CD4 and CD8 T cells and H&E staining to evaluate lamina propria (LP) infiltration of mononuclear cells in uninfected (open circles) and HIV-1-infected (HIV-infected; closed circles). Frequencies of colonic mucosal (A) CD38⁺HLA-DR⁺ CD4 T cells and (B) CD38⁺HLA-DR⁺ CD8 T cells (uninfected n=13; HIV-infected n=24) were evaluated (with background isotype values removed) as a percent of viable, CD45⁺ leucocytes and converted into a total number of activated CD4 or CD8 T cells per gram of tissue. Lines represent median values and statistical analysis was performed using the Mann-Whitney test. Correlations between CD40 expression levels (mean fluorescence intensity; MFI) on CD1c⁺ and CD1c^{neg} mDCs (shown with background isotype values removed; net MFI) and activated (A) CD4 T cells or (B) CD8 T cells (shown with background isotype values removed) in HIV-infected subjects (n= 19) were performed using the Spearman test. Dotted line is a visual representation of the significant associations. (C) Mononuclear infiltrate assessed as the relative cellularity of the LP infiltrate consisting of lymphocytes, plasma cells, eosinophils and occasional neutrophils and scored on a scale

of 0 = Not present, Minimal = 0.5, Mild = 1, Moderate = 2, and Severe = 3. Values are shown as the average score of 3 sections of colon biopsy from uninfected (open circles, n=7) and HIV-1-infected (HIV-infected; n=21) subjects. Lines represent median values and statistical analysis was performed using the Mann-Whitney test. Correlations between CD40 expression levels (mean fluorescence intensity; MFI) on CD1c⁺ and CD1c^{neg} mDCs (shown with background isotype values removed; net MFI) and mononuclear infiltrate scores in HIV-infected subjects (n= 16) were performed using the Spearman test. Dotted line is a visual representation of the significant associations.

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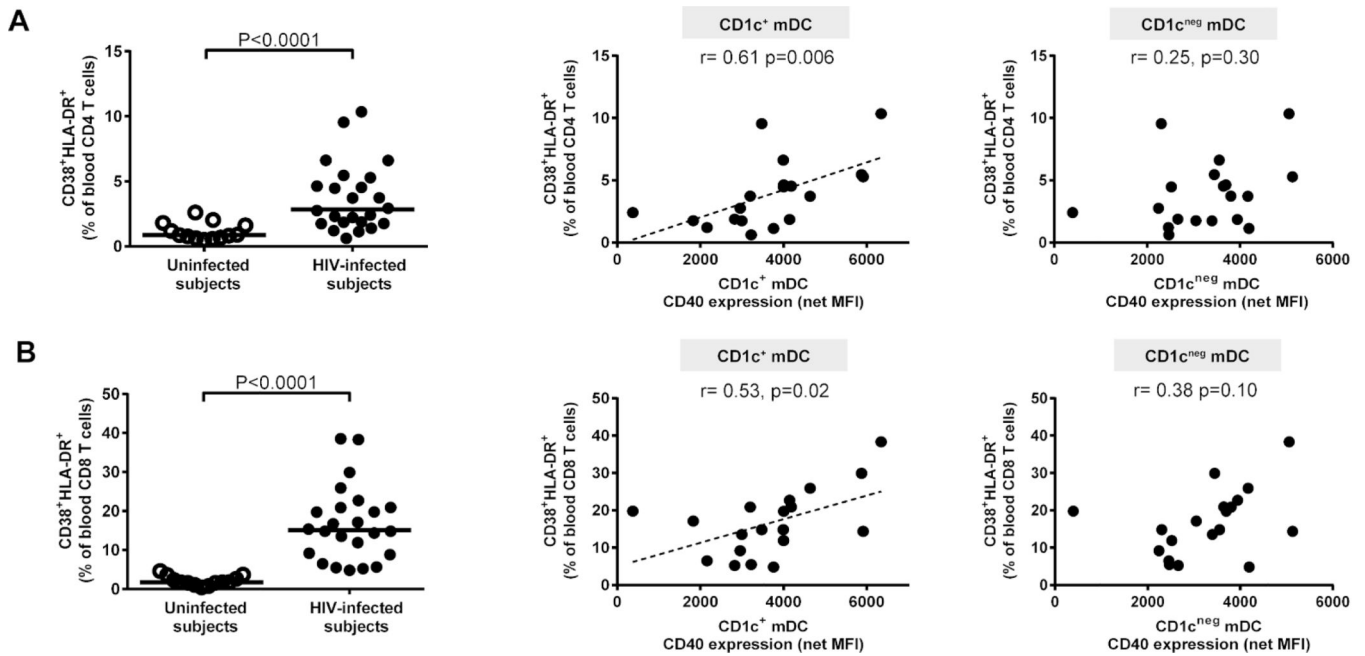


Figure 4. Activated colon CD1c⁺ myeloid dendritic cells (mDCs) correlate with systemic T cell activation

Multi-color flow cytometry techniques were used to determine frequencies of activated (percent CD38⁺ HLA-DR⁺) blood CD4 and CD8 T cells in uninfected (open circles; n=13) and HIV-1-infected (HIV-infected; n=24; closed circles). Percentages of (A) CD38⁺HLA-DR⁺ blood CD4 T cells and (B) CD38⁺HLA-DR⁺ blood CD8 T cells as a fraction of blood CD4 or CD8 T cells (shown with background isotype values removed). Lines represent median values and statistical analysis was performed using the Mann-Whitney test. Correlations between CD40 expression levels (mean fluorescence intensity; MFI) on CD1c⁺ and CD1c^{neg} mDCs (shown with background isotype values removed; net MFI) and activated blood (A) CD4 and (B) CD8 T cells (shown with background isotype values removed) in HIV-infected subjects (n= 19) were performed using the Spearman test. Dotted line is a visual representation of the significant associations.

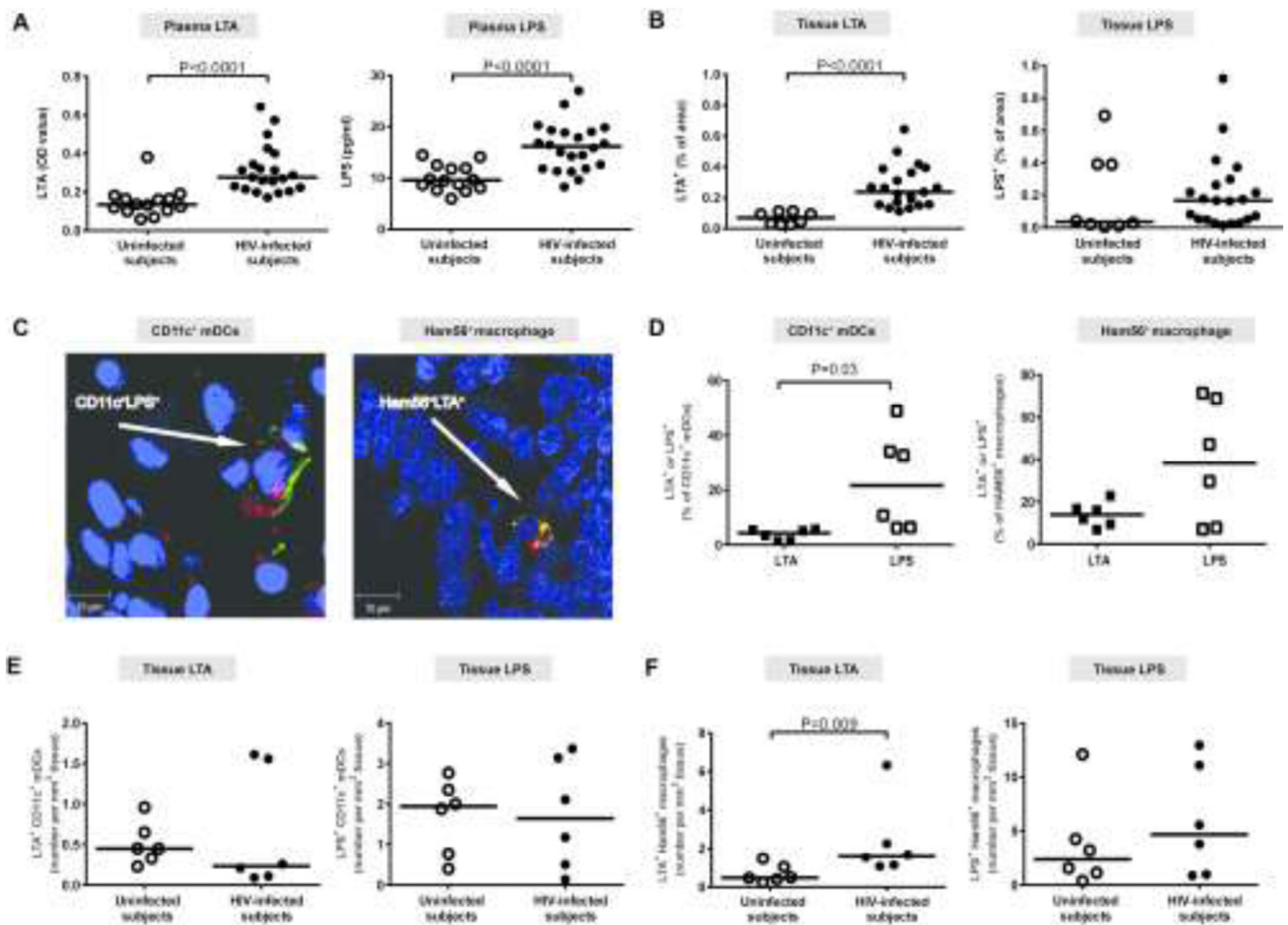


Figure 5. Colonic tissue and systemic levels of microbial products are increased in HIV-1-infected subjects and colonic myeloid dendritic cells (mDCs) associate with tissue LPS to a greater extent than LTA

Levels of LTA and LPS were evaluated in the (A) plasma of uninfected ($n=14$) and HIV-1-infected (HIV-infected; LTA $n=21$; LPS $n=22$) subjects and in the (B) colonic lamina propria (LP) of uninfected ($n=8$) and HIV-1-infected (HIV-infected, $n=21$) subjects. Lines represent median values and statistical analysis was performed using the Mann-Whitney test. (C) Representative images demonstrating localization of mDCs (CD11c/green) or macrophages (Ham56/yellow) with either LTA or LPS (red) in formalin-fixed, paraffin-embedded colon biopsy tissue of an HIV-1-infected subject. (D) Comparisons between percentages of LTA⁺ or LPS⁺ CD11c⁺ mDCs and HAM56⁺ macrophages in HIV-infected subjects ($n=6$). Lines represent median values and statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. (E, F) Number of LTA⁺ or LPS⁺ (E) CD11c⁺ mDCs and (F) Ham56⁺ macrophages per mm² of tissue in uninfected ($n=6$) and HIV-infected subjects ($n=6$). Lines represent median values and statistical analysis was performed using the Mann-Whitney test.

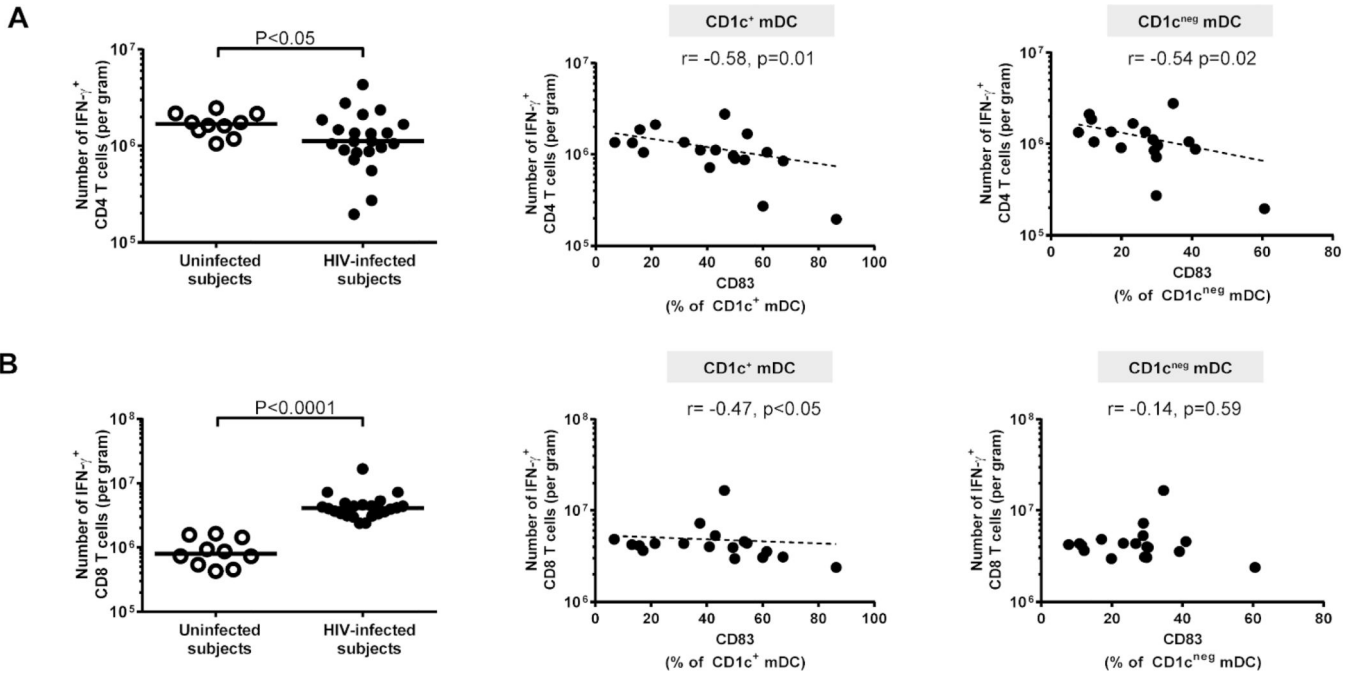


Figure 6. CD83⁺ myeloid dendritic cells (mDCs) negatively correlate with colonic IFN- γ -producing CD4 and CD8 T cells

Multi-color flow cytometry techniques were used to determine frequencies of IFN- γ -producing colonic CD4 and CD8 T cells following mitogenic stimulation in uninfected (open circles; n=10) and HIV-1-infected (HIV-infected; n=22) subjects. Frequencies of colonic (A) IFN- γ ⁺ CD4 T cells and (B) IFN- γ ⁺ CD8 T cells were evaluated (background isotype values removed) as a percent of viable, CD45⁺ leucocytes and converted into a total number of activated CD4 or CD8 T cells per gram of tissue. Lines represent median values and statistical analysis was performed using the Mann-Whitney test. Correlations between percent of CD83⁺ CD1c⁺ and CD1c^{neg} mDCs (shown with background isotype values removed) and number of IFN- γ ⁺ (A) CD4 T cells or (B) CD8 T cells (shown with background isotype values removed) in HIV-infected subjects (n= 18) were performed using the Spearman test. Dotted line is a visual representation of the significant associations.

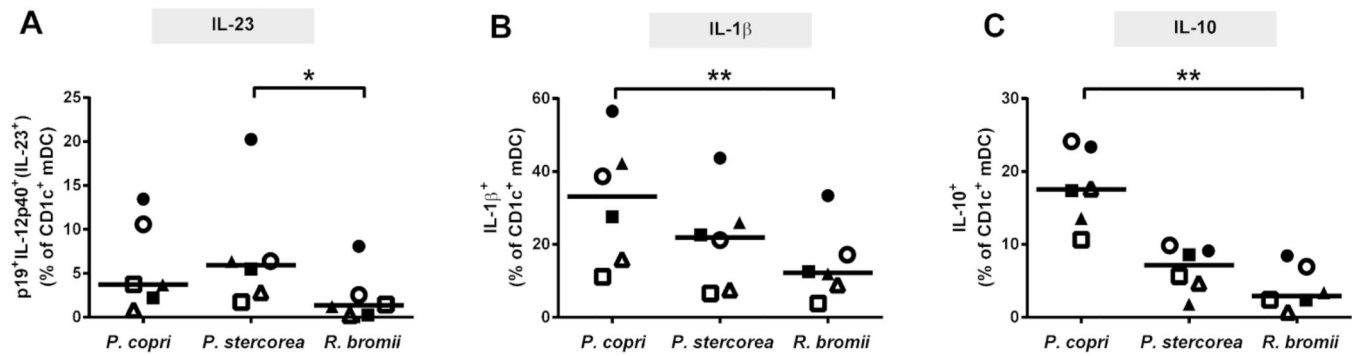


Figure 7. CD1c⁺ myeloid dendritic cell (mDC) cytokine production in response to *in vitro* stimulation with HIV-altered mucosal bacteria (HAMB) species

Colonic LPMC (n=7 samples) were exposed to *Prevotella copri*, *P. stercorea* or *Ruminococcus bromii* for 18–20hrs and multi-color intracellular cytokine flow cytometry techniques used to enumerate IL-23⁺ (IL-12p40⁺p19⁺), IL-1β⁺ and IL-10⁺ CD1c⁺ mDCs. Appropriate isotype controls were removed to control for background staining. Values are shown as HAMB-specific cytokine⁺ mDCs determined by removing the percent of cytokine⁺ CD1c⁺ mDCs detected in unstimulated cultures. Each symbol is a unique donor. Lines represent median values and statistical analysis was performed using the Friedman test for matched-paired comparisons across multiple groups, with a Dunn's multiple comparison test performed when the overall p value was <0.05. *p<0.05, **p<0.01.

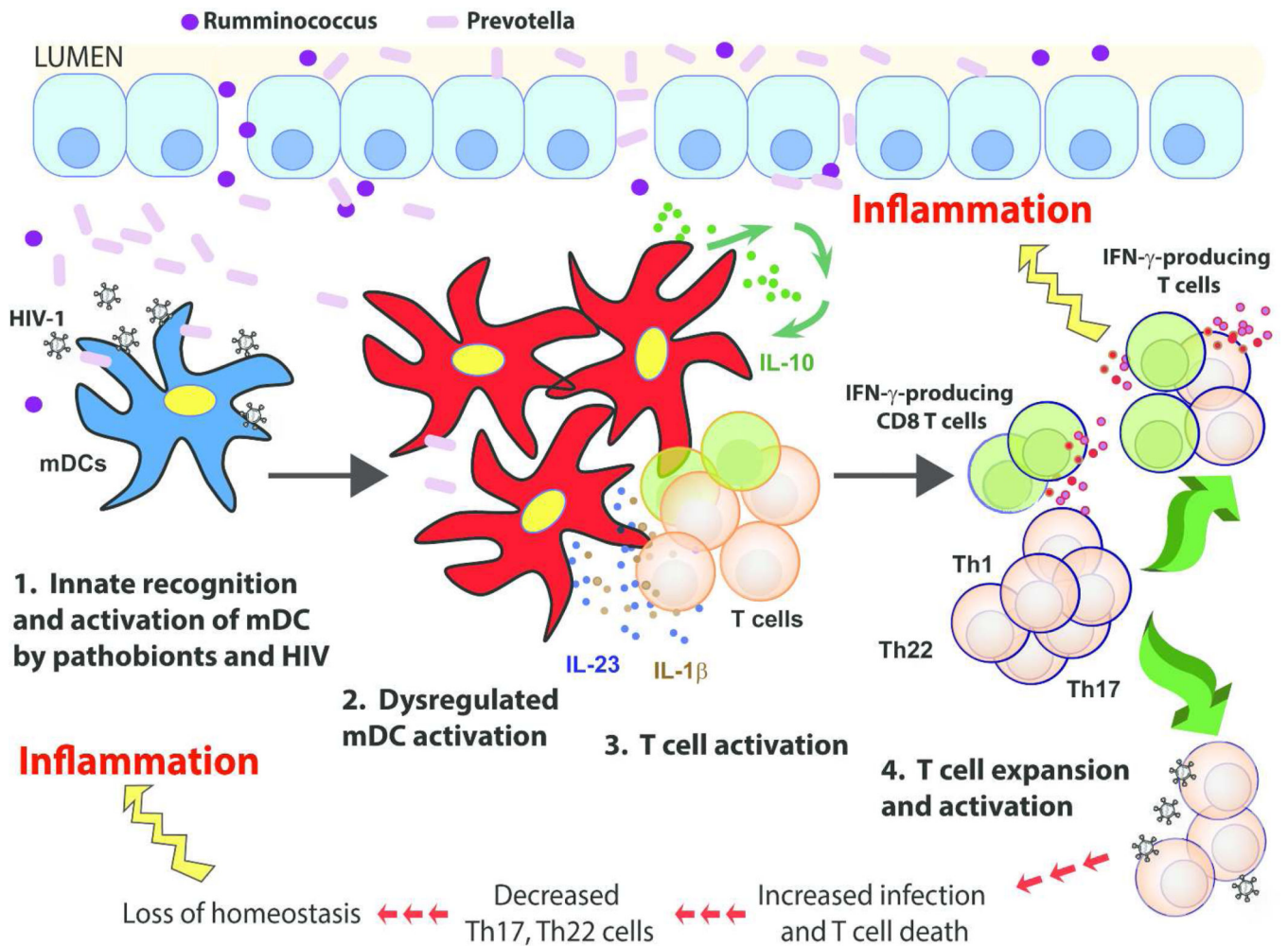


Figure 8. Proposed model illustrating colonic myeloid dendritic cells (mDCs) driving mucosal immune activation and inflammation during chronic untreated HIV-1 infection
 HIV replication in the lamina propria (LP) results in epithelial barrier disruption, leading to the 1) increased translocation of gram-negative *Prevotella* into the LP which synergizes with HIV-1 to induce 2) a dysregulated mDC activation profile characterized by increased levels of CD40 and decreased CD83 expression. Activated mDCs subsequently induce 3) increased T cell activation via stimulation of bacteria-specific CD4 T cells^{19,49} through cell-cell contact (e.g. CD40/CD40L), production of inflammatory cytokines (IL-23, IL-1 β) and potentially via CD83-mediated loss of T cell regulation.³⁵ mDCs produce IL-10 to compensate for increased pro-inflammatory cytokine production;⁴³ however, this may also exacerbate IFN- γ production.⁴² In total, this culminates in 4) increased T cell activation and expansion of T helper (Th)1, Th17 and Th22 and IFN- γ -producing CD8 T cells. Activated Th1/17/22 cells are targets for viral replication¹⁹ which ultimately results in their infection and depletion. Increased mucosal mDC and T cell activation and inflammation, a loss of mDC-mediated regulation, and a lack of “protective” Th17 and Th22 cells further contribute to epithelial barrier breakdown and microbial translocation, thereby potentiating a vicious

cycle that ultimately leads to systemic inflammation and immune activation and their attendant comorbidities.

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Table 1

Subject Characteristics

	Uninfected subjects	HIV-1-infected subjects
Number of subjects	14	24
Age (yrs)	31 (23–54)	33.5 (22–58)
Male/Female Ratio	9/5	18/6
CD4 count (cells/μl)	724 (468–1071)	445 (221–1248) *
Plasma Viral Load (HIV-1 RNA copies/ml)	-	51350 (2880 – 207000)
Years since first HIV-1 seropositive test	-	3.25 (0.17–15)
Ethnicity:^{n/s}		
Non-Hispanic	11 (78.6%)	19 (79.2%)
Hispanic	3 (21.4%)	5 (20.8%)
Race:^{n/s}		
White/Caucasian	10 (71.4%)	17 (70.8%)
Black/African American	2 (14.2%)	6 (25.0%)
Asian	2 (14.2%)	1 (4.2%)

Values are shown as median (range) except for Ethnicity and Race which are shown as the number and percentage of each cohort. Statistical analysis was performed using the Mann-Whitney test for comparisons between uninfected and HIV-1-infected subjects and the Fisher Exact test or Chi-square test for comparison of categorical data.

*p=0.001.

Table 2

Activated CD1c⁺ myeloid dendritic cell (mDC) or CD1c^{neg} mDC associations with constitutive mucosal cytokines in HIV-1-infected subjects

	Cytokine (pg/ml) (median, range)*	CD40 expression levels (MFI) on CD1c ⁺ mDC (n=15)	CD40 expression levels (MFI) on CD1c ^{neg} mDC (n=15)
IL-23	47.7, 0–1264	r=0.67, p=0.008	r=0.55, p=0.03
IL-1β	67.8, 7.2–2730	r=0.72, p=0.003	r=0.36, p=0.19
IL-6	513.1, 0–8711	r=0.68, p=0.006	r=0.38, p=0.16
TNF-α	48.9, 0–2301	r=0.66, p=0.009	r=0.40, p=0.14
IL-10	26.6, 7.4–759.9	r=0.54, p=0.04	r=0.36, p=0.19
IFN-γ	18.8, 0–5921	r=0.71, p=0.004	r=0.54, p=0.04
IL-17	11.6, 0–125.5	r=0.56, p=0.03	r=0.27, p=0.33

Statistical analysis was performed using the Spearman test.

*N=19 HIV-1-infected subjects. Bold values highlight statistically significant correlations. MFI: mean fluorescence intensity.

Table 3

Activated CD1c⁺ myeloid dendritic cell (mDC) or CD1c^{neg} mDC associations with HIV-altered mucosal bacteria (HAMB) species

HAMB species	Prevalence *	↑ or ↓ in HIV-1 infected subjects [#]	CD40 expression levels (MFI) on CD1c ⁺ mDC [‡]	CD40 expression levels (MFI) on CD1c ^{neg} mDC
<i>Prevotella copri</i>	100%	↑ (p=0.02)	r= 0.55, p=0.0525	r=0.16, p=0.60
<i>Prevotella stercorea</i>	88.2%	↑ (p=0.01)	r= 0.48, p=0.0997	r=0.01, p=0.97
<i>Bacteroides acidifaciens</i>	58.8%	↓ (p=0.02)	r= -0.51, p=0.0592	r= -0.29, p=0.28
<i>Blautia schinikii</i>	52.9%	↓ (p=0.005)	r= -0.45, p=0.0863	r= -0.21, p=0.38
<i>Ruminococcus bromii</i>	82.3%	↓ (p=0.03)	r= -0.55, p=0.0542	r= -0.50, p=0.0798

* Percent of HIV-1-infected subjects with each species: statistical analysis to determine associations between activated colonic mDC. Given the small samples size, these associations were only assessed when each species was detected in greater than 50% of HIV-1-infected subjects;

[#] Increased (↑) or decreased (↓) abundance of each bacteria in HIV-1-infected subjects (n=17) compared to uninfected controls (n=14) determined using the Mann-Whitney test.

[‡] Associations between activated colonic CD1c⁺ mDC (n=13) or CD1c^{neg} mDC (n=13) and abundance of HAMB were determined using the Spearman test. MFI: mean fluorescence intensity.