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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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/maturity status of colonic myeloid DC (mDC) subsets (CD1c+ and CD1c−) 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. Percentage of CD83+CD1c+mDCs negatively correlated with frequencies of interferon-γ-producing colon CD4+ and CD8+ T cells. CD40 expression on CD1c+mDCs positively associated with abundance of high prevalence mucosal Prevotella copri and Prevotella stercorea but negatively associated with a number of low 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.

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 progression.2–4 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.5 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 lipopolysaccharide (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, 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 tract. Within days of infection, irrespective of the route of transmission, HIV-1 replication results in severe and rapid depletion of intestinal memory CD4 T cells, including preferential depletion of T helper type 17 (Th17) 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 gastrointestinal 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 interleukin (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 on 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 have 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 naive or had not been on treatment for >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, three had stopped ART at least 8 years (range 8–14 years) prior to the study, one 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 online. 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

| Table 1 Subject characteristics |
|--------------------------------|
| **Uninfected subjects** | **HIV-1-infected subjects** |
| Number of subjects | 14 | 24 |
| Age (years) | 31 (23–54) | 33.5 (22–58) |
| Male/female ratio | 9/5 | 18/6 |
| CD4 count (cells μL−1) | 724 (468–1071) | 445 (221–1248)* |
| Plasma viral load (HIV-1 RNA copies ml−1) | — | 51350 (2880–207000) |
| Years since first HIV-1 seropositive test | — | 3.25 (0.17–15) |
| Ethnicity | | |
| Non-Hispanic | 11 (78.6%) | 19 (79.2%) |
| Hispanic | 3 (21.4%) | 5 (20.8%) |
| Race | | |
| 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 Mann–Whitney test for comparisons between uninfected and HIV-1-infected subjects and Fisher's exact test or Chi-square test for comparison of categorical data. *P = 0.001.
and CD11c but were delineated by the expression of CD1c (Supplementary Results, Supplementary Figure S1). Similar frequencies of both CD1c+ and CD1cneg mDCs were observed in uninfected and HIV-1-infected subjects when enumerated as either a percentage of viable, CD45+ cells (Supplementary Table S1) or as an absolute number of DCs per g 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 mm−2, range 3.8–19.1; uninfected controls: 12.4 CD11c+ cells mm−2, 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 mm−2, 8.7–20.3) compared with uninfected controls (3.9 HAM56+ cells mm−2, 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 toward higher numbers of pDCs in HIV-1-infected subjects (median: 9354 pDC per g, 1835–59658) compared with uninfected subjects (4243, 2104–14155; P = 0.09) was noted.

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), CD1cneg mDCs and CD303+ plasmacytoid DCs (pDCs) in uninfected (open circles) and HIV-1-infected (HIV-infected; closed circles) subjects. (a) Frequencies of CD1c+ mDCs, CD1cneg mDCs (uninfected n = 10; HIV-infected n = 19) and CD303+ pDCs (uninfected n = 12; HIV-infected n = 22) were evaluated as a percentage of viable, CD45+ leucocytes and converted into a total number of DC per g of tissue. (b) CD40 expression levels (mean fluorescence intensity (MFI)) and (c) percentage of CD83+ DCs were assessed on CD1c+ mDCs, CD1cneg 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 Mann–Whitney test.
Colonic CD1c+ mDC and CD1cneg mDC activation based on CD40 expression was significantly higher in HIV-1-infected subjects compared with 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+ pDCs per g, 561–56192; n = 21) compared with 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 percentage of CD1c+ mDCs, CD1cneg 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 CD1cneg mDC CD40 expression did not (Figure 2a). Unlike our previous observations of activated blood DCs,21 CD40 expression on colon CD1c+ mDCs and CD1cneg mDCs did not correlate with either plasma viral load (Figure 2b) or with peripheral CD4 count (CD1c+ mDCs: r = −0.12, P = 0.60; CD1cneg 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 CD1cneg 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 CD1cneg mDCs were positively associated with the degree of mononuclear cell infiltration (Figure 3c).

Significantly 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 with controls (Supplementary Table S3). However,

![Figure 2](Image)

**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 CD1cneg 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 Spearman’s test. Dotted line is a visual representation of the significant association.
no significant associations were found between the 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-1β, IL-6, and tumor necrosis factor (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⁺ mDC activation levels and mucosal levels of IL-23 and IFN-γ.

Plasma IL-6 levels were significantly increased in HIV-1-infected subjects (1.43 pg ml⁻¹, 0.43–5.09; n = 24) compared

**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 (percentage of CD38⁺ HLA-DR⁺) colonic mucosal CD4 and CD8 T cells and hematoxylin and eosin 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 percentage of viable, CD45⁺ leucocytes and converted into a total number of activated CD4 or CD8 T cells per g of tissue. Lines represent median values and statistical analysis was performed using Mann–Whitney test. Correlations between CD40 expression levels (mean fluorescence intensity (MFI)) on CD1c⁺ and CD1c⁻ 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 Spearman’s 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 three 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 Mann–Whitney test. Correlations between CD40 expression levels (MFI) on CD1c⁺ and CD1c⁻ mDCs (shown with background isotype values removed; net MFI) and mononuclear infiltrate scores in HIV-infected subjects (n = 16) were performed using Spearman’s test. Dotted line is a visual representation of the significant associations.
with controls (0.70 pg ml\(^{-1}\), 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-\(\alpha\) 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-\(\alpha\) 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-\(\alpha\) (\(r = 0.63, P = 0.02; n = 14\)) and IL-10 (\(r = 0.76, P = 0.002; n = 14\)), whereas CD1c\(^{neg}\) mDC activation was associated with plasma IFN-\(\gamma\) (\(r = 0.59, P = 0.03; n = 14\)) and weakly with IL-10 (\(r = 0.54, P = 0.05; n = 14\)).

**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\(^{-1}\)) | 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                                                 | \(r = 0.67, P = 0.008\)                                     |
| IL-1\(\beta\)             | 67.8, 7.2–2730                                               | \(r = 0.72, P = 0.003\)                                     |
| IL-6                      | 513.1, 0–8711                                                | \(r = 0.68, P = 0.006\)                                     |
| TNF-\(\alpha\)            | 48.9, 0–2301                                                 | \(r = 0.66, P = 0.009\)                                     |
| IL-10                     | 26.6, 7.4–759.9                                              | \(r = 0.54, P = 0.04\)                                     |
| IFN-\(\gamma\)            | 18.8, 0–5921                                                 | \(r = 0.71, P = 0.004\)                                     |
| IL-17                     | 11.6, 0–125.5                                                | \(r = 0.56, P = 0.03\)                                     |

\(\text{IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; TNF, tumor necrosis factor. Statistical analysis was performed using Spearman's test. Bold values highlight statistically significant correlations.}\)

\(\text{a}\, n = 19\, \text{HIV-1-infected subjects.}\)
Colonic mDCs are identified in association with tissue LPS to a greater extent than lipoteichoic acid (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, 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 the levels of mucosal IL-1β ($r = 0.58$, $P = 0.02$) and 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 LTA were also observed in HIV-1-infected subjects compared with 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 with 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$^+$ mDCs and HAM56$^+$ macrophages ($n = 6$). Lines represent median values and statistical analysis was performed using Wilcoxon’s matched-pairs signed rank test. The number of LTA$^+$ or LPS$^+$ CD11c$^+$ mDCs and HAM56$^+$ macrophages per mm$^2$ of tissue in uninfected ($n = 6$) and HIV-infected subjects ($n = 6$). Lines represent median values and statistical analysis was performed using Mann–Whitney test.

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 and 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).

Percentage of CD83-expressing colonic mDCs is negatively associated with IFN-γ-producing colonic T cells
In HIV-1-infected subjects, the percentage of CD83⁺ CD1c⁺ mDCs negatively correlated with the number of IFN-γ⁺ CD4⁺ T cells and CD8⁺ T cells (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 with controls. Levels of CD40 on CD1c⁺ mDCs trended (P < 0.1) toward positive associations with high-abundance Prevotella copri and P. stercordae and toward negative associations with low-abundance Bacteroides acidifaciens, Blautia schinikii, and Rumminococcus bromii (Table 3). CD1c⁺ mDC activation also trended toward a negative association with R. bromii, but no clear associations with any other HAMB species were noted (Table 3).

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). Production of IL-23, IL-1β, and IL-10 by CD1c⁺ mDC following in vitro stimulation of total LP mononuclear cells (LPMC) with P. copri and P. stercordae (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-1β-, 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. stercorae induced a higher percentage of IL-23⁺ CD1c⁺ mDCs compared with R. bromii, with this reaching statistical significance for P. stercorae (Figure 7a). P. copri induced the highest fraction of IL-1β⁺ CD1c⁻ mDCs, and this difference was highly significant (P < 0.01) when compared with R. bromii (Figure 7b).

P. copri induced the highest percentage of IL-10⁺ CD1c⁺ mDCs, which was, on average, 3.3 % and 7.7 % that induced by P. stercorae 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 % ± 3.9 % (s.e.m.)), whereas a higher percentage of IL-10⁺ CD1c⁺ mDC co-produced IL-1β⁺ (62 ± 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 simian immunodeficiency virus (SIV) infection, 24–27 no significant differences were observed in the frequency of colon CD1c⁺ or CD1c⁻ mDCs in HIV-1-infected subjects compared with 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. 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, ulcerative colitis, and arthritis.

Although typically indicative of “maturation”, the precise role for DC expression of CD83 in directing immune responses is not well understood. Downregulation of membrane-bound CD83 by RNA interference or by viruses such as human cytomegalovirus and herpes simplex virus type 1 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 with control and uninflamed areas, 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 whether this is an mDC-mediated process or, conversely, IFN-γ-producing T cells have 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. Moreover, activated CD1c⁺ mDCs in HIV-1-infected subjects were associated with numerous mucosal cytokines, including IL-23 and IL-1β. Within the mucosa, increased levels of IL-23 and IL-1β have been implicated in intestinal inflammation mediated, in part, through the promotion of T-cell-associated IFN-γ and IL-17 production. 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 LPS mDCs in the in vitro expansion and enhanced infection of Th1 and Th17 cells in response to exposure to commensal bacteria and HIV-1.

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 infection 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. 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. Systemically administered IL-10 stimulated the production of IFN-γ during human endotoxemia, 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 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 in vivo and in vitro observations suggest a role for intestinal mDCs in IL-10 production, the exact nature of the immune-regulatory vs. 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 Escherichia coli in the colon of chronically HIV-infected rhesus macaques, and increased levels of MT were due, in part, to ineffective phagocytosis by intestinal macrophages. 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. Despite an increase in LTA and LPS tissue levels and in the number of LTA⁺ macrophages in HIV-1-infected subjects, we only observed an increase in the number of LTA⁺ mDCs 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 this clinical study, levels of CD1c+ mDC activation correlated with mucosal HIV-1 viral load, suggesting that HIV-1 itself may have 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 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 manner via effects of HIV-1 on colonic pDCs 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 toward increased frequencies of intestinal pDCs, a significant increase in the numbers of CD40+ pDCs, and a decrease in the percentages of CD83-expressing pDCs were observed in HIV-1-infected subjects compared with uninfected controls. Further, the numbers of activated pDCs were directly associated with the 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 subjects as well as increased pDC frequencies with poly-functional cytokine phenotypes in pathogenic SIV infection models. Unlike mDCs, pDCs are rarely found in intestinal tissue under steady-state conditions, thus accumulation of activated pDCs during chronic HIV-1 infection is likely due to increased migration from the blood to the colon. In conclusion, we propose a model whereby colonic mDCs drive mucosal immune activation and inflammation during chronic untreated HIV-1 infection. HIV replication in the lamina propria results in epithelial barrier disruption, leading to the increased translocation of Gram-negative Prevotella into the LP, which synergizes with HIV-1 to induce a dysregulated mDC activation profile characterized by increased levels of CD40 and decreased CD83 expression. Activated mDCs subsequently induce increased T-cell activation via stimulation of bacteria-specific CD4 T cells through cell-cell contact (e.g., CD40/CD40L), production of inflammatory cytokines (interleukin (IL)-23, IL-1β), and potentially via CD83-mediated loss of T-cell regulation. mDCs produce IL-10 to compensate for increased pro-inflammatory cytokine production; however, this may also exacerbate interferon (IFN)-γ production. In total, this culminates in increased T-cell activation and expansion of Th helper type 1 (Th1), Th17, Th22, and IFN-γ-producing CD8 T cells. Activated Th1/17/22 cells are the targets for viral replication, 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.

**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 increased translocation of Gram-negative Prevotella into the LP, which synergizes with HIV-1 to induce a dysregulated mDC activation profile characterized by increased levels of CD40 and decreased CD83 expression. Activated mDCs subsequently induce increased T-cell activation via stimulation of bacteria-specific CD4 T cells through cell-cell contact (e.g., CD40/CD40L), production of inflammatory cytokines (interleukin (IL)-23, IL-1β), and potentially via CD83-mediated loss of T-cell regulation. mDCs produce IL-10 to compensate for increased pro-inflammatory cytokine production; however, this may also exacerbate interferon (IFN)-γ production. In total, this culminates in increased T-cell activation and expansion of Th helper type 1 (Th1), Th17, Th22, and IFN-γ-producing CD8 T cells. Activated Th1/17/22 cells are the targets for viral replication, 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.
IFN-γ-producing CD8 T cells. Activated Th1/17/22 cells are targets for viral replication, which ultimately results in their infection and depletion. 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 MT50–52 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 whether the microbiome is altered due to ongoing mDC-mediated mucosal inflammation or whether the increased abundances of pathobions induce mDC activation or whether both processes are involved.

**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 the study entry criteria, HIV-1-infected subjects were CART-naive or had not been on treatment for >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 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.13 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 data sets was obtained via BLAST53 of subject sequences against a database built from Silva23. Nomenclature classification of 16S rRNA sequence data sets was obtained via

**Plasma LTA, LPS, and sCD14 measurements.** Serum LTA levels were assessed using a custom enzyme-linked immunosorbent assay.16 LPS levels were measured in EDTA plasma samples using the Limulus Amebocyte Lysate assay (Lonza, Switzerland) following the manufacturer’s protocol as previously detailed.16 sCD14 levels were measured in heparin plasma using a commercially available enzyme-linked immunosorbent assay (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 enzyme-linked immunosorbent assay (R&D Systems).16

**Histological staining and analysis of colon biopsies.** Assessment of microbial product levels, CD11c+ mDCs, and Ham56+ macrophages in colonic LP is detailed extensively in Supplementary Materials. Using the Zeiss Zen Software (Zeiss, 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 the 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 CD11c+ mDCs to P. copri, P. stercoria, and R. bromii were assessed utilizing an ex vivo tissue culture model consisting of isolated colon LPMC from normal tissue16,18,19,49,55 and is detailed in Supplementary Materials.**

**Commensal bacteria stocks.** Expansion of P. copri (DSM# 18205, DSMZ, Braunschweig, Germany), P. stercoria (DSM# 18206), and R. bromii (ATCC# 27255, ATCC, Manassas, VA) was performed at 37°C under anaerobic conditions per the 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 g based on the frequency within viable, CD45+ cells, initial cell counts, and biopsy weights. Similarly, the percentage of activated colon CD4 and CD8 T cells as well as the percentage of cytokine+ CD4 and CD8 T cells were also converted to a total number per g 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 CD11c+ mDCs and CD11c+ mDCs and enumerate cytokine+ CD11c+ 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, San Jose, CA). Routine quality control using the Cytometer Setup and Tracking feature within the BD FACSDiva software version 6.1.2 (BD Biosciences) was performed daily as previously detailed.19
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 Mann–Whitney test and Friedman test with multiple Dunn comparison test for matched paired comparisons across multiple groups. To determine the differences between groups of matched paired data, Wilcoxon’s matched-pairs signed-rank test was performed. Correlations between variables were assessed using Spearman’s test. Fisher’s exact tests and Chi-squared tests were used for comparisons of categorical data. A P-value of < 0.05 was considered significant.

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

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DISCLOSURE
The authors declared no conflict of interest.

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