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HIV-1 Infection of Human Intestinal Lamina Propria CD4+ T Cells In Vitro Is Enhanced by Exposure to Commensal Escherichia coli

Stephanie M. Dillon,* Jennifer A. Manuzak,* Amanda K. Leone,* Eric J. Lee,* Lisa M. Rogers,* Martin D. McCarter,† and Cara C. Wilson*

Microbial translocation has been linked to systemic immune activation in HIV-1 disease, yet mechanisms by which microbes may contribute to HIV-associated intestinal pathogenesis are poorly understood. Importantly, our understanding of the impact of translocating commensal intestinal bacteria on mucosal-associated T cell responses in the context of ongoing viral replication that occurs early in HIV-1 infection is limited. We previously identified commensal Escherichia coli-reactive Th1 and Th17 cells in normal human intestinal lamina propria (LP). In this article, we established an ex vivo assay to investigate the interactions between Th cell subsets in primary human LP mononuclear cells (LPMCs), commensal E. coli, and CCR5-tropic HIV-1. Addition of heat-killed E. coli to HIV-1–exposed LPMCs resulted in increases in HIV-1 replication, CD4 T cell activation and infection, and IL-17 production. Conversely, purified LPS derived from commensal E. coli did not enhance CD4 T cell infection. E. coli exposure induced greater proliferation of LPMC Th17 than Th1 cells. Th17 cells were more permissive to infection than Th1 cells in HIV-1–exposed LPMC cultures, and Th17 cell infection frequencies significantly increased in the presence of E. coli. The E. coli-associated enhancement of infection was dependent on the presence of CD11c+ LP dendritic cells and, in part, on MHC class II-restricted Ag presentation. These results highlight a potential role for translocating microbes in impacting mucosal HIV-1 pathogenesis during early infection by increasing HIV-1 replication and infection of intestinal Th1 and Th17 cells.


One of the hallmarks of progressive HIV-1 disease is a gradual depletion of peripheral blood CD4+ T cells. However, HIV-1 infection is also characterized by a significant assault to the gastrointestinal (GI) tract, resulting in myriad structural and immunological complications. The impact of HIV-1 on the GI tract has been observed since the early days of research into the epidemic, when HIV-1 infection was associated with GI abnormalities such as diarrhea, weight loss, malnutrition, malabsorption, and villous atrophy (1). Recent studies have highlighted the central role played by the intestinal mucosal immune system in HIV-1 pathogenesis. Studies using a pathogenic SIV infection model demonstrated that significant and rapid depletion of intestinal CD4 T cells occurred as early as 7 d post infection (2–4) and was associated with early epithelial barrier dysfunction and high levels of viral replication (2, 4). Early in vitro studies demonstrated that human lamina propria (LP) CD4 T cells were also naturally permissive to HIV-1 infection and, unlike peripheral blood CD4 T cells, required no prior stimulation for productive infection (5). Indeed, >50% of human intestinal CD4 T cells are depleted during acute and early HIV infection, and significant depletion of these cells has been noted throughout all stages of HIV-1 disease (6–9). The susceptibility of LP T cells to HIV-1 or SIV infection at steady state likely relates to their increased activation status and expression of HIV/SIV coreceptors such as CCR5 and e4BP7 (10–14). Depletion occurs directly through lysis of infected cells (4, 15, 16) and indirectly through apoptosis of infected and uninfected CD4 T cells (15). With respect to HIV infection, higher HIV viral DNA and RNA levels were observed within GI tract CD4 T cells compared with peripheral blood CD4 T cells from subjects during acute and early infection, with infection detected in both activated and nonactivated mucosal CD4 T cells (9). In a recent study, d’Ettorre et al. (17) demonstrated that in chronically HIV-infected, untreated donors, HIV DNA load was greater in the gut mucosa than in peripheral blood.

Human Th17 cells are a subset of CD4 T cells that have been shown to produce IL-17A, IL-17F, IL-22, and IL-26 (18) and to play an important part both in mucosal defense against extracellular bacterial and fungal pathogens and in epithelial barrier maintenance and regeneration (19). Thus, the loss of these cells would be expected to have an impact on both intestinal homeostasis and immunity. A number of recent studies have highlighted that the specific depletion of Th17 cells is associated with disease progression in both SIV and HIV infections (20–24). Indeed, the preservation of Th17 cells during chronic SIV infection of sooty mangabeys has been associated with the nonpathogenic phenotype of this natural host of SIV (20).

HIV/SIV-associated epithelial barrier dysfunction and CD4 T cell depletion, in particular Th17 cell depletion, may contribute to
reduced protection from microbial products translocating from the lumen into the LP and into the systemic circulation. Using a model of intestinal inoculation of Salmonella typhimurium during acute SIV infection of rhesus macaques, Raffaelli et al. (25) observed depletion of Th17 cells, a decrease in epithelial barrier integrity, and the dissemination of S. typhimurium. In a recent study, Estes et al. (26) demonstrated the presence of not only microbial products such as LPS, but also Escherichia coli, in colonic LP and in lymph nodes of chronically infected rhesus macaques, providing more direct evidence for the occurrence of gut-associated microbial translocation in SIV infection. Indirect evidence for this phenomenon occurring in HIV-1 infection has also been demonstrated. Increased levels of microbial products were observed in the circulation of HIV-1–infected individuals and were found to be associated with T cell activation (27, 28).

Epithelial barrier dysfunction is known to occur early in SIV and HIV disease (2, 29–31) in association with mucosal translocation (26). However, the mechanisms by which translocated microbes induce mucosal immune dysfunction and impact viral replication remain poorly understood. We have previously identified significant frequencies of human LP effector CD4 T cells that produced IFN-γ and IL-17 in response to commensal bacteria in normal human intestinal tissue (32). The in vitro expansion of these bacteria-reactive T cells was dependent upon the presence of a subset of LP dendritic cells (DCs). We hypothesized that in the setting of HIV infection and a “leaky” gut barrier, LP mononuclear cells (LPMCs) would have increased exposure to bacteria and bacterial products. This exposure, in turn, would lead to the activation of bacteria-reactive T cells, increasing their permissiveness to HIV infection and replication. Using an in vitro assay that mimics the early interactions between HIV-1, commensal bacteria, and primary LPMCs, we present evidence to suggest not only that IL-17–producing intestinal CD4 T cells are preferentially infected, but also that productive infection is further enhanced in the presence of commensal E. coli. These results provide a mechanism for how microbial translocation may contribute to increased viral replication and CD4 T cell depletion in mucosal tissue.

Materials and Methods

**Tissue samples and preparation of LPMCs**

Human intestinal tissue samples (n = 10 jejenum, n = 11 colons) obtained from patients undergoing elective abdominal surgery were considered macroscopically normal, as previously described (32, 33). All patients undergoing surgery signed a release to allow the unrestricted use of discarded tissues for research purposes, and all protected patient information was de-identified to the laboratory investigators. This research was reviewed by the Colorado Multiple Institutional Review Board at the University of Colorado Anschutz Medical Campus and was granted exempt research status. LPMCs were isolated from tissue samples, and released LPMCs were cryopreserved and stored in liquid nitrogen, as detailed elsewhere (32, 33). Within all LPMC samples (n = 21), the median percentage of total viable cells was 75.5% (range 30.6–96.6%), and the median percentage of viable CD45+ LPMCs was 86.6% (range 54.5–97.7%), as assessed using flow cytometry staining protocols. Percentages of T cells as a fraction of viable CD45+ LPMCs were evaluated in the majority of samples: CD3+ T cells (76.4%, 53.7–95.2%; n = 20), CD4+ T cells (61.0%, 43.4–75.2%; n = 18), and CD8+ T cells (12.2%, 4.8–41.4%).

**Preparation of PBMCs**

Peripheral blood samples were collected from healthy adults, self-identifying as HIV-1 negative, who voluntarily gave written informed consent to participate. Collection of blood samples was approved by the Colorado Institutional Review Board at the University of Colorado Anschutz Medical Campus. PBMCs were isolated from heparinized blood samples by standard Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation, as described previously (34), and were cryopreserved and stored in liquid nitrogen, as detailed elsewhere (32, 33).

**Surface and intracellular flow cytometry staining assays**

Standard flow cytometry staining protocols for surface markers and for intracellular cytokine or HIV-1 p24 expression are detailed elsewhere (32, 33, 35). LP T cells were identified using CD45 (PerCP-Cy5.5; eBioscience, San Diego, CA), CD3 (FITC; BD Biosciences, San Jose, CA) and PE–Texas Red X, FCD; Beckman Coulter, Fullerton, CA, APC, APC-CY7, AF700; all BD Biosciences), and CD8 (APC; FICT; both BD Biosciences; and AF405, Invitrogen, Carlsbad, CA). PE-Cy5 CD38 and a matched isotype control (BD Biosciences) were used to evaluate T cell activation. Intracellular cytokines were detected using the following: V550 IL-17 and AF700 IFN-γ with V450 mouse IgG1 and AF700 mouse IgG1 used as isotype controls (all BD Biosciences). Intracellular HIV-1 p24 was detected using PE (BD Biosciences) (specific for the 25–35-, 34–37-, 24–29-, and 24–34-kDa proteins of the core Ags of HIV-1) and PE (RD1) mouse IgG1 as a matched isotype control (both Beckman Coulter). To evaluate CD11c and CD19 percentages in total LPMC populations and in CD11c/CD19-depleted LPMCs, the following Abs were used: PerCP-Cy5.5 CD45, PE-Cy5 CD11c, and APC-H7 CD19 (both from BD Biosciences). In all staining protocols, a Live/Dead Fixable Dead Cell Stain (Invitrogen) was included. At the completion of the staining procedure, LPMCs were resuspended in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) prior to acquisition on an LSRII Flow Cytometer (BD Biosciences).

**Commensal heat-killed E. coli and Bacteroides fragilis stocks and CCR5-tropic HIV-1Δifs stock**

E. coli stocks (no. 25922; ATCC, Manassas, VA) were expanded overnight in RPMI 1640 (Invitrogen) + 10% FBS (Sigma-Aldrich) at 37°C, 5% CO₂, or were plated on Brain Heart Infusion agar (BD Diagnostics, Sparks, MD) and incubated at 37°C for 1 d. B. fragilis stocks (no. 25285; ATCC) were expanded by culturing on Brucella plates (BD Diagnostics) and incubated at 37°C for 2 d in anaerobic conditions using a BD GasPack EZ Anaerobe Pouch System (BD Diagnostics). After expansion, bacteria were heat killed (HK) at 56°C for 2 h, washed, and resuspended at 3 × 10^6 bacteria per millilitre in Dulbecco’s PBS and stored in single-use aliquots at −20°C.

To prepare HIV-1 viral stocks for use in the in vitro assays, PBMCs were resuspended at 2 × 10^6 cells per millilitre in RPMI 1640 + 1% penicillin/streptomycin/glutamine (Sigma-Aldrich) + 10% human AB serum (complete medium [CM]; Gemini Bio-Products, West Sacramento, CA) and stimulated with 5 μg/ml PHA (Sigma-Aldrich) for 3 d at 37°C, 5% CO₂. PHA-stimulated PBMCs were collected and resuspended at 1 × 10^6 cells per millilitre in CM + 10 U/ml rIL-2 (Roche, Indianapolis, IN) and cultured with CCR5-tropic HIV-1Δifs (catalog no. 510, National Institutes of Health AIDS Research and Reference Reagent Program, Germantown, MD) for 7 d at 37°C, 5% CO₂. Fresh PHA-stimulated PBMCs, resuspended at 1 × 10^6 cells per millilitre in CM + 10 U/ml rIL-2 were added daily as additional “feeder cells.” At day 14 after initial viral infection, supernatants were collected and centrifuged at 1400 rpm for 10 min to remove cells and cellular debris. CCR5-tropic HIV-1Δifs supernatants were frozen in single-use aliquots at −80°C. HIV-p24 content of these viral stocks was determined using the HIV-1 p24 ELISA (PerkinElmer, Waltham, MA), and viral stock concentrations ranged from 0.11 to 0.26 μg/ml.

**Mitogenic stimulation of LPMCs**

LPMCs were resuspended at 1 × 10^6 cells per millilitre in CM + 500 μg/ml piperacillin/tazobactam (Wyeth, Madison, NY) ≤ 250 μg/ml amphotericin B (Invitrogen) and stimulated with 100 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of 1 μg/ml brefeldin A (Golgi Plug; BD Biosciences) for 5 h at 37°C, 5% CO₂. LPMCs were collected and intracellular percentages of IFN-γ and IL-17–producing cells determined by intracellular flow cytometry (IFC) assay.

**In vitro infection assays**

PBMCs or LPMCs were resuspended at 1 × 10^6 cells per millilitre in CM + 500 μg/ml piperacillin/tazobactam and cultured for 15–20 h with 0.08–0.2 μg per millilitre of CCR5-tropic HIV-1Δifs. LPMCs or PBMCs were washed in warm Dulbecco’s PBS and resuspended at 1 × 10^6 cells/ml in CM + 500 μg/ml piperacillin/tazobactam and cultured with or without HK bacteria at 5 bacteria: 1 LPMC or PBMC in 48-well plates for 2–3 d at 37°C, 5% CO₂. In some assays, ultrapure LPS derived from commensal E. coli strain K12 (10 μg/ml; InvivoGen, San Diego, CA) was added to HIV-1Δifs-exposed LPMCs in 96-well plates. To block MHC class II presentation, LEAF Purified mouse IgG2a isotype (BioLegend, San Diego, CA) was added to LPMCs in 96-well plates 30 min prior to the addition of E. coli and again up to 24 h later. LEAF Purified mouse IgG2a isotype (BioLegend) was used as the control Ab.
At the completion of the culture period, culture supernatants were collected and frozen at −20°C for subsequent assessment of secreted INF-γ, IL-17, and HIV-1 p24. LPMCs or PBMCs were collected and either immediately assessed for surface expression of CD38 and/or intracellular expression of HIV-1 p24 by flow cytometry or underwent mitogenic stimulation, with intracellular HIV-1 p24 percentages within cytokine-producing cells determined by IFC assay.

**T cell proliferation assays**

LPMCs were prelabeled with 5 μM CFSE (Invitrogen) in HBSS for 15 min at 37°C and washed with CM. CFSE-labeled LPMCs were resuspended at 1 × 10⁶ cells/ml in CM and cultured with or without HK E. coli (5 E. coli:1 LPMCs) for 7 d at 37°C, 5% CO₂. PHA (5 μg/ml; Remmel, Lenexa, KS) was used as a positive control, and all samples had PHA-induced CD4⁺ T cell proliferation >50% (range: 52.7–98.8%; data not shown). To determine proliferation of cytokine-producing cells, total LPMCs were then collected following the addition of 7 d of in vitro culture, underwent mitogenic stimulation, and were assessed for intracellular cytokine expression by IFC assay.

**Identification of cytokine-producing bacteria-reactive LP CD4 T cells**

LPMCs were resuspended at 1 × 10⁶ cells/μl in CM and cultured for 4 h with E. coli or B. fragilis (5 bacteria:1 LPMC) prior to the addition of 1 μg/ml brefeldin A. After an additional 14–16 h, LPMCs were collected and frequencies of CD4⁺ T cells producing INF-γ or IL-17 were determined by IFC assay.

**Depletion of CD11c⁺ and CD19⁺ LP cells**

LPMCs were stained with biotinylated CD11c (Miltenyi Biotec, Auburn, CA) and biotinylated CD19 (eBioscience). Control LPMCs were resuspended in buffer only. In all cases, FCR blocking reagent (Miltenyi Biotec) was added. Biotinylated Ab-bound cells were incubated with 32 °C Dynabeads (Invitrogen) per 1 × 10⁶ LPMCs for 30 min at 4°C under constant rotation. Dynabead-bound LPMCs were magnetically removed following the manufacturer’s recommended protocol. CD11c⁺ LPMCs were depleted by a median 80.3% (range: 71.4–93.8%), and CD19⁺ LPMCs were depleted by 91.4% (82.3–95.6%), compared with total viable CD4⁺ LPMCs (n = 7). Following depletion of CD11c⁺ and CD19⁺ LPMCs, the percentage of CD3⁺ T cells increased from 78.7% (range: 60.9–88.8%) to 85.2% (range: 69.6–93.9%) of viable CD4⁺ LPMCs. For LPMCs samples depleted of CD11c only (n = 3), CD11c⁺ LPMCs were depleted by 85.5% (range: 82.6–95%). LPMCs were either labeled with CFSE and cultured with or without HK E. coli (5 E. coli:1 LPMC), as described above, for 5 d or were pre-exposed to 10⁵ E. coli (0.08 μg p24 per million) for 2–3 d. As the ratio of E. coli:1 LPMC decreased from 5:1 to 0.2:1, a trend toward decreasing levels of CD3⁺ T cells was ascertained by 38 gating. In our initial in vitro infection assays, we observed significant downregulation of CD4 on CD3⁺ LP T cells in infected LPMC cultures (data not shown); therefore, we assessed expression of intracellular HIV-1 p24 in CD3⁺ CD8⁻ T cells (Fig. 1C). Addition of E. coli to HIV-1 infected LPMCs also resulted in significantly higher percentages of CD8⁻ T cells expressing intracellular HIV-1 p24 than in infected cultures without bacteria (Fig. 1D).

**Flow cytometric acquisition and analysis**

For all in vitro assays, a lymphocyte gate was established using forward scatter-area and side scatter-area properties within viable LPMCs. Doublets were then excluded using a forward scatter-height versus forward scatter-width dot plot. A total CD3 gate was established from within this population gated total CD3⁺ LPMCs. In all assays, except for the in vitro infection assays, CD4 T cells were then gated from within the CD3 gate. In our initial in vitro infection assays, we observed significant downregulation of CD4 on CD3⁺ LP T cells in infected LPMC cultures (data not shown); therefore, a CD8⁺ gate was established to include all CD4 T cells for these assays. To evaluate expression of CD38, intracellular p24, and intracellular cytokines, matched isotype Abs were used to establish background staining and then specific expression was determined by removing the background staining. Specific proliferation in response to E. coli was ascertained by establishing a CFSE depletion gate based on the unstimulated condition. All flow cytometry data were acquired on an LSR II Flow Cytometer. To control for the accuracy and precision of measurements taken over the course of the study, 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 (35).

**IFN-γ, IL-17, and HIV-1 p24 ELISAs**

The recommended manufacturer’s protocols were followed for IFN-γ (BD Biosciences), IL-17 (eBioscience), and HIV-1 p24 ELISAs. The lower detection limits were 4.7 pg/ml for IFN-γ, 4 pg/ml for IL-17, and 12.5 pg/ml for HIV-1 p24.

**Statistical analysis**

Nonparametric statistics were used. The Wilcoxon matched-pairs signed rank test was used to evaluate paired data. The Friedman test was used for matched-paired comparisons across multiple groups, with a multiple Dunn comparison test performed when the overall p value was < 0.05. When nonparametric tests failed owing to very small sample sizes, parametric tests were used. A paired t test was used to evaluate paired data. The repeated-measures ANOVA was used for matched-paired comparisons across multiple groups, with a Bonferroni multiple comparison test performed when the overall p value was < 0.05. All statistical analyses were done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

**Results**

**Exposure to commensal E. coli increases HIV-1 replication and infection of LP T cells in vitro**

In agreement with previous studies (5), we observed productive infection of LPMCs exposed to HIV-1_bal in the absence of exogenous stimulation (Fig. 1). LPMCs pre-exposed to HIV-1_bal + E. coli significantly produced higher levels of HIV-1 p24 compared with those with virus only (Fig. 1A). To determine if increased viral replication and infection in the presence of commensal E. coli was specific to intestinal T cells, we obtained PBMCs from unmatched donors and exposed them to HIV-1_bal and E. coli, using the same in vitro infection protocol (n = 9). Evaluation of HIV-p24 levels within culture supernatants failed to demonstrate an increase in HIV-p24 levels when PBMCs were cultured with HIV-1_bal + E. coli versus HIV-1_bal alone, and, rather, a decrease in HIV-p24 was noted (Fig. 1B). Thus, in contrast to HIV-1_bal + E. coli–exposed LPMCs, we did not observe a similar enhancing effect of bacteria on HIV replication in HIV-exposed PBMCs, demonstrating the gut specificity of this process.

To enumerate the percentage of productively infected LP CD4 T cells, we assessed intracellular p24 expression, using flow cytometry. We observed significant downregulation of CD4 on CD3⁺ LP T cells in infected LPMC cultures (data not shown); therefore, we assessed expression of intracellular HIV-1 p24 in CD3⁺ CD8⁻ T cells (Fig. 1C). Addition of E. coli to HIV-1_bal-exposed LPMCs also resulted in significantly higher percentages of CD8⁻ T cells expressing intracellular HIV-1 p24 than in infected cultures without bacteria (Fig. 1D).

**Exposure to HIV-1_bal and commensal E. coli increases T cell activation and cytokine production in vitro**

The T cell activation state is closely linked to the efficiency of HIV-1 infection and replication; therefore, we also assessed CD38 expression, an indicator of T cell activation (14), on T cells in LPMC cultures stimulated in vitro, as described above, using flow cytometry. The addition of E. coli to HIV-1–exposed LPMCs significantly increased CD38 expression on CD8⁻ T cells observed in HIV-1_bal–only cultures (Fig. 2A). CD38 expression on CD8⁻ T cells exposed to HIV-1_bal and E. coli [median mean fluorescence intensity (MFI): 547; range: 404–1736] was also greater than that on CD8⁻ T cells exposed to HIV-1_bal only (MFI: 577; range: 317–1101), although this difference failed to reach statistical significance (p = 0.07). The absolute levels of CD38 expression on CD8⁻ T cells (MFI: 886; range: 745–3011) were higher in response to HIV-1_bal + E. coli, than levels observed on CD8⁻ T cells (MFI: 547; range: 404–1736; p = 0.004).

Low levels of IFN-γ and IL-17 were detected in culture supernatants from LPMCs exposed to HIV-1_bal only (Fig. 2B, 2C). However, both IFN-γ and IL-17 significantly increased in the presence of E. coli (Fig. 2B, 2C). In a subset of experiments, LPMCs were pre-exposed to HIV-1_bal and cultured with a range of doses of E. coli for 2–3 d. As the ratio of E. coli to LPMCs decreased from 5:1 to 0.2:1, a trend toward decreasing levels of
CD38 expression on CD8\(^+\) T cells and decreasing IFN-\(\gamma\) and IL-17 production was observed (data not shown), showing bacterial dose-dependent effects on CD8\(^+\) T cell activation.

Statistically higher levels of IFN-\(\gamma\) and IL-17, and a trend toward higher levels of HIV-1 p24, were detected in culture supernatants from jejunal LPMCs compared with colon LPMCs stimulated with E. coli after pre-exposure to HIV-1Bal (Supplemental Table I). However, irrespective of anatomical location, the amount of cytokines produced and HIV-1 p24 levels were always greater following combined HIV-1Bal and bacterial exposure than those observed in HIV-1Bal only.

**Purified LPS from commensal E. coli does not increase HIV-1 infection within LPMCs**

Increased levels of LPS in the blood of HIV-infected individuals has been associated with systemic immune activation (27), and increased LPS levels were observed in the colon of SIV-infected rhesus macaques during the late acute stage of SIV infection (26).
Therefore, we wished to determine whether exposure of LPMCs to purified commensal *E. coli* increases HIV-1 replication within CD8+ T cells, as was observed with whole *E. coli*. As in previous assays, the addition of whole *E. coli* increased HIV-1 infection and replication within CD8+ T cells and production of IFN-γ and IL-17 within total LPMCs (Fig. 3A–D). However, the addition of purified *E. coli*-derived LPS did not increase levels of HIV-1 p24, indicating an inherent preference of HIV-1Bal for LP Th17 cells. High percentages of CD4+ T cells capable of producing IFN-γ were measured in primary LPMCs (median: 65.2% of CD4+ T cells; range: 33.6–73.2%) after 5-h stimulation with PMA/ ionomycin, with lower frequencies found of CD4+ T cells capable of producing either IL-17 alone (median: 1.9%; range: 1.4–10.6%) or coproducing both IL-17 and IFN-γ (median: 1.0%; range: 0.1–5.6%) (Supplemental Fig. 1).

To determine the proliferative capacity of the cytokine+ CD4+ T cell subsets in response to bacteria, LPMCs were initially labeled with CFSE, infected with HIV-1, then cultured with bacteria. *E. coli*-induced proliferation was detectable by flow cytometry after 5–7 d, but viability of HIV-exposed LPMCs substantially decreased after 5 d in culture. Thus, the proliferative capacity of specific cytokine+ CD4+ T cell subsets in response to *E. coli* was subsequently determined after 7 d of stimulation with *E. coli* in the absence of HIV-1Bal. In agreement with our previous study (32), we observed a significant increase in the percentage of CFSE+ proliferating CD4+ T cells in LPMCs cultured in the presence of *E. coli*, compared with unstimulated cultures (Fig. 4A). An example of the gating strategy used to evaluate proliferation of the cytokine+ CD4+ T cell subsets is shown in Fig. 4B. A hierarchy of proliferation within the specific cytokine populations in response to *E. coli* was observed (Fig. 4C). The highest fraction of proliferating cells was observed among the IL-17/ IFN-γ-coproducing CD4+ T cells, with lower percentages in the IL-17+ IFN-γ+ CD4+ T cell population and the lowest fraction occurring within IFN-γ+ IL-17+ CD4+ T cells (Fig. 4C). A trend toward greater proliferation of all IL-17–producing CD4 T cells than of total IFN-γ–producing T cells was noted (p = 0.06; data not shown).

Infection frequencies of IFN-γ+ and IL-17+–producing T cell subsets increase in response to exposure to HIV-1Bal and commensal *E. coli* in vitro

We next assessed whether expansion of IL-17+ CD4+ T cells in response to *E. coli* (Fig. 4) corresponded to greater levels of productive infection within this population of cytokine-producing T cells. Although intracellular HIV-1 p24 was detected in both IFN-γ and IL-17–producing CD8+ T cells following HIV-1 infection, a significantly higher percentage IL-17–producing T cells expressed HIV-1 p24, indicating an inherent preference of HIV-1Bal for LP Th17 cells (Fig. 5A). The percentage of infected IL-17+ and IFN-γ+ CD8+ T cells increased post infection with HIV-1Bal in the presence of *E. coli*, with the fraction of infected IL-17+ CD8+ T cells remaining statistically higher than IFN-γ+ CD8+ T cells (Fig. 5A). We next evaluated intracellular HIV-1 p24 expression within each cytokine-producing T cell subset in response to HIV-1Bal + *E. coli*, using the gating shown in Fig. 5B. The highest median percentage of HIV-1 p24+ cells was observed in the IL-17+IFN-γ+ CD8+ T cell population and the next highest percentage observed within CD8+ T cells producing IL-17 only (Fig. 5C). Elevated percentages of HIV-1 p24+ cells were observed in IFN-γ+ IL-17+ CD8+ T cells compared with non-cytokine-producing T cells, but these were lower than those noted for either of the IL-17–producing populations (Fig. 5C).

The ability of specific bacteria to enhance HIV-1 infection is related to frequencies of bacteria-reactive CD4 T cells in LPMCs

To determine whether enhancement of HIV-1 replication in LPMCs was unique to *E. coli* or was generalizable to other enteric bac-
teria, we compared T cell infection frequencies and cytokine responses to E. coli versus those to B. fragilis, another Gram-negative commensal organism. The Bacteroides group has been shown to be an abundant taxa in intestinal mucosal biopsies (36) and therefore might be likely to translocate into the intestinal LP during epithelial barrier breakdown. However, when LPMCs were pre-exposed to HIV-1Bal and then cultured with either E. coli or B. fragilis, B. fragilis failed to induce the increases in HIV-1 replication, T cell activation and infection, and cytokine production that were observed following E. coli exposure (Fig. 6A–D). To determine whether these differential responses were potentially related to differences in frequencies of resident bacteria-reactive LP CD4+ T cells, the frequencies of IFN-γ– and IL-17–producing CD4+ T cells responding to each bacterial species were measured in normal LP CD4+ T cells, the frequencies of IFN-γ– and IL-17–producing CD4+ T cells that recognized E. coli than those that recognized B. fragilis (Fig. 6E).

**Commensal E. coli-associated proliferation, cytokine production, and enhancement of infection of LP CD4+ T cells require the presence of CD11c+ LP cells and are, in part, MHC class II restricted**

We previously showed that bacteria-specific LP T cell proliferation was dependent on the presence of a subset of LP DCs (32). We wished to determine if the E. coli-associated increase in HIV-1 infection of LP T cells observed in our current in vitro assays was also dependent on the presence of APCs or, rather, represented a direct effect of E. coli on T cells. To enrich for T cells, LPMCs were pre-exposed to HIV-1Bal and then cultured with or without HK E. coli or purified LPS derived from commensal E. coli (Strain K12) for 2–3 additional days. (A–D) Cumulative data showing (A) p24 levels within culture supernatants, (B) net percentages of intracellular HIV-1-p24+ CD3+ CD8+ T cells within LPMC cultures, and (C) IFN-γ and (D) IL-17 levels within culture supernatants. Each symbol represents an individual sample (n = 4 jejunums). Statistical significance was determined using the repeated measures ANOVA (overall p value) with a Bonferroni multiple comparison test.

**E. coli**-derived LPS does not increase HIV-1 replication and infection of LP T cells or IFN-γ and IL-17 production in vitro. LPMCs were pre-exposed to CCR5-tropic HIV-1Bal and cultured with or without HK E. coli or purified LPS derived from commensal E. coli (Strain K12) for 2–3 additional days. (A–D) Cumulative data showing (A) p24 levels within culture supernatants, (B) net percentages of intracellular HIV-1-p24+ CD3+ CD8+ T cells within LPMC cultures, and (C) IFN-γ and (D) IL-17 levels within culture supernatants. Each symbol represents an individual sample (n = 4 jejunums). Statistical significance was determined using the repeated measures ANOVA (overall p value) with a Bonferroni multiple comparison test. **p < 0.01, ***p < 0.001.

**FIGURE 3.** Exposure to HIV-1Bal and commensal E. coli-derived LPS does not increase HIV-1 replication and infection of LP T cells or IFN-γ and IL-17 cytokine production in vitro. LPMCs were pre-exposed to CCR5-tropic HIV-1Bal and cultured with or without HK E. coli or purified LPS derived from commensal E. coli (Strain K12) for 2–3 additional days. (A–D) Cumulative data showing (A) p24 levels within culture supernatants, (B) net percentages of intracellular HIV-1-p24+ CD3+ CD8+ T cells within LPMC cultures, and (C) IFN-γ and (D) IL-17 levels within culture supernatants. Each symbol represents an individual sample (n = 4 jejunums). Statistical significance was determined using the repeated measures ANOVA (overall p value) with a Bonferroni multiple comparison test. **p < 0.01, ***p < 0.001.
within CD3+ CD4+ T cells in unstimulated cultures and within cultures stimulated with E. coli and IL-17–expressing CD4+ T cells were determined using isotypes. The CFSElo gate within IL-17+ IFN-γ+ population, followed by a 5-h mitogenic stimulation. (**Gating strategy used to determine the percentages of proliferating cells within each cytokine+ T cell population in response to E. coli, CD3+ CD4+ T cells were gated from within a viable lymphocyte gate with a doublet discrimination gate applied (profiles not shown). IFN-γ- and IL-17–expressing CD4+ T cells were determined using isotypes. The CFSElo gate within IL-17+ IFN-γ-, IL-17+ IFN-γ-, and IFN-γ- IL-17+ CD4+ T cells was established from unstimulated cultures. (C) Cumulative results from 6 samples (n = 3 jejunums; ● n = 3 colons, ○). Statistical significance determined using the Friedman test with a Multiple Dunn comparison test. **p < 0.01.

We have previously shown that expansion of E. coli–specific LP T cells was partially dependent on HLA-DR molecules (32). To further delineate potential mechanisms behind the current observations that LP DCs were required for the E. coli–associated enhancement of LP T cell infection, we evaluated levels of infection in the presence of an HLA-DR blocking Ab. Minimal effect of HLA-DR blocking on the levels of HIV-1 infection was observed in the absence of E. coli (Fig. 7D). However, significantly reduced levels of HIV-1 p24 were detected in culture supernatants after stimulation of HIV-1-p24–exposed LPMCs to E. coli in the presence of the HLA-DR blocking Ab (Fig. 7D), with levels in the presence of anti–HLA-DR Ab decreasing by 56.2% (SEM: 9.5%) relative to levels detected in the presence of the isotype control. Taken together, these results show that the bacterial enhancement of HIV replication and infection within LP CD4 T cells does not occur through direct stimulation of T cells but, rather, requires the presence of CD11c+ DCs and is partially MHC class II restricted.

Discussion
In recent years, Th17 cell dynamics during HIV infection have been extensively studied, and it is well established that significant depletion of these cells occurs both in peripheral blood (20, 37–40) and in the intestinal mucosa (20, 22, 24, 41). Decreases in intestinal Th17 cells during HIV infection may be a factor contributing to the translocation of bacteria and bacterial products into the LP (26) and into the systemic circulation of HIV-1–infected individuals (27, 28). Indeed, microbial translocation occurs throughout the course of HIV disease and has been associated with systemic immune activation, a predictor of disease progression (27, 28, 42, 43). A link between microbial translocation and poor clinical outcomes, such as cardiovascular disease, dementia, and mortality, has also been suggested (44–46).

The mechanisms underlying the depletion of Th17 cells are being actively investigated. HIV infection of peripheral blood Th17 cells has previously been demonstrated both in vivo (20) and in vitro (37, 39, 47). To evaluate whether Th17 cells were preferentially infected in vivo, Brenchley et al. (20) stimulated PBMCs from HIV-1–infected donors with anti-CD3 and observed no preferential infection of peripheral blood Th1 or Th17 cells. In chronically SIV-infected rhesus macaques, spleen IFN-γ+ and IL-17+ T cells had similar levels of SIV DNA (21). In contrast, Gosselin et al. (37) identified IL-17–producing T cells by surface expression of CCR6 and observed higher levels of integrated DNA within CCR6+ memory T cells relative to CCR6− memory T cells isolated from untreated, viremic HIV-infected individuals, suggesting that preferential infection of Th17 cells in vivo may in fact occur. Further, in vitro infection studies have suggested that peripheral blood Th17 cells were more permissive to HIV infection than were Th1 cells (37, 47). However, studies to address the mechanisms responsible for HIV-associated depletion of intestinal Th17 cells have been hampered by the small numbers of LPMCs typically obtained from clinical gut biopsies.

In the current study, we evaluated how HIV-1 replication and infection of resident LP T cells were influenced by exposure of LPMCs to commensal bacteria, to better understand the mechanisms by which microbial translocation might contribute to HIV pathogenesis at the mucosal level, particularly during early infection, when epithelial barrier breakdown, increased translocation of intestinal microbes, and increased viral replication are known to occur. Using an in vitro system modeling these early interactions between LPMC, HIV-1, and translocated whole bacteria, we demonstrate that exposure to commensal E. coli increases the activation and expansion of resident E. coli-reactive T cells resulting in increased HIV-1 replication and infection of IFN-γ− and IL-17–producing CD4 T cells. A hierarchy of infection was noted, with the greatest fraction of HIV-1 p24-expressing cells observed within the IL-17+ IFN-γ− population, followed by cells producing only IL-17, and finally those that produced IFN-γ only. This infectivity profile paralleled the proliferation profile of Th1 and Th17 subsets in response to E. coli. Furthermore, the ability of E. coli to enhance HIV-1 replication in LPMCs was...
linked to the frequency of resident LP CD4 T cells able to recognize that specific bacterial species, as HIV-1 replication was not enhanced in response to *B. fragilis*, a commensal organism recognized by few LP T cells.

It is well established that activated/memory T cells, including HIV-1–specific CD4 T cells, are preferentially infected by HIV-1 (14, 48–51). Studies have also implicated pathogenic bacteria in enhancing HIV-1 replication. Bacterial vaginosis-associated microbiota were shown to enhance HIV replication within a promonocytic cell line chronically infected with HIV-1 provirus (52); and in HIV-1–infected subjects with active tuberculosis, higher levels of HIV gag DNA were observed in *Mycobacterium tuberculosis*-specific peripheral CD4 T cells than in total memory CD4 T cells (53). In addition, this group demonstrated higher HIV infection rates within *M. tuberculosis*-specific CD4 T cells following in vitro mitogenic stimulation (53). Within the intestine, the activated phenotype of LP T cells has been linked to their natural susceptibility to HIV infection and to their rapid subsequent depletion (5, 14). The activated state of intestinal T cells may be partially explained by the presence of commensal bacteria-reactive T cells (32). Our current study expands on these previous findings by addressing the potential impact of translocating commensal bacteria on the local immune response within the intestinal LP during HIV-1 infection. Notably, our study suggests that the translocation of certain enteric bacteria across a leaky epithelial barrier into the intestinal LP could increase the pool of activated resident LP CD4 T cells, particularly Th17 cells, resulting in increased infection rates. These in vitro findings provide a physiological basis by which translocating microbes might contribute to mucosal Th17 cell depletion, but this theoretical process remains to be verified as a true mechanism of HIV-1 pathogenesis in vivo.

In studies to address the potential mechanisms behind the bacteria-associated enhancement of HIV-1 infection and replication within LP T cells, we demonstrated that exposure of infected LPMCs to purified LPS, a known TLR4 agonist (54), did not result in greater productive HIV-1 infection of LP Th17 cells coproducing IFN-γ. LPMCs (n = 4 jejunums, ○; n = 3 colons, □) were pre-exposed to CCR5-tropic HIV-1 and cultured with or without HK *E. coli* for 3 additional days, followed by a short-term mitogenic stimulation. (A) Net percentages of IL-17+ CD3+ CD8− (Total IL-17) or IFN-γ− CD3+ CD8− (Total IFN-γ) T cells expressing HIV-1 p24 within LPMC cultures in response to HIV-1 and to HIV-1 + *E. coli*. Statistical significance was determined using the Wilcoxon matched-pairs signed rank test. (B) Gating strategy to determine net HIV-1 p24 expression within CD3+ CD8− T cell cytokine+ subsets (IL-17+ IFN-γ−, IL-17+ IFN-γ+, IL-17− IFN-γ−, and IL-17− IFN-γ+) in response to HIV-1 + *E. coli*. (C) Cumulative percentages of net CD3+ CD8− cytokine+ HIV-1 p24+ T cells within LPMC cultures in response to HIV-1 + *E. coli*. Statistical significance was determined using the Friedman test with a Multiple Dunn comparison test for each of the cytokine+ T cell populations relative to IFN-γ− IL-17− CD3+ CD8− T cells. *p < 0.05, **p < 0.001.
in the increases in HIV-1 replication that were observed using whole bacteria. We previously identified MHC class II-restricted, Th1 and Th17 bacteria-reactive T cells within the LP of normal human intestinal tissue (32) and showed that their proliferation was DC dependent. We now show that the ability of bacteria to enhance HIV-1 replication in LP CD4 T cells is also dependent on the presence of LP DCs, and is, in part, MHC class II restricted. Taken together, these findings suggest that innate stimulation of single bacterial TLR alone is insufficient to result in T cell activation and increased infection and that Ag presentation by DCs is likely critical to the expansion of resident bacteria-reactive CD4 T cells necessary for increased HIV-1 replication. However, other factors, such as cytokines, are also probably involved in the enhancement of HIV-1 replication, as MHC class II blockade only partially inhibited this process. The bacterial Ags, either alone or in combination, that are responsible for the observed enhancement of infection and replication, as well as the DC-derived cytokines or chemokines involved, remain to be determined.

One of the more intriguing observations made in our study is the bacteria-induced selective expansion and infection of Th17 cells that coproduce IFN-γ. Although not extensively researched, this Th17/Th1 subset has been described in other investigations addressing human peripheral blood Th17 responses in the setting of HIV-1 infection. IL-17/IFN-γ–coproducing T cells expressed CCR5 and were productively infected after exposure to CCR5-tropic HIV-1 in vitro (37, 47), and in one study, the highest levels of in vitro infection and depletion were noted in IL-17+ IFN-γ+ CD4 T cells (47). During early HIV infection, higher frequencies of HIV-specific IL-17+ IFN-γ+ blood CD4 T cells have also been noted in vivo (55). To the best of our knowledge, this current study is the first to show that commensal E. coli induced expansion of human LP IL-17+ IFN-γ+ CD4 T cells, increasing the frequency of productively infected cells when concurrently exposed to CCR5-tropic HIV-1 in vitro.

In a recent study tracking IL-17–producing cells in an in vivo mouse model, Hirota et al. (56) demonstrated that IL-23 was necessary not only for the induction of full effector function of Th17 cells, but also for induction of the Th1-associated transcription factor Tbet and the subsequent switch of these cells to an IL-17+ IFN-γ+ T cell phenotype. Importantly, this plasticity of IL-17–producing cells appeared only during experimental autoimmune encephalomyelitis, a chronic inflammatory disease, and not
postinfection of the skin with *Candida albicans*, which resulted in acute inflammation, rapid clearance of the fungus, and a relatively anti-inflammatory environment. A proinflammatory environment as a requirement for “switching” of Th17 cells to IL-17/IFN-γ-coproducing T cells has also been observed in a mouse model of ocular inflammation (57). Thus, in the context of our HIV-1-infection assay, we speculate that the expansion of these cells and their increased infection in the presence of HIV-1 and *E. coli* in vitro may result in part from the proinflammatory milieu generated. However, further detailed analysis of LP Th1/Th17 cells, including their expression of Th1 and Th17 transcription factors and dependence on specific cytokines induced by enteric bacteria, is necessary to define a possible role for them in HIV-1-associated mucosal immune dysfunction.

Why do IL-17–producing LP CD4 T cells, and in particular those that coproduce IL-17 and IFN-γ, constitute the greatest fraction of infected cells after exposure to HIV-1 and *E. coli*? Several studies have shown that intestinal Th17 cells are highly infectable, which may be in part attributable to the high level of baseline activation of LP CD4 T cells (14), as well as the expression of HIV coreceptors. LP CD4 T cells express high levels of CCR5 (5, 58), and CCR5-expressing LP Th17 cells were shown to be preferentially depleted in HIV-infected subjects (20). In addition, peripheral blood IL-17+ IFN-γ+ CD4 T cells were shown to highly express CCR5 (37, 47). Recently, HIV and SIV have been demonstrated to bind the gut-homing receptor α4β7 on peripheral blood CD4+ T cells in vitro and in vivo (10, 11), and the α4β7hi T cells contained a substantial proportion of IL-17–producing cells (11). Intestinal α4β7hi CD4 T cells were also preferentially infected in the early stages of SIV infection (59). Thus, expression of α4β7, as well as CCR5, may explain the greater infection frequency of intestinal Th17 cells in our in vitro infection assay. In addition, peripheral blood Th17 cells were shown to have a reduced ability to produce MIP-1α and MIP-1β, CCR5 ligands associated with blocking of HIV replication, relative to IFN-γ–producing T cells, thus providing another potential mechanism for a greater infectivity rate of IL-17–versus IFN-γ–producing T cells (47).

Our data suggest that the intrinsic targeting of IL-17–producing cells by HIV-1 can be further enhanced upon exposure to *E. coli*, a process that appears to be dependent upon the baseline frequency of bacteria-reactive cells and their capacity to proliferate upon bacterial exposure. In particular, the selective activation and expansion of IL-17/IFN-γ-coproducing cells in response to bacteria likely contributes to their selective targeting by HIV-1 in our in vitro model system. Given the pivotal role that Th17 cells play in mucosal homeostasis and epithelial barrier function (19), the infection and depletion of a relatively small number of Th17 cells could have a significant impact on mucosal immunity and the clinical outcome of HIV infection. Additional in vivo work is
necessary to determine whether commensal bacteria enhance the targeting of Th17 subsets in the setting of HIV-1 infection.

Although depletion of LP CD4+ T cells during HIV infection is likely multifactorial, our study provides one potential mechanism by which translocation of selected enteric bacteria from the intestinal lumen into the LP could enhance the infection and depletion of LP CD4+ T cells, especially those producing IL-17 or coproducing IL-17 and IFN-γ. On the basis of our results, we propose that during early HIV infection—as HIV replicates in the intestinal LP, resulting in inflammation and damage to the integrity of the epithelial barrier—increased exposure of LP CD4+ T cells to translocating commensal bacteria would lead to the activation, expansion, and increased infection and depletion of bacteria-reactive Th17 and Th17/Th1 LP cells. In this study, an in vitro model was used to address the dynamic interactions between HIV-1, commensal bacteria, and LPMCs that would be harder to capture in "snapshots" using clinical samples. However, clinical studies are necessary to confirm that these in vitro findings are relevant to mucosal pathogenesis in either early or chronic HIV-1 infection. An understanding of the role of microbial translocation in mucosal pathogenesis and its attendant mechanisms will be important in facilitating the development of therapeutic approaches aimed at blocking this process at the mucosal level.

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Disclosures

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