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Mucosal Th17 Cell Function Is Altered during HIV Infection and Is an Independent Predictor of Systemic Immune Activation

Connie J. Kim,^{*} Lyle R. McKinnon,^{*,†} Colin Kovacs,[‡] Gabor Kandel,^{*,§} Sanja Huibner,^{*} Duncan Chege,^{*} Kamnoosh Shahabi,^{*} Erika Benko,[‡] Mona Loutfy,^{*,‡,¶} Mario Ostrowski,^{*,§,||} and Rupert Kaul^{*,||,#}

Mucosal Th17 cells maintain the gut epithelial barrier and prevent invasion by luminal bacteria through a delicate balance of immunosuppressive and proinflammatory functions. HIV infection is characterized by mucosal Th17 depletion, microbial translocation, and immune activation. Therefore, we assessed the function of blood and sigmoid Th17 cells during both early and chronic HIV infection, as well as the impact of short- and long-term antiretroviral therapy. Th17 cells were defined as IL-17a⁺ CD4 T cells, and their functional capacity was assessed by the coproduction of the inflammatory cytokines IL-22, TNF- α , and IFN- γ , as well as the immunoregulatory cytokine IL-10. Gut Th17 cells had a much greater capacity to produce proinflammatory cytokines than did those from the blood, but this capacity was dramatically reduced from the earliest stages of HIV infection. Immunoregulatory skewing of mucosal Th17 cell function, characterized by an increased IL-10/TNF- α ratio, was uniquely seen during early HIV infection and was independently associated with reduced systemic immune activation. Antiretroviral therapy rapidly restored mucosal Th17 cell numbers; however, normalization of mucosal Th17 function, microbial translocation, and mucosal/systemic immune activation was much delayed. These findings emphasize that strategies to preserve or to more rapidly restore mucosal Th17 function may have important therapeutic benefit. *The Journal of Immunology*, 2013, 191: 2164–2173.

Protective immunity and immune regulation is provided by the balanced function of various CD4 T cell subsets, and their depletion in HIV and SIV infection leaves the host susceptible to a range of opportunistic infections that define AIDS (1). The progression of HIV and SIV infection is mediated by a rapid depletion of gastrointestinal CD4 T cells, followed by a deterioration of the gut epithelium and increased microbial translocation (2–4). IL-17a-producing Th17 cells are an important subset of CD4 T cells that maintains the gut mucosa by inducing the proliferation of epithelial cells (5), promoting antibacterial defensin production (6), and recruiting neutrophils in the context of bacterial invasion (7, 8). Th17 cells are highly susceptible to HIV and are preferentially

depleted in the gut mucosa (9–11), whereas HIV infection is associated with an increase in mucosal immunosuppressive regulatory CD4 T cells (Treg) that develop reciprocally to Th17 cells (9, 10). These alterations in CD4 T cell subsets impair mucosal protection against luminal bacteria and may lead to microbial translocation into the systemic circulation (10). Increased levels of circulating bacterial products, such as LPS and peptidoglycan, elicit potent proinflammatory innate immune responses and drive persistent immune activation (3, 12, 13). Importantly, HIV-infected individuals have elevated systemic immune activation; in an antiretroviral therapy (ART)-naive individual the degree of this activation is the best predictor of HIV disease progression to AIDS (14). Furthermore, despite the significant survival benefits of ART, gut immune reconstitution is often incomplete, and ongoing immune activation contributes to persistent neurocognitive and cardiovascular dysfunction (15–17).

Although murine Th17 cell differentiation has been well defined, human Th17 cell development is distinct and incompletely understood (8, 18). Several studies indicate the necessity of TGF- β together with a proinflammatory cytokine, such as IL-6, IL-21, IL-1 β , or IL-23, for human Th17 development, although a common differentiation pathway has not been identified (8, 19, 20). In mice, the cytokine milieu in which Th17 cells differentiate can lead to polarization of subsequent Th17 function. Specifically, stimulation with TGF- β and IL-6 generated IL-17a- and IL-10-producing CD4 T cells with a reduced proinflammatory capability (21–23). However, the addition of IL-23 led to the expansion of IL-17a⁺ CD4 T cells that produced an abundance of proinflammatory cytokines, such as IL-22, IFN- γ , and TNF- α (18, 21). These proinflammatory Th17 cells have been associated with autoimmune diseases, such as inflammatory bowel disease, psoriasis, and rheumatoid arthritis (24–26), but they play an important role in defense against microbial translocation from the gut (5, 6, 27, 28). For

^{*}Department of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada; [†]Department of Medical Microbiology, University of Nairobi, Nairobi 00202, Kenya; [‡]Maple Leaf Medical Clinic, Toronto, Ontario M5G 1K2, Canada; [§]St. Michael's Hospital, Toronto, Ontario M5B 1W8, Canada; [¶]Women's College Hospital, Toronto, Ontario M5S 1B1, Canada; ^{||}Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A8, Canada; and [#]Department of Medicine, University Health Network, Toronto, Ontario M5G 2C4, Canada

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Address correspondence and reprint requests to Dr. Rupert Kaul and Connie J. Kim, Department of Medicine, University of Toronto, Medical Sciences Building, Room #6356, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada. E-mail addresses: rupert.kaul@utoronto.ca (R.K.) and kim.connie@gmail.com (C.J.K.)

The online version of this article contains supplemental material.

Abbreviations used in this article: ART, antiretroviral therapy; s, soluble; Treg, regulatory CD4 T cell; VL, viral load.

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example, although a key role of Th17 cells is to recruit neutrophils to the site of bacterial invasion, human neutrophils do not express IL-17aR; instead, they depend on the coproduction of IFN- γ and TNF- α by Th17 cells (7). Interestingly, a recent study demonstrated that Th17 cell functionality was pathogen specific: human Th17 cells primed with the bacteria *Staphylococcus aureus* produced IL-17a and IL-10, whereas those primed with the fungus *Candida albicans* produced IL-17a and IFN- γ under the regulation of IL-1 β (28). Therefore, the coproduction of specific cytokines by mucosal Th17 cells may be a critical determinant of their ability to maintain gut mucosal defense.

The ability of HIV-specific CD8⁺ T cells to manifest multiple functions, including the coproduction of perforin and different cytokines, is central to host virus control (29, 30). Polyfunctional virus-specific T cells are associated with improved control of HIV replication and with delayed HIV disease progression (29, 31, 32). In addition, elite HIV nonprogressors who maintain normal CD4 T cell counts and an undetectable HIV blood viral load (VL) in the absence of ART demonstrate more polyfunctional and more complex HIV-specific CD8 T cells in the blood and rectal mucosa than do HIV-infected noncontrollers (29, 33). Although recent studies of the Th17 subset also demonstrated considerable functional diversity, the relevance of this polyfunctionality to gut mucosal defense and the impact of HIV infection on this function have not been explored. Therefore, we investigated the functional profile of blood and sigmoid colonic Th17 cells during HIV infection and ART, as well as the relationship of these parameters with microbial translocation and host immune activation.

Materials and Methods

Study participants

Sixty study participants were recruited through the Maple Leaf Medical Clinic and provided written informed consent. The Research Ethics Boards at the University of Toronto and St. Michael's Hospital approved the study protocol. Participants belonged to one of the following study groups: HIV-uninfected controls (HIV⁻, $n = 9$); ART-naïve HIV-infected participants in early and chronic phases of infection (early HIV⁺, $n = 24$; chronic HIV⁺, $n = 12$), and long-term ART-treated aviremic participants (HIV+ART, $n = 15$). A subset of early ($n = 6$) and chronic ($n = 5$) HIV-infected individuals was followed longitudinally for a median of 12 mo after ART initiation.

Cell isolation from blood and sigmoid colon

Peripheral blood was collected into Acid Citrate Dextran solution A vacutainer tubes (BD Biosciences), and PBMCs were isolated by Ficoll-Hypaque density centrifugation. Sigmoid biopsies were collected ~25–30 cm from the anal verge, as previously described (9), immediately placed into RPMI solution (RPMI 1640 media containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 \times GlutaMAX-1; Invitrogen), and weighed. Mucosal tissue mononuclear cells were isolated by two sequential Collagenase type II digestions at 0.5 and 1.0 mg/ml (Clostridiopeptidase A; Sigma-Aldrich) for 30 min each on a shaking heated block at 37°C. After tissue digestion, cells were passed through a 100- μ m strainer. The median cell number was 45.2 million/g of tissue (range: 21.0–105.5 million cells/g), and it did not differ with regard to HIV or treatment status.

Flow cytometry

Isolated cells were stimulated with PMA (1 ng/ml) and ionomycin (1 μ M/ml; Sigma) or with media alone for 6 h at 37°C, with the last 5 h containing brefeldin A (1 μ M/ml) in RPMI 1640 solution supplemented with 10% FBS. Cells were washed with 1% FBS-1 \times PBS, permeabilized, stained for 30 min with fluorochrome-labeled mAbs and a violet or aqua LIVE/DEAD viability dye (Invitrogen), and fixed in 2% paraformaldehyde. Abs included CD3, CD4, CD8, HLA-DR, CD38, IL-17a, IL-22, IFN- γ , IL-10, and TNF- α (BD BioSciences, eBioscience, and Beckman Coulter). Cells were acquired on a FACSCanto II (BD Systems) and analyzed using FlowJo analytical software v9.0.2 (TreeStar). Flow cytometric gates were set based on media alone and/or all fluorescence minus one control, and values for functional cell subsets were background corrected where applicable. Dead cells and doublets were excluded based on live/dead

staining and side scatter cell-granularity properties. Th17 cells were defined as IL-17a⁺ CD4 T cells, and Tregs were defined as FOXP3 and CD25 dually positive CD4 T cells. The absolute number of gut cells was determined by multiplying the percentage of live lymphocytes, determined by flow cytometry, by the number of gut cells/g of tissue, as previously described (2, 34). Th17 cell polyfunctionality analysis was generated by Boolean gating analysis using FlowJo software, and a minimum event number of 50 was set for Th17 polyfunctionality analyses. SPICE software v5.22 (National Institute of Allergy and Infectious Diseases/National Institutes of Health) was used to graph these data using the average value for each cytokine category.

Microbial translocation markers

Assays were performed according to the manufacturers' instructions, in duplicate. Plasma samples were diluted 5 \times in endotoxin-free water, heat inactivated at 65°C for 15 min, and assayed to quantify LPS levels using the limulus amoebocyte lysate assay kit (Cambrex). A commercially available ELISA kit (R&D Systems) was used to measure levels of soluble (s)CD14.

Statistical analysis

The Spearman rank-correlation test, Mann-Whitney *U* test, and Wilcoxon signed-rank test were performed using IBM SPSS Statistics 20.0 for Mac (SPSS), and Th17 polyfunctionality analysis was performed using SPICE Student *t* test (v5.22). Linear regression analyses were used to measure independent predictors of immune activation. The dependent variable (blood CD38+DR+CD8⁺ T cells) was logit transformed, and backward stepwise linear-regression analyses were performed for multiple independent covariates using SPSS. Values of $p < 0.05$ were considered significant.

Results

Participant clinical and immune characteristics

Sigmoid colon biopsies and peripheral blood were collected from 60 participants: HIV-uninfected individuals (HIV⁻, $n = 9$), ART-naïve individuals during early (early HIV⁺; $n = 24$) and chronic (chronic HIV⁺; $n = 12$) stages of HIV infection, and long-term ART-treated individuals (long-term ART; $n = 15$). Early HIV-infected individuals either had documented seroconversion ($n = 12$) or a known high-risk HIV sexual exposure followed by symptoms compatible with seroconversion illness within the past 7 mo ($n = 12$); all were IgG seropositive, and the median duration of infection was 4 mo. Participants on long-term ART had been treated for a median of 13 y (range, 6–20 y), with an undetectable blood VL for ≥ 6 y. Participant clinical and immune characteristics are outlined in Table I.

Absolute and relative CD4 T cell numbers were depleted in the blood and sigmoid colon during both early and chronic HIV infection, and long-term ART restored all parameters except for the absolute number of CD4 T cells in the sigmoid mucosa. The number of Th17 cells, defined as CD4 T cells producing IL-17a after mitogen stimulation, was decreased in the sigmoid mucosa during chronic HIV infection ($p = 0.002$), with a similar trend during early HIV infection ($p = 0.075$). Immune activation was defined by the coexpression of HLA-DR and CD38 by CD8⁺ T cells and was substantially increased in the blood and sigmoid mucosa during both early and chronic stages of HIV infection (Supplemental Fig. 1A–C). Immune activation during early and chronic HIV infection was comparable in both the blood and gut ($p = 0.473$ and $p = 0.754$, respectively). Plasma LPS levels, a marker of microbial translocation, were increased during the chronic stage of HIV ($p = 0.025$), but not during the early HIV stage ($p = 0.817$), whereas plasma levels of sCD14 were increased during both early and chronic stages ($p = 0.031$ [early HIV⁺] and $p = 0.002$ [chronic HIV⁺], Supplemental Fig. 1D, 1E).

Reduced functional capacity of sigmoid Th17 cells during untreated HIV infection

The functional capacity of Th17 cells was assessed by the coproduction of the proinflammatory cytokines IL-22, IFN- γ , and/or

Table I. Clinical and immune characteristics of study participants

	HIV ⁻ (n = 9)	Early HIV ⁺ (n = 24)	Chronic HIV ⁺ (n = 12)	Long-Term ART (n = 15)
Age (y)	40 (26–43)	33 (28–40)	33 (26–44)	51 (47–57)**
Blood CD4 count (/ml)	780 (645–1235)	440 (242–688)*	275 (210–435)**	700 (400–770)
Blood VL (c/μl)	N/A	16,481 (3,192–77,848)	29,628 (6,688–113,687)	<49
Blood CD4 T cells (%)	62 (49–70)	38 (32–48)*	24 (21–35)**	48 (34–56)
Gut CD4 T cells (%)	58 (45–67)	21 (15–28)**	13.4 (10–16)**	49 (41–55)
Abs gut CD4 T cells (10 ⁶ cells/g tissue)	1.7 (1.1–3.4)	0.7 (0.4–1.1)*	0.4 (0.2–1.1)*	0.9 (0.3–1.5)*
Blood Th17 cells (%)	0.3 (0.2–1.0)	0.6 (0.4–1.4)	0.7 (0.4–1.5)	0.5 (0.3–1.0)
Gut Th17 cells (%)	2.1 (1.1–3.1)	2.3 (1.2–4.5)	1.3 (0.9–2.4)	3.0 (1.3–4.4)
Abs gut Th17 cells (10 ² cells/g tissue)	4.0 (1.7–6.7)	1.7 (0.1–3.3)	0.9 (0.5–1.5)*	1.9 (1.2–5.8)
Blood Tregs (%)	1.0 (0.9–1.5)	1.8 (1.0–3.1)*	1.4 (0.7–2.3)	0.3 (0.1–0.7)
Gut Tregs (%)	2.4 (1.5–4.2)	4.8 (3.9–6.4)*	4.4 (2.3–6.6)	1.6 (0.8–3.3)

All data are median (interquartile range).

All comparisons were made to the HIV⁻ group.

Th17 cells were defined as IL-17a-producing CD4 T cells, and Tregs were defined as CD4 T cells coexpressing FOXP3 and CD25.

* $p \leq 0.05$, ** $p \leq 0.001$.

Abs, Absolute number; N/A, not applicable.

TNF- α , as assessed through Boolean gating. First, we compared the function of Th17 cells from the sigmoid colon and blood in HIV-uninfected individuals. Gut Th17 cells had a much greater capacity to produce proinflammatory cytokines than did those from the blood ($p = 0.018$; Fig. 1A), with higher frequencies of triple cytokine-producing Th17 cells ($p = 0.006$) and dual TNF- α and IL-22-coproducing Th17 cells ($p = 0.002$, data not shown). However, proinflammatory cytokine production capacity by sigmoid Th17 cells was dramatically reduced during both the early and chronic stages of HIV infection ($p < 0.001$ [HIV-uninfected versus early HIV⁺] and $p = 0.019$ [HIV-uninfected versus chronic HIV⁺], Fig. 1B). Although there was substantial heterogeneity within study groups (Fig. 1C), the mean number of proinflammatory cytokines produced per Th17 cell was substantially reduced during both early and chronic HIV infection (1.21 cytokines [HIV-uninfected], 0.70 cytokines [early HIV⁺], and 0.73 cytokines [chronic HIV⁺], Fig. 1D). This reduction was primarily due to a decrease in triple and dual TNF- α and IL-22 cytokine-producing Th17 cells (Fig. 1E). Sigmoid Th17 polyfunction was reduced by a similar amount during early and chronic HIV infection ($p = 0.416$ [Fig. 1B] and $p = 0.542$ [Fig. 1D]). HIV infection was not associated with changes in the proinflammatory function of blood Th17 cells (data not shown).

Although CD8⁺ T cells were also documented to produce IL-17a (called Tc17 cells) (35, 36), a subset analysis including one HIV-uninfected control and one ART-treated individual demonstrated that sigmoid colonic CD4 T cells produced an abundance of IL-17a compared with CD8 T cells (median, CD4: 1.17%; CD8: 0.15%).

Immunoregulatory skewing of mucosal Th17 function

Because the capacity of mucosal Th17 cells to produce proinflammatory cytokines was dramatically reduced during HIV infection, we next assessed production of the immunoregulatory cytokine IL-10. In HIV-uninfected participants, mucosal Th17 cells most commonly produced the proinflammatory cytokine TNF- α (median, 69.7%), whereas production of IL-10 was rare (0.3%); however, there was immunoregulatory Th17 skewing during untreated HIV infection (TNF- α median, 43.4%; IL-10 median, 3.5%; Fig. 2A). Immunoregulatory skewing was calculated as the ratio of immunoregulatory (IL-10⁺) Th17 cells/proinflammatory (TNF- α ⁺) Th17 cells and was particularly increased during early HIV stages ($p < 0.001$, Fig. 2B). The IL-10/TNF- α Th17 ratio was comparable between the HIV-uninfected group and the chronic HIV-infected or long-term ART-treated group ($p = 0.676$

and $p = 0.387$, respectively), but with some interindividual heterogeneity. Mucosal IL-10⁺ Th17 cells did not coexpress the Treg marker FOXP3⁺; the frequency of IL-10 production by gut Th17 cells or bulk CD4 T cells did not correlate with mucosal Tregs in ART-naive individuals (Spearman rank coefficient, $p = 0.237$, $r_s = -0.205$ and $p = 0.255$, $r_s = -0.195$ respectively). Although IL-10⁺ sigmoid Th17 cells did not typically coproduce other proinflammatory cytokines, a small fraction coproduced IFN- γ and TNF- α (<5% for each).

We hypothesized that immunoregulatory mucosal Th17 skewing would be associated with reduced mucosal immune activation during untreated HIV infection, including both early and chronic stages. An increase in the mucosal Th17 immunoregulatory ratio was associated with reduced CD8⁺ T cell immune activation in both the gut ($p = 0.034$, $r_s = -0.383$) and blood ($p = 0.002$, $r_s = -0.518$; Fig. 2C, 2D). Furthermore, an increased immunoregulatory Th17 ratio correlated with reduced plasma LPS levels ($p = 0.002$, $r_s = -0.612$; Fig. 2E) and a lower blood HIV VL ($p = 0.026$, $r_s = -0.376$; Fig. 2F). No associations were seen with plasma sCD14 levels ($p = 0.538$, $r_s = -0.142$).

We also assessed IL-10 production by gut Th1 cells (IFN- γ ⁺ CD4 T cells). Again, early HIV infection was associated with increased IL-10 by gut Th1 cells compared with the HIV-uninfected group (median, 5.43% versus 2.15%, $p = 0.001$) and chronic HIV infection (2.72%, $p = 0.020$); however, the level of IL-10 production by gut Th1 cells was not associated with blood ($p = 0.121$, $r_s = -0.275$) or gut immune activation ($p = 0.820$, $r_s = -0.042$) or with plasma LPS levels ($p = 0.159$, $r_s = -0.297$) in ART-naive individuals.

Cytokine production by bulk CD4 T cells in the gut mucosa

Given that HIV infection and immune activation were associated with the altered function of gut Th17 cells, we also evaluated the frequency and number of bulk CD4 T cells producing the cytokines IL-10, IFN- γ , TNF- α , and IL-22 in the gut. Similar to gut Th17 cells, bulk CD4 T cells producing IL-10 were also elevated during early HIV infection but not during chronic HIV infection ($p < 0.001$ and $p = 0.477$, respectively), whereas the absolute numbers remained unchanged compared to HIV-uninfected individuals (Fig. 3A). The proportion of IFN- γ -producing CD4 T cells, referred to as Th1 cells, was comparable between HIV-uninfected and HIV-infected individuals, but their absolute numbers were reduced during early HIV infection ($p = 0.001$), and a similar trend followed during the chronic stage ($p = 0.088$, Fig. 3B). TNF- α -producing CD4 T cells were reduced in proportions and numbers

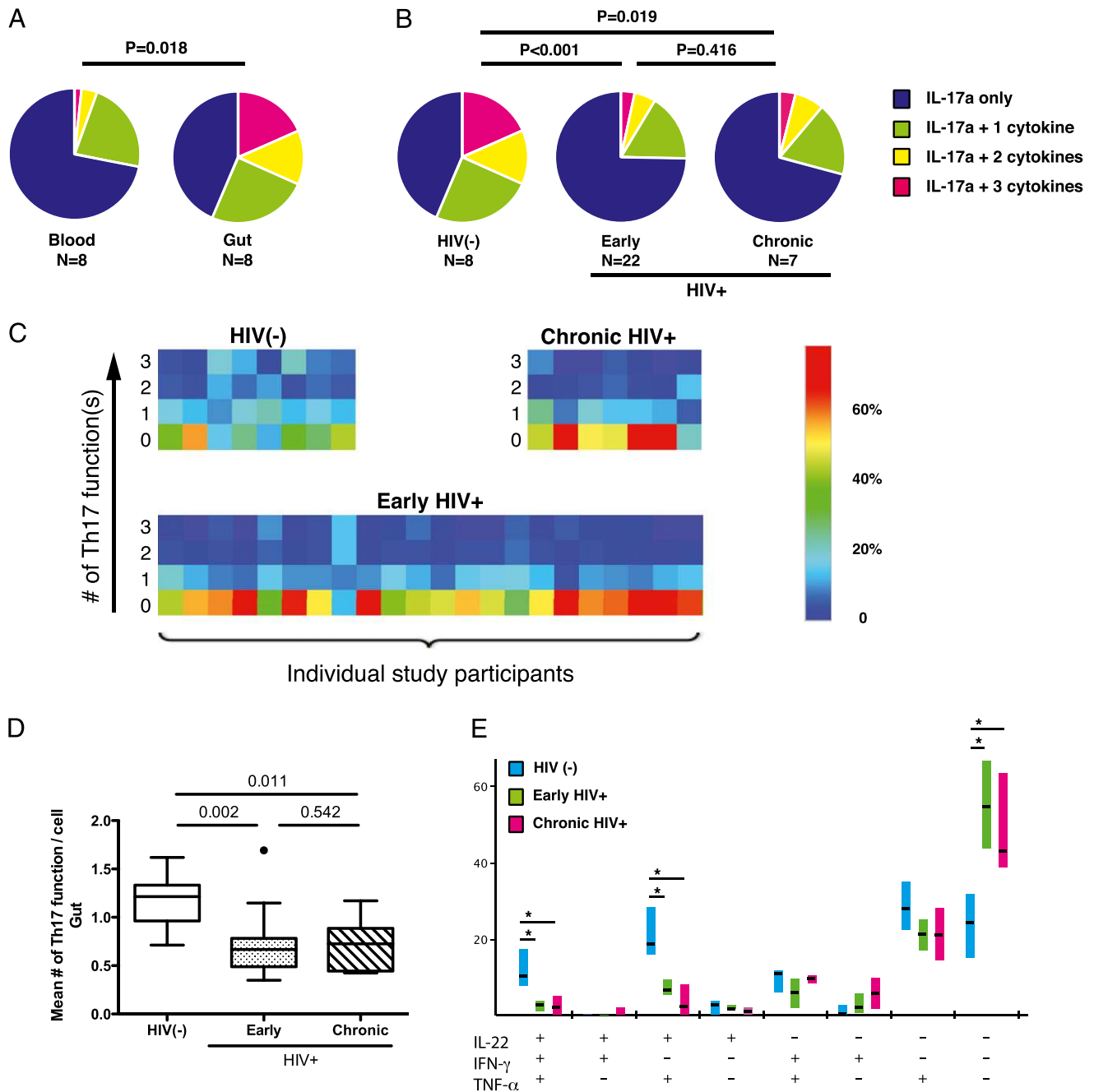


FIGURE 1. Polyfunctionality of mucosal Th17 cells, as measured by the coexpression of IL-22, IFN- γ , and/or TNF- α , was reduced in untreated HIV infection. Sigmoid Th17 cells in HIV-uninfected individuals were much more polyfunctional than were blood Th17 cells (A), but this was profoundly reduced during all stages of untreated HIV infection (B). Heat map-style plots demonstrate the vast heterogeneity within each study group (C); however, the mean number of sigmoid Th17 function/cell was reduced in early and chronic HIV infection (D). (E) Each possible combination of IL-22, IFN- γ , and TNF- α production by Th17 cells is shown; horizontal lines indicate the median, and box plots depict the interquartile range. Th17 functional analysis was only available in a subset of study participants in each group and was based on cell availability. $p \leq 0.05$.

only during early HIV infection ($p = 0.039$ for both comparisons, Fig. 3C), whereas IL-22-producing CD4 T cell numbers and proportions were reduced in both early and chronic stages of HIV infection (Fig. 3D).

Plasma LPS and sCD14 levels were not associated with the proportion of any of the cytokines produced by bulk gut CD4 T cells in ART-naïve individuals (LPS, $p > 0.214$; sCD14, $p > 0.443$; data not shown), as well as blood immune activation ($p > 0.269$). However, there was a trend of IL-10-producing CD4 T cells correlating with reduced blood immune activation ($p = 0.097$, $r_s = -0.294$), and TNF- α -producing CD4 T cells were

associated with increased gut immune activation ($p = 0.007$, $r_s = +0.467$).

Independent associations of systemic immune activation

Systemic immune activation is a well-defined predictor of HIV disease progression (14) and was elevated to comparable levels during early and chronic untreated HIV infection (Supplemental Fig. 1). In our participants, systemic immune activation was associated with the blood VL and mucosal immune activation and was inversely correlated with the blood CD4 count, the frequency of IL-10⁺ gut Th17 cells, and the mucosal immunoregulatory

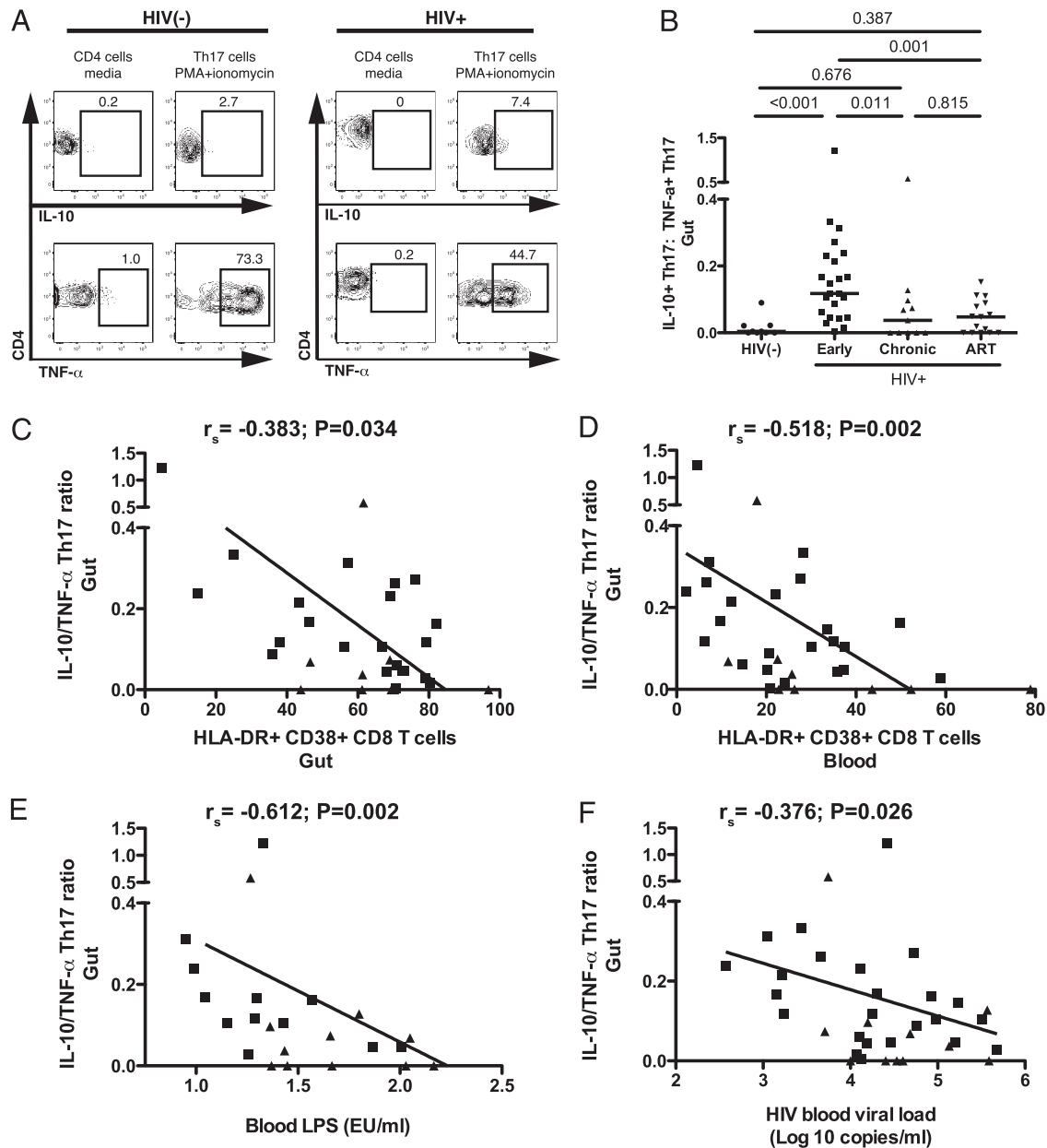


FIGURE 2. The ratio of immunoregulatory Th17 cells (IL-10⁺)/proinflammatory Th17 cells (TNF- α ⁺) was increased in early HIV-infected individuals. **(A)** In HIV-uninfected controls, sigmoid Th17 cells commonly produced TNF- α , whereas IL-10 production was scarce. However, this was reversed in untreated HIV infection: IL-10 production by sigmoid Th17 cells was increased, and TNF- α was reduced. Unstimulated media control of CD4 T cells was used for gating purposes. **(B)** Immunoregulatory skewing of sigmoid Th17 cells (calculated as the ratio of IL-10⁺ Th17/TNF- α ⁺ Th17 cells) was unique to early HIV infection. The IL-10/TNF- α Th17 ratio was associated with reduced gut **(C)** and blood **(D)** immune activation (HLA-DR and CD38 copositive CD8 T cells), reduced plasma LPS levels **(E)**, and reduced blood VL **(F)** in all HIV-infected, ART-naïve individuals, including early and chronically infected individuals. Immune activation markers were available in $n = 22$ early and $n = 9$ chronic HIV-infected participants, and plasma LPS and sCD14 levels were available in $n = 12$ early and $n = 11$ chronic HIV-infected participants.

Th17 ratio (Table II). Therefore, we performed a stepwise multivariate linear-regression model to define the best clinical and immune variables that were independent predictors of systemic immune activation. In this model, only the blood CD4 count and the mucosal immunoregulatory Th17 ratio were independently associated with systemic immune activation ($p = 0.002$ and $p = 0.004$, respectively, Table II).

Impact of long-term ART on mucosal Th17 number and function

In participants on long-term ART with complete gut CD4 reconstitution ($p = 0.183$), blood immune activation and plasma LPS

levels were comparable to those in HIV-uninfected controls ($p = 0.156$ and $p = 0.188$, respectively; data not shown). In addition, mucosal Th17 numbers ($p = 0.355$), relative frequency ($p = 0.180$), and function ($p = 0.111$) did not differ from HIV-uninfected individuals (Fig. 4A–C). There was complete restoration of triple cytokine-producing Th17 cells in the sigmoid mucosa and of dual IL-22 and TNF- α -coproducing Th17 cells (data not shown). The production of TNF- α ⁺ by mucosal Th17 cells exceeded that of HIV-uninfected controls (HIV⁻, 69.7% versus ART, 88.0%; $p = 0.002$); however, the immunoregulatory mucosal IL-10/TNF- α Th17 ratio in the long-term ART group was similar to that in HIV-uninfected individuals ($p = 0.319$). Long-term ART

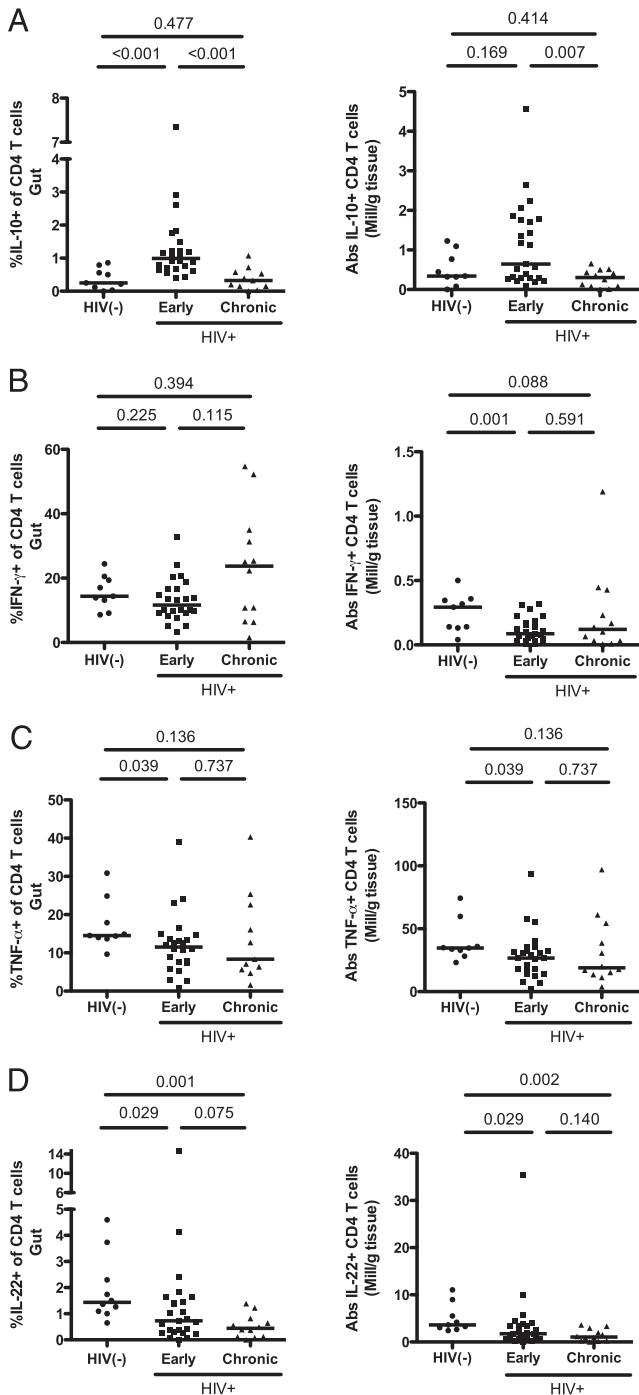


FIGURE 3. Proportion and number of bulk gut CD4 T cells producing the cytokines IL-10 (A), IFN- γ (B), TNF- α (C), and IL-22 (D) in HIV-uninfected and early and chronic HIV-infected individuals.

had similar cytokine production by bulk CD4 T cells in the gut (IL-10, IFN- γ , TNF- α , and IL-22) compared with HIV-uninfected individuals (data not shown), with the exception of gut Th1 cell numbers being less than those of HIV-uninfected controls (HIV⁻, 0.293 versus ART, 0.104 cells/g tissue; $p = 0.042$).

We next dichotomized the ART-treated group based on the degree of gut CD4 reconstitution to determine whether effective CD4 restoration positively impacted the function of gut CD4 cells and HIV disease progression. High gut CD4 reconstitution was defined as ART-treated individuals with CD4 T cell frequency above the median (>48.8%). Although the level of CD4 reconstitution in the gut did not impact blood or gut immune activation

(HLA-DR⁺ on CD8 T cells, $p > 0.563$), sCD14 levels were greater in individuals with low CD4 reconstitution (<48.8%) (2.34 $\mu\text{g/ml}$ [low] versus 1.93 $\mu\text{g/ml}$ [high], $p = 0.049$), and a similar trend followed with plasma LPS levels (1.47 EU/ml [low] versus 1.32 EU/ml [high], $p = 0.105$). Those with high CD4 reconstitution on ART had a similar proportion and number of gut Th17 cells ($p = 0.643$ and $p = 0.418$ respectively), and similar Th17 polyfunction ($p = 0.682$, Fig. 4D). Furthermore, the level of gut CD4 reconstitution on ART did not impact the relative and absolute number of IL-22 (both $p = 0.248$), IL-10 ($p = 0.431$ and $p = 0.189$, respectively), IFN- γ (Th1 cells; $p = 0.298$ and $p = 0.165$, respectively), or TNF- α (both $p = 0.115$) production by bulk CD4 T cells in the gut mucosa.

Impact of short-term ART on mucosal Th17 cells and immune activation

We then assessed these mucosal parameters in a subset of HIV-infected individuals ($n = 11$) who initiated ART during chronic ($n = 5$) and early ($n = 6$) stages of HIV infection, with repeat sampling after a median of 12 mo. The blood VL became undetectable at a median of 4 mo (range, 1–6 mo) after ART initiation. Gut and blood CD4 T cell frequencies increased after short-term ART (paired analysis: $p = 0.003$ [gut], $p = 0.006$ [blood]), although the CD4 T cell frequency remained below the level of HIV-uninfected controls at both sites (gut: 39% versus 58%, $p = 0.009$; blood: 36% versus 62%, $p = 0.030$).

During untreated chronic HIV infection, plasma LPS levels were elevated, and sigmoid Th17 cell function and numbers were lost. After short-term ART, both the frequency and the number of gut Th17 cells were reconstituted (paired analysis, both $p = 0.043$, Fig. 5A, 5B); interestingly, the frequency of gut Th17 cells exceeded that of HIV-uninfected individuals ($p = 0.020$), whereas their numbers were comparable ($p = 0.549$). Their functional capacity was not altered ($p = 0.898$); this remained lower than both the HIV-uninfected group ($p = 0.049$) and the long-term ART group ($p = 0.002$, Fig. 5C). In addition, the IL-10/TNF- α Th17 ratio was not altered by short-term ART ($p = 0.815$, data not shown). Although there was a reduction in systemic immune activation (paired analysis, $p = 0.043$, Fig. 5D, 5E), as well as a trend toward reduced mucosal immune activation ($p = 0.080$, Fig. 5E) after ART initiation, gut immune activation remained significantly elevated compared with HIV-uninfected participants (30.9% versus 14.4%, $p = 0.009$), and a similar trend was seen in the blood (10.3% versus 2.6%, $p = 0.076$). Neither plasma LPS nor sCD14 levels changed after ART initiation (paired analysis, $p = 0.175$ and $p = 0.313$, respectively; data not shown).

Because ART treatment during the chronic stage of HIV infection did not reconstitute gut Th17 cell function or immune activation in the short-term, we next explored the impact of initiating ART during the early stages of HIV infection. Early initiation of ART reduced immune activation in the blood and gut (paired analysis, both $p = 0.028$); however, they still remained higher than in HIV-uninfected individuals in the blood (13.3% versus 2.6%, $p = 0.045$), and a similar trend followed in the gut (31.0% versus 14.4%, $p = 0.100$). Moreover, short-term ART during early infection did not enhance the functionality of gut Th17 cells ($p = 0.391$, data not shown).

Discussion

Progressive HIV infection is characterized by a preferential loss of mucosal Th17 cells, a CD4 T cell subset that plays an important role in mucosal defense against potentially pathogenic microbes from the gut lumen (9, 11, 37). In the absence of HIV, mucosal Th17 cells produce several cytokines with a range of effector

Table II. Univariate and multivariate linear regression of variables correlated with systemic immune activation in untreated HIV-infected individuals

	Univariate Linear Regression ^a			Multivariate Stepwise Linear Regression ^a		
	Unstandardized B Coefficient	95% CI	p Value	Unstandardized B Coefficient	95% CI	p Value
Clinical parameters						
Age (y)	-1.243	-0.027 to 0.050	0.557	<i>b</i>	<i>b</i>	<i>b</i>
Blood CD4 count (/ml)	-0.002	-0.003 to -0.001	<0.001	-0.002	-0.004 to -0.001	0.002
Blood viral load (c/μl)	4.54×10^{-6}	2.0×10^{-6} to 7.0×10^{-6}	<0.001	<i>b</i>	<i>b</i>	<i>b</i>
Plasma LPS (EU/ml)	0.829	-0.707 to 2.364	0.273	<i>b</i>	<i>b</i>	<i>b</i>
Plasma sCD14 (μg/ml)	-0.522	-1.772 to 0.729	0.394	<i>b</i>	<i>b</i>	<i>b</i>
Mucosal parameters						
HLA-DR+CD38+ CD8 T cells (%)	0.025	0.008 to 0.042	0.005	<i>b</i>	<i>b</i>	<i>b</i>
Th17 cells (%)	0.039	-0.153 to 0.232	0.680	<i>b</i>	<i>b</i>	<i>b</i>
IL-10+Th17 cells (%)	-0.070	-0.121 to -0.019	0.009	<i>b</i>	<i>b</i>	<i>b</i>
TNF-α Th17 cells	0.003	-0.018 to 0.025	0.769	<i>b</i>	<i>b</i>	<i>b</i>
IL-22 Th17 cells	-0.001	-0.026 to 0.025	0.968	<i>b</i>	<i>b</i>	<i>b</i>
IFN-γ Th17 cells	-1.234	-0.069 to 0.026	0.372	<i>b</i>	<i>b</i>	<i>b</i>
IL-10/TNF-α Th17 cell ratio	-2.162	-3.646 to -0.678	0.006	-2.188	-3.580 to -0.795	0.004

^aDependent variable (blood immune activation) was logit transformed.

^bVariable was not independently associated with blood immune activation and was eliminated from the stepwise linear-regression model.

CI, Confidence interval.

functions (28, 38). Because HIV infection is associated with a dramatic reduction in the polyfunctional capacity of HIV-specific CD8⁺ T cells (39, 40), we examined the effector functions of mucosal Th17 cells and their relationship with HIV-associated microbial translocation and immune activation. In the absence of HIV, Th17 cells in the gut mucosa expressed a much wider array of functions than did those from blood. However, this polyfunction was dramatically reduced from the very earliest stages of HIV infection (<7 mo of infection), even before the mucosal Th17 number had been significantly reduced. Although ART quickly increased Th17 numbers, restoration of their polyfunctional capacity was only apparent after a prolonged period of treatment. Interestingly, among ART-naïve participants, there also was skewing of gut Th17 cells toward an immunoregulatory phenotype (increased IL-10/TNF-α ratio), which was only seen during the early stages of HIV infection. Moreover, in a linear-regression model that included key mucosal and clinical parameters, only

the gut immunoregulatory Th17 ratio and CD4 count were independent predictors of systemic immune activation. Therefore, the mucosal immunoregulatory skewing of Th17 cells may play an important role in maintaining the mucosal barrier during HIV infection.

In addition to IL-17a, Th17 cells produce several effector cytokines: TNF-α and IFN-γ recruit neutrophils to the site of infection (7), and IL-22 promotes epithelial renewal and enhances the expression of antimicrobial peptides by epithelial cells (5, 6). Our results suggest that the HIV-associated loss of Th17 cell effector functions in the gut mucosa may be causally related to the increased translocation and systemic dissemination of luminal bacteria (37). However, increased systemic microbial translocation (i.e., plasma LPS levels) was only apparent during chronic HIV infection, whereas a dramatic impairment of Th17 function was apparent very early after HIV acquisition. We hypothesize that this delayed impact of Th17 functional impairment on mi-

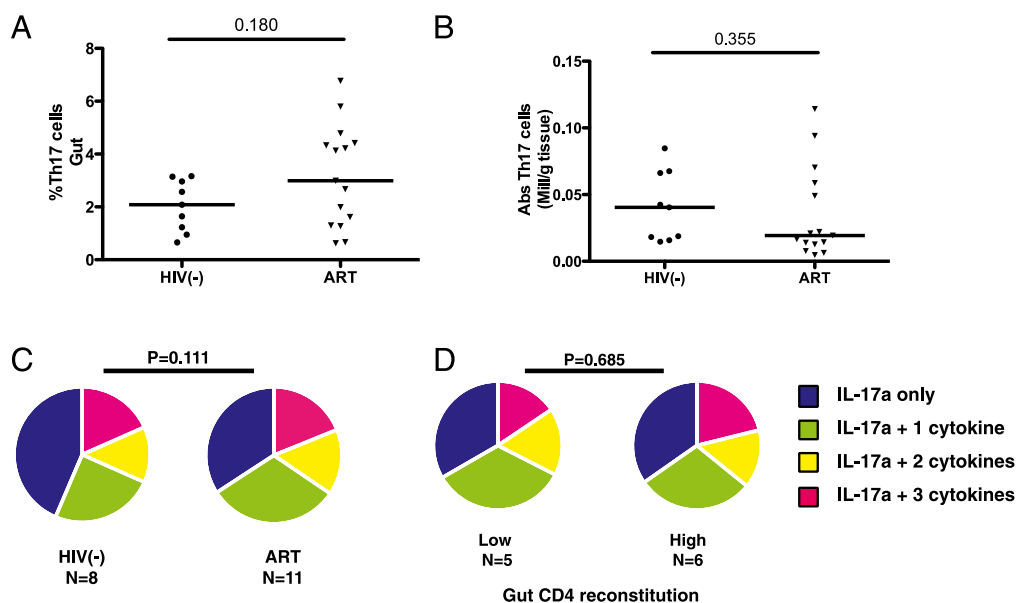


FIGURE 4. Reconstitution of sigmoid Th17 cell number and their function after long-term ART. The absolute (A) and relative frequency (B) of sigmoid Th17 cells and their polyfunctional capacity (C) in long-term ART-treated individuals were comparable to those in HIV-uninfected controls. (D) Sigmoid Th17 polyfunction after long-term ART was similar between those with high CD4 reconstitution and low CD4 reconstitution (based on gut CD4 reconstitution above or below the median value of 48.8% in the ART group).

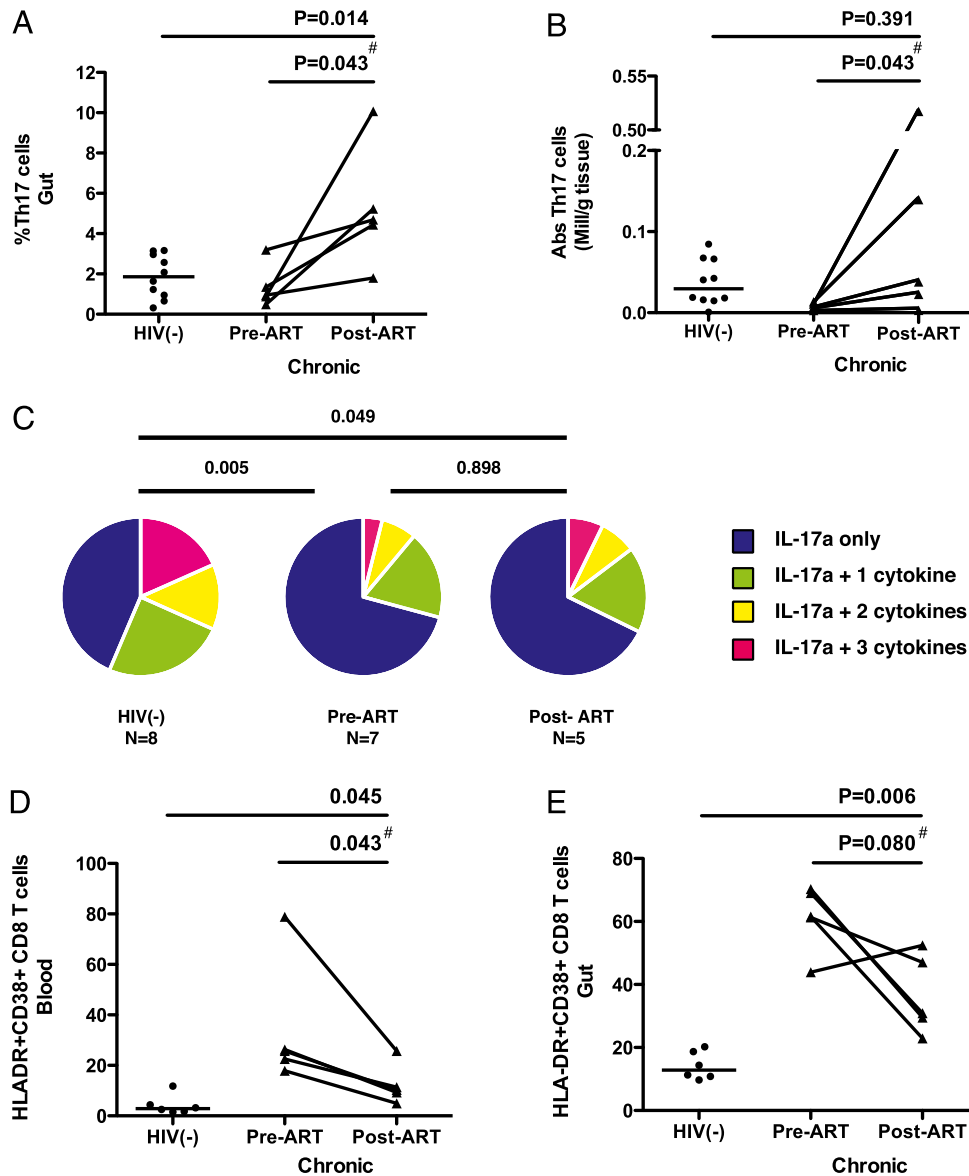


FIGURE 5. Numerical and functional profile of sigmoid Th17 cells and level of immune activation soon after initiating ART during the chronic stage ($n = 5$) of HIV infection. Short-term ART initiated during chronic HIV infection normalized the relative (**A**) and absolute number (**B**) of sigmoid Th17 cells; however, their polyfunctional capacity was not different from their respective ART-naïve baseline time points (**C**). Blood immune activation was reduced in all individuals soon after ART initiation (**D**), and gut immune activation was reduced in four of the five individuals but remained greater than in HIV-uninfected controls (**E**). #Paired analysis.

crobal translocation may be because a loss of epithelial integrity is also needed for microbial translocation to occur, and this integrity is only compromised later in HIV infection, coincident with the loss of mucosal IL-22 production capacity (2, 4).

Although the polyfunctional capacity of mucosal Th17 cells was reduced during untreated HIV infection, this was not directly associated with microbial translocation or systemic immune activation. Instead, the only mucosal immune parameter that was independently associated with systemic immune activation in our study was the immunoregulatory skewing of Th17 cells (increased IL-10/TNF- α ratio). The latter was most apparent during the early stages of HIV infection and was also inversely correlated with mucosal immune activation, microbial translocation, and the blood VL. HIV infection was associated previously with an increase in IL-10 production by several cell subsets, but their role in HIV disease progression or protection has not been delineated (41–44), and the impact of IL-10-producing mucosal Th17 cells on HIV

pathogenesis has not been explored. Our data suggest that, although retaining the overall polyfunction of mucosal Th17 cells may be important, maintaining a fine balance between the proinflammatory and immunoregulatory functions of mucosal Th17 cells may even be more critical.

However, this is a cross-sectional study, and the direction of causality is unclear. Immunoregulatory skewing of gut Th17 cells may either be protective against or the result of mucosal microbial translocation and inflammation. Bacteria, such as *S. aureus*, were shown to induce human blood Th17 cells that coproduce IL-10 (28), and so the immunoregulatory Th17 phenotype might simply reflect the colocalization of translocated luminal bacteria. Thus, Th17 cells within the mucosa may have little impact on epithelial integrity. In contrast, the self-regulating expression of IL-10 by Th17 cells was important in preventing Th17-induced colitis in a murine model (21); therefore, these cells might be playing a direct role in the reduced levels of mucosal and systemic in-

flammation that we observed in individuals with skewed gut regulatory Th17 cells. IL-10 deriving from Th17 cells in the gut mucosa may function synergistically with other Th17-associated cytokines to fine tune and balance the proinflammatory capacity of Th17 cells; however, further studies, likely in animal models, are needed to determine the direction of causality.

Our study cannot define the mechanism behind the loss of gut Th17 cell polyfunction. It is possible that polyfunctional Th17 cells are more susceptible to HIV infection, which may impact their cell number or functionality (11); thus, slow HIV clearance in the gut mucosa after ART initiation may hinder and delay functional reconstitution of Th17 cells (45). Moreover, Th17 development and/or recruitment may be affected by local or systemic factors during HIV infection. This will be an important area for future research. In addition, our assays measured a limited repertoire of Th17 functions, specifically the production of cytokines IL-17a, IL-22, IL-10, TNF- α , and IFN- γ , and were unable to assess the functionality of IL-17a-producing CD8 T (Tc17 cells). It is likely that HIV is also associated with alterations in the production of other cytokines or the performance of additional Th17 functions. Future studies will need to address this, as well as the precise timing of Th17 functional restoration in relation to ART, which our study can only narrow to somewhere between 1 and 13 y.

In summary, we show that HIV infection causes dramatic alterations in the functional capacity of mucosal Th17 cells. These alterations occur even earlier in the HIV disease course than do the previously described reductions in Th17 number and are less readily reversed after the initiation of effective ART. Their association with mucosal and systemic immune activation and microbial translocation suggests that strategies to preserve or to more rapidly restore mucosal Th17 function will have important therapeutic benefit.

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Disclosures

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