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Parallel studies of mucosal immunity in the reproductive and gastrointestinal mucosae of HIV-infected women

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Abstract

Problem: The effects of HIV on the gastrointestinal tract (GIT), including CD4 depletion, epithelial disruption, and collagen deposition, are well documented and only partially reversed by combination antiretroviral therapy (cART). However, the effects of HIV on the female reproductive tract (FRT) are poorly understood, and most studies have focused on ectocervix and vagina without assessing the upper tract. Here, we investigated $CD4^+$ T-cell frequency, phenotype, and HIV-specific T-cell responses in the endocervix and endometrium of HIV-infected women, comparing these tissues to the GIT.

Method of Study: Mucosal samples and blood were obtained from 18 women: 4 who were HIV positive and not on cART for at least three years prior to sampling, including 2 natural controllers (viral load [VL] undetectable and CD4 >350); 9 women on cART with low to undetectable VL; and 5 HIV-uninfected women. Mucosal samples included terminal ileum, sigmoid colon, endocervical cytobrush, endocervical curettage, and endometrial biopsy. T-cell frequency, phenotypes, and HIV-specific T-cell responses were analyzed by multiparameter flow cytometry.

Results: T-cell activation, measured by CD38/HLA-DR co-expression, remained significantly elevated in endometrium following cART, but was lower in gastrointestinal tissues. HIV-specific $CD8^+$ T-cell responses were detected in ileum, colon and endometrial tissues of women both on and off cART, and were of higher magnitude on those not on cART.

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Conclusions: Our findings reveal differences in CD4$^+$ T-cell frequencies, immune activation and HIV-specific T-cell responses between the gastrointestinal and reproductive tracts, and highlight differences between HIV controllers and women on cART.

Keywords

T-cell; CTL; HIV; GALT; MALT; endometrium; endocervix

INTRODUCTION

The mucosal tissues of the reproductive and gastrointestinal tracts act as the major portals of HIV entry and are considered the first line of host defense against sexually transmitted pathogens. Both tissues house populations of CCR5-expressing, activated effector memory CD4$^+$ T-cells that are highly susceptible to HIV infection[1]. Within days of infection gastrointestinal CD4$^+$ T-cells are depleted, in striking contrast to the gradual decline of CD4$^+$ T-cells in peripheral blood[1,4,5]. The emergence of HIV-specific cellular immune responses in the gut is considered to be “too little and too late” to prevent extensive early CD4$^+$ T-cell depletion in this tissue[2]. Early depletion of gastrointestinal CD4$^+$ T-cells, including Th17 cells, contributes to loss of epithelial barrier integrity; this in turn leads to translocation of microbial products into the systemic circulation, which is hypothesized to contribute to generalized immune activation[3].

The extensive depletion of CD4$^+$ T-cells from the gastrointestinal lamina propria is well described, and has been attributed in part to the increased availability of activated, CCR5$^+$ T-cells, which constitute a primary target for HIV[4, 5]. Disruption of gut-associated lymphoid tissue (GALT) is strongly implicated as a contributor to HIV disease progression, and it has been suggested that maintaining a healthy GALT may be key to limiting HIV immunopathogenesis[6–8]. The dynamics of CD4$^+$ T-cell depletion in the female reproductive tract (FRT) after HIV infection, and reconstitution of these cells following initiation of combination antiretroviral therapy (cART), have been less thoroughly studied. Like the gastrointestinal tract (GIT), the FRT houses numerous CCR5$^+$ activated, effector memory CD4$^+$ T-cells[9, 10], and experimental infection of rhesus macaques with simian immunodeficiency virus (SIVmac) leads to rapid depletion of these cells[11]. The reproductive and gastrointestinal tracts share numerous immunological features[1]; however, few studies of HIV disease have explored both compartments simultaneously, likely due in part to the difficulties associated with tissue acquisition in humans.

Advances in the efficacy of cART regimens have resulted in dramatic increases in the life expectancy and survival of HIV-infected individuals[12]. Despite the success of cART in reducing plasma viremia and promoting recovery of CD4$^+$ T-cells numbers in peripheral blood, immune reconstitution in the GIT is often incomplete or delayed[13–15]. The effects of cART on CD4$^+$ T-cell recovery in the gut remain somewhat unclear, with reports ranging from minimal to complete restoration depending upon the area of the GIT studied, morbidity at the time of treatment initiation and duration of cART[16–20]. With few exceptions[15, 21], most studies in humans have been limited to a single region of the GIT[4, 14, 16–18, 22–26]. Little information exists concerning the effects of cART on T-cell populations in the FRT;

moreover, most studies of HIV pathogenesis in the FRT have focused on tissues of the lower (ectocervix and vagina) rather than the upper tract (endocervix and endometrium), and it has not been determined whether restoration of $CD4^+$ T-cells and immune function in the FRT parallels restoration in the GIT[27–31] or blood.

A small subset of HIV infected individuals who control plasma viremia in the absence of cART are termed elite or "natural" controllers[32]. These individuals frequently have higher $CD4^+$ T-cell counts and less pronounced immune activation compared to typical progressors[23, 33–35]. Some individuals who meet virologic criteria as natural controllers nevertheless experience significant $CD4^+$ T-cell loss[36, 37], and demonstrate increased immune activation[34, 38, 39] and a range of non-AIDS-defining morbidities[40–43]. Studies of mucosal tissues in these subjects have been limited; however, available data reveal that many individuals identified as controllers have strong, polyfunctional HIV-specific T-cell responses in rectal mucosa[23]. It is not known whether similarly robust HIV-specific responses are present in the upper female reproductive tract.

We now report the results of a study of $CD4^+$ T-cell percentages, T-cell activation/maturation phenotypes and HIV-specific T-cell responses in small and large intestine, endocervix and endometrium, measured at a single time point in a group of long-term HIV-infected women. These women included natural controllers; women not taking cART despite progressive disease; recipients of cART and uninfected comparison participants (Table 1). To our knowledge, this study is the first to directly compare immune parameters in the GIT and the upper reproductive tract of HIV-positive women.

MATERIALS AND METHODS

Study Subjects and Specimen Collection.

Study participants included women recruited from the Women's Interagency HIV Study (WIHS) and participants recruited in the San Francisco Bay area specifically for this study. The WIHS is a multisite observational cohort study of HIV among women which has been described previously[44]; all WIHS participants who contributed to this report were enrolled at the San Francisco Bay area WIHS site. Eight HIV infected and 5 HIV uninfected participants of WIHS enrolled in this study (Table 1). An additional 5 San Francisco Bay area women, who were not enrolled in WIHS, were recruited specifically for this study. We defined cART as described in the Department of Health and Human Services panel on Antiretroviral Guidelines for Adults and Adolescents[45]. HIV disease stage, HIV RNA viral loads and $CD4^+$ T-cell counts over time were obtained from WIHS data, or through self-reporting with medical record verification for participants who were not recruited from WIHS. Of the 13 HIV seropositive participants, 9 reported receiving cART. Seven of the 9 demonstrated viral suppression (below 75 vRNA copies/mL); of these women, all but one had peripheral blood $CD4^+$ T-cell counts >350 cells/μl (the outlier had a single $CD4^+$ T-cell count of 278). Two women who reported using cART had detectable plasma HIV RNA (234 and 100 copies/ml, respectively); both had $CD4^+$ T-cell counts >500 cells/ml. The other 4 HIV seropositive women reported that they had not received cART: these included two natural controllers with plasma HIV RNA <75 copies/mL and $CD4^+$ T-cell counts >350 cells/μl; and two women with a past history of viremia >1,000 copies/ml and $CD4^+$ T-cell

counts between 250 and 350. We also studied 5 consistently HIV uninfected participants of WIHS whose peripheral blood $CD4^+$ T-cell counts all exceeded 800 cells/ml. Median estimated duration of HIV infection was 19 years for both HIV-infected groups. The study protocol was approved by the Institutional Review Boards of the University of California, San Francisco (UCSF) and the University of California, Davis.

Clinical Laboratory Parameters.

Plasma HIV RNA quantification and determination of $CD4^+$ T-cell counts were performed using standard assays in laboratories that participate in the NIAID DAIDS Laboratory Quality Assurance Program. Serological testing for HIV was performed via FDA-approved enzyme-linked immunoassays with Western blot confirmation.

Study Visits.

All participants attended a screening visit during which data on medical history, current medications and use of intravaginal products was assessed. Eligibility criteria included a history of regular menstrual cycles, or a history of menopausal cessation of bleeding, non-use of sex steroid treatments and IUDs, ability to follow study instructions and procedures, low risk for procedural bleeding (no use of anticoagulant treatments or history of bleeding dyscrasias) and no history of inflammatory bowel disease. For women enrolled in WIHS, screening occurred within a six-week window of a WIHS visit. If it occurred outside this time, or the woman was not enrolled in WIHS, CD4 counts and HIV RNA quantitation on peripheral blood and plasma were performed as described above. Routine pelvic examinations were performed to rule out acute reproductive tract infections, or other contraindication to endometrial biopsy. Cervical swab samples were obtained for nucleic acid amplification tests for infection with *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Participants were instructed in the use of urine LH surge tests (Clearblue brand, Proctor & Gamble, Cincinnati, OH) and colonoscopy bowel preparation procedures cessation of P.O intake and bowel cleansing (polyethylene glycol, GoLYTELY solution jug, Braintree Laboratories, Braintree, MA). Participants contacted study staff at the time of a positive LH surge results, or if no such result occurs. Colonoscopy visits were scheduled to occur 7–9 days after LH surge to coincide with the mid-luteal phase of the ovulatory cycle. If no LH surge was observed, colonoscopy was scheduled to occur at the estimated time of the mid-luteal ovulatory phase based on the participant's prior menstrual record. Post-menopausal women were scheduled for colonoscopy at their convenience; reproductive mucosal samples were not collected from post-menopausal women. Participants were contacted prior to beginning the bowel preparation to determine if they were acutely ill (for example an upper respiratory infection or genital herpes recurrence) and if so, the procedure was rescheduled.

Approximately 20mL of blood was collected in vacutainer tubes coated with EDTA (BD Pharmingen, SA Jose, CA). Colonoscopy was performed in a hospital endoscopy suite using standard procedures under conscious sedation. Six to 10 endoscopic biopsies were obtained from healthy appearing tissues of each of the sigmoid colon and terminal ileum. After completion of colonoscopy, the participant, while sedated, was placed in the lithotomy position and reproductive tract specimens were collected. Following collection of

endocervical wick samples, endometrial biopsies were obtained using 3 mm Miltex brand Softflex endometrial biopsy cannulas (1–2 passes). Subsequently, endocervical cytobrush and curettage specimens were collected using Cytobrush Plus® Cell collectors and Kevorkian curettes (both from CooperSurgical, Trumbull, CT), respectively. Tissue samples were placed in RPMI 1640 medium supplemented with 15% fetal calf serum, penicillin (100 U/mL), streptomycin (100ug/mL) and L-glutamine (2mM), designated as R15 medium. Specimens were immediately transported at ambient temperature to the laboratory at the University of California at Davis for processing and analysis.

Isolation of Mononuclear Cells.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Pfizer, New York, NY), and washed in phosphate-buffered saline (PBS). The mononuclear cells from cytobrush were isolated by rubbing the brushes together, rinsing the brushes with medium, passing the cell suspension through a 70-μM nylon cell strainer (Becton Dickinson Discovery Labware, Bedford, MA) and washing in R-15 medium. Mononuclear cells from endocervical curettage were isolated by repeated pipetting, followed by straining and washing, as described for cytobrushes. A previously published protocol, optimized for high leukocyte yield and viability without compromising the detection of most surface antigens, was followed for isolation of lymphocytes from mucosal biopsies[9, 46, 47]. Briefly, biopsies from endometrium, ileum and sigmoid colon were subjected to digestion with 0.5 mg/mL collagenase type II (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO) in RPMI/5% fetal calf serum for 30 minutes in a shaking incubator at 37°C. After 30 minutes, the undigested pieces were passed through a 16-gauge blunt-end needle and a 70-μM nylon cell strainer (Becton Dickinson Discovery Labware, Bedford, MA); cells were then immediately washed in R15 medium and PBS. Remaining tissue pieces were digested an additional 1–2 times, as needed, until no observable fragments remained.

Antibodies and Peptide Pools.

Fluorochrome-labeled monoclonal antibodies used for the phenotypic and intracellular cytokine staining (ICS) assay included CD3 (UCHT1), CD8 (SK1), CCR7 (3D12), CXCR4 (12G5), CCR5 (2D7), IFNγ (B27), TNF-α (MAb11) and unlabeled co-stimulatory antibodies to CD28 (L293) and CD49d (L25) (purchased from Becton-Dickinson Pharmingen, San Diego, CA). CD45RA (2H4) and CD4 (T4D11) were purchased from Beckman Coulter (Fullerton, CA). CD38 (HB7), CD107a (H4A3) were obtained from BD Biosciences (San Jose, CA). HLADR (TU36), Aqua amino reactive dye from Invitrogen (Carlsbad, CA), and IL-2 (MQ1–17H12) were purchased from eBioscience (San Diego, CA). HIV Gag (p55, HXB2 sequence) peptide pools consisting of 15-mer peptides with an 11-amino acid overlap and CMV, EBV and Influenza (CEF) peptide pools were purchased from JPT Peptide Technologies (Berlin, Germany).

Phenotypic and ICS assay and Flow Cytometry.

Leukocytes from whole blood (WB), FRT and GIT were analyzed for cell surface phenotype on the day of collection[46]. The remaining mononuclear cells from PB, endometrium, ileum and sigmoid colon were rested overnight in R15 medium prior to ICS assay. GIT tissue cells suspended in R15 medium were supplemented with piperacillin-tazobactam (0.5 mg/mL)

(Zosyn; Wyeth Pharmaceuticals, Philadelphia, PA) to limit bacterial growth during incubation. ICS assay was performed as described previously[22, 26, 35]. To measure antigen-specific immune responses, cells were stimulated with pooled peptides (3.5μg/mL) spanning the HIV Gag protein or a commercial peptide pool containing immunodominant peptides from cytomegalovirus, Epstein-Barr virus and influenza A virus ("CEF"). Medium containing the peptide vehicle (dimethyl sulfoxide) and co-stimulatory antibodies served as a negative control and staphylococcus enterotoxin B (SEB, 5μg/mL) was used as a positive control. During a 5-hr antigen stimulation that included co-stimulatory antibodies to CD28 and CD49d, cells were stained for CD107a. Cells were further stained for surface markers such as CD4, CD8 and for viability. Cells were fixed with 4% paraformaldehyde and permeabilized using FACS Perm 2 (BD Biosciences) prior to intracellular staining for CD3, IFNγ, TNFα and IL-2. Samples were read on an LSR II flow cytometer using FACSDiva software (BD Biosciences) within 24 hrs of assay completion. Data were analyzed using FlowJo software (TreeStar, Ashland, OR). Boolean gating was used to separate cells into functional categories. Response data were then graphed using SPICE software (version 5.35, provided by Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Data Analysis.

Examples of cell surface phenotyping data for $CD4^+$ and $CD8^+$ T-cells in all tissues are provided in Supplementary Figure S1. Comparisons of T-cell percentages and expression of activation, memory and chemokine markers between and within study groups were performed using the Mann-Whitney test and Wilcoxon matched pairs signed rank test, as appropriate, in Graph Pad Prism software (V5.0, Graph Pad, San Diego, CA). Correlations between variables were done using the Spearman correlation, and linear regression was used to graph a best-fit line to the data (GraphPad Prism).

Antigen-specific T-cell response data were analyzed as previously described[22]. Examples of CD8+ T-cell response data for all tissues are provided in Supplementary Figure S2. Briefly, we determined whether antigen-specific responses differed significantly from the negative control using a formula that assumed a Poisson distribution for both responses, accounting for the actual numbers of gated events in each case. Net antigen-specific responses were then calculated by subtracting the negative control values from antigen-specific responses. Statistical analysis of Boolean-gated data was performed in SPICE software using the Wilcoxon rank test to compare individual responses at a significance level of $P < 0.01$, and a permutation test, based on χ^2 statistics, to compare pie charts.

RESULTS

In women on cART, mean $CD4^+$ T-cell percentages are lowest in endometrium.

Thirteen HIV infected and 5 uninfected women were included in the study. Characteristics of these women are detailed in Table 1 and summarized in Materials and Methods. We measured $CD4^+$ T-cell percentages in blood and multiple sites of GIT and FRT collected from HIV-positive women and healthy controls (Supplementary Figure S1); comparisons between subject groups and between tissue sites are shown in Figure 1. In women on cART

with viral suppression, we detected significantly lower percentages of $CD4^+$ T-cells in blood, ileum, sigmoid colon and endometrium compared to HIV uninfected women. These findings suggest incomplete $CD4^+$ T-cell reconstitution in the GIT and FRT tissues of women on cART. There were also some differences between tissues: in women on cART, $CD4^+$ T-cell percentages in endometrium were significantly lower than in blood or sigmoid colon (Fig. 1). We also looked for strong associations between $CD4^+$ T-cell percentages in different tissues in women on cART, and found two significant correlations: between blood and sigmoid $CD4^+$ T-cell percentages (Spearman r = 0.73, P = 0.031); and between endometrial and endocervical cytobrush $CD4^+$ T-cell percentages (Spearman r = 0.89, P = 0.033) (data not shown). Somewhat surprisingly, the three women on cART with the lowest blood $CD4^+$ T-cell counts (participants S06, S07 and S08) did not consistently reveal lower $CD4^+$ T-cell percentages in tissues compared to women on cART with blood $CD4^+$ T-cell counts >500 (Fig. 1) (data not shown).

HIV controllers maintain $CD4^+$ T-cell percentages in FRT and GIT similar to healthy controls.

Within the group of participants who were not receiving cART at the time of sampling, two HIV positive women (S05 and W57) met virologic and immunologic criteria as HIV controllers (i.e., $CD4^+$ >350 cells/μL and VL <75 copies/mL). Strikingly, these women maintained tissue $CD4^+$ T-cell percentages similar to those of HIV uninfected women (Figure 1). In contrast, two other participants who were not receiving cART (W56 and W58) had low but detectable viremia and blood CD4 counts below 350 cells/μL. These women had noticeably lower percentages of $CD4^+$ T-cells in mucosal tissues compared to S05 and W57 (Figure 1). Although the sample size was too small to establish statistically significant differences between groups, these findings indicate differential maintenance of mucosal $CD4^+$ T-cell populations in individuals with different HIV disease progression phenotypes.

High percentages of activated T-cells in endometrium of women on cART.

To assess mucosal T-cell activation status, we measured coexpression of HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T-cells from blood and tissues (Supplementary Figure S1). Previous studies have shown that T-cell activation is relatively low in GIT of controllers and individuals on cART as compared to untreated progressors[15, 23]; however, to our knowledge T-cell activation in the upper FRT of HIV-infected women has not been studied, and comparisons between GIT and FRT have not been previously reported. In the present study, the percentage of activated T-cells in the GIT was generally comparable between subject groups. However, we observed a significantly higher percentage of activated $CD4^+$ and $CD8^+$ T-cells in the endometrium of participants on cART compared to uninfected women (Figures 2A, 2B). Comparison between tissues within the cART-treated group also indicated a significantly higher percentage of activated $CD8^+$ T-cells in endometrium than in endocervix (curettage and cytobrush), GIT tissues (ileum and sigmoid), or blood (Figure 2B). In the uninfected group, T-cell activation marker expression was also greater in endometrial $CD8^+$ T-cells compared to ileal $CD8^+$ T-cells.

Higher percentages of effector memory T-cells in ileum of women not on cART, including controllers, compared to uninfected women.

To assess the preservation and restoration of memory T-cell subsets in mucosal tissues, we determined the breakdown of memory/effector T-cell subsets based on expression of surface markers CCR7 and CD45RA (Supplementary Figure S1; Figure 3A–D). Using these standard markers, naïve T-cells are defined as $CCR7^+/CD45RA^+$; central memory cells as $CCR7^+$/CD45RA-; effector memory cells as CCR7-/CD45RA-, and terminally differentiated effector cells as CCR7-/$CD45RA^+$ [48, 49]. We found large variation in expression of memory/effector markers on mucosal T-cells, particularly among women on cART (Figure 3). Also, as compared to uninfected participants, women not on cART had significantly higher percentages of effector memory $CD4^+$ and $CD8^+$ T-cells in ileal mucosa (Figs. 3B, 3D), with correspondingly lower percentages of central memory T-cells (Figs. 3A, 3C). Similar trends were observed in sigmoid mucosa, although they did not reach significance. These trends were not observed in either blood or FRT tissues; however, fewer reproductive tract samples were available than GIT samples for these analyses.

HIV Gag-specific T-cell responses in tissues are consistently lower in women on cART compared to those not on cART.

To measure HIV-specific T-cell responses, lymphocytes freshly isolated from FRT, GIT and blood were stimulated with pooled peptides spanning HIV Gag; $CD8^+$ T-cells producing IL-2, IFNγ, TNFα and CD107a were measured by flow cytometry (Supplementary Figure S2). The total percentage of responding T-cells (i.e., exhibiting any response or combination of responses), and the percentages of cells producing individual factors, were determined after background subtraction. These results are summarized in Figures 4A–4D. The percentage of $CD8^+$ T-cells expressing either CD107 or IFNγ in response to HIV Gag stimulation was greater in sigmoid colon of women not on cART compared to those on cART; similar trends were observed in ileum, but did not reach significance due to small sample size (Fig. 4B, C). Production of IL-2 was generally weak in mucosal samples (Fig. 5) and was not included in Figure 4. Together, these findings are consistent with our previous reports of strong HIV Gag-specific T-cell responses in gastrointestinal mucosa of individuals not on antiretroviral therapy, including HIV controllers[15, 22, 23, 35, 50]. HIV-specific T-cell responses in mid-luteal endometrial tissues were generally comparable in magnitude and range to those detected in blood (Figure 4). Unfortunately, insufficient T-cells ($<1\times10^5$) were obtained from endocervical curettage and cytobrush to reliably perform antigen-specific T-cell response assays by flow cytometry using these samples.

As a control, we also measured $CD8^+$ T-cell responses to a cocktail of immunodominant peptides from CMV, EBV and influenza (abbreviated CEF) in blood, endometrium, and GIT tissues. CEF-specific $CD8^+$ T-cell responses ranging from 1–3% of $CD8^+$ T-cells were detected in all three subject groups, including uninfected women, indicating the presence of memory T-cell responses to other pathogens in gastrointestinal and reproductive tissues (data not shown)[51].

Boolean gating reveals fewer polyfunctional HIV-specific CD8+ T-cells in women on cART.

Using Boolean gating and SPICE analysis, HIV-specific T-cells were assigned to 15 different functional categories (excluding the non-responding subset) based upon patterns of co-expression of the four analytes: IFNγ, TNFα, IL2 and CD107. Figures 5A and 5B show the breakdown of $CD8^+$ T-cell responses by analyte(s) expressed by mucosal $CD8^+$ T-cells from women who were not on cART (Figure 5A) or on cART (Figure 5B). In women not on cART, polyfunctional HIV-specific T-cells, defined as those cells co-expressing 3 or 4 analytes in response to stimulation, were more abundant in ileum and sigmoid colon than in endometrium (Figure 5A), although this finding is limited by the small number of samples in each group. A comparison of Figures 5A and 5B reveals that generally, HIV Gag-specific T-cell responses in women on cART (Fig. 5B) were lower in magnitude across all functional groups, in agreement with previous work[15, 22, 23, 35, 50].

DISCUSSION

The effects of HIV infection on the FRT remain to be fully characterized, and most previous studies evaluating mucosal tissues in HIV disease have focused on a single tissue site[14, 17, 18]. Similar to the gastrointestinal tract, tissues of the lower (ectocervix, vagina) and upper (endocervix) FRT contain partially activated, memory $CD4^+$ T-cells expressing CCR5 and/or CXCR4, which can serve as targets for HIV[52]. In this study, we compared T-cell frequency, phenotype, immune activation and HIV-specific T-cell responses in multiple regions of the GIT and upper FRT in a group of long-term HIV-positive women, both on and off cART.

To the best of our knowledge, this is the first study to address the effects of cART on $CD4^+$ T-cell recovery, immune activation and HIV-specific $CD8^+$ T-cell responses in parallel in both the GIT and upper FRT of HIV-infected women. Wide variation in the degree of $CD4^+$ T-cell reconstitution in the gut mucosa, ranging from minimal to near complete, has been reported in response to cART, and initiation of cART during chronic HIV infection generally does not lead to complete $CD4^+$ T-cell restoration[14, 17, 19, 20, 53]. Consistent with previous reports, we observed incomplete $CD4^+$ T-cell recovery in mucosal tissues of cART-treated women compared to uninfected participants. There were subtle differences in $CD4^+$ T-cell percentages between mucosal sites, reaching significance only for the comparison between endometrium (with the lowest mean percentages) and sigmoid colon (with the highest). It should be noted that in the absence of data on the number of $CD4^+$ T-cells per unit area in each tissue, it remains unclear the extent to which observed differences in T-cell percentage reflect differences in absolute T-cell numbers.

Although the mechanisms controlling $CD4^+$ T-cell recovery during cART are not fully defined, potential contributing factors include chronic immune activation, residual viral replication, altered expression of mucosal homing and retention molecules and inflammation-associated fibrosis[54–57]. Chronic immune activation is a better predictor of survival in HIV disease than either HIV RNA or $CD4^+$ T-cell count[8, 58, 59], and the efficacy of cART in reconstituting the immune system is strongly correlated with its ability to reduce this activation[60–63]. Notably, in this study, the highest levels of co-expression of T-cell activation markers CD38 and HLA-DR were found on endometrial $CD8^+$ and $CD4^+$ T-cells

in women on cART. The specific mechanisms underlying the maintenance of chronic T-cell activation in this tissue following cART are unclear and will require further study. HIV replication may induce immune activation by directly stimulating innate and adaptive immune responses[64]; female reproductive hormones also influence immune activation in the FRT[65–67]. Age may also contribute to these differences, as the mean/median ages of women in this study were 38/39, 48/47.5, and 45.8/45 for HIV-negative, HIV^+ without cART, and HIV^+ with cART groups, respectively, although the three post-menopausal participants did not contribute reproductive mucosal samples.

An effective $CD8^+$ T-cell response contributes to reduction in peak viremia during acute HIV infection[68, 69]. In many HIV controllers, strong and polyfunctional HIV Gag-specific T-cell responses are strongly associated with viral control[22, 23, 26, 35, 70, 71]. cART is associated with decreased HIV-specific T-cell frequency and breadth, largely due to reduced antigen load[15, 22, 23, 35, 72, 73]. Polyfunctional HIV-specific T-cells have been reported at individual mucosal sites including bronchoalveolar tissues, the gastrointestinal tract and the lower reproductive tract[15, 23, 28, 35, 74]. However, very little information is available on HIV specific $CD8^+$ T-cell responses in the upper FRT[75]. In the present study, HIV Gag-specific $CD8^+$ T-cells were detected in parallel in gastrointestinal and reproductive tissues of the same individuals. While the number of endometrial samples obtained from women not on cART was unfortunately too low to draw general conclusions regarding the relative abundance of these cells compared to other mucosal tissues, we were able to demonstrate HIV-specific $CD8^+$ T-cells expressing multiple cytokines in luteal phase endometrium of women both on and off cART.

Cyclic changes in female sex hormones influence $CD4^+$ and $CD8^+$ T-cell abundance, CCR5 expression and cytotoxic T-cell activity in the upper FRT[76, 77], with potentially important consequences for HIV susceptibility and disease progression[78]. $CD8^+$ T-cell responses in the upper FRT are suppressed by high levels of estrogen and progesterone secreted during the luteal phase (days 14 to 28) of the menstrual cycle[77]. In contrast, such hormonal fluctuations have minimal effects on T-cells in the lower reproductive tract, which remain constant throughout the menstrual cycle[79]. In the present study, it was not possible to obtain samples at multiple time points; however, given the reported effects of female sex hormones on T-cell responses in the upper FRT, it would be of interest to directly compare T-cell activation and HIV-specific T-cell responses in samples acquired during the proliferative and luteal phases.

In conclusion, these studies reveal important parallels as well as significant differences in $CD4^+$ T-cell frequencies, immune activation and HIV-specific T-cell responses between two major mucosal tissues: the gastrointestinal and reproductive tracts. Importantly, T-cell phenotypes and HIV-specific response patterns differed between blood and mucosal tissues. This finding underscores the importance of studying lymphocyte distribution and antigen-specific responses in tissues, as blood is not always an accurate surrogate marker. This study also calls attention to the potential role of the upper FRT, currently an under-studied tissue, in HIV disease. Future work should attempt to further elucidate the ability of this tissue to serve as a potential reservoir for replicating virus, and to address the role of hormonally regulated, cyclic variation of immune responses in the host's susceptibility to HIV infection and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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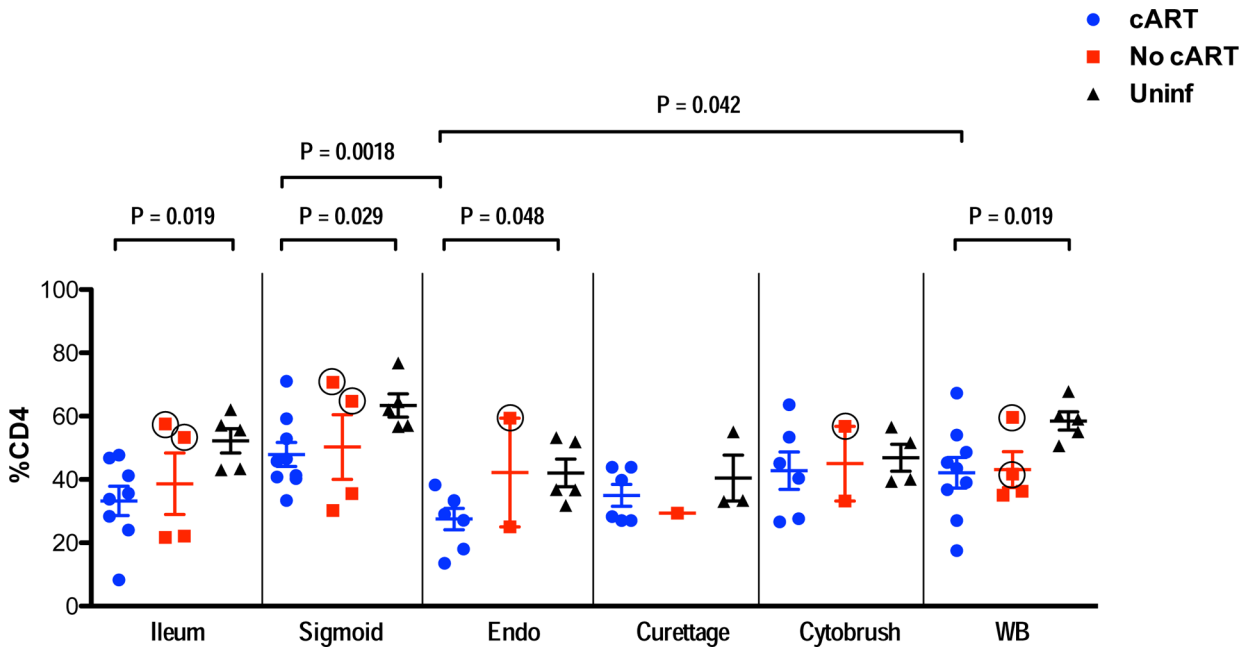

Figure 1. Percentages of CD4$^+$ T-cells in GIT, FRT and WB.

CD4$^+$ T-cell percentage, relative to all viable CD3$^+$ cells, is indicated on the y-axis. Tissue types are indicated on the x-axis. After initial gating based on scatter characteristics and doublet discrimination, viable cells (based on Aqua dye exclusion) were gated for expression of CD3, followed by CD4[9]. As described in the text, the No cART group included two HIV controllers; data points for these two participants are identified as red squares with black circles. Abbreviations: Endo, endometrium; Uninf, uninfected; WB, whole blood. Comparisons between groups and tissues were performed with Mann-Whitney and Wilcoxon matched pairs signed rank tests, respectively. Horizontal and vertical bars represent mean and standard deviation, respectively. Significant differences, either between participant groups within a single tissue type or between tissues within a participant group, are indicated by horizontal brackets.

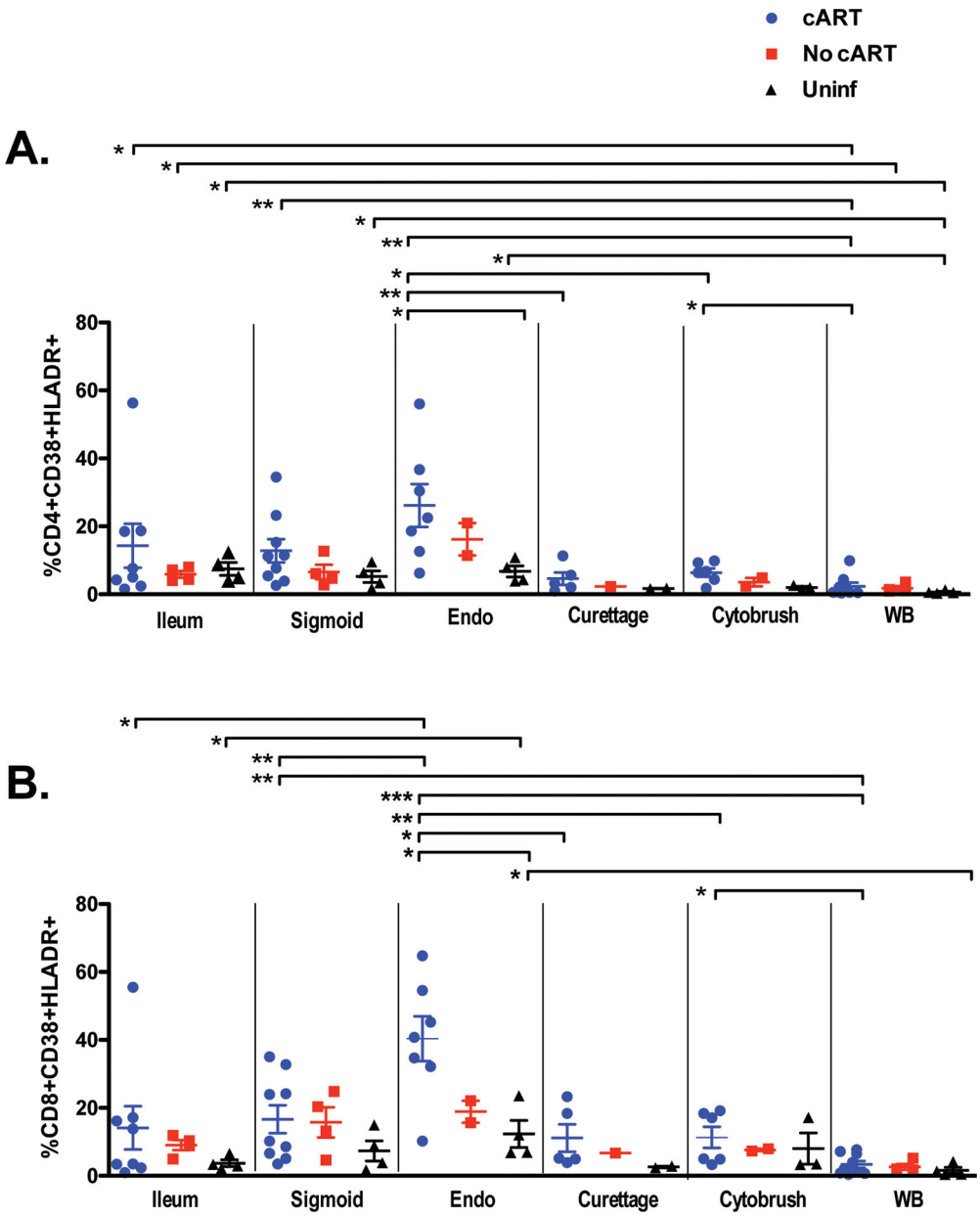

Figure 2. Percentages of activated T-cells.

Shown are the percentages of **(A)** $CD4^+$ and **(B)** $CD8^+$ T-cells co-expressing HLA-DR and CD38. After initial gating based on scatter characteristics and doublet discrimination, viable cells (based on Aqua dye exclusion) were gated for expression of CD3, followed by CD4 and/or CD8, and finally HLA-DR and/or CD38[9]. bbreviations: Endo, endometrium; Uninf, uninfected; WB, whole blood. Comparisons between participant groups and tissues were performed with Mann-Whitney and Wilcoxon matched pairs signed rank test, respectively. The horizontal and vertical bars represent mean and standard deviation, respectively. Significant differences, either between participant groups within a single tissue type or between tissues within a participant group, are indicated by horizontal brackets with asterisks to indicate P values as follows: *P < 0.05; **P < 0.01; ***P <0.001.

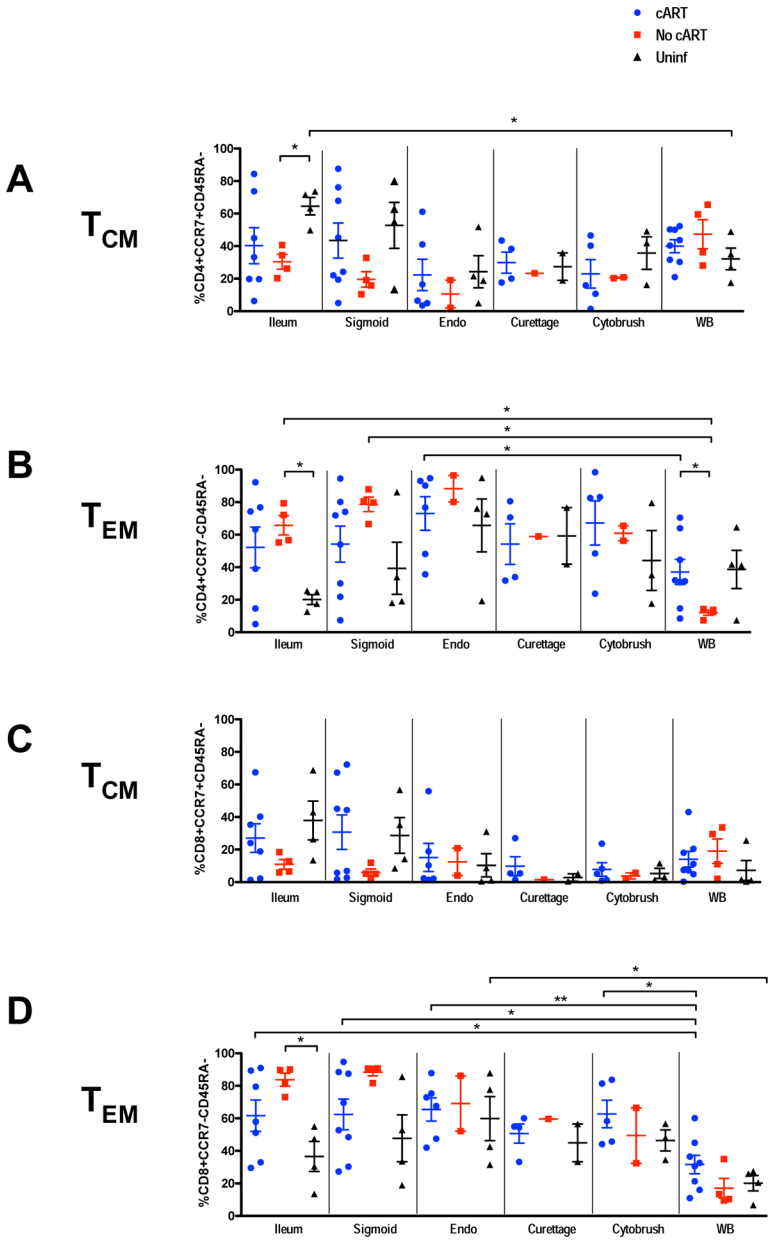

Figure 3. Memory T-cell subsets.

Percentages of central **(A, C)** and effector memory **(B, D)** T-cells. Panels A and B correspond to $CD4^+$ T-cells; C and D are $CD8^+$ T-cells. After initial gating based on scatter characteristics and doublet discrimination, viable cells (based on Aqua dye exclusion) were gated for expression of CD3, followed by CD4 and/or CD8, and finally CD45RA and/or $CCR7^9$. Abbreviations: Endo, endometrium; Uninf, uninfected; WB, whole blood. Comparisons between groups and tissues were performed with Mann-Whitney and Wilcoxon matched pairs signed rank test, respectively. The horizontal and vertical bars represent mean and standard deviation, respectively. Significant differences, either between participant groups within a single tissue type or between tissues within a participant group,

are indicated by horizontal brackets with asterisks to indicate P values as follows: *P < 0.05; **P < 0.01.

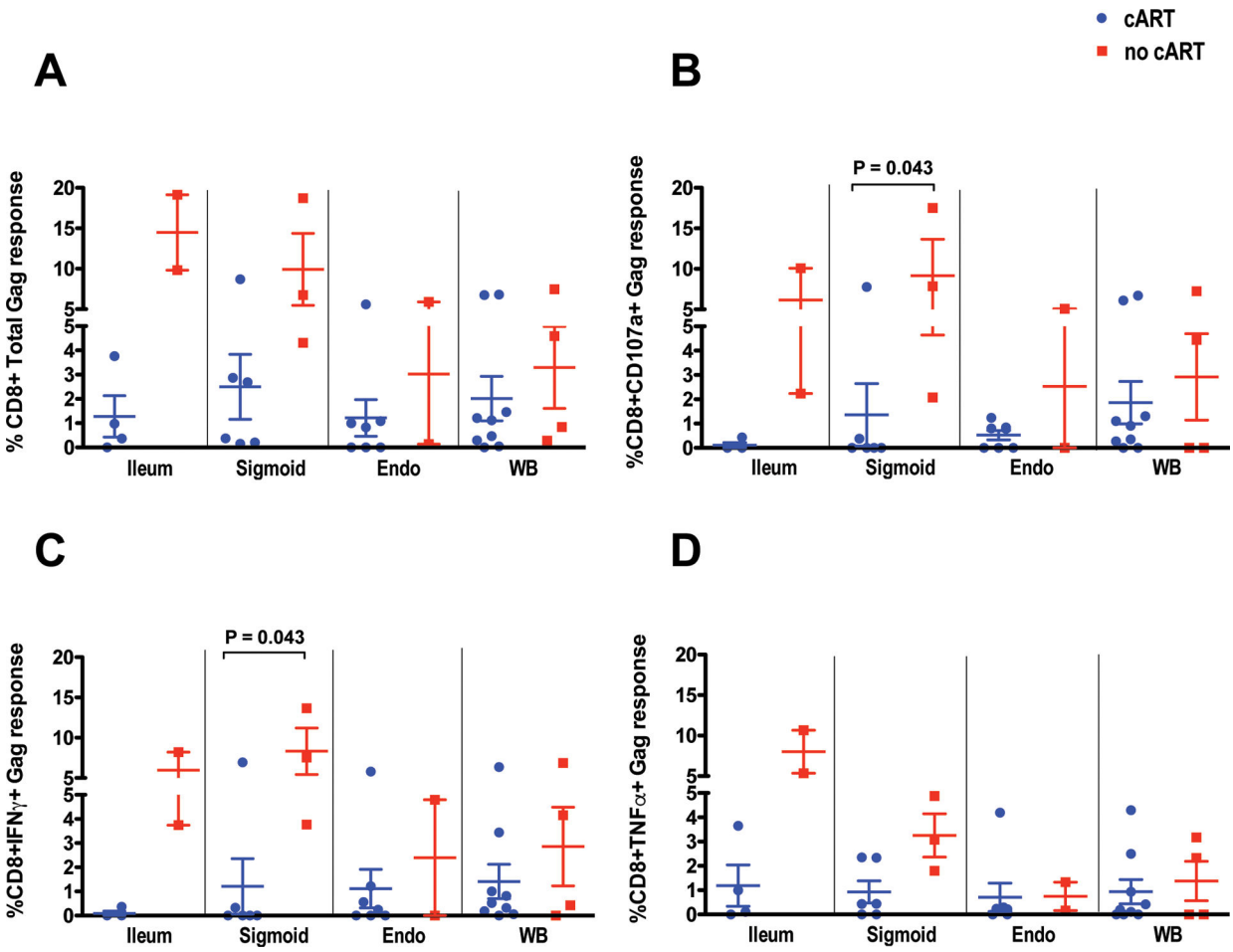

Figure 4. HIV Gag-specific CD8$^+$ T-cell responses.

Panel (A) summarizes the total percentage of Gag-specific T-cells in each tissue based on cytokine flow cytometry results; cells producing multiple analytes are only counted once. Panels B thru D show the percentages of cells producing (B) CD107a, (C) IFNγ, or (D) TNFα, respectively, in response to HIV Gag stimulation. After initial gating based on scatter characteristics and doublet discrimination, viable cells (based on Aqua dye exclusion) were gated for expression of CD3, followed by CD4 and/or CD8, and finally Boolean gating for production of each analyte[9]. Abbreviations: Endo, endometrium; WB, whole blood. Comparisons between groups and tissues were performed with Mann-Whitney and Wilcoxon matched pairs signed rank test, respectively. The horizontal and vertical bars represent mean and standard deviation, respectively. Significant differences between participant groups within a single tissue type are indicated by horizontal brackets.

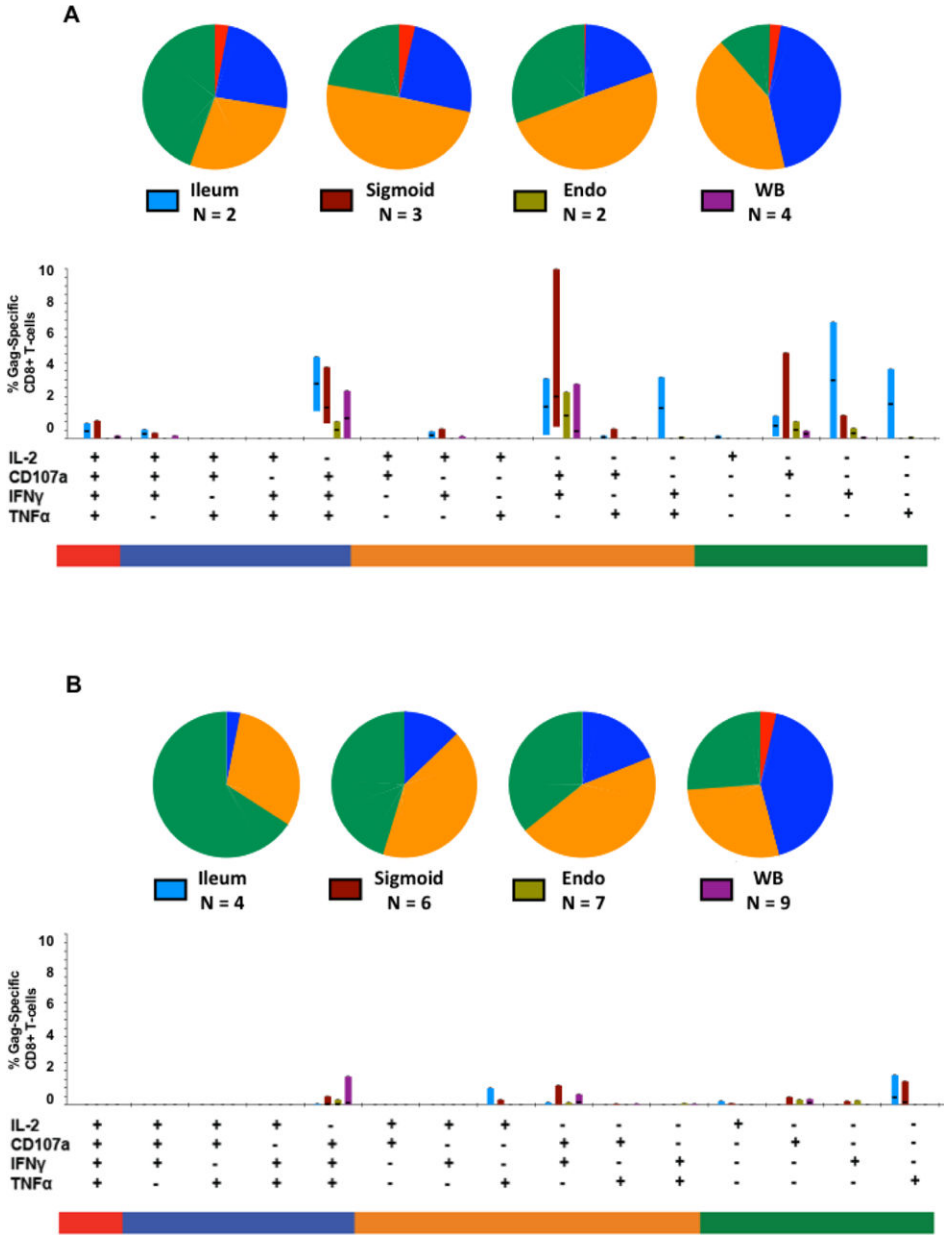

Figure 5. Polyfunctional analysis of HIV Gag-specific CD8$^+$ T-cell responses.

The Figure summarizes responses in terminal ileum, sigmoid colon, endometrium (Endo), and whole blood (WB). As described in the legend to Figure 4, after initial gating based on scatter characteristics and doublet discrimination, viable cells (based on Aqua dye exclusion) were gated for expression of CD3, followed by CD4 and/or CD8, and finally Boolean gating for production of each analyte[9]. Figure (5A) shows responses in women not on cART; Figure (5B) includes only responses from women on cART. The total HIV Gag-specific response was broken down into 15 individual categories, as indicated by plus and minus signs. Vertical bars show the percentage of HIV Gag-specific CD8$^+$ T-cells from each tissue (which are color coded and indicated in the chart legend) after subtraction of background counts. The median response for each functional category is indicated by a horizontal black

line; bar height spans inter-quartile ranges (25^{th} thru 75^{th} percentile). Pie charts are color-coded to indicate responses comprised of a single function (green), two functions (orange), three functions (blue) or four functions (red). Responses involving 3 or 4 functions are considered as polyfunctional.

Table 1:

Characteristics of study participants.

Patient Identification No./Group	Ethnicity	Age (yrs)	Time since first known pos HIV test (yrs)	CD4 count (Cells/μl)	Plasma Viral load (copies/mL)[§]	Cervical viral load (copies/mL)
HIV uninfected (n=5)						
W51	AA	35	-	905	-	-
W52	AA	39	-	1051	-	-
W53	AA	35	-	846	-	-
W63	AA	40	-	1705	-	-
W66	AA	43	-	1042	-	-
HIV infected and off antiretroviral treatment (n=4)						
S05	AA	47	16	1345	<40	<40
W56[**]	C	54[*]	25	320	266	NA
W57[**]	AA	48[*]	19	450	<75	NA
W58	AA	43	20	275	2500	1035
HIV infected and receiving cART (n=9)						
S04[**]	AA	46	16	535	26	<40
S06	C	45	25	397	<20	<40
S07	C	49	21	454	<20	<40
S08[**]	C	59[*]	23	278	<40	<40
W54[•]	AA	42	18	1000	<75	<40
W55	AA	38	9	896	<75	<40
W59	AA	42	14	689	234	<40
W60	AA	44	12	720	100	<40
W62	C	48	21	559	<40	<40

Abbreviations:

Identification numbers beginning with “W” indicate WIHS participants; Numbers beginning with “S” are non-WIHS participants. AA-African American, C-Caucasian, NA-not available.

[*] Post-menopausal;

[**] No FRT tissues collected;

[•] No ileum collected.

[§] Plasma viral load data were obtained from WIHS or through self-reporting with medical record verification; accordingly, assay detection thresholds vary.