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Increased Frequency of Regulatory T Cells Accompanies Increased Immune Activation in Rectal Mucosae of HIV-Positive Noncontrollers[▽]

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Gut-associated lymphoid tissue (GALT) is a major site of HIV replication and CD4$^+$ T cell depletion. Furthermore, microbial translocation facilitated by mucosal damage likely contributes to the generalized immune activation observed in HIV infection. Regulatory T cells (Treg) help maintain homeostasis and suppress harmful immune activation during infection; however, in the case of persistent viral infections such as HIV, their role is less clear. Although a number of studies have examined Treg in blood during chronic infection, few have explored Treg in the gastrointestinal mucosa. For this study, paired blood and rectal biopsy samples were obtained from 12 HIV noncontrollers (viral load of >10,000 copies/ml plasma), 10 HIV controllers (viral load of <500 copies/ml plasma for more than 5 years), and 12 HIV seronegative control subjects. Noncontrollers had significantly higher percentages of Treg in rectal mononuclear cells (RMNC), but not in blood, compared to seronegative subjects ($P = 0.001$) or HIV controllers ($P = 0.002$). Mucosal Treg positively correlated with viral load ($P = 0.01$) and expression of immune activation markers by CD4$^+$ ($P = 0.01$) and CD8$^+$ ($P = 0.07$) T cells. Suppression assays indicated that mucosal and peripheral Treg of noncontrollers and controllers maintained their capacity to suppress non-Treg proliferation to a similar extent as Treg from seronegative subjects. Together, these findings reveal that rather than experiencing depletion, mucosal Treg frequency is enhanced during chronic HIV infection and is positively correlated with viral load and immune activation. Moreover, mucosal Treg maintain their suppressive ability during chronic HIV infection, potentially contributing to diminished HIV-specific T cell responses and viral persistence.

Regulatory T cells (Treg) are important mediators of immune homeostasis that act by suppressing the activation and effector functions of innate and adaptive immune cells (7, 58, 61, 65, 86, 90, 93, 96). Although first described in the 1970s as "suppressor T cells" (38, 39), little was known of these cells until the mid-1990s, when Sakaguchi et al. identified a minor subset of CD4$^+$ T cells responsible for protection from autoimmunity in mice. These cells were characterized by expression of the high-affinity interleukin-2 (IL-2) receptor alpha chain, CD25 (74). CD25 proved to be a reliable Treg marker in mice; however, human effector memory T cells can also express intermediate levels of CD25, blurring the distinction between regulatory and nonregulatory cells (81). In 2003, several groups linked defects in CD4$^+$ CD25$^+$ T cells to mutations in the *Foxp3* gene of "scurfy" mice, which suffer from aggressive X-linked autoimmune disease (15, 35, 46, 52). In humans, similar mutations in *FOXP3* cause IPEX (*i*mmune dysregulation, *p*olyendocrinopathy, *e*nteropathy, *X*-linked syndrome), a multiorgan autoimmune disease often accompanied by severe intestinal inflammation (9, 18, 36). With the addition of FOXP3, the identification of human Treg was significantly improved. This transcription factor is necessary for Treg generation and function and remains the most definitive marker of Treg to date (35, 48, 99). Nonetheless, transient, low-level expression of FOXP3 has been observed in human T cells after T cell receptor (TCR) stimulation; thus, FOXP3 expression alone is not always sufficient to differentiate Treg from non-Treg (75).

Importantly, Treg not only act to suppress autoreactive cells, but also play a central role in protecting host tissues from immune-mediated damage by limiting immune activation and proliferation during infection (8). In the case of persistent viral infections such as HIV, however, the benefits of a vigorous Treg response are debatable (7, 32, 60). HIV presents several unique challenges to fully understanding how Treg impact disease progression. The central question involves determining whether Treg are predominantly beneficial or harmful to the infected individual. Studies with the macaque model suggested an early Treg response may be responsible for premature and persistent suppression of effector responses, contributing to the establishment of a chronic disease state (29, 30). In agreement with this hypothesis, Treg have been shown to inhibit HIV-specific T cell responses *ex vivo* and thus may act *in vivo* by suppressing HIV-specific immunity, allowing HIV to persist

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(1, 53, 55, 98). Transient Treg depletion during chronic Friend virus infection in mice resulted in the reactivation of virus-specific CD8 T cells and a reduction in virus load, again indicating a potentially harmful role for Treg in retroviral infection (22). Furthermore, Favre et al. recently postulated that in gut-associated lymphoid tissue (GALT), expansion of Treg in response to inflammation may support disease progression by preventing the recovery of Th17 cells, resulting in persistent mucosal barrier dysfunction and failure to clear translocated microbial products (31). However, other reports have suggested that Treg play a largely beneficial role, either by suppressing HIV-replication in conventional $CD4^+$ cells (66) or by limiting nonspecific immune activation (17, 51). Indeed, because immune activation closely correlates with disease progression (19, 40, 63, 88), it is possible that in the context of chronic infection the beneficial aspects of the Treg response outweigh the disadvantages.

To date, the majority of studies on Treg in HIV infection have focused on peripheral blood; however, HIV also affects lymphoid tissues, notably the gastrointestinal mucosa (12, 14, 41, 43, 44, 83, 84, 95). $CD4^+$ T cells in the gut are depleted early and rapidly after infection with HIV or simian immunodeficiency virus (SIV) (14, 43, 59, 64, 87, 94). The depletion of gut $CD4^+$ T cells, including Th17 cells, is associated with decreased integrity of the mucosal barrier, resulting in gut "leakiness" and microbial translocation, a likely contributor to increased immune activation (11, 13, 28, 31, 37, 44, 77). Although studies of peripheral Treg levels have had varied results (3, 24, 54, 56, 92, 101), a general consensus appears to be emerging regarding Treg in tissues. Several studies have found evidence of increased Treg frequencies in lymph nodes and tonsils of untreated SIV-positive (SIV^+) macaques and HIV^+ patients with high viral loads (2, 3, 10, 27, 29, 31, 69, 78); however, very few have examined mucosal Treg from HIV-infected humans (27, 31), and to our knowledge, none have directly tested their functional capacity. Moreover, while Treg may accumulate in SIV- and HIV-infected tissue sites, the causes and effects of this increase remain unclear. By comparing levels of peripheral and mucosal Treg present in HIV controllers, who are able to maintain low-to-undetectable levels of viremia in the absence of antiretroviral therapy, to those in noncontrollers with progressive infection, we may begin to answer some of these challenging questions.

This study examined Treg phenotype, frequency, and function in both blood and rectal tissue of HIV controllers and noncontrollers. We observed increased frequencies of Treg in rectal mucosae of noncontrollers compared to controllers or seronegative individuals regardless of the method used to identify Treg. In contrast, no significant differences between groups were detected in blood. Moreover, although the number of $CD4^+$ T cells was decreased in rectal mucosae of noncontrollers, Treg numbers did not differ significantly between patient groups. The percentage of rectal Treg correlated positively with plasma viral load and with mucosal immune activation, suggesting that these factors may drive mucosal accumulation and/or conversion of Treg. We also demonstrated, for the first time, the suppressive capacity of mucosal Treg isolated from HIV^+ patients. Although mucosal Treg of noncontrollers appeared more highly activated, they suppressed proliferation of autologous $CD4^+$ $CD25^-$ T cells to a similar extent as Treg from controllers or seronegatives. Thus, noncontrollers maintain a functional Treg response to viral infection that is nonetheless unable to effectively control mucosal immune activation. Collectively, these results point to the gastrointestinal mucosa as a central player in HIV pathogenesis throughout the course of disease. Increases in Treg frequency in rectal mucosa may contribute to T effector cell dysfunction and viral persistence in HIV noncontrollers by perpetuating a destructive cycle of effector cell suppression, virus replication, and immune activation.

MATERIALS AND METHODS

Study subjects and clinical samples. HIV-positive ($n = 23$) and -negative ($n = 12$) subjects were enrolled and samples collected at the University of California—Davis Medical Center (UCDMC) in Sacramento, CA, or at San Francisco General Hospital (SFGH), University of California—San Francisco (UCSF). Informed consent was obtained from all subjects under protocols approved by the Committee on Human Subjects Research, UCSF, and the University of California—Davis Institutional Review Board. Each patient provided approximately 20 ml of blood, collected in Vacutainer tubes coated with EDTA (BD Pharmingen, San Jose, CA), and 20 to 25 rectal biopsy specimens, collected in R15 medium (RMPI 1640 containing 15% fetal bovine serum, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2 mM glutamine). The HIV^+ participants, none of whom were on antiretroviral therapy, were considered "noncontrollers" if they maintained plasma HIV RNA levels of >10,000 copies/ml and "controllers" if they maintained plasma HIV RNA levels of <2,000 copies/ml, as previously defined by Emu et al. (20, 26). All controllers in our study had plasma HIV RNA levels <500 copies/ml, well below the established criteria for controllers.

Cell isolation. Patient blood and rectal biopsy samples were processed the same day they were collected. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Hypaque (GE Healthcare, United Kingdom) gradient centrifugation. Rectal biopsies were obtained by flexible sigmoidoscopy as described previously (34) and subjected to three rounds of tissue digestion in medium containing 0.5 mg/ml collagenase II (Sigma-Aldrich, St. Louis, MO) followed by mechanical disruption with a 12-ml syringe equipped with an 18-gauge blunt-end needle, and passage through a 70-μm-pore cell strainer to yield a single-cell suspension of rectal mononuclear cells (RMNC). RMNC were pooled and washed in R15. Red blood cell lysis was performed as needed on PBMC and RMNC using ammonium chloride-potassium carbonate-EDTA (ACK). Cells destined for immunophenotyping were rested overnight at 37°C with 5% CO_2, while all remaining cells were used to establish the suppression assays. Zosyn (piperacillin-tazobactam; Wyeth, Madison, NJ) was added at 0.5 mg/ml to all rectal cell cultures to prevent bacterial growth.

Treg immunophenotyping. Following isolation, 1×10^6 PBMC and 1×10^6 RMNC were immunophenotyped using multiparameter flow cytometry. Surface staining for CD3, CD4, CD8, CD25, CD127, CD38, and programmed death 1 receptor (PD-1), followed by intracellular staining for FOXP3 was performed using the eBioscience method and FOXP3 staining buffer set (eBioscience, San Diego, CA). LIVE/DEAD fixable Aqua dead cell stain (Invitrogen, Carlsbad, CA), was included in the surface stain in order to identify viable cells. Fluorescence minus one (FMO) controls (72) were included as needed. BD CompBeads (BD Pharmingen) were used to assess compensation settings prior to acquisition and to establish a compensation matrix for data analysis. All samples were fixed in 1% paraformaldehyde before acquisition on an LSRII flow cytometer (BD Pharmingen). Flow cytometry data were analyzed using FlowJo software (Tree-Star, Inc., Ashland, OR). The number of Treg per 10,000 $CD3^+$ T cells was calculated from flow data using the following formula: (total $CD4^+$ $FOXP3^+$ $CD25^+$ cell count/total $CD3^+$ cell count) × 10,000, as previously described (24, 68).

Purification of T cell subsets and suppression assays. PBMC and RMNC were separated into $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ fractions using EasySep particles and magnets according to the manufacturer's suggested protocol (StemCell Technologies, Inc., Vancouver, British Columbia, Canada). Briefly, after incubation with the EasySep human CD4 enrichment cocktail, magnetic beads ("D particles") were used to negatively select for $CD4^+$ T cells. The CD4-enriched cells were then incubated with EasySep human CD25 selection cocktail and magnetic nanoparticles to positively select for the $CD4^+$ CD25-bright population. The $CD4^+$ $CD25^-$ responder cells remaining in the supernatant fraction were resuspended in prewarmed phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and incubated with 2 μM carboxyfluorescein

succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37°C with 5% CO_2. Staining was arrested by the addition of 5 times the volume of ice-cold R15 followed by a 5-min incubation on ice. Responder cells were washed twice more in R15 before counting.

For the suppression assays, wells of a 96-well round-bottom plate were either left uncoated or coated with 0.2 μg/ml anti-CD3, clone OKT3 (eBioscience), for 4 h at 37°C. Autologous PBMC were irradiated at 4,000 rads; 1×10^5 irradiated feeder cells were added to the culture wells along with 1×10^4 to 11×10^4 CFSE-labeled responder cells and the indicated number of $CD4^+$ $CD25^+$ Treg in R15 supplemented with 10 mM HEPES, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Plates were centrifuged at 700 rpm for 5 min to initiate cell contact and incubated at 37°C with 5% CO_2 for 60 to 84 h. After the indicated culture period, cells were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD25, and anti-FOXP3 using the FOXP3 staining buffer set according to the manufacturer's instructions. Anti-OX40/CD134 antibodies were included in the stain for the majority of samples. Cell phenotype and proliferation of responder cells (via CFSE dye dilution) were determined by flow cytometry. In order to control for Treg remaining in the responder cell population, the ratio of CFSE-negative ($CFSE^-$) $CD4^+$ $CD25^+$ $FOXP3^+$ to CFSE-positive ($CFSE^+$) $CD4^+$ $CD25^+$ $FOXP3^+$ cells was used in the correlation analyses where the percentage of proliferation was determined directly from histogram plots of $CFSE^+$ cells.

Antibodies. The fluorochrome-labeled antibodies used for this study include anti-CD3 (clone UCHT1, Pacific Blue labeled; BD Pharmingen), anti-CD4 (SFCI12T4D11, electron-coupled dye (ECD) labeled; Beckman Coulter, Brea, CA; or L200, peridinin chlorophyll protein [PerCP]-Cy5.5 labeled; BD Pharmingen), anti-CD8 (SK1 or 3B5, Qdot605 labeled; Invitrogen), anti-CD25 (M-A251, phycoerythrin [PE]-Cy7 labeled; BD Pharmingen), anti-CD127 (hIL-7R-M21, Alexa Fluor 647 labeled; BD Pharmingen), anti-PD-1 (eBioJ105, PE labeled; eBioscience), anti-CD38 (HIT2, PerCP-Cy5.5 or PE-Cy5 labeled; BD Pharmingen), anti-FOXP3 (PCH101, Alexa Fluor 700 labeled; eBioscience), and anti-OX40/CD134 (ACT35, PE labeled; BD Pharmingen).

Statistical analysis. GraphPad Prism (GraphPad Software, La Jolla, CA) was used to graph and analyze data for statistical significance. Intergroup comparisons were analyzed using a two-tailed Mann-Whitney test. The Wilcoxon matched-pairs test was used for intragroup comparisons between blood and rectal samples. The Spearman rank test was used for bivariate correlations, while linear regression analysis was used to produce an accompanying best-fit line. P values between 0.1 and 0.05 were considered trends; P values of ≤ 0.05 were considered significant.

RESULTS

Patient groups. This study examined paired blood and rectal biopsy specimens from three groups of individuals: 12 HIV-positive noncontrollers with a viral load over 10,000 copies/ml plasma, 10 HIV controllers able to maintain a viral load of less than 500 copies/ml plasma for more than 5 years in the absence of antiretroviral therapy, and 12 HIV-seronegative control subjects. No subjects were on therapy at the time of sample collection, although three had documented prior antiretroviral treatment (Table 1).

Regulatory T cell frequency is increased in the mucosae of HIV^+ patients with high viral loads. Human Treg are typically identified as either $CD4^+$ $CD25^+$ $CD127^{lo}$ (5, 62, 80) or $CD4^+$ $FOXP3^+$ $CD25^+$ (35, 73, 99). The designation $FOXP3^+$ $CD25^{hi}$ has also been used in an attempt to avoid inclusion of recently activated non-Treg that express an intermediate level of CD25, although this approach typically underestimates the total number of Treg (32, 80). In order to investigate the relationship between Treg frequency and progressive infection, we first identified Treg using multiple approaches, as shown in Fig. 1A. Regardless of the gating strategy employed, the frequency of Treg within the $CD3^+$ $CD4^+$ gate was consistently higher in the rectal mucosae of noncontrollers than controllers ($P < 0.009$ for all gating strategies) or seronegative subjects ($P < 0.004$ for all gating strategies) (Fig. 1B). In the peripheral blood, however, significant differences in Treg percentages were only observed when $CD25^+$ $CD127^{lo}$ was used to define the subset, where $CD25^+$ $CD127^{lo}$ Treg were significantly decreased in the blood of controller samples compared to uninfected seronegative subjects ($P = 0.04$). Additionally, within the controller and seronegative groups, Treg frequencies in the periphery and rectal mucosa were similar, whereas the noncontroller group consistently exhibited an increased frequency of Treg in rectal mucosa compared to blood ($P = 0.11$ for $FOXP3^+$ $CD25^+$, $P = 0.01$ for $FOXP3^+$ $CD25^{hi}$, $P = 0.02$ for $FOXP3^+$ $CD127^-$, and $P = 0.06$ for $CD25^+$ $CD127^{lo}$). Notably, all four Treg gating strategies indicated that subjects with progressive HIV infection experienced an increase in Treg frequency in mucosal tissue to a degree that was not seen in the periphery. In contrast, controllers maintained levels of Treg that were similar to those of uninfected controls in both compartments.

FOXP3 expression level varies depending on the method used to identify Treg but is not significantly different between patient groups. Although a similar distribution of FOXP3 expression was found in all patient groups, there was considerable variation in the FOXP3 median fluorescence intensity (MFI), depending on the Treg gating strategy examined. Within the various Treg populations, expression of FOXP3 was brightest in the $FOXP3^+$ $CD25^{hi}$ population, followed by the $FOXP3^+$ $CD25^+$ and $FOXP3^+$ $CD127^-$ groups, and lowest in the $FOXP3^+$ $CD25^-$ and $CD25^+$ $CD127^{lo}$ populations for all patient groups (Fig. 1C). CD127 is known to be dysregulated in HIV infection (16, 23), and loss of CD127 expression has been used as a measure of immune activation. Additionally, a previous study of Treg found that the $CD25^{hi}$ $CD127^{lo}$ phenotype appeared to be reflecting the high levels of immune activation found in viremic progressors and did not correlate with levels of $FOXP3^+$ $CD25^{hi}$ T cells (21). Thus, while the $CD25^{hi}$ $CD127^{lo}$ Treg phenotype may have discriminatory value in patient populations with lower levels of immune activation (i.e., HIV controllers and HIV-uninfected individuals) (47), it is a less consistent Treg marker in untreated viremic participants. For these reasons, the $FOXP3^+$ $CD25^+$ population was chosen for all subsequent analyses of Treg phenotype in order to avoid a falsely high estimation by using $CD25^+$ $CD127^{lo}$ or falsely low estimation by using $FOXP3^+$ $CD25^{hi}$. Although no significant differences between patient groups were observed, FOXP3 MFI was significantly higher in rectal $FOXP3^+$ $CD25^+$, $FOXP3^+$ $CD25^{hi}$, and $FOXP3^+$ $CD127^-$ Treg in all patient groups compared to their peripheral blood counterparts ($P \leq 0.05$ for all; data not shown), similar to what has previously been described in the lymph nodes of HIV^+ individuals (53).

The number of mucosal Treg does not differ significantly between noncontrollers, controllers, and seronegative subjects. The gastrointestinal mucosa experiences a significant loss in the percentage and total number of $CD4^+$ T cells after HIV or SIV infection coupled with an increase in $CD8^+$ T cells as the body attempts to control the virus (85, 94). As expected, in our cohort HIV noncontrollers revealed the greatest loss of blood and rectal $CD4^+$ T cells, but controllers also had significant CD4 loss compared to seronegative subjects (Fig. 2A). Because such perturbations in the major lymphocyte populations occur, it is important to assess not only the frequency of

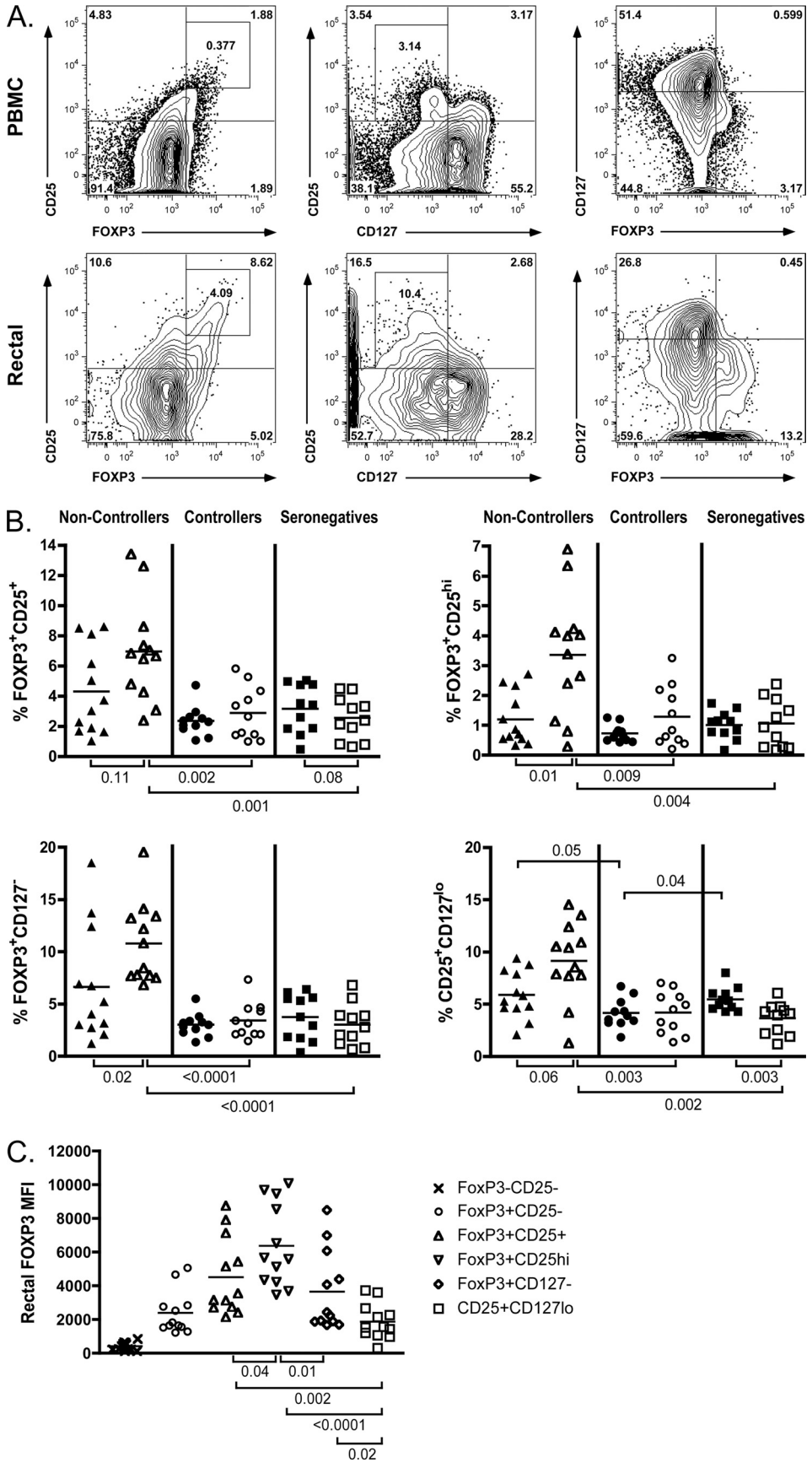
TABLE 1. Characteristics of the patients in this study

Patient identification no. and group	Gender	Ethnicity[a]	Age (yr)	No. of yr infected	Viral load (copies/ml)	CD4 count (cells/μl)
Seropositive						
Noncontrollers						
106[b]	Male	AA	36	6.3	35,800	502
G126	Male	White	36	8.6	23,000	157
131	Male	White	47	3.6	14,600	512
143	Male	AA	48	11.4	26,300	238
G135	Female	AA	38	17.4	20,606	396
G138[c]	Male	White	53	23.2	10,073	350
G137	Female	AA	37	5.7	13,200	523
S1335	Male	AA	53	24.2	48,105	482
S1597	Male	H/L, Iberian	49	12.8	12,593	510
S1580	Male	White	43	1.8	31,966	467
S1613	Male	AA, NAm	40	0.3	41,812	561
S1359	Male	H/L	34	2.7	52,459	714
Median			42	7	24,650	492
Controllers						
48	Female	White	54	16.6	50	1,710
97	Male	H/L	49	18.8	114	927
105	Male	White	52	5.6	738	500
G15[d]	Female	AA	55	21.6	50	679
S1116	Male	White	53	25.8	69	535
S1198	Male	White	54	23.3	40	728
S1581	Male	White	56	23.8	40	1,227
S1548	Female	AA	47	18.3	73	718
S1572	Male	AA	56	19.3	40	909
S1495	Male	White	55	18.3	323	542
S1541	Male	White	47	14.3	47	1,895
Median			54	19	50	728
Seronegative						
129	Female	White	41	NA[e]	NA	695
130	Male	White	42	NA	NA	642
135	Female	White	46	NA	NA	1,499
137	Male	AA	46	NA	NA	651
138	Male	AA	38	NA	NA	772
142	Male	White	24	NA	NA	1,183
G53	Female	AA	44	NA	NA	866
G64	Male	AA	56	NA	NA	892
G54	Female	White	47	NA	NA	813
S1939	Male	White	30	NA	NA	769
S1812	Male	White	51	NA	NA	699
S1913	Male	AA	48	NA	NA	873
Median			45			793

[a] AA, African American; H/L, Hispanic or Latino; NAm, Native American.
[b] Was on antiretroviral therapy (ART) previously.
[c] Was off ART for 4 months previous to the time of sample collection.
[d] Was on ART during pregnancy only, 18 years ago.
[e] NA, not applicable.

$CD4^+$ Treg present within the $CD4^+$ T cell population, but also their frequency relative to total $CD3^+$ cells. Although the mean value was highest for seronegative subjects in both blood and rectal mucosa, no significant difference in the number of Treg normalized per 10,000 $CD3^+$ T cells was observed between groups in the mucosa. These data suggest that multiple, competing factors influence Treg abundance in mucosal tissues. Treg can be infected by HIV (67, 70), yet they may not be as readily depleted as conventional $CD4^+$ T cells, resulting in a stable number but increased frequency of Treg within the $CD4^+$ subset. Treg accumulation in lymphoid tissues is associated with disease progression, and their survival may be promoted by interactions between CD4 and gp120 (50, 69).

Interestingly, HIV controllers had a significantly lower number of Treg in the blood compared to seronegative subjects ($P = 0.04$), which was likewise reflected in a higher ratio of


A.
PBMC
Rectal
CD25
FOXP3
CD127
4.83
1.88
0.377
91.4
1.89
3.54
3.17
3.14
38.1
55.2
51.4
0.599
44.8
3.17
10.6
8.62
4.09
75.8
5.02
16.5
2.68
10.4
52.7
28.2
26.8
0.45
59.6
13.2
B.
Non-Controllers
Controllers
Seronegatives
% FOXP3+CD25+
0.11
0.002
0.08
0.001
% FOXP3+CD25hi
0.01
0.009
0.004
% FOXP3+CD127-
0.02
<0.0001
<0.0001
% CD25+CD127lo
0.05
0.04
0.06
0.003
0.003
0.002
C.
Rectal FOXP3 MFI
0.04
0.01
0.002
<0.0001
0.02
FoxP3-CD25-
FoxP3+CD25-
FoxP3+CD25+
FoxP3+CD25hi
FoxP3+CD127-
CD25+CD127lo

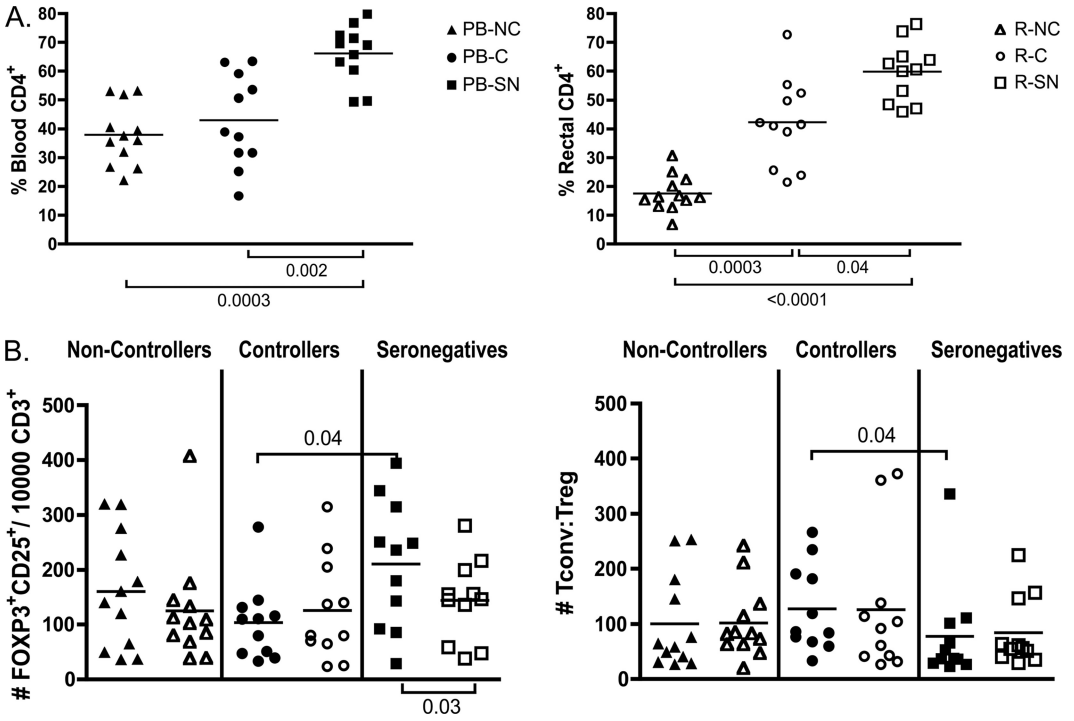


FIG. 2. Numbers of $CD4^+$ $FOXP3^+$ $CD25^+$ Treg are not significantly different between patient groups despite a decline in total $CD4^+$ T cells of noncontrollers and controllers. (A and B) Treg frequencies in PBMC (PB-; solid symbols) and RMNC (R-; open symbols) of noncontrollers (NC; triangles), controllers (C; circles), and seronegative subjects (SN; squares) were determined by staining as described in Materials and Methods, and data were acquired on an LSRII flow cytometer. (A) Percentage of $CD4^+$ T cells in the blood (left) and rectal mucosae (right). (B) The number of Treg per 10,000 $CD3^+$ T cells was determined by dividing the number of $CD4^+$ $FOXP3^+$ $CD25^+$ T cells acquired during flow cytometry sample acquisition by the total number of $CD3^+$ T cells acquired and multiplying by 10,000 (right). The ratio of conventional T cells to Treg was determined by the following formula where the number of cells was determined by flow cytometry data: [$CD8^+$ T cells + ($CD4^+$ T cells − $CD4^+$ $CD25^+$ $FOXP3^+$ Treg)]/$CD4^+$ $CD25^+$ $FOXP3^+$ Treg (left). Horizontal lines within the groups correspond to the mean. The numbers above and below the brackets are *P* values. *P* values of ≤0.05 were considered significant; *P* values between 1 and 0.05 were considered trends.

conventional $CD4^+$ and $CD8^+$ T cells to Treg ($P = 0.04$) (Fig. 2B). These data are consistent with prior findings from our group, which involved different subjects and different phenotypic panels to define the Treg population (47). As previously argued, these lower numbers of Treg may allow for the more robust, polyfunctional HIV-specific responses often seen in HIV controllers, while also contributing to higher generalized immune activation (47). Despite these consistent differences in blood, we saw no differences in Treg frequencies or numbers (defined multiple ways) in the gut mucosa when comparing HIV-seronegative subjects and controllers. A larger study focused on HIV controllers may be needed to define the complex impact that Treg might have on HIV progression in these individuals.

Markers of immune activation are elevated in conventional T cells and Treg of noncontrollers. High-level expression of markers of immune activation has been shown to correlate with clinical measures of progressive infection (40, 63, 88). Recently, it was demonstrated that the coexpression of activation marker CD38 and the inhibitory molecule PD-1 on T cells is highly enriched in HIV-specific CD8 T cells of progressors compared to controllers and closely correlates with viral load and $CD4^+$ T cell loss (25, 97). PD-1 expression in HIV-infected subjects has also been linked to persistent viral replica-

FIG. 1. Treg frequency is increased in the rectal mucosae of noncontrollers regardless of the gating strategy used to identify Treg. (A) Representative flow cytometry plots depicting four different approaches for identifying $CD4^+$ Treg in human PBMC (top panels) and rectal cells (bottom panels) in the $FOXP3^+$ $CD25^+$ (left), $FOXP3^+$ $CD25^{hi}$ (left, inset), $CD25^+$ $CD127^{lo}$ (middle), and $FOXP3^+$ $CD127^-$ (right) groups. Numbers within the gates represent the frequencies of the gated $CD4^+$ subpopulations. (B) Treg frequencies in PBMC (solid symbols) and RMNC (open symbols) of noncontrollers (triangles), controllers (circles), and seronegative subjects (squares) were analyzed using the gating strategies described above. (C) FOXP3 expression in the rectal mucosae was determined by MFI and compared between each of the four Treg gating approaches. $FOXP3^-$ $CD25^-$ non-Treg and the $FOXP3^+$ $CD25^-$ subsets were included in the analysis as reference groups. FOXP3 MFI was highest within the $CD4^+$ $FOXP3^+$ $CD25^{hi}$ and $CD4^+$ $FOXP3^+$ $CD25^+$ gates. This graph contains data from the noncontroller patients; a similar pattern of expression was seen in controllers and seronegative subjects. Horizontal lines within the groups correspond to the mean. The numbers above and below the brackets are *P* values. *P* values of ≤0.05 were considered significant; *P* values between 1 and 0.05 were considered trends.

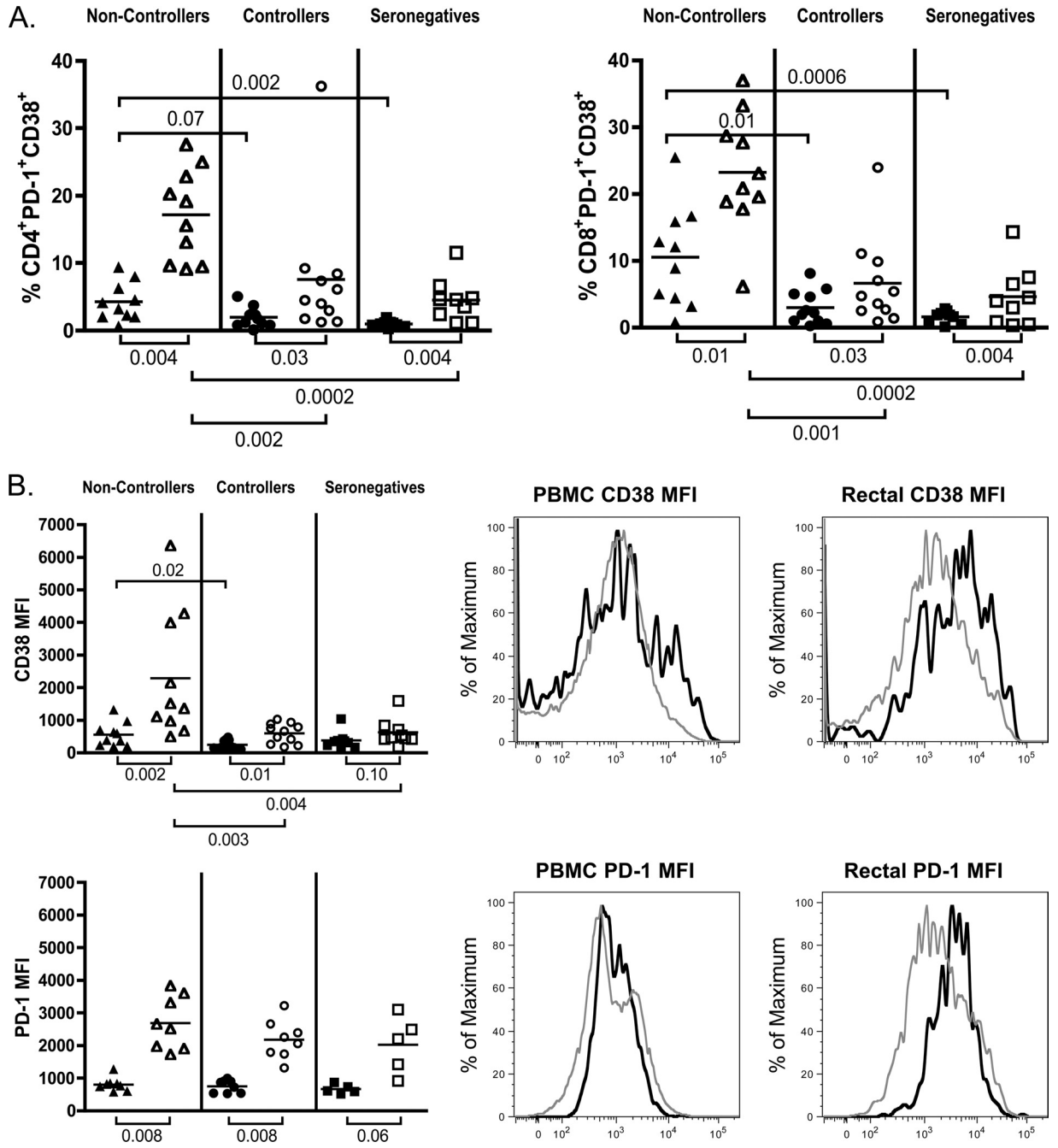


FIG. 3. Markers of T cell activation are elevated in total $CD8^+$, $CD4^+$, and Treg populations in the mucosae of noncontrollers. (A and B) Treg frequencies in PBMC (solid symbols) and RMNC (open symbols) of noncontrollers (triangles), controllers (circles), and seronegative subjects (squares) were stained as described in Materials and Methods, and data were acquired on an LSRII flow cytometer. Horizontal lines within the groups correspond to the mean. The numbers above and below the brackets are *P* values. *P* values of ≤0.05 were considered significant; *P* values between 1 and 0.05 were considered trends. (A) Coexpression of CD38 and PD-1 was used to assess T cell activation in total $CD4^+$ (left) and $CD8^+$ (right) T cells in the blood and rectal mucosae of the three patient groups. (B) MFI of CD38 (top panel) and PD-1 (bottom panel) in Treg populations of noncontrollers, controllers, and seronegative subjects (left). Shown are representative histograms from a noncontroller comparing MFI in $CD4^+$ $FOXP3^-$ $CD25^-$ non-Treg (gray lines) to $CD4^+$ $FOXP3^+$ $CD25^+$ Treg (black lines) for CD38 (top panels) and PD-1 (bottom panels) in the peripheral blood and rectal mucosae (right).

tion as well as $CD4^+$ T cell activation (25, 76). In our patient cohort, the frequency of activated $CD4^+$ and $CD8^+$ $PD\text{-}1^+$ $CD38^+$ cells was very significantly increased in rectal mucosae of noncontrollers compared to controllers ($P = 0.002$ and $P = 0.001$) or seronegative subjects ($P = 0.0002$ and $P = 0.0002$) (Fig. 3A). In the periphery, activation measured by coexpression of CD38 and PD-1 was always significantly lower than in the mucosa and showed the same trends that were seen in the

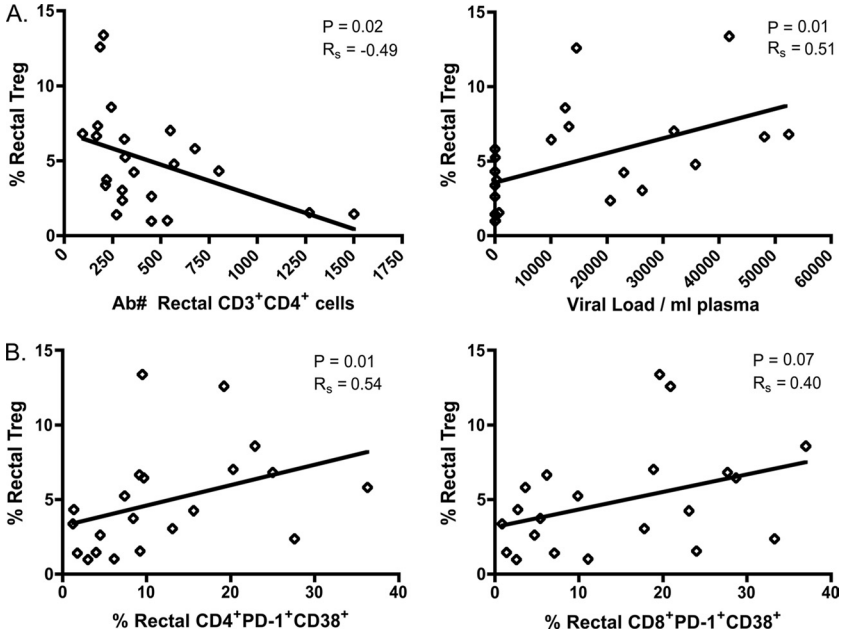


FIG. 4. Mucosal Treg frequency negatively correlates with mucosal CD4 numbers and positively correlates with plasma viral load and mucosal T cell activation. (A) Relationship between rectal Treg frequencies in HIV^+ subjects and mucosal $CD3^+$ $CD4^+$ cell number based on events acquired by flow cytometry, calculated by dividing the number of $CD4^+$ cells by the number of live cells and multiplying by 10,000 (left) or the viral load determined by quantitative PCR (qPCR) (right). Ab#, absolute number. (B) Relationship between rectal Treg frequencies in HIV^+ subjects and percentage of mucosal $CD38^+$ $PD\text{-}1^+$ CD4 T cells (left) or $CD38^+$ $PD\text{-}1^+$ CD8 T cells (right). Regression analysis was used to produce best-fit lines on all graphs, while the *P* values and rho values were determined using the Spearman rank test for bivariate correlations.

mucosa with respect to intergroup comparisons: noncontrollers maintained the highest levels of activated $CD4^+$ and $CD8^+$ T cells in blood compared to controllers ($P = 0.07$ and $P = 0.01$) or seronegative subjects ($P = 0.002$ and $P = 0.0006$) (Fig. 3A). In agreement with the study referenced above (97), CD38–PD-1 coexpression on total $CD8^+$ cells in PBMC and mucosae negatively correlated with CD4 count ($P = 0.003$ and $P = 0.001$ for PBMC and mucosa, respectively) and positively correlated with plasma viral load ($P = 0.003$ and $P = 0.004$, respectively). Similar relationships were observed for activated $CD38^+$ $PD\text{-}1^+$ $CD4^+$ T cells, where $P = 0.003$ in the blood and $P = 0.02$ in the mucosae are with respect to CD4 count, and $P = 0.06$ in the blood and $P = 0.004$ in the mucosae are with respect to viral load. Notably, utilizing coexpression of CD38 and PD-1 in the correlation analyses consistently resulted in greater significance than use of CD38 alone (data not shown). In summary, compared to controllers and seronegative subjects, T cells of noncontrollers displayed increased expression of CD38 and PD-1, which was particularly striking in rectal mucosa. Controllers, in contrast, did not display significant activation compared to seronegative subjects.

Further analysis of the $CD4^+$ $CD25^+$ $FOXP3^+$ Treg population also revealed increased immune activation. Although both CD38 MFI and PD-1 MFI were consistently higher on rectal Treg versus peripheral Treg for all patient groups, only CD38 expression was specifically increased in mucosal Treg of noncontrollers compared to controllers or uninfected controls ($P = 0.003$ and $P = 0.004$) (Fig. 3B). In comparison to $CD25^-$ $FOXP3^-$ conventional $CD4^+$ cells (non-Treg) or $CD4^+$ $CD25^+$ $FOXP3^-$ cells, Treg consistently expressed higher levels of PD-1, with an overall expression pattern that differed little between groups, whereas CD38 expression exhibited greater variation (data not shown).

Correlation analyses between Treg, clinical parameters of infection, and immune activation. Although Treg frequency was increased in the mucosae of noncontrollers, it is difficult to determine what effect this increase may have on disease progression. By examining the relationship between Treg frequency, viral load, CD4 count, and immune activation in HIV^+ patient samples, we can begin to assess the impact of Treg on chronic HIV infection and vice versa. Rectal Treg frequency was negatively correlated with the absolute number of mucosal $CD4^+$ T cells ($P = 0.02$) and positively correlated with plasma viral load ($P = 0.01$) (Fig. 4A). In contrast, no significant relationships existed for blood Treg and plasma viral load or peripheral CD4 count (data not shown).

Increasing immune activation is also known to correlate with high viral load and low CD4 counts, but the relationship between immune activation and Treg levels is less clear (17, 31, 51). Although regression analysis revealed a positive relationship for both rectal and peripheral Treg frequencies with respect to $CD38^+$ $PD\text{-}1^+$ T cells, once again a significant correlation was present only in the mucosa. The relationship was strongest for rectal Treg and mucosal $CD4^+$ activation ($P = 0.01$); however, a strong trend also existed between rectal Treg and mucosal $CD8^+$ activation ($P = 0.07$) (Fig. 4B).

Rectal Treg of noncontrollers are suppressive and are able to suppress polyclonally stimulated non-Treg to a similar extent as Treg of controllers or seronegatives. Given that both mucosal Treg frequency and immune activation are elevated in chronic infection of noncontrollers, we hypothesized that mucosal Treg of noncontrollers might be functionally impaired in

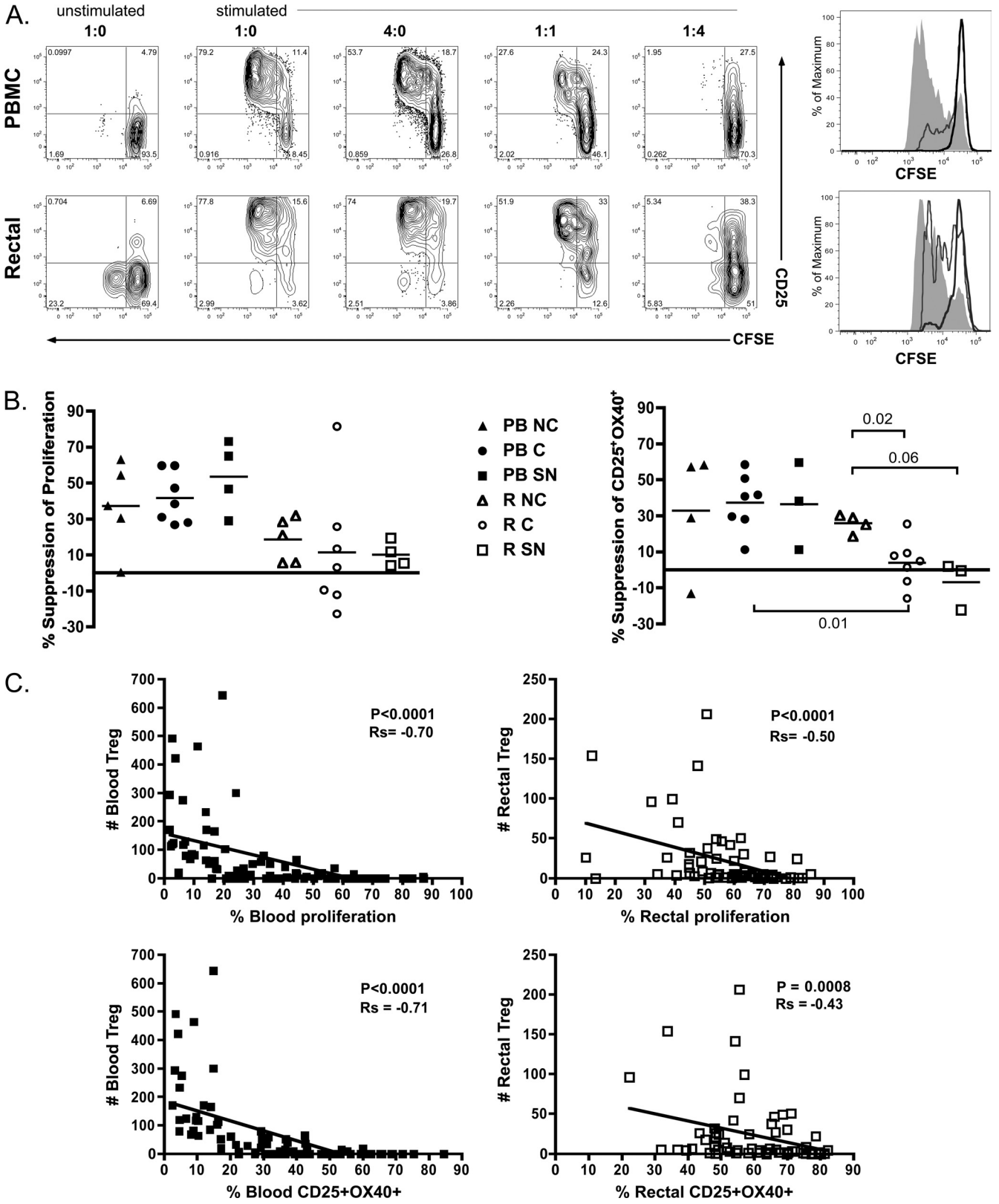


FIG. 5. Peripheral and mucosal Treg of noncontrollers, controllers, and seronegative subjects are suppressive *ex vivo*. $CD4^+$ $CD25^-$ non-Treg and $CD4^+$ $CD25^+$ Treg were separated from PBMC and RMNC using magnetic beads as described in Materials and Methods. A total of 1×10^4 CFSE-labeled non-Treg were added to 96-well round-bottom plates containing 1×10^5 irradiated, autologous PBMC and cultured alone or with increasing numbers of Treg. Cells were unstimulated or stimulated with 0.2 μg/ml immobilized anti-CD3 for 60 to 84 h. (A) Representative flow cytometry gating on $CFSE^+$ cells from one experiment. Proliferation in cultures containing PBMC (top panels) or rectal cells (bottom panels) was

their ability to limit immune activation compared to Treg from controllers or HIV-negative individuals. Alternatively, Treg in noncontrollers might more effectively (yet inappropriately) suppress HIV-specific adaptive responses, providing a competitive advantage to the virus. To compare the functionality of Treg from controllers, noncontrollers, and seronegative subjects, we performed a series of suppression assay experiments by coculturing $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^-$ non-Treg isolated from peripheral blood and rectal cell suspensions. Non-Treg were incubated alone or with increasing numbers of Treg and left unstimulated or stimulated with anti-CD3 for 3 days; proliferation was assessed using CFSE dye dilution (Fig. 5A). Although peripheral Treg suppressed proliferation to a greater extent than did mucosal Treg (Fig. 5B), no significant differences in the suppression of proliferation were observed between noncontrollers, controllers, and seronegative subjects in either blood or rectal cell cultures.

A previous study found that simultaneous expression of CD25 and CD134 (OX40) was induced after TCR engagement and that CD25-OX40 coexpression strongly correlated with antigen-specific gamma interferon secretion and proliferation (100). We also observed a highly significant correlation between the proliferation and $CD25^+$ $OX40^+$ expression in stimulated T cell cultures ($P < 0.0001$ and Spearman's rank order coefficient [R_s] = 0.89 for PBMC and $P < 0.0001$ and $R_s = 0.53$ for RMNC; data not shown). Using coexpression of CD25 and OX40 as a measure of the T cell response to stimulation, we again observed no significant differences in the suppressive abilities of peripheral Treg isolated from noncontrollers, controllers, or seronegative subjects. In mucosal cultures, however, Treg from noncontrollers were significantly more suppressive than those from controllers ($P = 0.02$) and exhibited a strong trend toward increased suppression compared to Treg from seronegative subjects ($P = 0.06$) (Fig. 5B). It is interesting to note that this effect was seen for non-Treg CD25-OX40 coexpression but not proliferation inhibition. This may indicate Treg can modulate effector functions to different degrees depending on the tissue microenvironment and specific pathways engaged.

Although the degree of Treg-mediated suppression was greater in cells isolated from peripheral blood compared to rectal mucosa, regardless of patient group (Fig. 5B), our data suggest that this was most likely due to reduced purity of non-Treg and Treg fractions from rectal cell suspensions compared to PBMC (data not shown). Factors potentially affecting purity include clumping of mucosal mononuclear cells and reduced CD4 staining intensity following collagenase digestion (our unpublished observation). However, in preliminary experiments, cell viability and yield were greater following magnetic bead isolation than flow cytometry-based cell sorting of rectal Treg. To account for the reduced purity of Treg and non-Treg fractions in rectal samples, correlation analyses were performed comparing the percentage of non-Treg proliferation to the number of $FOXP3^+$ $CD25^+$ Treg present in the $CD4^+$ $CFSE^-$ population divided by the number of $FOXP3^+$ $CD25^+$ Treg present in the $CD4^+$ $CFSE^+$ (non-Treg) population (Fig. 5C). These analyses clearly showed a very close correlation between total number of Treg present in a culture and suppression for both the peripheral blood and rectal mucosa ($P < 0.0001$ for PBMC and $P < 0.0001$ for RMNC), confirming the suppressive nature of the isolated Treg. Similar results were obtained for the $CD25^+$ $OX40^+$ coexpression and Treg number ($P < 0.0001$ for PBMC and $P < 0.0008$ for RMNC) (Fig. 5C).

DISCUSSION

The impact of Treg on HIV progression has been the subject of some debate (24, 27, 32, 45, 69, 79). Adding to the confusion is the variety of approaches used to identify Treg. The combination of markers most commonly used includes $CD4^+$ $CD25^+$, $CD4^+$ $CD25^+$ $CD127^{lo}$, $CD4^+$ $FOXP3^+$, $CD4^+$ $CD25^+$ $FOXP3^+$, and $CD4^+$ $CD25^+$ $FOXP3^+$ $CD127^{lo}$. Additionally, the majority of studies have focused on peripheral Treg, while comparatively few have sampled mucosal tissues despite the severe impact HIV infection is known to have on the gut (12, 84). For these reasons, we used a multiparameter flow cytometry approach to determine the phenotype of Treg present in the blood and rectal mucosae and established a suppression assay to evaluate Treg function in subjects from across the clinical spectrum.

Regardless of the method we used to identify Treg, a significantly increased frequency was evident in the rectal mucosae of noncontrollers compared to controllers or seronegative subjects. This is in agreement with previous studies examining Treg in lymphoid and mucosal tissues of HIV^+ progressors (3, 27, 31, 53, 56, 78). We did not observe a corresponding increase in peripheral blood of noncontrollers, in contrast to what has been reported previously (71, 78, 89). This discrepancy may be due to differences in patient characteristics, specifically a lower median CD4 count in the noncontroller cohort, and/or due to differences in Treg gating strategies.

This increase in frequency was observed as a percentage of $CD4^+$ T cells; however, the number of Treg normalized per 10,000 $CD3^+$ T cells was not increased in noncontrollers relative to the other groups. This finding suggests that HIV may preferentially deplete conventional $CD4^+$ T cells while sparing

assessed using CFSE. Contour plots show the results of cultures containing the indicated ratio of non-Treg to Treg. Histograms depict CFSE expression in non-Treg for cultures at non-Treg/Treg ratios of 1:0 (shaded areas), 1:1 (gray lines), and 1:4 (black lines). (B) Treg-mediated suppression was measured in PBMC (PB-; solid symbols) and RMNC (R-; open symbols) of noncontrollers (NC; triangles), controllers (C; circles), and seronegative subjects (SN; squares). Percent suppression was determined by dividing the level of proliferation (left) or CD25-OX40 coexpression (right) in the 1:1 cocultures by the same parameter in the 1:0 cultures, multiplying the result by 100, and subtracting this percentage from 100. Horizontal lines within boxes correspond to the mean. The numbers above and below the brackets are *P* values. *P* values of ≤ 0.05 were considered significant; *P* values between 1 and 0.05 were considered trends. (C) Graphs illustrate the relationship between $FOXP3^+$ $CD25^+$ Treg numbers present in PBMC (solid symbols) and RMNC (open symbols) cultures and the percent proliferation (top panels) or percent CD25-OX40 coexpression (bottom panels). Regression analysis was used to produce a best-fit line for both graphs; *P* values and rho values were determined using the Spearman rank test for bivariate correlations.

the regulatory population; alternatively, both populations may be infected and depleted to a similar extent, but Treg may be more quickly replaced. Indeed, multiple mechanisms may influence Treg numbers in the mucosae, including recruitment and trafficking (3, 50), increased survival (50, 69), active proliferation (3, 27), and peripheral conversion of non-Treg into Treg (56). A recent study of SIV-infected rhesus macaques found high levels of HIV DNA present in Treg but low levels of HIV RNA, indicating Treg were permissive to viral entry and integration, but not productively infected. Additionally, Treg proliferated along with the total $CD4^+$ T cell pool but were less susceptible to SIV-related cell death, resulting in an increased percentage and absolute number of Treg (2). A similar situation may be at play in chronic HIV infection. Treg themselves are a target of viral infection (67, 70), and the balance between proliferation, immune activation, and death may be what keeps Treg numbers stable as non-Treg decline.

Importantly, controllers maintain low mucosal Treg frequencies similar to seronegative subjects. Although this might simply be a reflection of the low level of plasma viremia and relative $CD4^+$ T cell preservation, controllers do experience a significant loss of gut $CD4^+$ T cells compared to uninfected persons without a corresponding increase in Treg frequency. Our group has previously reported that HIV-specific $CD4^+$ and $CD8^+$ T cell responses in the rectal mucosae of controllers were of greater magnitude and more polyfunctional than responses in noncontrollers (33, 34). Additionally, controllers had a significantly lower number of Treg and higher ratio of conventional T cells to Treg in the periphery compared to HIV-negative controls, a situation that could potentially allow for more robust anti-HIV responses.

The level of T cell activation was significantly higher in noncontrollers compared to controllers or seronegative patients in both the blood and mucosal compartments, as expected. CD38–PD-1 coexpression was higher in the rectal compartment compared to the blood for all patient groups, and the difference in activation was comparable for $CD4^+$ T cells and $CD8^+$ T cells. Mucosal Treg likewise expressed increased levels of CD38 and PD-1, measured by MFI, compared to blood Treg. Noncontroller Treg had a significantly higher CD38 MFI in the mucosae compared to controllers or seronegative subjects as well as increased CD38 MFI in the periphery compared to controllers only. Thus, it appears that mucosal Treg of noncontrollers are highly activated and may be responding to increased levels of immune activation in the gut. In support of this, the frequency of rectal Treg exhibited a positive correlation with both plasma viral load and CD4 and CD8 mucosal T cell activation. Importantly, significant correlations between Treg frequency, viral load, and immune activation were seen only in the gut, suggesting that in addition to the early assault endured during primary infection, this compartment continues to be a dynamic site of HIV replication throughout the course of disease.

The gastrointestinal mucosa must maintain a delicate balance between tolerance to dietary and commensal organisms and responsiveness to pathogenic intruders. A number of studies have underscored the importance of Treg in maintaining a healthy gut environment (48). Treg suppressive function in the blood and lymph node of HIV^+ subjects has been examined in numerous studies (1, 4, 6, 49, 53–55, 57, 82, 91, 92, 98), but to our knowledge, this is the first to perform functional experiments on mucosal Treg in HIV infection. Studies of peripheral Treg function found a decreased suppressive capacity in Treg from patients with high viral loads and low CD4 counts (54, 92). In contrast, Kinter et al. described an increased capacity for suppression in Treg isolated from lymph nodes of HIV^+ progressors (53). Despite difficulties purifying mucosal Treg, we did observe Treg-mediated suppression of non-Treg proliferation as well as a decrease in coexpression of CD25 and OX40. The degrees of suppression of proliferation were similar in all patient groups for both blood and mucosal cultures.

Interestingly, noncontrollers did have significantly greater suppression of CD25-OX40 coexpression in mucosa compared to controllers. Griseri et al. recently described an important role for OX40 expression on Treg in a mouse model of colitis (42). OX40 provided survival signals necessary for the accumulation of Treg in the colon, and under inflammatory conditions, $OX40^+$ Treg were able to suppress colitogenic T cell responses, presumably by competing with effector T cells for access to OX40L, thereby limiting their ability to sustain an effective response. Further studies will be necessary to determine whether expression of OX40 on mucosal Treg of humans has a role in the suppression of effector responses in HIV.

As the body of work examining Treg in HIV infection continues to grow, a model of how Treg are impacted by infection and how they in turn impact disease progression is beginning to emerge. Although Treg can be infected and depleted by HIV, their frequency relative to conventional $CD4^+$ T cells increases in progressive infection. This increase is most evident in the lymphoid tissues and gastrointestinal tract, where the majority of early HIV replication occurs. The relationship between mucosal Treg frequency, viral load, and immune activation in our study suggests that the frequency of Treg increases in the gastrointestinal mucosa in response to high levels of immune activation in HIV noncontrollers. They may then suppress antiviral immune responses, contributing to an environment permissive to ongoing viral replication, which in turn further exacerbates immune activation. Additional studies will be required to address the mechanisms driving the accumulation of Treg in mucosal tissues and to test whether strategies designed to modulate mucosal Treg function could boost adaptive responses and/or limit immune activation in HIV noncontrollers.

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