Suppression of HIV-specific T cell activity by lymph node CD25$^+$ regulatory T cells from HIV-infected individuals

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CD25$^+$ CD4$^+$ FoxP3$^+$ regulatory T (Treg) cells isolated from the peripheral blood of asymptomatic HIV-infected individuals have been demonstrated to significantly suppress HIV-specific immune responses *in vitro*. CD25$^+$ Treg cell suppressor activity in the peripheral blood seems to diminish with progression of HIV disease, and it has been suggested that loss of Treg cells contributes to aberrant immune activation and disease progression. However, phenotypic studies suggest that Treg cells may migrate to, and be maintained or even expanded in, tissue sites of HIV replication. Currently, it is not known whether tissue-associated Treg cells maintain suppressive activity in the context of HIV infection, particularly in individuals with advanced disease. The present study demonstrates that CD25$^+$ Treg cells isolated from lymph nodes and peripheral blood of HIV$^+$ subjects, even those with high viral loads and/or low CD4$^+$ T cell counts, maintain potent suppressive activity against HIV-specific cytolytic T cell function. This activity was better in lymph node as compared with peripheral blood, particularly in patients with high levels of plasma viremia. In addition, the expression of certain CD25$^+$ Treg-associated markers on CD4$^+$ T cells isolated from lymph nodes differed significantly from those on CD4$^+$ T cell subsets isolated from the peripheral blood. These data suggest that CD25$^+$ Treg cell-mediated suppression of HIV-specific responses continues throughout the course of HIV disease and, because of their particularly potent suppression of HIV-specific CTL activity in lymphoid tissue, may considerably impact the ability to control HIV replication *in vivo*.

cytolytic T lymphocyte | cytokine | proliferation

Under conditions of persistent antigen exposure and immune activation numerous immunosuppressive mechanisms function to limit potential immune-mediated damage to the host. Among the best characterized of these mechanisms are CD25$^+$FoxP3$^+$ regulatory T (Treg) cells, a suppressor CD4$^+$ T cell subset initially described as playing a critical role in containing certain types of autoimmunity in animal models (1). A potentially deleterious consequence of CD25$^+$ Treg cells is the suppression of appropriate antigen-specific immune responses. In this regard, CD25$^+$ Treg cells have been implicated in playing a role in disease/pathogen persistence, such as certain solid tumors and parasitic and bacterial infections (2–8). Furthermore, CD25$^+$ Treg cells accumulate and expand at sites of antigen expression/ inflammation as well as in draining lymph nodes where they seem to exert particularly strong site-localized immunosuppression (2, 4, 9).

Chronic HIV infection is characterized by loss of CD4$^+$ T cells, a broad array of immune dysfunctions, and persistent immune activation (10–12). Whereas the direct and indirect roles of HIV and its gene products in immune dysfunction are well documented (13, 14), recent evidence suggests that normal host-mediated negative immunoregulatory mechanisms, triggered by persistent antigenimia and immune activation, may also impact the immune competence of infected individuals (15). In this regard, numerous *in vitro* studies have demonstrated that CD25$^+$ Treg cells isolated from the peripheral blood of asymptomatic HIV-infected individuals significantly suppress HIV-specific CD4$^+$ and CD8$^+$ T cell immune responses (16–18). However, progressive HIV disease has been associated with reduced frequencies or suppressor activity of CD25$^+$ Treg cells in the peripheral blood (PB) (17, 19–21). These observations have led to the hypothesis that HIV infection is associated with a selective loss of CD25$^+$ CD4$^+$ Treg cells leading to a reduced ability to control HIV-associated aberrant immune activation and associated immune dysfunctions, ultimately resulting in more rapid disease progression. An alternative hypothesis is that CD25$^+$ Treg cells are redistributed to tissue sites of HIV expression (19, 22–24). Currently, the data supporting this later. hypothesis is largely phenotypic and it is not known whether tissue-associated Treg cells, particularly in individuals with advanced disease, maintain their ability to suppress HIV-specific immune responses. Localization to and perhaps activation of CD25$^+$ Treg cells at lymphoid tissue sites might have a particularly negative impact on the ability of HIV-specific immune responses to control viral replication and spread. The present study investigates the suppressive activity of CD25$^+$ Treg cells isolated from lymph nodes (LN) and PB of chronically HIV-infected individuals at various stages of disease progression, including those with high viremia and/or CD4$^+$ T cells $<150/\mu$l.

Results

Phenotypic Analyses of CD25$^+$ Treg Cells Isolated from Lymphoid Tissue and Peripheral Blood. Total PB mononuclear cells (MC) and LNMC were stained for CD4, CD25, intracellular FoxP3 and additional putative CD25$^+$ Treg markers (CD127, SCTLA-4, or GITR). Previous studies with human PBMC have clearly correlated suppressor activity with a CD25^{hi} phenotype (25) and therefore, in the present study, CD25$^+$ Treg cells were identified by CD25^{hi} [mean fluorescence intensity (MFI) $\geq$ 70] and FoxP3 (26, 27) coexpression (Fig. 1*A*). The purity of the CD4$^+$ T cell subsets that were isolated on the basis of $\pm$CD25hi expression and subsequently added back to various cultures in functional assays (see below) is illustrated in Fig. 1*B*. Comparing matched LNMC and PBMC (both obtained within 24 h) isolated from 11 HIV-infected subjects (Table 1), several significant differences were observed in the phenotype of the CD4$^+$ T cell subsets. First,

Author contributions: A.K. and A.S.F. designed research; J.M., L.R., and R.J. performed research; G.R. contributed new reagents/analytic tools; G.R. coordinated patient recruitment and procedures; and A.K. and A.S.F. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: Treg, regulatory T; LN, lymph node; PB, peripheral blood; MFI, mean fluorescence intensity; MC, mononuclear cell; VL, viral load.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0611423104/DC1.

www.pnas.org/cgi/doi/10.1073/pnas.0611423104

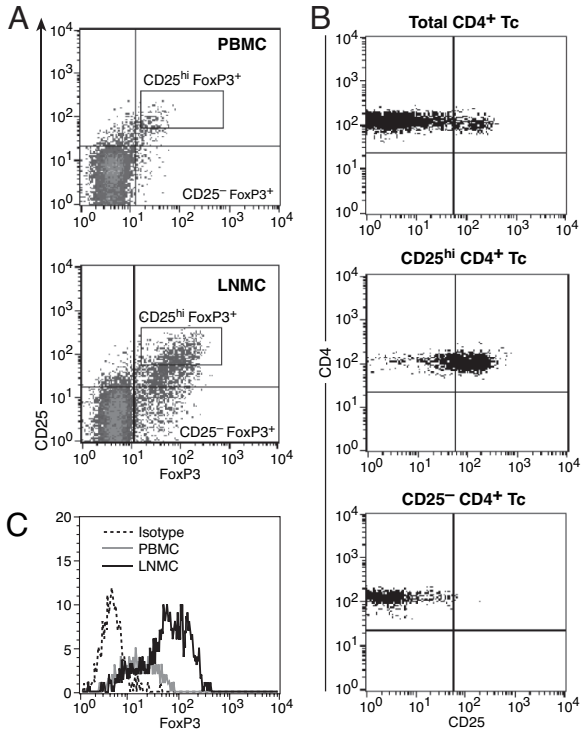

Fig. 1. Representative phenotypic analysis of CD25 and FoxP3 expression in $CD4^+$ T cells of PBMC compared with LNMC. (*A*) Freshly isolated PBMC and LNMC were stained for CD3, CD4, CD25 and intracellular FoxP3. CD25 and FoxP3 expression was analyzed in the $CD3^+CD4^+$ population. (*A*) $CD25^+$ Treg cells in PBMC and LNMC were quantified as $FoxP3^+$ and $CD25^{hi}$ (MFI $\geq$70) as indicated in the box in the upper right quadrant. (*B*) Representative example of $CD25^{+hi}$ expression on purified $CD4^+$ T cell subsets used in functional assays. (*C*) Representative example of FoxP3 expression intensity (MFI) on $CD25^+$ $CD4^+$ T cells in LN and PB.

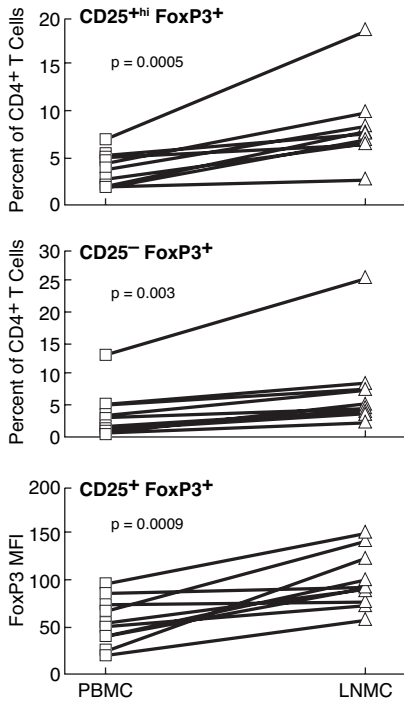

Fig. 2. The frequency and MFI of FoxP3 expression in $CD4^+$ T cell subsets within LNMC is significantly greater than within PBMC. $CD25^{hi}$ (*Top*) and $CD25^-$ (*Middle*) $FoxP3^+$ $CD4^+$ T cell frequencies and FoxP3 MFI in $CD25^+$ $CD4^+$ T cells (*Bottom*) in parallel LNMC and PBMC isolated from 11 chronically HIV-infected individuals.

the frequency of $CD25^{hi}FoxP3^+$ Treg cells was higher in LNMC than in PBMC (Fig. 1*A* and Fig. 2 *Top* plot). An increased frequency of $CD25^{hi}FoxP3^+$ $CD4^+$ Treg cells in LNMC versus PBMC was observed in all subjects regardless of the level of plasma viremia (data not shown). Second, the intensity of FoxP3 expression (MFI) was significantly higher in $CD25^+$ $FoxP3^+$ Tregs cells from LN compared with PB (Figs. 1*C* and 2 *Bottom* plot). There was no significant correlation between plasma viremia or $CD4^+$ T cell count and $CD25^{hi}FoxP3^+$ Treg frequency or FoxP3 MFI in either the PB or LN (data not shown). Finally, as reported (19), the frequency of $CD25^-$ $CD4^+$ T cells expressing FoxP3 was significantly greater in LNMC than in PBMC (Fig. 2 *Middle* plot).

Several surface(s) markers have been reported to be differentially expressed on $CD25^+$ Treg cells compared with normal $CD4^+$ T cells in the peripheral blood. Human peripheral blood $CD25^{hi}$ $FoxP3^+$ Treg cells are reported to be exclusively $CD127^{lo/neg}$ and enriched for $sCTLA\text{-}4^+$ and $GITR^+$ whereas normal resting (CD25-FoxP3-) $CD4^+$ T cells are $CD127^{hi}$ and negative for sCTLA-4 and GITR (28, 29). sCTLA-4 and GITR expression failed to adequately discriminate between $CD25^{hi}$ $FoxP3^+$ Treg cells and normal resting (CD25-FoxP3-) $CD4^+$ T cells (identifying $\leq$50% of $CD25^{hi}$ $FoxP3^+$ subset) (Fig. 3 *Middle* and *Bottom* panels). There was a significantly higher frequency of $CD25^{hi}$ $FoxP3^+$ $CD4^+$ T cells expressing GITR in the LN compared with the PB (Fig. 3 *Bottom* panel). CD127 was the most clearly differentially expressed marker with only 5–7% of the $CD25^{hi}$ $FoxP3^+$ $CD4^+$ T cell subset in the peripheral blood expressing a $CD127^{hi}$ (MFI $\geq$ 40) phenotype. However, this phenotypic profile of CD127 expression was not as clear-cut in LNMC where up to 30% of $CD25^{hi}$ $FoxP3^+$ $CD4^+$ T cells expressed $CD127^{hi}$ (Fig. 3 *Top* panel). The expression of sCTLA-4, GITR and CD127 on $CD25^-FoxP3^+$ $CD4^+$ T cells is shown in supporting information (SI) Fig. 7.

Functional Studies. In the present study, freshly isolated, matched PBMC and LNMC of chronically HIV-infected subjects 1–5 (see Table 1) were assessed for $CD25^+$ cell-mediated suppression of HIV-specific T cell function. Two of the study subjects had advanced disease (patients 1 and 5) with $CD4^+$ T cell counts/μl of <150; patient 5 had a viral load (VL) of 58,791 copies/ml whereas patient 1 was on long-term antiretroviral therapy (ART), had a VL <50 copies/ml, and had Kaposi sarcoma. The remaining patients had $CD4^+$ T cell counts of >350 and variable VL (<50–203,495 copies per ml). $CD25^+$ Treg activity was assessed by comparing HIV-specific T cell function in unfractionated versus $CD25^+$ cell-depleted MC, unless otherwise indicated.

Lymphoproliferation Assay. The most common method to assess $CD25^+$ Treg suppressor activity has been to demonstrate inhibition of normal $CD25^-$ $CD4^+$ T cell proliferation. Using such functional assays, we previously reported on the difficulty in detecting suppressive activity of PB-derived $CD25^+$ Treg cells isolated from chronically HIV-infected individuals with advanced disease compared with HIV^+ subjects with a favorable clinical status (17). In the present study, total and $CD25^{+hi}$ cell-depleted $CD4^+$ T cells (see Fig. 1*B*) isolated from PBMC and LNMC of patients 1–5 (Table 1) were compared for proliferation in response to HIV p24. As expected, $CD4^+$ T cells from the two individuals with advanced disease (subjects 1 and 5) failed either to proliferate to HIV p24 or failed to exert CD25+ cell-mediated suppression ($\geq$ 40% inhibition) (Fig. 4). Although subject 1 had been on ART and had an undetectable

Table 1. Clinical profiles of study subjects

Patient	VL (HIV RNA copies per ml)	$CD4^+$, Tc/μl	$CD8^+$, Tc/μl	CD4:CD8 Tc ratio	ART
1	<50	143	2,264	0.06	Y
2	<50	592	1,613	0.37	Y
3	24,618	422	991	0.43	N
4	203,495	369	819	0.45	N
5	58,791	143	1,378	0.10	N
6	29,870*	672	1,071	0.63	N
7	123,000*	317	337	0.94	N
8	62,000*	517	873	0.59	N
9	<10,000*	798	495	1.61	N
10	13,760*	767	2,013	0.38	N
11	50,725*	394	816	0.48	N

ART, antiretroviral therapy; Tc, T cells .
*VL measured using assays with a limit of detection of 10,000 HIV RNA copies per ml.

viral load for some time, both these individuals had <150 $CD4^+$ T cells per μl, suggesting that $CD4^+$ T cell count was most relevant to Treg activity in this assay. As reported (17), *in vitro* $CD25^+$ Treg activity was most potent within both PBMC and LNMC of the individual (subject 2) with the best clinical status ($CD4^+$ T cells >500/μl and VL <50) (Fig. 4). The effect of $CD25^{+hi}$ cell depletion on allogeneic antigen-induced proliferation in LNMC and PBMC is shown in SI Fig. 8.

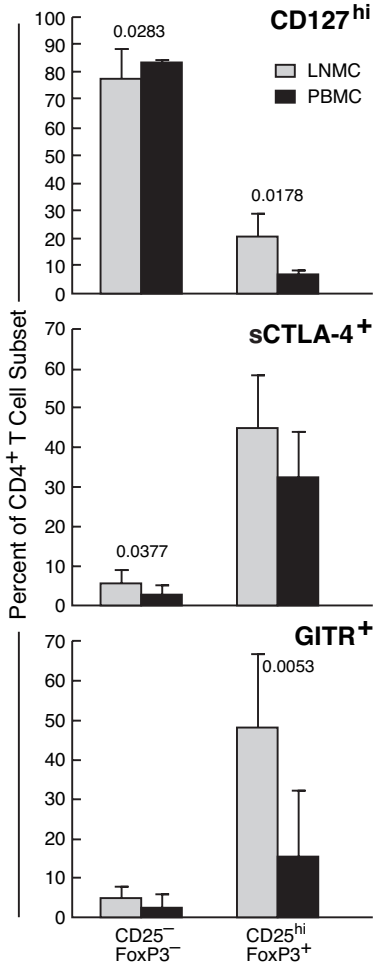

Fig. 3. Comparison of PB and LN for the expression of surface markers used to distinguish $CD25^+$ Treg cells and normal $CD4^+$ T cells. LNMC and PBMC were stained for CD4, CD25, intracellular FoxP3, and either sCTLA-4, GITR, or CD127. $CD25^-FoxP3^-$ and $CD25^{hi}FoxP3^+$ $CD4^+$ LNMC and PBMC subsets were analyzed for expression of CD127, sCTLA-4, and GITR. *P* values compare LNMC and PBMC subsets. Data are of mean percent ± SD of data obtained from LNMC and PBMC isolated from 11 chronically HIV-infected individuals.

Assays of Cytolytic Activity. As HIV-specific proliferation assays proved to be problematic for the assessment of CD25+ Treg activity, we sought to measure CD25+ Treg suppressor capacity using another relevant functional assay. Virus-specific $CD8^+$ T cell-mediated cytolytic (CTL) activity is thought to be critical in the control or elimination of most viral infections, including HIV (30–34). As the lymphoid tissue is the major site of active HIV replication (35–37), HIV-specific CTL activity in the lymph nodes likely represents an important immunologic response with regard to control of HIV replication *in vivo*. In the present study we compared unfractionated and $CD25^{+hi}$ cell-depleted PBMC and LNMC from patients 1–5 for HIV-specific cytolytic activity (Granzyme B substrate cleavage) in HIV Gag peptide restimulation assays. In addition, with cells from patients 3–5, we were able to assess relative Treg suppressive activity in $CD25^-$ MC plus $CD25^{+hi}$ versus $CD25^-$ $CD4^+$ T cell add-back experiments.

As seen in a representative experiment (Fig. 5), HIV Gag-specific target cell "killing" was enhanced in $CD25^+$ cell-depleted (*Middle* plots) compared with unfractionated (*Top* plots) LNMC and was dramatically suppressed by the readdition of 30% $CD25^{hi+}CD8^-$ (≥85% $CD4^+$ T cells; data not shown)

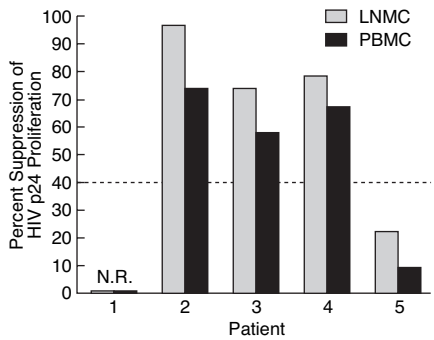

Fig. 4. CD25+ Treg suppressive capacity assessed in HIV p24-specific proliferation assays. Total and $CD25^+$ cell-depleted $CD4^+$ T cells isolated from LNMC and PBMC of subjects 1–5 were stimulated with HIV p24 protein for 6 days, then pulsed with ^3H thymidine 16 h. Percent suppression of proliferation by $CD25^+$ cells was determined by comparing net cpm obtained in total versus $CD25^+$ cell-depleted $CD4^+$ T cell cultures (≥40% was arbitrarily considered to be a significant level of suppression). N.R., no response.

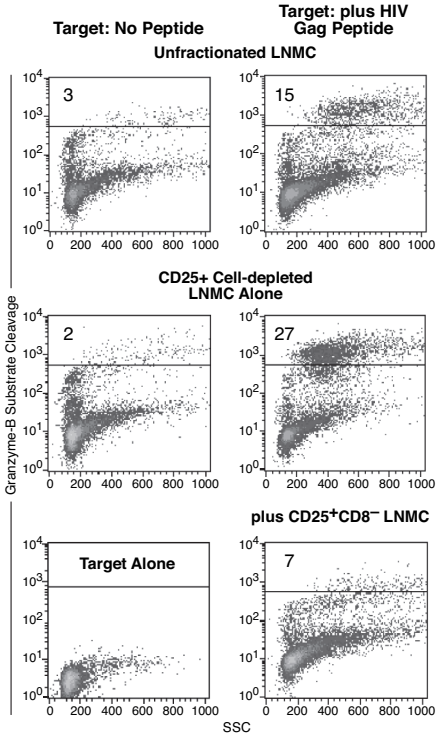

Fig. 5. Representative example of $CD25^+$ Treg suppressive capacity assessed in flow cytometry-based CTL assays by using Granzyme-B substrate cleavage as a read out. HIV Gag pre-stimulated effector unfractionated (*Top* plots) and $CD25^+$ cell-depleted LNMC, alone or plus 30% $CD25^+CD8^-$ MC, (*Middle* and *Bottom Right* plots, respectively) were cultured for 1 h with FL-4-labeled un-pulsed or HIV Gag peptide-pulsed autologous $CD25^-CD8^-$ target cells in the presence of a Granzyme-B substrate that fluoresces when cleaved. Percent target cell killing (indicated within each plot) was defined by the frequency of substrate cleavage high events within the $FL\text{-}4^+$ target cell gate; analyses are of $FL\text{-}4^+$ target cells only. Substrate cleavage in target cells cultured in the absence of effector cells is shown in the *Bottom Left* plot.

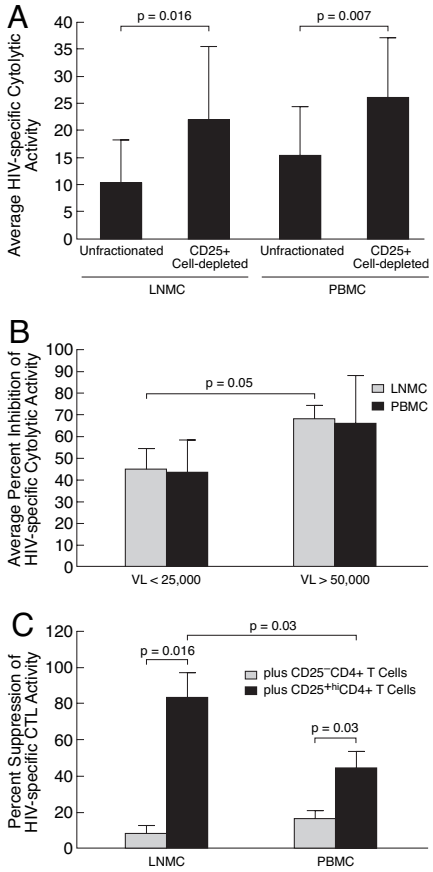

Fig. 6. Comparison of PB and LN $CD25^+$ Treg cell-mediated suppression of HIV-specific CTL activity. (*A*) Mean (± SD) HIV-specific CTL activity in unfractionated and $CD25^+$ cell-depleted PBMC and LNMC. (*B*) Mean (± SD) percent inhibition of HIV-specific CTL activity mediated by $CD25^+$ cells in LNMC and PBMC of patients stratified by VL (HIV RNA copies per ml). (*C*) Comparison of the suppressive effects of LN and PB-derived $CD25^{hi+}$ $CD4^+$ T cells on HIV-specific CTL activity. Purified $CD25^-$ or $CD25^{hi+}$ $CD4^+$ T cells were isolated from the PB and LN (subjects 3–5) and added to autologous $CD25^+$ cell-depleted MC (at 10%) at the time of primary stimulation with HIV Gag peptides. CTL assays were performed 6 days later after exposure to HIV Gag peptide-pulsed target cells. Data are of mean (±SD) percent suppression of HIV-specific CTL activity present in control $CD25^+$ cell-depleted MC cultured alone.

LNMC to $CD25^+$ cell-depleted LNMC (*Bottom Right* plot). HIV-specific CTL activity was detected in both PBMC and LNMC of all study subjects and, comparing unfractionated versus $CD25^+$ cell-depleted MC, significant $CD25^+$ cell-mediated suppression of HIV-specific CTL activity was observed with both PBMC and LNMC (Fig. 6*A*). Of interest, a difference in the ability of LN CD25+ Tregs to suppress HIV-specific CTL activity was seen between patients with high, compared with patients with low, levels of viremia. In this regard, $CD25^+$ cell-mediated suppression of HIV-specific CTL activity in LNMC of the two subjects with the highest VL (VL >50,000; patients 4 and 5) was significantly greater than that observed in LNMC of subjects with VL <25,000 HIV RNA copies/ml (Fig. 6*B*). This difference in $CD25^+$ Treg activity related to level of viremia was not significant with PBMC (Fig. 6*B*). To determine whether $CD25^+$ Treg cells isolated from the PB and LN differed in regard to their suppressive capability, LN or PB-derived $CD25^{hi+}$ or $CD25^-$ $CD4^+$ T cells were added back (at 10%) to $CD25^-$ MC effector populations at the time of initial HIV Gag peptide stimulation. Both PB and LN-derived $CD25^{hi+}$, but not $CD25^-$, $CD4^+$ T cells suppressed HIV-specific CTL activity (Fig. 6*C*); however, the suppressive effect of LN-derived $CD25^{hi+}CD4^+$ T cells was significantly greater than that observed with PB-derived $CD25^{hi+}$ $CD4^+$ T cells (Fig. 6*C*).

Discussion

The present study was designed to determine the functional capability of lymphoid tissue-derived $CD25^+$ Treg cells, particularly those isolated from individuals with advanced HIV disease, with regard to their suppressive effects on HIV-specific T cell responses. The answer to this question is critical to determining whether immunosuppressive $CD25^+$ Treg cells represent a reasonable target for immune-based approaches for enhancing HIV-specific immune responses and, if so, whether such an approach is appropriate at all stages of HIV disease. The results of this study indicate that $CD25^+$ Treg cells maintain significant suppressive activity against HIV-specific cytolytic T cell responses even in the advanced stage of HIV disease. Furthermore, LN-derived $CD25^+$ Treg cells suppressed HIV-specific responses to a greater degree than did PB-derived $CD25^+$ Treg cells, particularly in those individuals with high viral loads.

Natural $CD25^{hi}$ $FoxP3^+$ Treg cells are an important component of the immune surveillance system designed to control autoimmunity (1); however, their suppressive activity has been shown to also impact appropriate foreign antigen-specific immune responses. In this regard, $CD25^+$ Treg cell activity has been shown to contribute to the persistence of certain infections and tumors *in vivo* (2–8). In addition, suppressive antigen-specific $CD25^+$ Treg cells can be generated ("induced" $CD25^+$ Treg cells) from presumably normal

$CD4^+$ T cells under certain pathogenic/micro environmental conditions (38, 39). Whether natural or induced, $CD25^+$ Treg cells have been demonstrated to accumulate and expand at tissue sites of inflammation/antigen expression where cell contact-mediated suppression may be particularly effective (2, 4, 9). In HIV disease, HIV replication/antigen expression largely occurs in the lymphoid tissue (36, 37), the site at which primary immune responses to most antigens are generated. Therefore, antigen-driven accumulation and activation of $CD25^+$ Treg cells in lymphoid tissue sites could have a considerable impact not only on effector cell function but also on the generation of lymphocyte responses to new antigens or antigenic epitopes, as might arise in the case of highly mutable HIV.

Results from early studies using PB suggested that $CD25^+FoxP3^+$ Treg cell function and/or frequency decline with HIV disease progression (17, 19–21). Evidence that $CD25^+$ Treg cells are not necessarily lost over the course of HIV disease but rather accumulate in the lymphoid tissue has been generated by *in situ* phenotypic and mRNA studies (19, 22, 24). High levels of HIV replication have been associated with the accumulation of cells with a Treg-like phenotype and/or FoxP3, TGF-β and IDO mRNA in tonsils (19, 24) and lamina propria (22). In our phenotypic studies, $CD25^{hi}FoxP3^+$ cell frequencies in LNMC were consistently higher than in PBMC regardless of viral load or $CD4^+$ T cell counts of the study subject. In contrast, the frequency of $CD25^{+hi}$ Treg cells is reported to be similar in lymph node and PB of healthy HIV uninfected controls (40). Of interest, we also observed that FoxP3 intensity (MFI) in the $CD25^+$ $CD4^+$ T cell subset of LNMC was significantly higher than that in PBMC. It has been reported that human $CD25^+$ Treg cells expressing high levels of FoxP3 exhibit more rapid suppressive activity compared with $FoxP3^{mid}$ $CD25^+$ Treg cells (41). With regard to additional surface markers associated with $CD25^+$ Treg cells, $CD25^{hi}FoxP3^+CD4^+$ T cells exhibited higher GITR expression in the LN than in PB, which suggests that LN Treg cells would be more likely to be activated and expand in response to interaction with dendritic cells that express GITR-L (42, 43). $CD127^{neg/lo}$ (29) was the most specific surface phenotype for $CD25^{hi}FoxP3^+$ $CD4^+$ T cells in both PB and LN; however, a greater proportion of $CD25^{hi}FoxP3^+$ Treg cells expressed $CD127^{hi}$, rather than $CD127^{neg/lo}$, in the LN compared with the PB. Overall , no surface marker was sufficient to fully and accurately discriminate between $CD25^{hi}FoxP3^+$ Treg cells and normal $CD4^+$ T cells in the LN.

Importantly, our functional studies demonstrate that $CD25^+$ Treg cells isolated from both the PB and LN of all subjects, even those with advanced disease, retain significant suppressive activity as detected by HIV-specific CTL assays. Although the sample size in the present study was low, the data suggest that $CD25^+$ Treg cell-mediated suppression of HIV-specific CTL activity is more effective in lymphoid tissue than in PB, particularly in those patients with moderate/high compared with undetectable/low viral loads. Whereas *in vitro* human CD25+ Treg cells have generally been found to suppress $CD4^+$ T cell proliferation via an ill-defined cell contact-dependent, IL-10/transforming growth factor (TGF)-β-independent mechanism (44), *in vivo* $CD25^+$ Treg cells are thought to exert immunosuppression by numerous mechanisms, including TGF-β (28, 45, 46). Increased $CD25^+$ Treg activity in LNMC compared with PBMC may be due to differences in the frequency of $CD25^+$ Treg cell subsets that suppress by the production or induction of soluble immunosuppressive factors (47, 48), particularly TGF-β. Although definitive comparative studies of TGF-β production by PBMC and LNMC in chronically HIV-infected individuals have not been performed, TGF-β is elevated in the lymphoid tissue of viremic HIV-infected subjects (19, 22, 24). Of note, Treg-mediated suppression of antigen-specific CTL activity in certain murine models has been shown to be TGF-β-dependent (49, 50).

Using proliferation assays as a read-out, we previously reported on a cohort of patients that exhibited a loss of $CD25^+$ Treg suppressive activity against HIV-specific $CD4^+$ T cell responses in the PB with HIV disease progression (17). We proposed, as have others (19), that "HIV-specific" Treg cells might migrate out of the blood into the lymph nodes during periods of viremia and we hypothesized that $CD25^+$ Treg activity against HIV-specific T cell function would be retained in lymphoid tissue. However, in the present study, $CD25^+$ cell-mediated suppression of HIV-specific $CD4^+$ T cell proliferation was not observed in either PBMC or LNMC isolated from individuals with more advanced disease, although suppression of HIV-specific CTL activity was detected in both PBMC and LNMC of all subjects. The apparent differential sensitivity of antigen-specific proliferation and cytotoxicity to CD25+ Treg-mediated suppression has not been reported in the context of HIV infection. It is possible that factors that reduce sensitivity of normal T cells to $CD25^+$ Treg-mediated suppression, such as TCR signal strength, GITR expression and IL-6, (28, 43, 51–53)} differ in the two assay systems. Alternatively, different CD25+ Treg subsets (47) or mechanisms (45, 46) may suppress CTL effector function more efficiently than other T cell functions. In this regard, there is evidence in murine models indicating that CD25+Treg-mediated suppression of CD8+ effector functions, but not of T cell proliferation, requires intact TGF-β signaling (45, 50, 54). Furthermore, it has been suggested that cytolytic activity may be the most Treg-sensitive T cell function *in vivo* (49). The data from the present study would suggest that CTL activity in humans is also more sensitive to $CD25^+$ Treg-mediated suppression than is proliferation or other effector functions, at least in the context of progressive HIV disease.

In summary, the present study provides proof of concept that $CD25^+$ Treg cells maintain the ability to suppress HIV-specific responses, particularly CTL function, throughout the course of HIV disease. CD25+ Treg-mediated suppression of HIV-specific cytolytic activity in lymphoid tissue, the major site of HIV replication, may be particularly detrimental to the ability to control virus replication *in vivo*. It remains to be established whether immune-based therapies designed to transiently deplete $CD25^+$ Treg cells *in vivo* would ultimately be beneficial or detrimental in the context of HIV infection.

Materials and Methods

Cellular Subset Isolation. Inguinal or axillary LN biopsies and lymphophereses were obtained after informed consent from 11 HIV-infected individuals under National Institutes of Health (NIH) approved protocols (NIH 92-I-25, 02-I-0202, and 81-I-0164). The clinical profiles of study subjects are shown in Table 1. Fresh LNMC and PBMC of individuals 1–5 were used in functional assays and frozen LNMC and PBMC from subjects 6–11 were used for phenotypic studies only. LNMC were obtained by gentle physical disruption (scalpel microdissection) and collagenase treatment. PBMC were isolated by centrifuge density gradient. MCs or $CD4^+$ T cells [isolated by using a negative selection antibody mixture and immunomagnetic beads (Stem Cell Technologies, Vancouver, BC, Canada)] were retained as unfractionated or separated into $CD25^{+hi}$ and $CD25^-$ subsets. $CD25^{hi}$ cells were obtained by using anti-human CD25 PE conjugated Mab (3 μl/10^6 cells) followed by a short incubation (3 min) with anti-PE immunomagnetic beads (Miltenyi, Auburn, CA). For certain assays, the $CD25^-$ MC were further depleted of $CD8^+$ cells by using anti-CD8-coupled immunomagnetic beads (Dynal, Brown Deer, WI) to derive $CD8^-CD25^-$ control MC ($\geq$85% $CD4^+$ T cells). $CD25^+$ Treg phenotypic analyses were performed on total LNMC and PBMC by using anti-human FoxP3 (intracellular; EBiosciences, San Diego, CA), CD4, CD25, CD127, CTLA-4 and GITR. All antibodies were

purchased from BD PharMingen (San Jose, CA) unless otherwise indicated.

HIV-Specific Lymphocyte Proliferation Assay. Unfractionated (total) $CD25^-$ or $CD25^{+hi}$ $CD4^+$ T cells isolated from lymph nodes or the PB were plated at $1–2 \times 10^5$ cells per well in media [RPMI medium 1640 (Invitrogen, Carlsbad, CA), 10% human AB serum (Hyclone, Logan, UT) supplemented with 1 mM glutamine, antibiotics, and Hepes buffer] plus autologous γ-irradiated $CD25^-$ PBMC (APC; 1:1). LN-derived cells were cultured in laminin-coated plates (Invitrogen). Cells were either untreated or stimulated with HIV-1 p24 (5 μg/ml; Protein Sciences, Meriden, CT) or allo-antigens (a mix of allogeneic, γ-irradiated PBMC from three different donors cultured at a 1:1 ratio with responder populations). At day 6, post-stimulation cells were exposed to 0.5 μCi per well (1 Ci = 37 GBq) ^{3}H thymidine (PerkinElmer, Boston, MA) for 16 h to assess cellular proliferation (net cpm). Effective Treg-mediated suppression of proliferation was considered to be present if net cpm values of unfractionated MC were $\leq$60% of CD25-depleted MC net cpm values; this cut-off was based on a similar threshold used in a previous study (17).

HIV-Specific Cytolytic Activity. All assays were conducted in RPMI medium 1640 plus 10% human AB sera media. "Effector" (E) unfractionated or $CD25^+$ cell-depleted PBMC or LNMC (LNMC were cultured in laminin-coated plates) received primary stimulation with autologous $CD25^-CD8^-$MC (peptide-presenting cells: PPC) (1:10 ratio) that were untreated (no peptide) or were pulsed with a pool of overlapping 15mer HIV Gag (AIDS Reagent Repository, Rockville, MD). In certain experiments the $CD25^{+hi}$ and $CD25^-$ subsets of $CD4^+$ T cells or $CD8^-$ MC were added to $CD25^+$ cell-depleted MC (at 10–30%) before primary stimulation with peptides. Six days later, effector cells under each condition were harvested, washed, and 5×10^6 E cells were restimulated with 0.2×10^6 labeled (FL4 channel detection) unpulsed or HIV Gag peptide-pulsed, autologous $CD8^-CD25^-$ target (T) cells (T:E ratio = 1:25). Effector plus target cells were cultured for 1 h in the presence of a Granzyme B substrate that fluoresces in FL-1 when cleaved (GranToxiLux, OncoImmunin, Gaithersburg, MD). Granzyme B substrate cleavage was assessed in the $FL4^+$ target population as per manufactures recommendation by using a BD FACSCalibur. HIV-specific cytolytic activity was determined by subtracting substrate cleavage values obtained in cultures containing peptide-free target cells from those values obtained in cultures containing HIV Gag peptide-pulsed target cells.

Statistical Analyses. All statistical analyses were conducted by using unpaired or paired Student's *t* test and Spearman's (non-parametric) correlation.

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