$CD25^+CD4^+$ Regulatory T Cells from the Peripheral Blood of Asymptomatic HIV-infected Individuals Regulate $CD4^+$ and $CD8^+$ HIV-specific T Cell Immune Responses In Vitro and Are Associated with Favorable Clinical Markers of Disease Status

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Abstract

Human immunodeficiency virus (HIV) disease is associated with loss of $CD4^+$ T cells, chronic immune activation, and progressive immune dysfunction. HIV-specific responses, particularly those of $CD4^+$ T cells, become impaired early after infection, before the loss of responses directed against other antigens; the basis for this diminution has not been elucidated fully. The potential role of $CD25^+CD4^+$ regulatory T cells (T reg cells), previously shown to inhibit immune responses directed against numerous pathogens, as suppressors of HIV-specific T cell responses was investigated. In the majority of healthy HIV-infected individuals, $CD25^+CD4^+$ T cells significantly suppressed cellular proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells in response to HIV antigens/peptides in vitro; these effects were cell contact dependent and IL-10 and TGF-β independent. Individuals with strong HIV-specific $CD25^+$ T reg cell function in vitro had significantly lower levels of plasma viremia and higher $CD4^+$: $CD8^+$ T cell ratios than did those individuals in whom this activity could not be detected. These in vitro data suggest that $CD25^+CD4^+$ T reg cells may contribute to the diminution of HIV-specific T cell immune responses in vivo in the early stages of HIV disease.

Key words: cytokine • proliferation • human • suppression • FoxP3

Introduction

HIV-1 infection is characterized by a progressive loss of $CD4^+$ T cells, chronic immune activation, and an increasing array of immune dysfunctions (1–3). HIV-specific T cell immune responses are particularly impaired, and defects appear earlier in disease as compared with responses directed at other microbial antigens (4–6). The basis of HIV-specific T cell immune dysfunction has not been elucidated fully, and it is likely that both HIV-specific and nonspecific mechanisms are involved (7–12). In this regard, one avenue that has not been explored fully in relation to HIV-specific immune responses is the potential role of host-mediated immunosuppressive mechanisms that might be activated in the face of persistent antigenic exposure.

$CD25^+CD4^+$ suppressor/regulatory T cells (T reg cells), a cellular subset with constitutive immunosuppressive activity first described in the context of autoimmune disorders (13, 14), are one of several cellular subsets involved in controlling inappropriate or excessive immune activation (15–18). $CD25^+CD4^+$ T reg cells have been demonstrated to suppress antigen-specific $CD4^+$ and $CD8^+$ T cell responses directed against tumors (19, 20) and allografts (21, 22) as well as against parasitic (23–25), bacterial (26, 27), fungal (28, 29), and viral (30–32) antigens or infections in vivo in mice.

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Abbreviations used in this paper: CFSE, carboxyfluorscein diacetate succinimidyl ester; ICC, intracellular cytokine; LPA, lymphocyte proliferation assay; LTNP, long-term non progressor; SI, stimulation indexes; T reg cell, regulatory T cell.

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Furthermore, the immunosuppressive activity of $CD25^+CD4^+$ T reg cells has been implicated in the inability of mice to clear infection with certain pathogens (23, 29–32) or to mount effective responses to vaccination (26). Although studies on $CD25^+CD4^+$ T reg cells have been conducted primarily in murine systems, a population with a virtually identical phenotypic and functional profile has been described in humans (33). The present paper presents evidence supporting the potential role of $CD25^+CD4^+$ T reg–mediated immunosuppression in the diminution of HIV-specific $CD4^+$ and $CD8^+$ T responses in asymptomatic HIV-infected individuals.

Materials and Methods

Purification of Cellular Populations. Phlebotomy (National Institutes of Health [NIH] protocol no. 91-I-0140) or apheresis (NIH protocol no. 81-I-164) was performed on uninfected control individuals ($n = 20$) or on HIV-infected, untreated, or antiretroviral therapy-treated individuals ($n = 52$; see Table I). PBMCs, obtained by Ficoll-Hypaque density gradient centrifugation, were either depleted of $CD25^+$ cells using anti-CD25–coupled immunomagentic beads (Miltenyi Biotec) to obtain CD25-depleted PBMCs or exposed to a cocktail of immunomagnetic beads (StemCell Technologies Inc.) to obtain $CD4^+$ T cells (>95% purity) by negative selection. For lymphocyte proliferation assays (LPAs), $CD4^+$ memory T cells were obtained by depleting $CD45RA^+$ cells from $CD4^+$ T cells using anti-CD45RA mAb (BD Biosciences) coupled to goat anti–mouse immunomagnetic beads (Dynal); subsequently, $CD25^+$ and $CD25^-CD4^+$ memory T cell subsets were obtained using anti-CD25–coupled immunomagentic beads (Miltenyi Biotec). CD25 expression in CD25-depleted and $CD25^+CD4^+$ subpopulations was <5% and ≥87%, respectively (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20032069/DC1). Autologous APCs were γ-irradiated (5,000 R) $CD2^-$ or $CD25^-$ PBMCs. $CD8^+$ cells were isolated by positive selection using anti-CD8–coupled immunomagnetic beads (Dynal); beads were removed using DETACHaBEAD (Dynal). Autologous $CD4^+CD25^-$ T cells were used as the HIV-producing target cell population in assays to determine HIV-induced proliferation of $CD8^+$ effector T cells. Flow cytometric quantification of cellular subsets was performed using fresh PBMCs surfaced stained with mAbs recognizing CD3, CD4, CD25, CD45RO, CD103, or CD122, or stained for intracellular CTLA-4 (BD Biosciences).

LPA. PBMCs or memory $CD4^+$ T cell subsets were plated at $1–2 \times 10^5$ cells/well in media (RPMI 1640 supplemented with 1 mM glutamine, antibiotics, and Hepes buffer) plus 10% human AB serum. $CD25^-$ and $CD25^+CD45RA^-CD4^+$ T cells were cultured alone or at a 1:1 ratio. Autologous $CD25^-$ PBMCs (APCs) were γ-irradiated (4,000 R) and added at a ratio of 1:1 (APCs:total T cells) to wells containing $CD4^+$ memory T cell populations. Cells were either untreated or exposed to 5–10 μg/ml HIV-1 p24 (Protein Sciences). In certain experiments neutralizing 0.5 μg/ml of mouse anti–human IL-10 (R&D Systems), 10 μg/ml of chicken anti–human TGF-β (R&D Systems) or isotype control antibodies were added to cultures; in addition, transwell inserts were used to separate $CD25^+$ and $CD25^-CD4^+$ T cells to assess the requirement for cell–cell contact. Supernatant was harvested and frozen for later assessment of cytokine production, and at day 6, cells were exposed to 0.5 μCi/well [^{3}H]thymidine (PerkinElmer) for 16 h to assess cellular proliferation. In a subset of subjects, $CD25^+$ or $CD25^-CD45RA^-CD4^+$ T cells (10^4–10^5) were added to 10^5 $CD25^-CD45RA^-CD4^+$ T cells/well and stimulated with a pool of allogeneic γ-irradiated PBMCs (2×10^5), and proliferation was assessed at day 5. Proliferation stimulation indexes (SI) were calculated by dividing the cpm obtained in antigen-stimulated conditions by the cpm obtained in unstimulated (background) conditions.

Intracellular or Secreted Cytokine Production. For assessment of HIV p24-induced intracellular cytokine (ICC) expression in $CD4^+$ T cells, 10^6 PBMCs, $CD25^-$ PBMCs, or $CD45RA^-CD4^+$ subpopulations (plus 20% $CD2^-$ PBMCs) were untreated or exposed to 10 μg/ml HIV-1 p24 for 4–5 h. 1 μL/ml Golgi Plug (BD Biosciences) was added for an additional 9–10 h, and cells were harvested and stained for surface markers and intracellular IL-2 or IFN-γ as per manufacturer's recommendations (BD Biosciences). $CD8^+$ and $CD4^+$ T cells present in unfractionated or $CD25^-$ PBMCs were tested for rapid cytokine production (6 h) in response to a pool of overlapping 15-mer Gag peptides (AIDS Reagent Repository) as described previously (5). Assessment of antigen or anti-CD3 (immobilized 10 μg/ml)–induced supernatant-associated levels of cytokines and chemokines was performed using Multiplex assay systems (Biosource International or Upstate Biotechnology); assays were read using a Luminex reader (Mirabio). TGF-β levels were assessed in supernatants from cells cultured in media plus 1% FCS and stimulated with 10 μg/ml of immobilized anti-CD3 using a TGF-β ELISA (R&D Systems).

Proliferation of Effector (Perforin$^+$) CD8$^+$T Cells in Response to Autologous HIV Super-infected Target Cells. $CD25^+$ or $CD25^-CD4^+$ T cells, $CD8^+$ T cells, and autologous CD2-depleted PBMCs (APCs) were isolated as aforementioned. A portion of $CD25^-CD4^+$ T cells to serve as targets were exposed to VSV env-pseudotyped (Δ nef-luciferase) HIV virus (single round replication competent) at a multiplicity of infection of 0.5 for 6–14 h and washed three times. Purified $CD8^+$ T cells were labeled with the fluorescent dye carboxyfluorscein diacetate succinimidyl ester (CFSE) by standard methods. Uninfected or VSV env-pseudotyped HIV super-infected target cells were cultured with CFSE-labeled $CD8^+$ T cells alone or together with $CD25^+$ or $CD25^-CD4^+$ T cells at a ratio of 0.05–0.2:1:1, respectively, with 20% $CD2^-$ PBMCs in media plus 10% human AB serum. Cells were stained for CD69, CD3, or CD8 and intracellular perforin 5–11 d after coculture as described previously (34).

Assessment of FoxP3 Expression by Western Blot. Total cellular lysate from 5×10^5 freshly isolated PBMC subsets were run on 10% Tris/Glycine polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Pierce Chemical Co.), and membranes were probed with polyclonal rabbit anti–human FoxP3 antisera followed by horseradish peroxidase–conjugated goat anti–rabbit secondary Ab (Cell Signaling); signal detection was achieved with SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Co.).

Online Supplemental Material. Fig. S1 shows the purity of isolated $CD25^-$ and $CD25^+CD4^+$ T cell subsets; cells were gated on $CD3^+$ T cells (>97%) and assessed for surface CD4 and CD25 expression. Fig. S2 depicts the mean net cpm ± SD obtained in LPAs after exposure of $CD4^+$ T cell subsets from p24 LPA suppressors and nonsuppressors to 10 μg/ml HIV p24. Fig. S3 shows the mean net cpm ± SD obtained in LPAs after exposure of $CD4^+$ T cell subsets from p24 LPA suppressors, nonsuppressors, and nonresponders to *Candida albicans*. Proliferative responses of $CD4^+$ memory T cell subsets to *C. albicans* were performed using

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5 μg/ml of *C. albicans* (Sigma Aldrich). Fig. S4 depicts the mean ± SD copies of HIV DNA present in fresh $CD25^+$ versus $CD25^-CD4^+$ T cells isolated from seven HIV-infected donors. HIV DNA levels were determined by quantitative real-time PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20032069/DC1.

Results

Characterization of $CD25^+CD4^+$ T Cells in the Peripheral Blood of HIV-infected and Uninfected Individuals. Human $CD25^+CD4^+$ T cells have been shown to be a heterogeneous population that includes immunosuppressive T reg cells, anergic but nonsuppressive T cells, and normal activated T cells (35, 36). Until very recently (37), no surface markers had been reported to be uniquely expressed on suppressive $CD25^+CD4^+$ T reg cells, making identification and quantification of this population difficult. However, the cellular transcription factor FoxP3 has been demonstrated to be functionally relevant to immunosuppressive activity of $CD25^+CD4^+$ T cells in both mice and humans (38–42). In this regard, fresh $CD25^+$, but not $CD25^-$, $CD4^+$ T cells from both HIV-infected and uninfected individuals expressed FoxP3, and there were no clear qualitative differences in FoxP3 expression between these two groups of individuals (Fig. 1 A).

In addition, the expression of high-density CD25 has been correlated, in numerous studies, with immunosuppressive T reg cell activity in human $CD4^+CD25^+$ T cells (35, 36, 43–45). Using this marker, we attempted to quantify total $CD25^+CD4^+$ T reg cells in PBMCs from HIV-infected (n = 38) and uninfected (n = 20) individuals. PBMCs from HIV-infected and uninfected individuals contained similar frequencies of total $CD25^+$ cells in the memory ($CD45RO^+$) $CD4^+$ T cell population ($P > 0.05$; unpublished data); however, the frequency of cells expressing high density CD25 (Fig. 1 B, top) was modestly but significantly elevated in PBMCs of HIV-infected individuals (Fig. 1 B, bottom). The frequency of $CD25^{hi}$ cells in PBMCs of HIV^+ subjects remained relatively constant over a broad range of memory $CD4^+$ T cell percentages (Fig. 1 C), and the absolute numbers of this population declined proportionately with the loss of $CD4^+$ T (memory) cells and disease progression (Fig. 1 D). In both HIV-infected and uninfected subjects, $CD25^+CD45RO^+CD4^+$ T cells expressed significantly ($P < 0.002$) higher levels, as

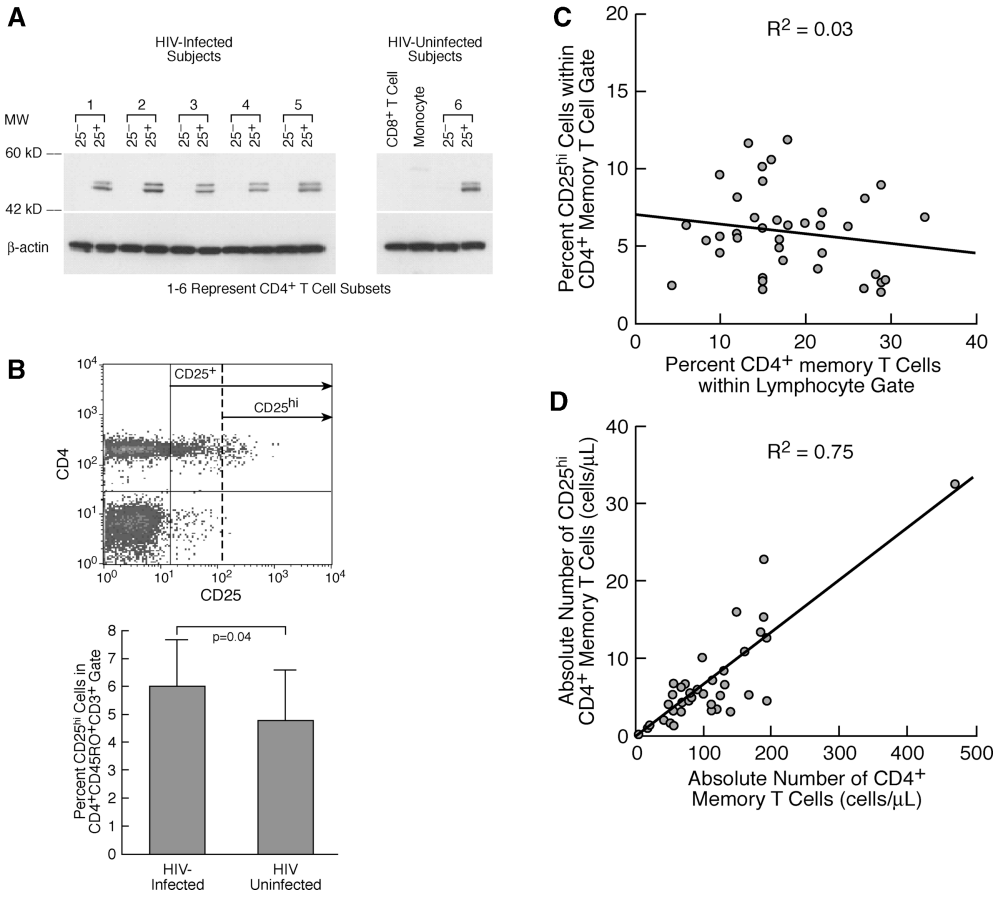

Figure 1. $CD25^+CD4^+$ T cells in PBMCs from HIV-infected individuals express phenotypic markers consistent with $CD25^+$ T reg cells: quantification of total $CD25^+CD4^+$ T reg cells in PBMCs of HIV-infected subjects by surface expression of $CD25^{+hi}$. (A) FoxP3 protein expression, detected by Western blot, is comparable in fresh $CD25^+CD4^+$ T cells isolated from HIV-infected versus HIV-uninfected individuals Fresh $CD25^-CD4^+$ T cells, $CD8^+$ T cells, and monocytes do not express FoxP3. (B, top) Representative example of FACS® profile and gates used to quantify $CD25^{+hi}CD4^+CD45RO^+$ T cells. Freshly isolated PBMCs were gated on $CD45RO^+CD3^+$ and analyzed for CD4 and CD25 expression. $CD25^{+hi}$ gates were set so that ≤0.05% of all PBMC subsets, other than $CD4^+$ T cells, fell to the right of the $CD25^{+hi}$ gate (dashed line). (bottom) Comparison of the frequency of $CD25^{+hi}$ cells within the $CD4^+CD45RO^+$ T cell subset of PBMCs from HIV-infected (n = 38) versus uninfected (n = 20) subjects. (C) The frequency of and the (D) absolute number of $CD25^{+hi}$ versus total $CD4^+$ memory T cells in PBMCs isolated from 38 HIV-infected individuals representing a broad percentage range of $CD4^+$ memory T cells.

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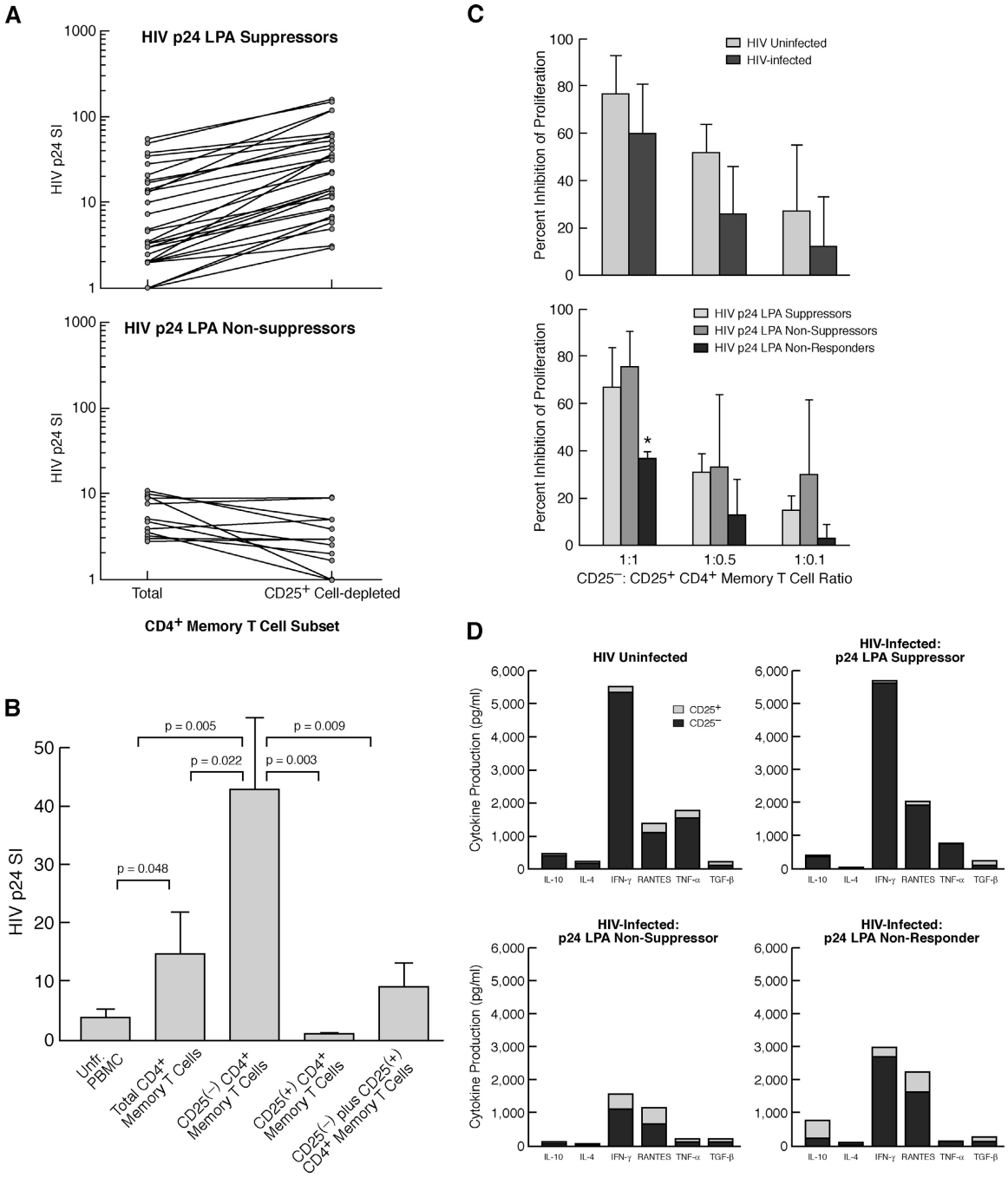

Figure 2. Functional analyses of the CD25$^+$ memory CD4$^+$ T cell subset from HIV-infected individuals. (A) Comparison of the HIV p24 proliferation SI of total versus CD25$^+$ cell subset-depleted CD4$^+$ memory T cells isolated from HIV-infected individuals with strong (top, HIV p24 LPA Suppressors) or no/weak (bottom, HIV p24 LPA Non-suppressors) HIV-specific CD25$^+$ T reg cell suppressor activity. The geometric mean HIV p24 SI of total and CD25$^+$ cellular subset-depleted populations from LPA suppressors versus LPA nonsuppressors was 5.6 and 20.4 versus 5.3 and 3.6, respectively. (B) HIV p24 SI in more extensive LPA assays using PBMCs and CD4$^+$ memory T cell subsets from LPA suppressors (n = 19). Data are represented as mean HIV p24 SI ± SD. (C) Comparison of the suppressive activity of CD25$^+$CD4$^+$ memory T cells isolated from (top) HIV-uninfected individuals (n = 6) versus all HIV$^+$ subjects (n = 9) and (bottom) from HIV$^+$ subjects comparing HIV p24 LPA suppressors, nonsuppressors, and nonresponders (n = 3 for each category) under polyclonal (pool of allogeneic PBMCs) stimulation conditions. Only CD25$^+$ memory CD4$^+$ T cells from HIV p24 LPA nonresponders exhibited significantly ($P \leq 0.04$) weaker polyclonal T reg cell activity as compared with HIV-uninfected subjects or other groups of HIV$^+$ subjects. Data are represented as mean ± SD percent inhibition of control (CD25$^-$ subset) SI. (D) Comparison of cytokine/chemokine production by anti-CD3–stimulated CD25$^+$ and CD25$^-$ subsets of CD4$^+$ memory cells isolated from HIV-uninfected individuals (n = 6) and HIV p24 LPA suppressors, nonsuppressors, and nonresponders (n = 4 for each category).

compared with their CD25$^-$ counterparts, of surface markers (CD103, CD122, and intracellular CD152 [CTLA-4]) shown previously to be preferentially expressed on human CD25$^+$CD4$^+$ T cells that exhibit T reg cell–like suppressive activity (references 35, 43, 45–48 and unpublished data).

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Functional Characterization of CD25$^+$CD45RA$^-$CD4$^+$ T Cells Isolated from HIV-infected Individuals: Suppression of HIV-specific and HIV-nonspecific CD4$^+$ T Cell Proliferation In Vitro. HIV p24 LPAs were performed using PBMCs and CD4$^+$CD45RA$^-$/RO$^+$ (memory) T cell subsets (total vs. CD25$^+$ subset depleted) from 52 HIV-infected individuals (Table I and Fig. 2 A). In experiments with PBMCs isolated from a portion of individuals (10/52; referred to as HIV p24 LPA nonresponders) the SI to HIV p24 failed to exceed a value of three under any cellular subset condition (unpublished data). In experiments with cellular subsets from the remaining individuals ($n = 42$) that did proliferate in response to HIV p24, CD25$^+$ subset depletion of memory CD4$^+$ T cells led to an average increase in the p24 SI of 3.2-fold (P = 0.0003; Table I). However, within this group, we observed two distinct patterns concerning the suppressive effect of the CD25$^+$ subset on HIV p24 proliferative responses. Using a cut-off index of >1.5-fold enhancement of the p24 SI after CD25$^+$ subset depletion from the total of memory CD4$^+$ T cell population, individuals were separated into two groups as follows: those with strong (HIV p24 LPA suppressors) or those with no or weak (HIV p24 LPA nonsuppressors) CD25$^+$ subset suppressor effect. Approximately 31% of the individuals tested

Table I. *Clinical and Functional Characteristics of HIV-infected Subjects*

Patient ID	CD4$^+$ Tc	CD8$^+$ Tc	CD4:CD8 ratio	Viral load	Change in p24 SI[a]	Percent CD25^{hi} [b]
	cells/μL	*cells/μL*		*HIV RNA copies/ml*		
All HIV$^+$ subjects[c]						
Mean	543	1,001	0.6	14,275	3.2	6.0
SD	259	474	0.4	21,845	3.2	2.7
Geometric mean	481	907	0.5	2,031	2.2	5.4
[A] LPA suppressors[d]						
Mean	619	956	0.8	5,839	4.2	6.2
SD	270	478	0.4	9,977	3.3	2.8
Geometric mean	565	844	0.7	821	3.5	5.6
Range	252–1,380	218–2,400	0.3–1.8	<50–29,000	1.6–18.5	2.1–11.9
[B] LPA nonsuppressors[e]						
Mean	476	1,186	0.4	20,112	0.9	5.5
SD	211	448	0.2	25,950	0.4	2.4
Geometric mean	417	1,121	0.4	6,175	0.8	5.0
Range	250–741	750–2,344	0.2–0.7	<50–86,888	0.3–1.4	2.3–9.2
[C] Nonresponders[f]						
Mean	361	1,117	0.4	34,319	NA	4.9
SD	248	638	0.3	11,909	NA	1.7
Geometric mean	308	969	0.4	29,867	NA	4.5
Range	160–781	461–2,093	0.1–1.1	<50–79,000	NA	2.2–7.3
p-value						
A vs. B	0.110	0.161	0.006	0.012	NA	0.45
A vs. C	0.026	0.990	0.133	0.0001	NA	0.19
B vs. C	0.370	0.272	0.593	0.260	NA	0.51

[a]HIV p24 SI of CD25$^-$/HIV p24 SI of total memory CD4$^+$ T cells.
[b]In CD4$^+$ memory Tc population.
[c]$n = 52$.
[d]$n = 29$.
[e]$n = 13$.
[f]$n = 10$.
NA, not applicable.

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(13/42) fell under the category of HIV p24 LPA nonsuppressors (mean ± SD HIV p24 SI of total memory $CD4^+$ T cells vs. $CD25^-$ memory $CD4^+$ T cells = 5.3 ± 3.4 vs. 4.6 ± 3.3, respectively; P = NS; Table I and Fig. 2 A, bottom). The majority of p24 responsive individuals (69%; 29/42) were HIV p24 LPA suppressors (mean ± SD HIV p24 SI of total memory $CD4^+$ T cells vs. $CD25^-$ memory $CD4^+$ T cells = 10.6 ± 16 vs. 44.7 ± 44, respectively; P = 0.0001; Table I and Fig. 2 A, top). No significant difference was found in the HIV p24 (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20032069/DC1) or the *C. albicans* (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20032069/DC1) proliferative response (mean net cpm) of total $CD4^+$ memory T cells from HIV p24 LPA nonsuppressors as compared with HIV p24 LPA suppressors. In more extensive HIV p24 LPA analyses conducted with cells from 19 HIV p24 LPA suppressors, $CD25^+CD45RA^-CD4^+$ cells were found to be anergic to stimulation with HIV p24 and the addition of this population to the $CD25^-$ memory population significantly suppressed HIV p24-induced proliferation of the latter memory subset (Fig. 2 B).

Individuals within the different HIV p24 LPA response categories were found to differ significantly in regard to several clinical parameters of HIV disease progression. Those individuals who displayed strong HIV-specific $CD25^+$ $CD4^+CD45RA^-$ T cell suppressor activity in vitro (HIV p24 LPA suppressors) had significantly lower viral loads and higher $CD4^+$:$CD8^+$ T cell ratios or $CD4^+$ T cell counts than HIV p24 nonsuppressors and nonresponders (Table I). These observations support the hypothesis that HIV-specific $CD25^+$ T reg cell activity is associated with immune competency and that HIV disease–related factors may reduce the activity of and/or the sensitivity to HIV-specific $CD25^+CD4^+$ T reg cells.

To assess whether the suppressive activity of $CD25^+$ $CD4^+$ T cells isolated from HIV-infected donors was also variable in the context of HIV nonspecific stimulation, $CD25^+CD45RA^-CD4^+$ T cells isolated from a subset of individuals (n = 9 HIV^+ and n = 6 HIV^- subjects) were tested for their ability to suppress proliferation under polyclonal stimulation conditions (allogeneic PBMCs). No significant differences ($P > 0.05$) were observed in the suppressive activity of $CD25^+CD45RA^-CD4^+$ T cells from uninfected versus HIV-infected subjects (as a group) (Fig. 2 C, top). However, upon segregation of HIV^+ subjects based on their p24 LPA designation, $CD25^+$ cells from HIV p24 LPA nonresponders exhibited significantly lower polyclonal suppressive activity than each of the other groups ($P < 0.05$; Fig. 2 C, bottom).

Cytokine Production Profiles of $CD25^+$ and $CD25^-$ $CD45RA^-CD4^+$ T Cells. Consistent with previous papers (35, 46, 47, 49), anti-CD3–stimulated $CD25^+$ $CD45RA^-CD4^+$ T cells from HIV p24 LPA suppressors and uninfected subjects produced dramatically lower levels of cytokines/chemokines, but similar levels of TGF-β, as compared with the $CD25^-$ subset (Fig. 2 D). Although not statistically significant (P = 0.07), there was a trend for higher cytokine production by the $CD25^+$ subset from HIV p24 LPA nonsuppressors and nonresponders as compared with LPA suppressors and HIV^- subjects (Fig. 2 D). Of interest, the $CD25^-$ subset isolated from HIV p24 LPA nonsuppressors produced significantly lower levels of IL-10, TNF-α, and IFN-γ as compared with $CD25^-$ cells isolated from HIV p24 LPA suppressors, suggesting that the function of the normal ($CD25^-$) memory $CD4^+$ T cell population may differ between these groups.

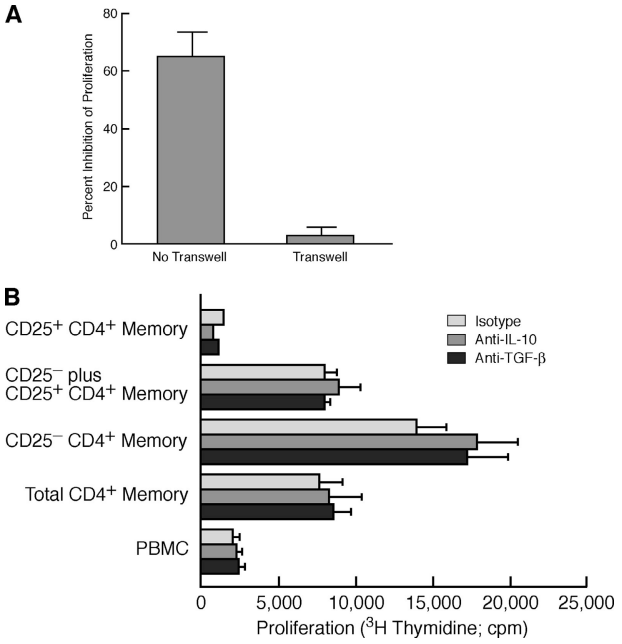

Figure 3. $CD25^+CD4^+$ memory T cell–mediated suppression of HIV p24-stimulated proliferation is cell contact dependent and IL-10 and TGF-β independent. (A) $CD25^-$ ± $CD25^+$ memory $CD4^+$ T cells from four p24 LPA suppressors were stimulated with HIV p24 (plus 10% $CD2^-$ PBMCs) either together (no transwell) or separated by a 0.4 μM transwell insert to prevent cell–cell contact. Data are presented as mean ± SD percent inhibition of HIV p24-stimulated proliferation achieved in the presence, as compared with the absence, of the $CD25^+$ subset. (B) A representative example of an HIV p24 LPA assay of PBMC subsets isolated from an HIV p24 LPA Suppressor conducted in the presence or absence of neutralizing anti–human IL-10 or TGF-β antibodies; data are presented as mean cpm ± SD of triplicate wells.

$CD25^+CD45RA^-CD4^+$ T Cells Suppress HIV p24 Proliferative Responses Via a Cell Contact–dependent and IL-10/TGF-β–independent Mechanism. Immunosuppression mediated by $CD25^+CD4^+$ T reg cells has been reported to be cell contact dependent, IL-10 independent and, depending on the experimental system, independent or partially dependent on TGF-β (50–53). To determine whether $CD25^+CD45RA^-CD4^+$ cell-mediated suppression of HIV p24-induced proliferation occurred via classic T reg cell mechanisms, HIV p24 LPA assays using cells from LPA suppressors were performed either in the context of transwells or in the presence of neutralizing anti–human IL-10 or anti–human TGF-β antibodies. Separation of $CD25^+$ and $CD25^-CD45RA^-CD4^+$ T cells with transwells com-

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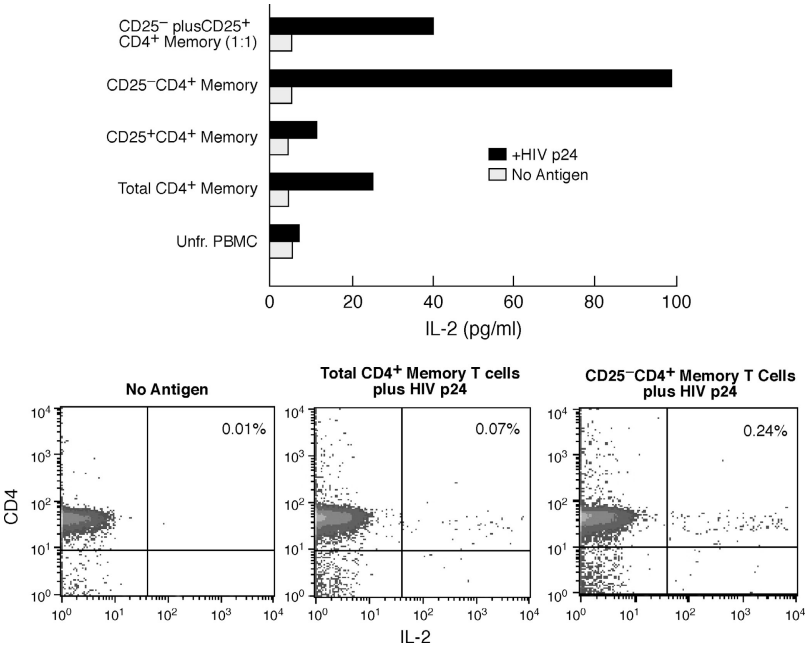

Figure 4. Suppression of HIV p24-stimulated proliferation by $CD25^+CD4^+$ memory T cells is associated with inhibition of IL-2 production. (A) IL-2 levels present in supernatants of an HIV p24 LPA using $CD4^+$ memory subsets isolated from an HIV p24 LPA suppressor. Data are representative of 19 independent experiments; the limit of detection for this assay was 15 pg/ml. (B) IL-2 ICC assay of total versus $CD25^+$ cell-depleted memory $CD4^+$ T cells (plus 10% $CD2^-$ PBMCs as APCs) isolated from an HIV p24 LPA suppressor stimulated with p24 protein for 13 h. Data are representative of eight independent experiments.

pletely eliminated the suppressive effects of the $CD25^+$ subset (Fig. 3 A), but neither anti–IL-10 nor anti–TGF-β neutralizing antibodies significantly abrogated suppression (Fig. 3 B).

$CD25^+$ PBMCs Suppress HIV Gag Protein or Peptide-induced Cytokine Production by $CD4^+$ and $CD8^+$ T Cells. The ability to inhibit IL-2 production by $CD4^+$ T cells is one of the first activities ascribed to $CD25^+CD4^+$ T reg cells (54). As expected, inhibition of HIV p24-stimulated memory $CD4^+$ T cell proliferation by $CD25^+CD45RA^-$ $CD4^+$ T cells from HIV p24 LPA suppressors was associated with reduction in antigen-induced IL-2 secretion; furthermore, the $CD25^+$ subset itself did not produce IL-2 in response to HIV p24 (Fig. 4 A). To rule out the possibility of IL-2 absorption by $CD25^+$ cells, ICC flow cytometry was performed (Fig. 4 B). Sufficient numbers of cellular subsets were obtained in eight p24 LPA suppressor and five p24 LPA nonsuppressor subjects to perform parallel LPA and IL-2 ICC assays using p24 protein. Considering all HIV^+ subjects, depletion of the $CD25^+CD4^+$ T cell subset resulted in a mean fold increase in the frequency of HIV antigen-induced IL-2^+ cells of 2.6 ± 2.0 (Table II). Although the frequency of total $CD4^+$ memory T cells producing IL-2 in response to HIV antigen was not significantly lower in HIV p24 LPA nonsuppressors as compared with suppressors (unpublished data), there was a significant difference between these two groups ($P = 0.04$) in regard to the enhancing effect of $CD25^+$ cellular subset depletion on this activity (Table II). A similar enhancement was observed in regard to HIV antigen-induced IFN-γ production by $CD4^+$ T cells after $CD25^+$ cellular subset depletion, however, statistical significance was not achieved (Table II). No significant IL-2 or IFN-γ production was observed in the $CD25^+$ memory $CD4^+$ T cell subset in any experiments (unpublished data).

$CD25^+$ $CD4^+$ T reg cells have also been found to potently inhibit antigen-specific $CD8^+$ T cell functions such as cytokine production, proliferation, and cytolysis (26, 31, 32, 55, 56). In the present paper, the effect of $CD25^+$ cell depletion on HIV Gag peptide-induced IFN-γ production was

Table II. *Effect of $CD25^+$ Cell Depletion on HIV Gag-induced Cytokine Production by $CD4^+$ and $CD8^+$ T Cells*

	Change in frequency of ICC^+ cells[a]		
	$CD8^+$ IFN-γ^+	$CD4^+$ IFN-γ^+	$CD4^+$ IL-2^+
All HIV^+ subjects			
Mean ± SD	2.1 ± 1.4	2.1 ± 1.3	2.6 ± 2.0
Frequency of subjects w/ increased ICC^+ cells	10/17	6/11	9/13
LPA suppressors			
Mean ± SD	2.5 ± 1.6	2.1 ± 1.5	3.5 ± 2.1
Frequency of subjects w/ increased ICC^+ cells	9/12	5/7	8/8
LPA nonsuppressors			
Mean ± SD	1.2 ± 0.4	1.9	1.4 ± 0.7
Frequency of subjects w/ increased ICC^+ cells	1/5	1/4	1/5
p-value[b]	0.020	NS	0.042

[a]Percent ICC^+ cells in $CD25^+$ cell-depleted population/percent ICC^+ cells in total population with Gag peptide or Gag protein stimulation.
[b]HIV p24 LPA suppressors versus nonsuppressors.

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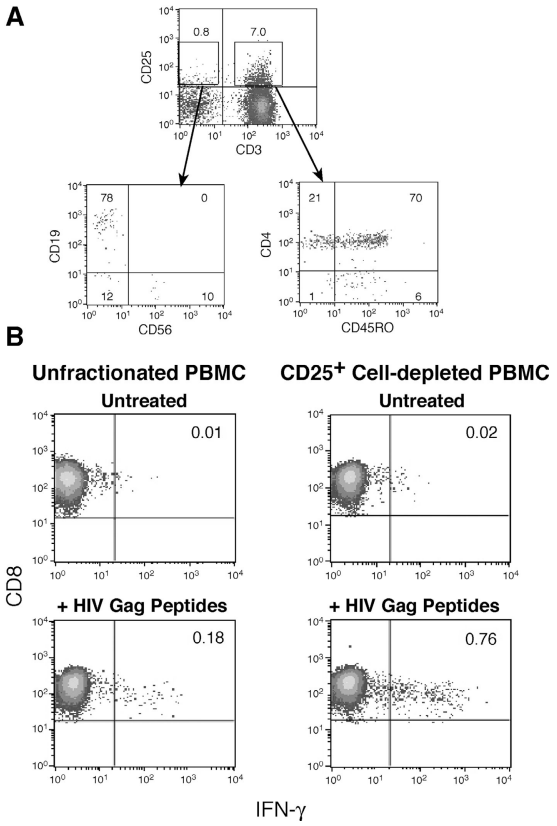

Figure 5. Depletion of CD25$^+$ cells enhances the frequency of CD8$^+$ T cells producing IFN-γ in response to HIV Gag peptides. (A) CD25$^+$ PBMCs are primarily CD4$^+$ memory T cells. PBMCs were stained for CD25 and CD3; CD3$^+$ and CD3$^-$CD25$^+$ populations (top) were analyzed (bottom) for the expression of other markers identifying the major PBMC cellular subsets. Approximately 90% of CD25$^+$ PBMCs are CD3$^+$ and the majority of these are CD4$^+$CD45RO$^+$ cells (bottom right); the remainder of the CD25$^+$ PBMCs are primarily CD19$^+$ B cells (bottom left). Data are representative of 25 independent experiments. (B) The frequency of CD8$^+$ T cells producing IFN-γ after 6 h of exposure to HIV Gag peptides in unfractionated versus CD25$^+$ cell-depleted PBMCs isolated from an HIV p24 LPA suppressor. Data are representative of nine independent experiments.

assessed in PBMCs and CD25$^+$ cellular subset-depleted PBMCs isolated from 17 HIV-infected individuals. As the CD25$^+$ PBMC subset is composed primarily of CD45RO$^+$ CD4$^+$ T cells (Fig. 5 A), depletion of PBMCs based solely on CD25 expression closely reflects the removal of CD25$^+$ memory CD4$^+$ T cells as seen when more purified populations are used. As reported previously (56), depletion of the CD25$^+$ cellular subset from PBMCs of HIV-infected individuals (analyzed as a group) resulted in an increase in the percentage of CD8$^+$ T cells producing IFN-γ in response to a pool of HIV Gag peptides (Table II). However, similar to data obtained in CD4$^+$ T cell functional assays, enhancement was observed almost exclusively with cells from HIV p24 LPA suppressors (Fig. 5 B), and there was a significant difference between HIV p24 LPA suppressors and nonsuppressors for this effect ($P = 0.02$; Table II).

CD25$^+$CD4$^+$ T Cells Suppress HIV-induced Proliferation of Perforin-expressing CD8$^+$ T Cells. One of the most distinctive qualitative features of CD8$^+$ T cells from HIV-infected individuals who effectively control viral replication in vivo (long-term nonprogressors [LTNPs]), as compared with those with progressive disease, is the potent proliferative capacity of the HIV-specific CD8$^+$ effector (perforin$^+$) T cell population after exposure to autologous HIV-infected cells (34). In the present paper, the effect of the CD25$^+$

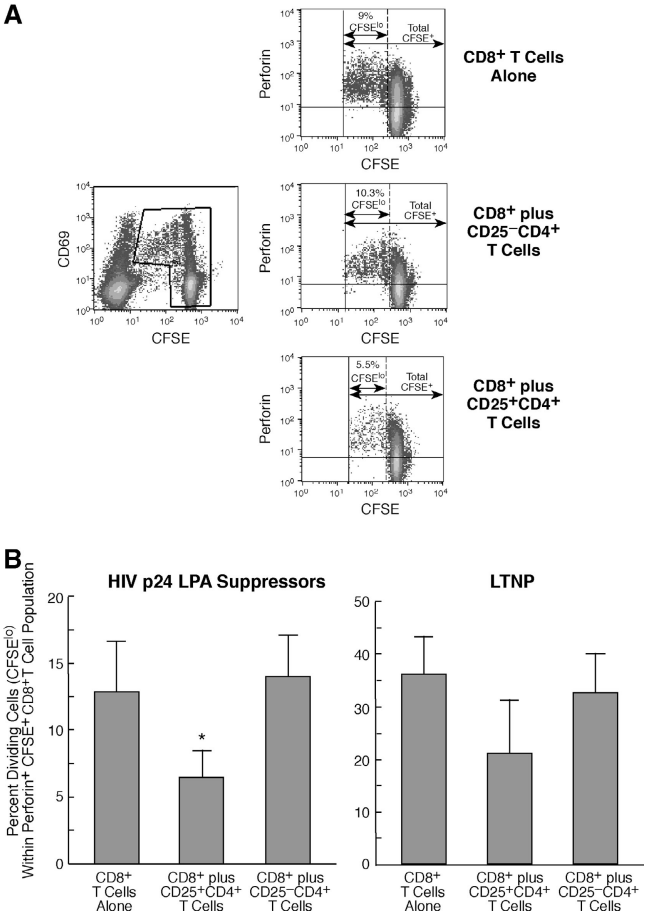

Figure 6. CD25$^+$, but not CD25$^-$, CD4$^+$ T cells suppress the proliferation of perforin-expressing effector CD8$^+$ T cells cultured in the presence of autologous HIV super-infected target cells. (A) HIV super-infected target cells were added to CFSE-labeled CD8$^+$ T cells alone, CFSE-labeled CD8$^+$ T cells plus CD25$^-$CD4$^+$ T cells, or CFSE-labeled CD8$^+$ T cells plus CD25$^+$CD4$^+$ T cells. Cultures were stained periodically for CD69 and CD3, and CFSE intensity was assessed over the course of 3–10 d after coculture (left). At the time by which at least four division cycles could be visualized (6–10 d), CD8 $^+$ cells from each culture condition were analyzed for perforin expression and for cellular division by CFSE (right). Values representing the percent CFSE^{lo} (dividing cells) perforin$^+$ cells within the total CFSE$^+$ perforin$^+$ cellular population are indicated. Data are representative of six independent experiments conducted using cells from HIV p24 LPA suppressors. (B) Mean ± SD percent dividing cells (CFSE^{lo}) within the perforin$^+$ CFSE$^+$ (CD8$^+$) T cell population in experiments with cells isolated from HIV p24 LPA suppressors with normal HIV disease progression (left, $n = 6$) or from long-term nonprogressors (LTNPs; right, $n = 2$). In experiments with cells from HIV p24 LPA suppressors, perforin$^+$ CD8$^+$ T cells cultured with CD25$^+$CD4$^+$ T cells proliferated to a significantly lesser degree in response to HIV-infected target cells than did those with target cells alone or those cultured with CD25$^-$CD4$^+$ T cells (*, $P < 0.03$).

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$CD4^+$ T cell subset on this activity was tested in cells isolated from six HIV p24 LPA suppressors and two LTNPs. $CD8^+$ T cells proliferating ($CFSE^{lo}$) in response to HIV super-infected target cells were largely confined to the $CD69^+$ population (Fig. 6 A, left) and, as reported previously (34), were exclusively associated with perforin expression (Fig. 6 A, right). The percentage of perforin$^+$ $CD8^+$ T cells that had undergone proliferation in response to HIV super-infected autologous target cells was significantly ($P = 0.03$) reduced in cultures containing $CD25^+$, as compared with $CD25^-CD4^+$ T cells or $CD8^+$ T cells alone (Fig. 6, A, right, and B, left). As expected, a greater proportion of $CD8^+$ T cells from HIV-infected LTNPs proliferated in response to HIV-infected target cells than did $CD8^+$ T cells from HIV p24 LPA suppressors with normal disease progression (Fig. 6 B, right); however, statistical analyses of the suppressive effect of $CD25^+CD4^+$ T cells in experiments with cells isolated from LTNPs was not possible due to the low number of subjects tested.

Discussion

The present paper presents evidence supporting the potential role of host-mediated immunosuppressive mechanisms, specifically $CD25^+CD4^+$ regulatory T cells, in the diminution of HIV-specific $CD4^+$ and $CD8^+$ T responses in asymptomatic HIV-infected individuals. HIV-infected individuals whose $CD25^+CD4^+$ T cells did not demonstrate HIV-specific T reg cell activity in vitro had significantly higher levels of plasma viremia and lower $CD4^+$ T cell counts or $CD4^+$:$CD8^+$ T cell ratios than did individuals with strong HIV-specific T reg cell activity. These data suggest that $CD25^+CD4^+$ T reg cell–mediated immunosuppression may play a role in the diminution of HIV-specific $CD4^+$ and $CD8^+$ T cell responses early in disease but that other factors associated with HIV infection obscure or reduce this activity in individuals with more progressed disease.

The primary purpose of the present paper was to determine whether HIV-specific $CD25^+$ T reg cells are present in infected individuals and, if so, to characterize their suppressive activity in regard to $CD4^+$ and $CD8^+$ HIV-specific T cell immune responses. Due to the low frequency of $CD25^+CD4^+$ T reg cells and the weak cytokine production by this population, quantifying antigen-specific subsets by classical MHC-peptide tetramers or ICC assays has not been feasible. As $CD25^+$ T reg cells must be activated via classic antigen-specific TCR triggering to exert suppressive activity (15, 18, 33, 57), the most effective method of assessing HIV-specific $CD25^+$ T reg cells is to determine the ability of $CD25^+CD4^+$ T cells to inhibit T cell responses after stimulation with HIV antigens. Our data demonstrate that HIV-specific T reg cells, capable of suppressing both $CD4^+$ and $CD8^+$ HIV-specific T cell function in vitro, are present in the majority of healthy HIV^+ individuals tested. However, the quality and/or quantity of HIV-specific $CD25^+CD4^+$ T reg cells appears to be diminished in certain individuals (HIV p24 LPA nonsuppressors; Tables I and II) before the loss of polyclonal T reg cell activity (Fig. 2 C) and the loss of normal ($CD25^-$) HIV-specific $CD4^+$ or $CD8^+$ T cell responses (Fig. 2 A). In HIV p24 LPA nonresponders, both polyclonal and HIV-specific T reg cell activity, as well as normal HIV-specific $CD4^+$ T cell function, is impaired.

Although variability in human $CD25^+$ T reg cell–mediated suppression of T cell responses can be seen depending on the in vitro systems used (33), HIV disease–associated dysfunction of both $CD25^-$ (normal) $CD4^+$ T cells and $CD25^+CD4^+$ T reg cells might be expected. In addition to the potential cytopathic effects of direct HIV infection of cells expressing CD4, particularly HIV-specific memory T cells (7), the interaction between HIV or envelope proteins and CD4 or chemokine receptors (HIV coreceptors) has been shown to significantly alter normal T cell function and sensitivity to apoptosis (9, 58–61). In this regard, loss of both HIV-specific and nonspecific $CD25^-CD4^+$ memory T cell and $CD25^+$ T reg cell responses were associated with significantly higher levels of plasma HIV viremia (Table I). Of interest, among individuals with intact HIV-specific $CD25^-CD4^+$ T cell function, those lacking HIV-specific $CD25^+$ T reg cell activity in vitro had significantly lower $CD4^+$:$CD8^+$ T cell ratios, a strong prognostic indicator in HIV disease progression (62), as compared with those who maintained this activity. These data suggest that, rather than playing an increasingly important role in HIV-associated immune dysfunction as disease progresses, HIV-specific $CD25^+$ T reg cell–mediated immunosuppression is most relevant in healthy HIV-infected individuals that are relatively immunocompetent. Furthermore, HIV-specific $CD25^+$ T reg cell function appears to be particularly sensitive to HIV disease–associated immune dysregulation and may be compromised even before normal ($CD25^-$) HIV-specific $CD4^+$ T cell responses.

Persistent antigens/pathogens, such as HIV, are believed to promote the expansion and activation of antigen-specific $CD25^+CD4^+$ T reg cells (16, 17, 63–66) and, under certain conditions, to induce normal $CD4^+$ T cells to gain $CD25^+$ T reg cell phenotype and function (15, 24). However, numerous factors associated with HIV infection, such as diminished capacity to produce IL-2 (1, 2, 67, 68), a cytokine critical for the development and the expansion of $CD25^+$ T reg cells (69); HIV-mediated disruption of $CD4^+$ T cell signaling (9, 58, 60); or alterations in Toll-like receptor signaling that can regulate T reg cell activity (29, 70–72), could limit the ability of HIV-specific T reg cells to become activated and proliferate in vivo. In addition, abnormal immune activation associated with HIV disease may alter the relative frequency of T reg cells, anergic cells, and normal activated T cells within the total $CD25^+CD4^+$ memory T cell population in the peripheral blood, as suggested by our cytokine data (Fig. 2 D). In this regard, the peripheral blood may not be the most appropriate compartment in which to accurately assess HIV-specific $CD25^+$ T reg cell activity as antigen-specific $CD25^+CD4^+$ T reg cells have been shown to accumulate or expand at tissue sites of

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antigen expression or pathogen replication, where they exert site-localized immunosuppression (44, 63, 65, 73). With this in mind, we are currently assessing whether, in individuals with more active viral replication, HIV-specific $CD25^+$ $CD4^+$ T reg cells reside largely in the lymphoid tissue, the primary site of HIV replication in vivo (74).

The data of the present paper suggest that HIV-specific $CD25^+$ T reg cell function may be compromised relatively early in HIV disease. Therefore, the question is raised whether or not this is reflective of a reduction in the numbers or function of the total $CD25^+$ T reg cell population. Several lines of evidence obtained in the present paper suggest that the total $CD25^+CD4^+$ T reg cell population is relatively intact in the majority of healthy HIV-infected individuals. First, the frequency of the $CD25^{+hi}$ subset within the $CD45RO^+CD4^+$ T cell population of HIV-infected individuals is stable across a broad percentage range of $CD4^+$ memory T cells (Fig. 1 C) and is, in fact, slightly elevated as compared with HIV-uninfected individuals (Fig. 1 B). Second, Fox P3 expression, a functionally relevant marker for immunosuppressive $CD25^+CD4^+$ T cells (38–42), was strongly expressed in $CD25^+CD4^+$ T cells from all HIV^+ subjects tested and did not appear significantly altered as compared with HIV uninfected donors (Fig. 1 A). Third, polyclonal $CD25^+$ T reg cell activity was not significantly different in the majority of healthy HIV^+ subjects tested as compared with HIV-uninfected individuals (Fig. 2 C). However, it should be noted that polyclonal $CD25^+$ T reg cell function was impaired in the subset of individuals (HIV LPA nonresponders) who, on average, had the lowest $CD4^+$ T cell counts and highest levels of plasma viremia (Table I). Additional studies that include a larger number of individuals with advanced disease will be necessary to confirm this observation. Finally, the levels of HIV provirus (DNA) detected in $CD25^+$ $CD4^+$ T cells were not significantly different than those detected in the $CD25^-$ subset (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20032069/DC1), indicating that there is not preferential infection of $CD25^+$ T reg cells. Together, these data support the hypothesis that there is a preferential alteration in the quantity, function, or tissue distribution of the HIV-specific, as compared with the total, $CD25^+$ T reg cell population with HIV disease progression.

Definitive data regarding the role of $CD25^+CD4^+$ T reg cells in pathogenic infections in humans is difficult to obtain at this point in time; however, the possibility that $CD25^+$ $CD4^+$ T reg cells, and perhaps other cellular populations, suppress HIV-specific $CD4^+$ and $CD8^+$ T cell responses in infected individuals in vivo is intriguing. $CD25^+CD4^+$ T reg cell–mediated suppression of antimicrobial immune responses clearly could have deleterious effects, but in certain infections, may also have potentially beneficial ramifications. In this regard, it has been proposed that the function of $CD25^+CD4^+$ T reg cells in the context of pathogenic infections is to control excessive, potentially dangerous, cellular immune responses and/or allow low level pathogen persistence as an effective strategy to prevent reinfection with more virulent strains (15, 16, 18, 23, 28, 75, 76). HIV antigen-stimulated $CD25^+CD4^+$ T reg cell–mediated suppression of HIV-specific and nonspecific T cell responses may be one mechanisms by which several detrimental processes, such as cellular activation-associated apoptosis/anergy, immune-mediated destruction, and, for $CD4^+$ T cells, susceptibility to productive HIV infection, are kept under control (10, 12, 77, 78). In this scenario, loss of HIV-specific $CD25^+$ T reg cell activity could prove to have an overall detrimental effect on the health of HIV-infected individuals. Additional studies will be needed determined whether HIV-specific $CD25^+$ T reg cell activity, if operative in vivo, hastens or delays HIV disease progression.

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