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*J Immunol* 2008; 180:5530-5536; doi: 10.4049/jimmunol.180.8.5530
http://www.jimmunol.org/content/180/8/5530

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Chemokine and Cytokine Mediated Loss of Regulatory T Cells in Lymph Nodes during Pathogenic Simian Immunodeficiency Virus Infection

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Regulatory T cells (Treg) play key roles in immune regulation through multiple modes of suppression. The effects of HIV-1 infection on Treg levels in lymphoid tissues remain incompletely understood. To explore this issue, we have measured the levels of forkhead box protein 3 (FOXP3)-positive cells and associated immunomodulatory genes in a pathogenic simian immunodeficiency virus/macaque model and found that a loss of Treg in lymph nodes occurred following simian immunodeficiency virus infection. Changes in expression of the ligands for CXCR3, CCR4, and CCR7 and the cytokines TGF-β and IL-2 were all linked to this loss of Treg, which in turn was linked with increased levels of cellular activation. Our findings identify three mechanisms that likely contribute to SIV-driven loss of T reg including reduced levels of cytokines associated with Treg differentiation and altered expression of agonist and antagonist chemokines. The loss of Treg and the associated cellular activation in lymphoid tissues is consistent with the events in HIV-1-infected individuals and suggest that components of the Treg differentiation and trafficking network could be targets for therapeutic intervention. The Journal of Immunology, 2008, 180: 5530–5536.

1 Abbreviations used in this paper: Treg, regulatory T cell; LN, lymph node; SIV, simian immunodeficiency virus; PI, postinfection; FOXP3, forkhead box protein P3.

Received for publication January 7, 2008. Accepted for publication February 8, 2008.

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1 This work was supported by Public Health Service Grants AI060422 (to T.A.R.), HL072682 (to D.E.K.), and U54 RR02241 (to S.C.W.).
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4 Abbreviations used in this paper: Treg, regulatory T cell; LN, lymph node; SIV, simian immunodeficiency virus; PI, postinfection; FOXP3, forkhead box protein P3.

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Materials and Methods

Animals and tissues

These studies were performed under the approval and guidance of the University of Pittsburgh Institutional Animal Care and Use Committee. They included 12 cynomolgus macaques (Macaca fascicularis) infected with SIV/DeltaB670 (18). Of these animals, six were sacrificed during acute infection (2 wk postinfection [PI]), five were sacrificed upon progression to AIDS, and five served as uninfected controls. Details regarding tissue processing and fixation have been previously described (8).
RNA isolation and real-time RT-PCR

Total RNAs from axillary LNs were isolated, treated with DNase (Ambion), and further purified with RNase free columns (Qiagen) as described previously (19). Four hundred nanograms of RNA from each specimen was reverse transcribed as previously described (19), with reverse transcriptase-negative controls included in parallel for each RNA sample.Primers and probes used for the real-time RT-PCR were either purchased from Applied Biosystems as ready-made sets or were designed using the Primer Express software package (probe sequences that were designed are available upon request). Real-time RT-PCR was used to measure relative mRNA expression levels by the comparative threshold cycle method of relative quantitation as described elsewhere (19, 20). The threshold cycle values for each gene were normalized to the endogenous control β2-microglobulin mRNA (× 1000).

Immunohistochemistry and immunocytochemistry

Immunohistochemical staining of LN tissue sections was performed as described previously (8, 19) using goat anti-CCR4 polyclonal antiserum (Abcam) or preimmune control serum (BD Biosciences). After washing in PBS twice, tissue sections were incubated with HRP-conjugated secondary antibody (Abcam) or preimmune control serum (BD Biosciences). After washing in PBS twice, tissue sections were incubated with HRP-conjugated secondary antibody (Abcam) or preimmune control serum (BD Biosciences). After washing in PBS twice, tissue sections were incubated with HRP-conjugated secondary antibody (Abcam) or preimmune control serum (BD Biosciences). After washing in PBS twice, tissue sections were incubated with HRP-conjugated secondary antibody (Abcam) or preimmune control serum (BD Biosciences).

Generation of macaque lymphoid tissue single-cell suspensions

Lymphoid tissues from cynomolgus macaques were minced in digestion medium (RPMI 1640) containing DNase I (20 mg/ml; Sigma-Aldrich) and collagenase A (1 mg/ml; Roche) for 60 min at 37°C, passed through a 100-μm cell strainer, and pelleted by centrifugation at 1200 rpm for 5 min. The pellet was resuspended in 1× RBC lysis solution (155 mM NH₄Cl, 10 mM NaHCO₃, and 0.1 mM EDTA, pH 7.4) for 5 min and centrifuged again at 1200 rpm for 5 min. The resulting cell pellet was resuspended in 1× PBS, counted on a hemacytometer, and aliquots of cell suspensions were frozen in freezing medium (90% FCS and 10% DMSO) and stored in liquid nitrogen. Gently thawed cells were exposed to Rfasγ (50 ng/ml; R&D Systems), or medium alone, and total RNAs were isolated at 24 or 48 h later. Real-time RT-PCR was performed to measure chemokine or chemokine receptor mRNA levels as described elsewhere (19).
Flow cytometric analysis

Anti-human mAbs were prescreened using multiple clonal and fluorochrometric combinations and selected on their ability to optimally discriminate antigenic expression by cynomolgus macaque LN cells. Live T cells were identified through successive gating strategies based on size and viability using the blue LiveDead apoptotic stain (Molecular Probes/Invitrogen Life Technologies). Immunophenotyping of extracellular Treg markers and chemokine receptors was performed on single-cell LN suspensions using the following Abs (purchased from BD Pharmingen, unless otherwise noted): CD3-Pacific Blue (SP34-2),

FIGURE 1. Changes in FOXP3 and cytokine levels in LN tissues during SIV infection of cynomolgus macaques. A, Real-time RT-PCR was used for measurement of FOXP3 mRNA in LN tissues from uninfected macaques or macaques in the early (acute (AC)) or late (AIDS) stages of disease (mean ± SEM). Immunofluorescence staining was performed to detect (C; original magnification, ×400) and quantitate (B) FOXP3⁺ cells in axillary LN tissue sections. D, Flow cytometry was used to detect and quantitate the percentage of total live cells that expressed FOXP3⁺ in the same LNs. E, F, H, and I, The relative levels of expression of the indicated mRNAs in macaque axillary LNs were examined by real-time RT-PCR (mean ± SEM). G, Linear regression of FOXP3 mRNA levels vs TGF-β mRNA levels is shown. J, Quantitative image analysis was performed on images from LN tissue sections immunostained simultaneously for CD3 and the proliferation marker Ki67 and the percentages of CD3⁺ cells that were also Ki67⁺ are shown. *, p < 0.05 and **, p < 0.01, compared with uninfected animals.

FIGURE 2. Changes in the relative expression levels of chemokine and chemokine receptor mRNAs during pathogenic SIV infection. A–I, Real-time RT-PCR was used to measure the levels of expression of the indicated mRNA in LNs from animals in the same disease states as described in the legend to Fig. 1. J and K, The effects of IFN-γ treatment of cynomolgus macaque primary LN cells on mRNAs encoding ligands for CXCR3 and CCR4 were measured using real-time RT-PCR. The mean (±SEM) values are shown from analyses performed on cells from three different animal. *, p < 0.05 and **, p < 0.01.
were decreased both early and late after SIV infection (Fig. 1). mRNA levels in LNs were determined at the RNA and protein levels. FOXP3 mRNA levels in LNs were decreased following SIV infection (Fig. 1, A–F). Overall proportions of Ki67– cells were also positive for the proliferation marker Ki67 in macaque LNs (16). The cytokine TGF-β1 is expressed by Treg cells that was also Ki67– and suppresses IFN-γ expression (22). Measurement of TGF-β1 and IL-2 mRNA levels in macaque LNs indicated that they also were decreased following SIV infection (Fig. 1, E and F), and they were highly correlated with FOXP3 levels (Fig. 1G; r = 0.91).

**Cell transfection and chemotaxis assays**

A human CCR4 cDNA in the pcDNA3.1 vector was obtained from the University of Missouri cDNA Resource Center. Cell transfections were performed as described previously (21). Briefly, the L1.2 murine pre-B cell line was electroporated with pcDNA3.1 (In Vitrogen Life Technologies) expressing human CCR4, and stably transfected cells were obtained after selection with 1 mg/ml G418 (Sigma-Aldrich). Chemotaxis was performed against CCL17 (10 μM) with or without coincubation with CXCCL11 or CXCCL8 (100 μM each). The 96-well ChemoTx chemotaxis system (5-μm pore; NeuroProbe) was used for these chemotaxis assays and chemokines were obtained from PeproTech. The lower wells were blocked with 31 μl of RPMI 1640/1% BSA for 30 min at room temperature, which was aspirated and replaced with 31 μl of the agonists, which were diluted in RPMI 1640/0.1% BSA. Then 2 × 10^5 CCR4–stably transfected L1.2 cells in 20 μl of RPMI 1640/0.1% BSA were placed above the membrane. After incubation for 5 h at 37°C in 5% CO2, the cells on top of the membrane were removed with a scraper and the migrated cells in the bottom wells were counted using a hemocytometer. Chemotaxis with macaque LN cells was performed in the same way, but with a 1.5-h incubation during migration.

**Statistical analyses**

All statistical analyses were performed using the Minitab software package (State College). Real-time RT-PCR data were analyzed using the two-sample t test to compare differences between disease states and Pearson’s correlation analyses were used to measure associations between relative mRNA expression levels. Paired t tests were used to examine the chemotaxis inhibition data. A p < 0.05 was considered significant.

**Results**

To determine whether pathogenic SIV infection affects Treg proportions in lymphoid tissues, we used a cynomolgus macaque (M. fascicularis) model and examined tissues at different stages after intrarectal infection with the pathogenic SIV/DeltaB670 isolate (18). Axillary LNs were examined from acutely infected (2 wk PI) and AIDS-developing (defined by decreasing CD4 counts, opportunistic infections, and wasting) animals, as well as uninfected controls (Table I). Because FOXP3 is considered to be a highly specific marker for Treg (3), changes in its expression were measured at the RNA and protein levels. FOXP3 mRNA levels in LNs were decreased both early and late after SIV infection (Fig. 1A). Immunoﬂuorescence staining for FOXP3+ cells in LN tissue sections also revealed a 60–70% decrease in the proportion of cells that were FOXP3+ after SIV infection (Fig. 1, B and C), which was highly correlated with the mRNA measurements (r = 0.721, p = 0.008). Additionally, flow cytometric analyses of LN single-cell suspensions conﬁrmed an ∼50% decrease in the FOXP3+ proportion of total live cells following SIV infection (Fig. 1D). These findings indicate there was a loss of FOXP3+ Treg in LNs after pathogenic SIV infection of cynomolgus macaques.

The cytokine TGF-β1 is expressed by Treg cells in concert with IL-2 in the differentiation and survival of inducible Treg (4) and suppresses IFN-γ expression (22). Measurement of TGF-β1 and IL-2 mRNA levels in macaque LNs indicated that they also were decreased following SIV infection (Fig. 1, E and F), and they were highly correlated with FOXP3 levels (Fig. 1G; r = 0.91).

Decreased expression of TGF-β1 and IL-2 could contribute to the reduction in FOXP3+ Treg levels in LNs during SIV infection by reducing differentiation of naive T cells into inducible Treg. Although there were decreases in FOXP3, TGF-β1, and IL-10 (data not shown) expression, another immunosuppressive element, IDO, was significantly increased after SIV infection, as was IFN-γ, an upstream inducer of IDO (23) (Fig. 1, H and I). This is consistent with reports that IDO is increased in tonsils of HIV-1–infected patients (24) and LNs of SIV-infected rhesus macaques (16).

Loss of Treg would be expected to lead to increased immune activation and this was observed. Immunoﬂuorescence detection and enumeration of CD3+ cells also positive for the proliferation marker Ki67 in macaque LNs revealed that the percentage of CD3+ cells that was also Ki67+ increased 3- to 5-fold after SIV infection (Fig. 1J). Overall proportions of Ki67+ cells were also signiﬁcantly increased after SIV infection (data not shown). These data indicated that T-cell activation levels increased concordantly with loss of Treg.

Human Treg highly express CCR4 (7) and CCR7 (5), and thus their ligands CCL17 and CCL21 could modulate homing of Treg to lymphoid tissues. Measurement of mRNA levels of these and other chemokines in macaque LNs revealed that mRNAs encoding ligands for CCR4 (CCL17 and CCL22) and CCR7...
CCL21 decreased (Fig. 2, A–C) following SIV infection, whereas mRNAs encoding IFN-γ-inducible CXCR3 ligands (CXCL9–11) increased (Fig. 2, D–F). The expression levels of the cognate chemokine receptors changed in parallel with their respective ligands (Fig. 2, G–I). The changes in CCR4 and CXCR3 ligand expression were likely driven by the increased IFN-γ levels (Fig. 1I), because ex vivo treatment of macaque LN cells with IFN-γ led to simultaneous induction of CXCR3 ligands and decrease of CCR4 ligands (Fig. 2, J and K). Correlation analyses revealed that in LNs, levels of CCL17 and CCL22 were positively correlated with those of their receptor CCR4 (r = 0.761 and r = 0.736, respectively), and levels of CCL21 were positively correlated with CCR7 (r = 0.646). In situ hybridization and immunostaining of tissue sections confirmed these changes in chemokine and chemokine receptor expression primarily in paracortical regions (Fig. 3).

More extensive correlation analyses revealed that FOXP3 mRNA levels were positively correlated with CCL22 (r = 0.627) and CCL21 (r = 0.682) levels (Fig. 4, A and B) and with their cognate receptors CCR4 (r = 0.493) and CCR7 (r = 0.745), respectively, but negatively correlated with CXCL9 (r = −0.615), CXCL10 (r = −0.552), and CXCL11 (r = −0.584) levels (Fig. 4, C–E) and local SIV viral RNA loads (r = −0.735). These findings indicate that loss of Treg in macaque LNs during SIV infection is associated with multiple changes in chemokine, cytokine and SIV levels.

Given that CCR4 and CCR7 are expressed by a large proportion of Treg (7) and have been shown to be important in Treg homing to LNs and other tissues (5, 6), we used flow cytometry to examine their expression on CD3+CD4+FOXP3+ cells in uninfected macaque LNs, the strategy of which is outlined in Fig. 5. Approximately 25% of CD3+CD4+FOXP3+ cells expressed CCR4 or CCR7 (Fig. 4F), whereas 35% expressed CXCR3 and 10% expressed CCR6. To determine whether CCL17, CCL21, and CXCL11 recruit FOXP3+ cells via CCR4, CCR7, and CXCR3, respectively, we performed chemotaxis with uninfected macaque LN cells. Immunostaining of FOXP3 in the input and migrated cells revealed that CCL17 and CCL21 recruited a population of cells that had a greater proportion of FOXP3+ cells than that recruited by CXCL11 (Fig. 4G), despite clear expression of CXCR3 (Fig. 4F). The lack of recruitment by CXCL11 could represent the net effect of positive and negative signaling through different receptors or possibly uncoupling of CXCR3 at the intracellular interface (25). These chemotactic data support the interpretation that CCL17 and CCL21 contribute to homing of FOXP3+ Treg into LNs and the reduced expression of these chemokines (Fig. 2) could contribute to loss or redistribution of FOXP3+ cells after SIV infection.

Given that ligands for CXCR3 antagonize the type 2 chemokine receptor CCR3 (26) and are up-regulated during SIV infection (Fig. 2), we examined whether a CXCR3 ligand would antagonize the type 2 and Treg chemokine receptor CCR4. The migration of cells stably expressing CCR4 in response to CCL17 (10 nM) or CCL22 (10 nM) was examined in the presence or absence of 1 μM CXCL11 or CXCL8 antagonist. The percentage of cells migrating was calculated relative to migration toward CCL17 or CCL22 alone. The data represent the mean ± SEM of four independent experiments.

FIGURE 4. Association between CXCR3 ligand and CCR4 ligand expression and function and FOXP3 levels in macaque LNs. Linear (A and B) and nonlinear (C–E) regression analysis plots of FOXP3 mRNA vs the indicated chemokine mRNA levels are shown. F. Flow cytometry was used to detect chemokine receptor expression on live CD3+CD4+FOXP3+ axillary LN cells from uninfected (n = 5) cynomolgus macaques. Axillary LN cells from uninfected macaques (n = 4 macaques) were subjected to chemotaxis to the indicated chemokines and immunocytochemical staining for FOXP3 was performed on the migrated cells. The chemotactic indices were calculated as the fraction of FOXP3+ cells in the migrated population relative to the input cells. *, p < 0.05 and **, p < 0.01. H. The migration of cells stably expressing CCR4 in response to CCL17 (10 nM) or CCL22 (10 nM) was examined in the presence or absence of 1 μM CXCL11 or CXCL8 antagonist. The percentage of cells migrating was calculated relative to migration toward CCL17 or CCL22 alone. The data represent the mean ± SEM of four independent experiments. ***, p < 0.01, compared with agonist alone. I. The percentages of live CD3+CD4+FOXP3+ cells expressing either CXCR3 or CCR4, as determined by flow cytometry, are shown as a function of disease state.
Discussion

In the present study, we found that FOXP3\(^+\) cells are lost early during pathogenic SIV infection and with loss evident also during AIDS, which is consistent with recent findings in rhesus macaques (15). This loss of T\(_{\text{reg}}\) was correlated with increased levels of local cellular activation and could be a key mechanism in the cumulative loss of immune function culminating in AIDS. In addition, we have identified multiple chemokine- and cytokine-mediated mechanisms that can account for the loss in T\(_{\text{reg}}\) in SIV-infected macaques.

In contrast to our findings, other recent studies have reported that rhesus macaques acutely and chronically infected with SIVmac251 (16, 17) and humans infected with HIV-1 (17, 24) had increased FOXP3\(^+\) T\(_{\text{reg}}\) levels and TGF-\(\beta\) expression in lymphoid tissues. These differences could be due to the different host systems studied, different viral strains, and different SIV inoculation routes, although our preliminary examination of rhesus macaque LNs following SIV/DeltaB670 infection also revealed a loss of T\(_{\text{reg}}\) (data not shown). Another explanation might be due to variation in the timing of the contraction and/or expansion of T\(_{\text{reg}}\) frequencies, as shown by Pereira et al. (15) in longitudinal studies of SIVmac239-infected rhesus macaques. In this study, the proportion of T\(_{\text{reg}}\) among CD4\(^+\) T cells decreased in LN and intestinal tissues of SIVmac239-infected rhesus macaques, but not in naturally infected sootey mangabey monkeys. Perhaps there are multiple avenues leading to the development of AIDS and, during HIV-1 or SIV infection, loss of T\(_{\text{reg}}\) or expansion of T\(_{\text{reg}}\) could represent separate paths that both ultimately contribute to the development of immunodeficiency when threshold levels are crossed. Loss of T\(_{\text{reg}}\) would lead to T cell hyperactivation and enhance HIV-1 and SIV replication and activation-associated cell death, whereas expansion of T\(_{\text{reg}}\) could lead to suppression of HIV-1-specific and other immune responses.

In this study, we have identified multiple, previously unrecognized mechanisms for the loss of T\(_{\text{reg}}\) during pathogenic SIV infection of cynomolgus macaques. Given that we found FOXP3 mRNA and protein levels decreased in LNs during SIV infection and FOXP3 mRNA levels were negatively correlated with local SIV viral loads, SIV infection has a role in the decline in T\(_{\text{reg}}\) in macaque LNs, perhaps through infection of these cells (27). In addition, our findings support three additional mechanisms contributing to this SIV-driven loss of T\(_{\text{reg}}\), which are outlined schematically in Fig. 6. First, reduced levels of TGF-\(\beta\) and IL-2 will lead to fewer naive T cells being differentiated into inducible T\(_{\text{reg}}\) (4). Second, reduced levels of CCR4 and CCR7 ligands, associated with increased IFN-\(\gamma\) levels, will lead to decreased recruitment of CCR4\(^+\) and/or CCR7\(^+\) T\(_{\text{reg}}\) into lymphoid tissues. Finally, potent up-regulation of CXCR3 ligand expression will provide abundant natural CCR4 antagonists that will also reduce recruitment of CCR4\(^+\) cells, including T\(_{\text{reg}}\). The intriguing sensitivity of FOXP3 levels to CXCR3 ligand levels as observed through regression analyses, supports their central role in loss of T\(_{\text{reg}}\) in macaque LNs. The increase of IFN-\(\gamma\) as an upstream regulator of CXCR3 ligand expression and the decrease in TGF-\(\beta\) as an upstream regulator of IFN-\(\gamma\) expression (22) reveal a complex set of interrelationships that control multiple positive and negative feedback systems.

Decline in T\(_{\text{reg}}\) numbers in LNs during SIV infection would reduce suppression of conventional T cells, contribute to their activation, and thereby provide enhanced opportunities for HIV-1 and SIV replication by modulating the composition and availability of cellular substrates. One hallmark of HIV-1 infection is immune activation (28), and loss of T\(_{\text{reg}}\) and TGF-\(\beta\) and IL-10

FIGURE 5. Flow cytometric gating strategy used to measure chemokine receptor expression on macaque FOXP3\(^+\)CD4\(^+\) T lymphocytes. Shown is the successive gating strategy used to ultimately visualize chemokine receptor levels on cynomolgus macaque LN T\(_{\text{reg}}\). Details are described in Materials and Methods.

FIGURE 6. Model for chemokine- and cytokine-mediated loss of T\(_{\text{reg}}\) in macaque LNs. Shown schematically are the relationships between IFN-\(\gamma\)-driven changes in CXCR3 and CCR4 ligand expression and IL-2/TGF-\(\beta\)-driven differentiation of T\(_{\text{reg}}\) on overall T\(_{\text{reg}}\) levels in LNs following SIV infection as detailed in Discussion. The arrows pointing up or down next to each component indicate the positive or negative effects of upstream players on the levels of the component.
expression, offer mechanisms by which this might occur. Non-
pathogenic SIV infections in African green monkeys (13, 29) and sooty mangabeys have been shown not to increase cellular activ-
ity despite substantial viral replication. Consistent with our
findings linking loss of Treg with increased immune activation
during pathogenic SIV infection, naturally infected nonhuman pri-
mates do not show a loss of Treg (15). Increased activation in
pathogenic SIV infections will provide cellular substrates and an
environment that will sustain viral replication and lead to greater
loss of immune function through direct and indirect killing as well
as activation-induced cell death. Our data indicate that T cell ac-
tivation levels increased concordantly with loss of Treg consistent
with the role of Treg in balancing overall local levels of cellular
activation. In addition, another disease outcome likely linked to
loss of Treg is the reported increase in susceptibility to autoimmune
activation. In addition, another disease outcome likely linked to
the role of Treg in balancing overall local levels of cellular
activation.

Acknowledgments
We thank Dr. Velpandi Ayyavoo for critically reading this manuscript, Dr.
Ashley Haase for insightful discussion and sharing advice on FOXP3 stain-
ing of tissue sections, and Melanie Pfeifer for assistance with generation of
macaque LN single-cell suspensions.

Disclosures
The authors have no financial conflict of interest.

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