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Tryptophan Catabolism by Indoleamine 2,3-Dioxygenase 1 Alters the Balance of TH17 to Regulatory T Cells in HIV Disease

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The pathogenesis of human and simian immunodeficiency viruses is characterized by CD4+ T cell depletion and chronic T cell activation, leading ultimately to AIDS. CD4+ T helper (Th) cells provide protective immunity and immune regulation through different immune cell functional subsets, including Th1, Th2, T regulatory (Treg), and interleukin-17 (IL-17)-secreting Th17 cells. Because IL-17 can enhance host defenses against microbial agents, thus maintaining the integrity of the mucosal barrier, loss of Th17 cells may foster microbial translocation and sustained inflammation. Here, we study HIV-seropositive subjects and find that progressive disease is associated with the loss of Th17 cells and a reciprocal increase in the fraction of the immunosuppressive Treg cells both in peripheral blood and in rectosigmoid biopsies. The loss of Th17/Treg balance is associated with induction of indoleamine 2,3-dioxygenase 1 (IDO1) by myeloid antigen-presenting dendritic cells and with increased plasma concentration of microbial products. In vitro, the loss of Th17/Treg balance is mediated directly by the proximal tryptophan catabolite from IDO metabolism, 3-hydroxyanthranilic acid. We postulate that induction of IDO may represent a critical initiating event that results in inversion of the Th17/Treg balance and in the consequent maintenance of a chronic inflammatory state in progressive HIV disease.

INTRODUCTION

Accumulating evidence suggests that the pathology associated with HIV infection may result from persistent and uncontrolled inflammation (1). This hypothesis is supported by the observations that chronic, untreated HIV infection is associated with systemic immune activation, including increases in nonspecific T cell activation and proliferation (2), elevated inflammatory cytokines and chemokines (3), and increased concentration of catabolic by-products such as neopterin and kynurenine in the circulation (4). The central role of T cell activation and inflammation in HIV disease pathogenesis is supported by the consistent observation that activated (CD8+CD38+HLA-DR+) T cells predicts disease progression independent of viral load (5). Other markers of inflammation [including interleukin-6 (IL-6) and high-sensitivity reactive protein] are also independent predictors of disease progression in both treated and untreated HIV infection (6).

Indoleamine 2,3-dioxygenase 1 (IDO1; previously referred as IDO or INDO) is the main inducible and rate-limiting enzyme for the catabolism of the amino acid tryptophan through the kynurenine pathway (7) (although there may be a separate and perhaps overlapping role for the newly discovered enzyme, IDO2) (8). Predominantly found in macrophages and dendritic cells (DCs), IDO1 is up-regulated by interferons (IFNs) and by agonists of Toll-like receptors (TLRs) (7). Increased catabolism of tryptophan by IDO1 suppresses T cell responses in a variety of diseases or states, including autoimmune disorders (9), allograft rejection (10), viral infections (11), cancer (12), and pregnancy (13). Such suppression is thought to occur either because IDO1 depletes the essential amino acid tryptophan or because it produces tryptophan catabolites that are toxic to T cells (or both) (14, 15). In either case, the ability of IDO1 to suppress immune responses has raised the possibility that it may contribute to the immunodeficiency seen in individuals with progressive HIV disease (4).

Although CD4+ T cell depletion is pathognomonic for HIV disease progression, the specific subsets of CD4+ T helper (Th) cells that are affected remain elusive. Four main lineages of CD4+ Th cells have been characterized, including IFN-γ–secreting Th1 cells, IL-4–secreting Th2 cells, FoxP3-expressing T regulatory (Treg) cells, and IL-17–secreting Th17 cells. These lineages derive from naïve CD4+ T cells under polarizing and mutually exclusive conditions in vitro, and presumably in vivo (16), and provide protective immunity against intracellular (Th1) or extracellular pathogens (Th1,2) as well as immune regulation and tolerance (Treg) or protection against bacterial infection at mucosal sites (Th1,17) (17). We recently reported that simian immunodeficiency virus (SIV) infection leading to AIDS in macaques was associated with a change in the balance of Treg and Th17 cells, whereas this balance...
was maintained in natural SIV infections that do not lead to AIDS in African green monkeys (18). TH17 cells are also lost in HIV infection, which has been suggested to account for a breakdown in mucosal immunity and an increase in microbial translocation across the gastrointestinal mucosa (19). Despite the selective depletion of TH17 cells during pathogenic SIV and HIV infection, there is no evidence that these cells are preferentially infected, and instead, bystander cell death may account for their loss (19). Studies in mice have suggested that IDO1 regulates the balance of TH17 to Treg cells, but the mechanism of such regulation remains unknown (20, 21). We hypothesized that elevated IDO1 activity may alter the balance of TH17 to Treg cells after infection by HIV, thereby establishing a positive feedback loop that increases systemic immune activation and accelerates disease progression. Here, we extend previous studies to show that enhanced IDO1 activity is associated with HIV disease progression and demonstrate that such activity results in an imbalance of TH17 and Treg cells in the peripheral blood and in rectosigmoid tissue that is both linked to HIV disease progression and mediated by the tryptophan catabolite 3-hydroxyanthranilic acid (3-HAA).

RESULTS

IDO1 activity is elevated in progressive HIV infection

IDO1 activity measured in plasma is elevated in HIV-infected subjects compared to healthy controls, especially in those who have progressed to AIDS (4, 11, 22). We confirmed and expanded these findings in well-characterized cohort of HIV-infected subjects in varying stages of disease progression and treatment. Untreated HIV-infected subjects were stratified into three groups on the basis of viral load and CD4+ T cell count at the time of the study: (i) controllers, defined as those with a steady-state viral load of <2000 HIV RNA copies per milliliter and a CD4+ T cell count of >500 cells/µl (n = 20); (ii) noncontrollers/high CD4, with viral loads of >10,000 copies/ml and CD4+ T cell counts of >350 cells/µl (n = 33); and (iii) noncontrollers/low CD4, with viral loads of >10,000 copies/ml and CD4+ T cell counts of <350 cells/µl (n = 33) (for further details, see Materials and Methods, Patient populations, Study A). Circulating concentrations of tryptophan were measured on plasma samples from each subject and found to be comparable in all groups (Fig. 1A, left). By contrast, kynurenine concentrations were significantly elevated in noncontrollers with low CD4+ T cell counts as was the ratio of kynurenine to tryptophan (K/T ratio). When all 60 subjects were considered as a single group, kynurenine concentrations were positively correlated with viral load and with the amount of CD4+ and CD8+ T cell activation, as measured by the percentage of T cells expressing HLA-DR and CD38, and negatively associated with CD4+ T cell counts (Fig. 1B).

Tryptophan and kynurenine concentrations were measured on a longitudinal basis within a subset of noncontrollers with high (n = 13) or low (n = 6) CD4+ T cell counts [median interval between mea-

![Fig. 1. Tryptophan catabolism is elevated in HIV disease progression. Plasma samples were obtained from chronically HIV-infected subjects from the SCOPE Cohort, who were either viral controllers (n = 20) or noncontrollers with high or low CD4+ T cell counts (respectively, higher or lower than 350 cells/µl, n = 33 in each group) (for further details, see Materials and Methods, Patient populations, Study A). (A) Plasma concentration of circulating tryptophan (left), kynurenine (middle), and K/T ratio (right). n.s., not significant. (B) Correlation of plasma kynurenine concentration with measures of HIV disease progression, including viral load, CD4+ T cell counts, as well as CD4+ and CD8+ T cell activation status, as measured by the fraction of cells positive for HLA-DR and CD38. The colors in these panels correspond to those in (A). (C) K/T ratio over time in noncontrollers with high or low CD4+ T cell counts (median, 7.8 months; IQR, 4.7 to 11.9 months). (D) Change of CD4+ T cell counts over time in viral noncontrollers with high CD4+ T cell counts (>500 cells/µl) and either high or low K/T ratios (respectively, higher or lower than 63.2, the median K/T ratio from all noncontrollers). The Mann-Whitney U test was used for group comparisons. The Spearman rank correlation test was used for correlations, with R_s being the Spearman correlation coefficient.
measurements, 7.8 months; interquartile range (IQR), 4.7 to 11.9; median K/T ratio = 63), and the K/T ratios were constant over time (Fig. 1C). Among noncontrollers with high CD4+ T cell counts (>500 CD4+ T cells/µl), those with high IDO1 activity as measured by a K/T ratio higher than the median value for noncontrollers (K/T > 63) at the first time point exhibited a greater subsequent decline in CD4+ T cell counts than those with low IDO1 activity at baseline (Fig. 1D). Consistent with a previous report (22), this observation indicates that high IDO1 activity is predictive of HIV disease progression.

**IDO1 is expressed in the peripheral lymph nodes and gastrointestinal lymphoid tissues of HIV-infected progressors**

Despite evidence that IDO1 correlates with HIV disease progression, incomplete knowledge exists about which cells produce the enzyme during the course of lentiviral infection. SIV-infected macaques exhibit a rapid increase of IDO1-positive CD4+ T cells in the lymph nodes (23). However, after HIV infection of human peripheral blood mononuclear cells (PBMCs) in vitro, IDO1 expression was up-regulated mostly in plasmacytoid DCs (pDCs) (24). In nonhuman primates, IDO1 expression is up-regulated during acute infection in blood, lymph nodes, and colon but only sustained at high concentrations during pathogenic infection (18, 25). We examined biopsies of lymph node and of rectosigmoid biopsy tissue from HIV+ and HIV− donors to determine the tissue localization and cell types responsible for IDO1 production during chronic HIV infection. As determined by quantitative polymerase chain reaction (PCR), HIV-infected noncontrollers (with both high and low CD4+ T cell counts) had significantly elevated IDO1 messenger RNA (mRNA) in rectosigmoid tissue compared to HIV-infected controllers (Fig. 2A). Immunohistochemical analysis of lymph node and rectosigmoid tissue from HIV-infected noncontrollers (with both high and low CD4) showed prominent IDO1 staining within cells with a dendritic morphology in both tissues as compared to healthy HIV-seronegative controls or HIV-infected controllers (Fig. 2B, top panels). Immunofluorescent analysis revealed that many of the IDO1-positive cells expressed the myeloid DC marker DEC205 but that they did not express markers for T cells (CD3), the monocytic lineage (CD68), or pDCs (BDCA2) (Fig. 2B, bottom panels, and fig. S1). Thus, the myeloid antigen-presenting DC (mDC) population appears to contain a substantial fraction of the resident antigen-presenting cells in which IDO1 activity is up-regulated both in peripheral lymph nodes and in rectosigmoid biopsy tissue.

**IDO1 activity in mDCs is activated by IFN-γ and enhanced by LPS**

Both mDCs and pDCs are important in regulating immune responses; each population, however, exhibits distinct requirements for activation. Thus, pDCs show up-regulation of IDO1 after direct engagement of CD4 with gp120 or after stimulation with IFNα or TLR7/9 agonists (24, 26). On the other hand, mDCs are more responsive to bacterial components, including lipopolysaccharide (LPS), which signals through...
A decreased TH17/Treg ratio is linked to HIV disease progression

Depletion of gut mucosal CD4+ T cells during pathogenic lentiviral infection is thought to lead to microbial translocation across the mucosal barrier and increases in the circulating concentrations of LPS (32). However, T cell loss in the gastrointestinal mucosa also occurs in nonpathogenic SIV infection without microbial translocation (33). We and others have shown that TH17 cells are selectively depleted in pathogenic SIV and HIV infection but maintained in nonpathogenic infections (18, 19). TH17 cells are important in controlling bacterial growth in mucosal tissues, and loss of TH17 cells in pathogenic SIV infection correlates with increased microbial translocation to peritoneal draining lymph nodes and the peripheral blood and lymphatic systems (34).

Despite strong evidence that a loss of TH17 cells is characteristic of pathogenic SIV and HIV infection, the underlying mechanisms accounting for the selective depletion of TH17 cells remain unclear. A clue to a potential mechanism for TH17 cell depletion was suggested by the observation that their loss in pathogenic SIV infection was accompanied by a concomitant rise in the frequency of induced Treg cells (18). Thus, although they perform substantially different roles during the course of an immune response, TH17 and Treg cells have reciprocal differentiation pathways from a common T cell progenitor (35–38). Given this relation, we analyzed the frequency of TH17 (IL-17A–secreting) cells and Treg (FoxP3+) cells among CD4+ T cells in PBMCs and rectosigmoid biopsy tissue from HIV-negative and HIV-infected subjects, including, as in the aforementioned Study A, viral controllers and noncontrollers, and those whose virus was suppressed as a result of long-term antiretroviral therapy (see Materials and Methods, Patient populations, Studies B and C, for further details on these cohorts). More than 95% of the CD4+ T cells from rectosigmoid tissue displayed a memory phenotype (CD45RA-CD27+), whereas only a fraction of peripheral blood CD4+ T cells had such a phenotype (fig. S3, A and B). Almost all TH17 cells also had a memory phenotype (CD45RA-CD27+) (fig. S3C) and produced IL-17A as well as significant amounts of IL-2, IL-22, and TNF-γ. The TH17 cells were substantially more frequent in CD4+ T cells from rectosigmoid biopsy tissue than in peripheral blood (about eight times higher in the representative example shown in Fig. 3A). Because the proportion of memory CD4+ T cells in the peripheral blood was different from that in the rectosigmoid biopsies, we measured the frequency of TH17 cells within the memory CD4+ T cell fraction in PBMCs. In peripheral blood, noncontrollers with more advanced disease (with CD4 of <350 cells/μl) showed a decreased proportion of memory TH17 cells than did HIV-negative subjects (fig. 3B, top panel) and an increased proportion of memory FoxP3+ Treg cells compared to controllers, HIV-positive subjects with suppressed viral loads, and HIV-negative subjects (fig. 3B, bottom panel). When the frequencies of these subpopulations were compared to one another, the TH17/Treg ratio was about 5 and 10 times lower in noncontrollers with advanced CD4 depletion when compared to noncontrollers with preserved CD4 counts and HIV-negative individuals, respectively (fig. 3C). The TH17/Treg ratio was inversely related to CD8+ T cell activation (as measured by CD38 and Ki67 expression in CD8+ T cells) (fig. 3D). Notably, the TH17/Treg ratio was also decreased among the antiretroviral-treated subset with undetectable viral loads, a result that was largely driven by five subjects whose TH17/Treg ratio did not return after treatment to a point comparable to that of HIV-negative individuals or viral controllers.

To determine the relation between TH17 cells and immune activation at early stages of HIV infection (during the first year), we studied PBMCs from 27 subjects 3 months (acute) and 12 months (chronic) after the estimated date of HIV infection (see Materials and Methods, Patient populations, Study C, and table S1). We determined the difference in T cell immune activation between these two time points (ΔKi67+ in CD8+ T cells) and calculated independent predictors of decreased or increased T cell immune activation with a multivariate mixed-effects analysis. Independent covariates included memory TH17 cells, CD4+ T cell counts, and log10-transformed plasma viral load during acute infection. We found that a higher frequency of memory TH17 cells during acute infection (3 months) was a predictor of decreased T cell immune activation over time (−4.56 lower CD8+Ki67% per each 1 percentage point higher of CD4+TH17%, P = 0.0098), independent of CD4+ T cell counts and viral load.

This relation was further analyzed in three groups defined by low or high viral load and immune activation (CD38 expression in CD8+ T cells) at 12 months (a time when viral and immunological set points are established). These groups were defined in the following manner: Group 1 with low viral load and low immune activation, Group 2 with high viral load and high immune activation, and Group 3 with high viral load but low immune activation (Fig. 3E, left). Previous results
The subjects were divided into three groups (of nine subjects each) on the basis of plasma viral load and T cell immune activation at the second time point: Group 1 with low viral load and low immune activation (purple circles), Group 2 with high viral load and high immune activation (red diamonds), and Group 3 with high viral load and low immune activation (blue squares) (for further details, see Materials and Methods, Patient populations, Study C). Memory (CD45RA+CD27+) IL-17A–expressing T<sub>h</sub>17 cells and FoxP3<sup>+</sup> T<sub>reg</sub> cells were enumerated as described in Materials and Methods. (A) Example of intracellular FACS detection of IL-17A– and IL-2–expressing CD4<sup>+</sup> T cells after PMA-ionomycin stimulation on total PBMCs (top panel) and on cells from rectosigmoid biopsies (bottom panel) from the same HIV controller. (B) Frequencies of memory (mem) T<sub>h</sub>17 cells (top panel) and T<sub>reg</sub> cells (bottom panel) in patient groups. (C) T<sub>h</sub>17/T<sub>reg</sub> ratio in patient groups. (D) Correlation between T<sub>h</sub>17/T<sub>reg</sub> ratio (expressed as log<sub>2</sub> memory T<sub>h</sub>17/T<sub>reg</sub> cells × 10) with systemic T cell immune activation, as measured by CD38<sup>+</sup> (left) and Ki67 expression (right) on peripheral blood CD8<sup>+</sup> T cells. (E) Viral load and immune activation set points at 12 months after primary HIV infection (left) delineate three groups with either low viral load and low immune activation (Group 1, purple circles), high viral load and high immune activation (Group 2, red diamonds), or high viral load but low immune activation (Group 3, blue squares). Correlation between the frequency of memory T<sub>h</sub>17 cells during acute infection (3 months after infection) and the change of T cell immune activation between acute to chronic infection, as measured (difference of Ki67<sup>+</sup> in CD8<sup>+</sup> T cells from 3 to 12 months after infection) (right). Mann-Whitney U test was used for group comparisons (*P < 0.05, **P < 0.005, ***P < 0.0005). The Spearman rank correlation test was used for the correlations, with R<sub>s</sub> being the Spearman correlation coefficient. The correlation (red curve) with P values and R<sub>s</sub> in (E) is indicated for Group 2.

Given the critical role of T<sub>h</sub>17 cells in maintaining host barriers and immune surveillance at mucosal sites, the frequency of T<sub>h</sub>17 and T<sub>reg</sub> cells was also examined in rectosigmoid biopsy tissue from 9 controllers and 11 noncontrollers (see Materials and Methods, Patient populations, Study D). Similar to the situation in peripheral blood, the frequency of T<sub>h</sub>17 cells (expressing IL-17A as well IL-2, IL-22, and TNFα) was lower, and the frequency of T<sub>reg</sub> cells (expressing FoxP3 and Ki67) was higher, in noncontrollers than in the controllers (Fig. 4, A and B), resulting in a lower T<sub>h</sub>17/T<sub>reg</sub> ratio in the former group of subjects with more advanced disease (Fig. 4C). This ratio was inversely related to CD8<sup>+</sup> T cell activation (Fig. 4D) and to circulating 16S ribosomal DNA (rDNA) (Fig. 4E). Thus, as
in the case of nonhuman primate lentiviral infection (18, 34), the degree of microbial translocation and T cell activation in progressive HIV disease is tightly associated with skewed maturation along the TH17/Treg axis.

**Tryptophan catabolites directly influence TH17/Treg cell ratios**

Tryptophan catabolism through the IDO1 pathway can regulate the balance between TH17 cells and other T cell subsets, including Treg cells (20, 21). In HIV infection, pDCs influence Treg frequencies in an IDO1-dependent fashion, but the mechanism underlying the generation of Treg cells is unclear (39). Treatment of mice with l-kynurenine influenced the ratio of TH17 and Treg cells in vivo (21). This effect was inhibited by a kynurenine-3-monooxygenase inhibitor, suggesting that tryptophan catabolites downstream of kynurenine are likely to be involved in controlling the TH17/Treg cell balance (21).

To address the role of different tryptophan catabolites in human T cell differentiation, we performed assay essays in vitro on human T cells from normal and HIV-infected patients in the presence of varying concentrations of the tryptophan catabolites: 3-hydroxykynurenine acid (3-HKA), 3-HAA, and picolinic acid (PA) (Fig. 5A). Notably, tryptophan catabolites such as quinolinic acid have been linked to the neurological defects associated with HIV infection but not to the regulation of immune function (40). By contrast, 3-HKA and 3-HAA have both been shown to influence T cell activation (10, 15, 21, 41). We noted a significant decrease in IL-17A–producing cells in the presence of both 3-HAA and 3-HKA but not in the presence of PA. Such decreases occurred primarily in TH17 cells that were IFN-γ–negative and were dose-dependent with increasing concentrations of catabolites, most notably 3-HAA (Fig. 5B and fig. S4A). We also determined the ability of different tryptophan catabolites to promote the differentiation of FoxP3+ Treg cells. Only 3-HAA enhanced the proportion of CD4+CD25+ T cells expressing FoxP3, an effect that also occurred in a dose-dependent manner (Fig. 5C). No dose-dependent change in IFN-γ production (Fig. 5D, left panel) or in cell proliferation, as assessed by carboxyfluorescein succinimidyl ester (CFSE) dilution (Fig. 5D, right panel), was observed within the CD4+ T cell population. Finally, we performed the same assay on PBMCs from HIV-infected controllers (n = 4) and found similar dose-dependent TH17 depletion by tryptophan catabolites (fig. S4B).

To determine whether tryptophan catabolism has a similar effect in vivo, we related the presence and function of IDO1 to HIV disease progression. In rectosigmoid tissue from controllers (n = 9) and noncontrollers (n = 11) (Study Group D), IDO1 mRNA expression was inversely related to the TH17/Treg ratio (Fig. 5E). When analyzed in plasma, a similar relation was found between higher IDO1 activity (as measured by K/T ratio) and low TH17/Treg ratios (Fig. 5F). These observations support the hypothesis that IDO1-mediated tryptophan catabolism plays a critical role in determining T cell differentiation pathways during HIV infection and, thus, permit microbial translocation that drives disease progression.

**DISCUSSION**

Research on aberrant immune system features in host-pathogen interactions, in inflammatory syndromes and autoimmune diseases, and on primary immune deficiencies has highlighted the importance of two immune cell lineages derived from a common progenitor under reciprocal and mutually exclusive differentiation pathways (35–38): TH17 cells, which produce the proinflammatory cytokine IL-17, and FoxP3+ Treg cells, whose function is immunosuppressive (42–45). TH17 cells, in particular, have been causally related both to chronic inflammatory diseases (46) and to host defenses against microbial agents (47). An intriguing developmental link also exists between the activity of the enzyme IDO1 and the differentiation of TH17 and Treg cells from naïve T cells. The products of IDO1, tryptophan catabolites such as kynurenines, can induce FoxP3 expression and the generation of Treg cells and can blunt the generation...
Fig. 5. IDO catabolites affect the $T_{\text{H}17}/T_{\text{reg}}$ ratio. PBMCs from healthy donors were cultured in vitro for 6 days in the presence of different IDO catabolites, including 3-HKA, 3-HAA, and PA. (A) Formulae of the IDO catabolites 3-HKA, 3-HAA, and PA and example of intracellular FACS detection of IL-17A and IFN-$\gamma$ expression in CD4$^+$ T cells after cell division (CFSE$^{low}$) for 3-HKA, 3-HAA, and PA compared to media. DMSO, dimethyl sulfoxide. (B) Frequency of IL-17A$^+$IFN-$\gamma^-$CD4$^+$ T cells after treatment with escalating doses of 3-HKA, 3-HAA, and PA. FACS results from one donor after 3-HKA and 3-HAA treatment are shown in the left panels. Results from four independent experiments with four different donors are at the right panel. (C) Dose-dependent changes in FoxP3$^+$ T_{reg} cells in the same experiments and example as in (B). (D) Dose-dependent changes in T_{H1} (IL-17A$^+$IFN-$\gamma^-$CD4$^+$ T cells) and in proliferation (CFSE$^{low}$). (E) Correlation between the $T_{\text{H}17}/T_{\text{reg}}$ ratio and IDO mRNA expression in rectosigmoid biopsy tissues from HIV controller and noncontroller subjects (Cohort Study D). (F) Correlation between the $T_{\text{H}17}/T_{\text{reg}}$ ratio in PBMCs and K/T ratio in plasma from HIV controller and noncontroller subjects (Cohort Study B). The Spearman rank correlation test was used for the correlations ($R_s$, Spearman correlation coefficient).
tomic immune activation during pathogenic HIV infection (44). We hypothesize that systemic inflammation in the acute stage of HIV infection, combined with the early loss of immune function caused by $T_{H17}$ cell depletion in the gastrointestinal tract, results in elevated IDO1 activity throughout the chronic phase of HIV infection. Such elevated activity, in turn, leads to the generation of catabolites (3-HAA) that alter T cell differentiation pathways in a manner that leads to further immunosuppression. Previous reports indicate that acute HIV and SIV infections result in a massive increase in IFN concentrations in part through direct activation of pDCs by HIV virions (24, 25). Activated pDCs are then prompted to up-regulate IDO1 through autocrine IFN signaling and TLR stimulation by HIV components (for example, ssRNA or CpG) (26). The early burst of IDO1 activity results in a transient alteration in the T cell response favoring the up-regulation of FoxP3 and generation of Treg cells over the differentiation of TH17 cells. In nonpathogenic SIV infection, the IFN response is eventually curtailed and IDO1 activity returns to baseline levels (18). However, in pathogenic SIV infection and chronic HIV infection, IFN remains high, leading to the persistence of elevated IDO1 activity, likely from both pDCs and, on the basis of our data here, mDCs as well (18, 25). This chronic activation of the IDO1 pathway diminishes the host’s capacity to generate $T_{H17}$ cells and favors the generation of $T_{reg}$ cells. The net outcome is a progressive loss of the mucosal immune barrier and increased susceptibility to mucosal infections, a result of fewer $T_{H17}$ cells, augmented by more $T_{reg}$ cells, which dampens T cell immunity to HIV and other pathogenic organisms (44).

Although we have demonstrated that 3-HAA can specifically invert the ratio of $T_{H17}$ and $T_{reg}$ cells, we have yet to determine the mechanism by which this occurs. Previous studies have shown that 3-HAA blocks T cell activation and promotes T cell death (15, 41). These studies generally used higher concentrations of 3-HAA than reported here (and we have also observed cellular toxicity at concentrations >100 mM). 3-HAA has also been found to inhibit $T_{H17}$ and $T_{H12}$ responses in a variety of in vivo settings, including allergy (55), organ transplantation (10), experimental autoimmune encephalomyelitis (9), and colitis (56). One study has shown that 3-HAA mediates its inhibitory effects on T cell activation and proliferation by directly inhibiting the phosphorylation of phosphoinositide-dependent kinase 1 and by preventing nuclear factor $k_B$ activation after T cell receptor stimulation (57). However, we did not observe increases in T cell death or inhibition of proliferation at lower concentrations of 3-HAA (25 to 100 mM) despite alterations in $T_{H17}$ and $T_{reg}$ cell differentiation.

IDO1-dependent tryptophan catabolism may be an important link between immune activation and the gradual decline of immune function seen in progressive HIV infection. Blockade of IDO1 with a pharmacological inhibitor (for example, 1-methy-D-tryptophan) in combination with antiretroviral therapy has shown some promise in lowering the viral load in pathogenic SIV infection (58) and enhancing the elimination of virus-infected macrophages in amurine model of HIV encephalitis (59). Clinical trials are currently under way to assess the efficacy of IDO1 inhibitors for cancer immunotherapy, and small-molecule inhibitors are being developed that may prove useful in a variety of clinical settings. Future efforts to determine whether blockade of IDO1 can alter the balance of T cell subsets in disease states represent an important goal for understanding HIV pathogenesis as well as other diseases characterized by chronic inflammation.

**MATERIALS AND METHODS**

**Patient populations**

PBMCs, plasma, and rectosigmoid biopsies were obtained from HIV-infected adults enrolled in the University of California, San Francisco (UCSF) SCOPE Cohort or the UCSF Options Cohort (5). SCOPE is an ongoing prospective cohort study aimed at investigating the long-term clinical and immunological consequences of HIV infections and their treatment. The UCSF Options Cohort is an early HIV infection cohort in which participants are enrolled within 12 months of HIV antibody seroconversion (typically within 6 months of seroconversion). Subjects were determined to be in early HIV infection via an algorithm using information on serial HIV antibody testing, less sensitive enzyme immunoassay antibody testing, RNA PCR detection, HIV protein Western blot banding patterns, and self-reported risk behaviors to estimate time since infection. All participants gave written informed consent using protocols approved by the Committee on Human Research, UCSF.

Four separate studies of HIV-infected subjects from SCOPE and Options Cohorts contributed to this analysis.

**Study A.** For the measurements of tryptophan and kynurenine shown in Figs. 1 and 2D, plasma was obtained from subjects in the SCOPE Cohort who were either (i) controllers, defined as those with a steady-state viral load of <2000 copies/ml and a CD4+ T cell count of >500 cells/µl ($n = 20$); (ii) noncontrollers/high CD4, with viral loads of >10,000 copies/ml and CD4+ T cell counts of >350 cells/µl ($n = 33$); or (iii) noncontrollers/low CD4, with viral loads of >10,000 copies/ml and CD4+ T cell counts of <350 cells/µl ($n = 33$). As per the SCOPE protocol, these subjects had contemporaneous viral loads, CD4+ T cell counts, and measurements of CD4+ and CD8+ T cell activation. Some of these plasma specimens had also previously been analyzed for endotoxin (LPS), sCD14, and EndoCAb (28).

**Study B.** For immunophenotyping and measurement of $T_{H17}$ and $T_{reg}$ cells in the peripheral blood of chronically infected subjects (Fig. 3), cryopreserved PBMCs were obtained from untreated subjects in the SCOPE Cohort who were controllers ($n = 14$) or noncontrollers ($n = 28$). As above in Study A, controllers were defined by undetectable or low plasma RNA HIV viral load (<2000 copies/ml) (median, <75; IQR, 75 to 75) and CD4+ T cell counts of >500 cells/µl of blood (median, 814; IQR, 713 to 1156). Noncontrollers were defined by a viral load of >10,000 copies/ml, with evidence of progressive disease (CD4 decline over time >50 cells per year), and further subdivided in groups with high or low CD4 counts [for example, CD4 counts of >350 cells/µl (median, 504; IQR, 473 to 541) or <350 cells/µl (median, 218; IQR, 179 to 260), respectively], indicating early- or late-stage disease progression [noncontroller/high CD4 ($n = 14$) or noncontroller/low CD4 ($n = 14$)]. We also identified subjects with undetectable viral loads on antiretroviral therapy (“Suppressed,” $n = 14$) with CD4 counts of >500 cells/µl (median, 720; IQR, 658 to 830) and a high-risk HIV-seronegative group (Negative, $n = 14$) (CD4 counts not determined).

**Study C.** For the longitudinal analysis of acutely infected subjects shown in Fig. 3E, cryopreserved PBMCs were obtained from 27 participants of the Options Cohort at two time points after infection. The first time point was selected during early infection (acute) at ~3 months after the estimated infection date (median, 3.03; IQR, 2.4 to 3.6) and the second time point at ~12 months of infection (median, 11.6; IQR, 9.1 to 14.3), a time when viral and immunological set
points are established (chronic). We further subdivided these subjects into three groups (Groups 1 to 3) on the basis of previous results demonstrating that both high viral load and high T-cell immune activation set points are independent predictors of more rapid CD4+ T-cell decline and disease progression (5). As illustrated in Fig. 3E, we selected three groups of subjects at the chronic time point based on plasma HIV RNA concentrations (viral load) and T-cell immune activation [measured by the median fluorescence intensity (MFI) of CD38 on total CD8+ T cells (Fig. 3E, left)]. Group 1 had low viral load (<10,000 copies/ml) and low immune activation, Group 2 had high viral load (>10,000 copies/ml) and high immune activation, and Group 3 had high viral load (>10,000 copies/ml) but low immune activation. Groups 2 and 3 had no significant differences in viral load, and Groups 1 and 2 had no significant differences in immune activation (P > 0.1, Mann-Whitney). Conversely, Groups 2 and 3 had significantly higher viral load than Group 1, and Group 2 had significantly higher immune activation than Groups 1 and 3 (P < 0.05, Mann-Whitney). This strategy was designed to select subjects who would show different rates of CD4 decline in the absence of antiretroviral treatment, with Group 2 subjects predicted to be more rapid progressors than those in Groups 1 and 3.

**Study D.** For the simultaneous analysis of TH17 and Treg cells in specimens of PBMCs and rectosigmoid biopsy tissue (Fig. 4) as well as for analysis of mRNA by PCR (Fig. 2A), immunohistochemistry (Fig. 2B), and 16S rDNA (Fig. 4A), paired rectosigmoid biopsies and blood samples were obtained from subjects in the SCOPE Cohort who were (i) noncontrollers (n = 11), defined as untreated individuals with plasma HIV RNA concentrations of >10,000 copies/ml (median, 24,734; range, 15,534 to 91,199) and CD4+ T-cell counts of ≥200 cells/μl (median, 253; range, 196 to 673), and (ii) controllers (n = 9), defined as untreated individuals with undetectable (<75 RNA copies/ml) or plasma HIV RNA concentrations of <2000 RNA copies/ml (median, 77; range, <75 to 1957) and CD4+ T-cell counts of ≥500 cells/μl (median, 701; range, 518 to 1507).

All cryopreserved samples were obtained after density centrifugation of acid citrate dextrose (ACD) solution–treated collection tubes following standard procedures. All samples in Studies B and C were thawed, processed, and analyzed at the same time to limit technical variations during processing and flow cytometry measurements. All data from Studies A to D were processed and analyzed in a blinded fashion.

**Tissue collection and processing**

Whole blood was collected into EDTA-containing tubes (BD Biosciences) for cell counts and into ACD-containing tubes (BD Biosciences) for purification of plasma and PBMCs. All HIV-infected participants in Study D underwent paired blood draw (except one) and flexible sigmoidoscopy. The sigmoidoscope was advanced to the rectosigmoid region, and 30 mucosal biopsies were obtained in a circumferential fashion at ~15 cm from the anal verge using a disposable biopsy forceps with a 3.3-mm outside diameter. Focal areas with visible evidence of inflammation were avoided. For each participant, 4 biopsy specimens were fixed in buffered formalin and paraffin-embedded (for immunohistochemistry), 2 biopsies were immediately frozen in optimal cutting temperature buffer and transferred to a −80°C freezer, 4 biopsies were placed in RNA Later (Ambion) (for RNA extraction), and the remaining 20 biopsy specimens were placed in 15 ml of R-10 (RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Hyclone) and 50 U/50 μg of penicillin-streptomycin per milliliter) and transported within 2 hours to the laboratory for immediate processing. A suspension of mucosal cells from rectosigmoid biopsies was obtained after mechanical digestion and then three successive collagenase type II treatments (0.5 mg/ml) (Sigma-Aldrich). Cell viability in all samples was assessed by trypan blue exclusion. After purification, PBMCs and rectosigmoid biopsy cells were counted and resuspended at 3 × 10^6 to 8 × 10^6 cells/ml for subsequent phenotyping and functional analysis in R-10 medium [RPMI 1640 medium supplemented with 10% FCS, 10 mM Hepes, 2 mM L-glutamine, penicillin-streptomycin (50 U/50 μg ml−1), and 0.1 mM Gibco minimum essential medium nonessential amino acid solution (all from Invitrogen)].

**Measurement of tryptophan and kynurenine concentrations in plasma**

Tryptophan and kynurenine concentrations in plasma were measured by high-performance liquid chromatography. In brief, 100 μl of plasma was combined with 100 μl of 3-nitro-tyrosine (5 μg/ml), which served as an internal standard. Trichloroacetic acid (25 μl) (20%) was then added to precipitate all proteins, and the sample was centrifuged and ~100 μl of the supernatant was collected for analysis. From the supernatant, 20 μl of sample was injected into the column [Nova-Pak C18 30 cm × 3.1 mm (Waters)] and run through in mobile phase [15 mM potassium phosphate (diacid) (pH 7.0), with 2.75% acetonitrile]. Tryptophan concentrations were measured by fluorescence (Shimadzu RF 530 fluorescence detector: excitation, 285 nM; detection, 365 nM), and kynurenine concentrations were measured with an ultraviolet detector (Waters M486 UV detector: detection, 360 nM). Standard curves and quality control samples [phosphate-buffered saline-bovine serum albumin (BSA) (5%) with 50 μM tryptophan and 2.5 μM kynurenine] were included in each run, and final concentrations were determined based on internal standards and standard curves.

In cell culture supernatants, tryptophan and kynurenine concentrations were measured instead by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The plasma sample (100 μl) was added to 100 μl of internal standard, 3-nitro-tyrosine (5 μg/ml), and vortexed for 1 min, and 20 μl of trifluoroacetic acid was added to precipitate the proteins. After vortexing for 1 min, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was transferred to autosampler vial, and 5 μl was injected to the LC-MS/MS system. The mass detector was a Micromass Quattro Ultima using electrospray positive ionization mode. The multiple reaction monitor was set at 205.1 to 188.0 mass/charge ratio (m/z) for Trp, 209.1 to 192.0 m/z for kynurenine, and 227.0 to 181.1 m/z for internal standard, respectively. The column was Synergi Polar RP (4.6 × 75 mm, 4-μm particle size) with mobile phase consisting of 2% acetonitrile, 5.4% methanol, and 0.1% formic acid. The flow rate was 1.0 ml/min, one-fourth split into the mass system. The standard curve was generated by adding tryptophan and kynurenine standard solutions to water and treated in the same way as the plasma sample.

**Immunohistochemistry and immunofluorescence**

IDO1 expression was measured by immunohistochemistry and immunofluorescence with a rabbit polyclonal antibody that was prepared as described (60). Lymphoid tissues and rectal biopsies were fixed in formalin (10% normal buffered formalin) and embedded in paraffin, and 5-μm-thick sections were prepared for staining. Tissue sections were
Human DCs were generated from CD14+ monocytes purified from whole blood over Ficoll-Hypaque density gradients and washed in fresh RPMI 1640 culture medium. For detection of cytokine production by flow cytometry, the cells were stimulated on day 6 for 5 hours at 37°C with PMA (10 ng/ml) and ionomycin (1 µg/ml) in tris-buffered saline–BSA. Cytokine analysis was performed after stringent gating on singlet live (AQUA) CD4+ or CD8+ T cell lymphocytes from rectosigmoidal biopsies or from PBMCs and reported as background-subtracted values from the unstimulated cell population from each patient and for each stimulus, as described (62). Notably, whenever possible, we used the MFI of CD38 on CD8+ T cells as the reference marker for T cell immune activation, as described (5). However, when CD38 MFI measurements were not possible (for example, at a time when samples were not analyzed together), the frequency of CD8+ T cells that were gated positive for CD38 was used instead. Fluorescence-activated cell sorting (FACS) analysis was performed on a four-laser BD LSR-II flow cytometers using High Throughput System plate readers (BD Biosciences). Data were analyzed with FlowJo software v6.8 (Treestar) and transferred into analysis and graphic software including Excel (Windows), StatView (Abacus Concepts), SPICE (provided by M. Roederer), and/or GraphPad Prism 4.

In vitro T cell activation assays

PBMCs were isolated from healthy volunteers by centrifugation of whole blood over Ficoll-Hypaque density gradients and washed in freshly prepared R-10. The cells were subsequently labeled with the dye CFSE (Invitrogen) and cultured at a concentration of 3 × 10^5 cells per well in 96-well U-bottom tissue culture plates that had previously been coated with antibody to CD3 (clone SP34-1; 0.5 µg/ml; BD Pharmingen). Soluble antibody to CD28 (0.5 µg/ml; BD Pharmingen) and irradiated CD3-depleted PBMCs from an unrelated (allogeneic) donor were added to each well at a final concentration of 1 × 10^5 cells per well. Finally, graded concentrations of different tryptophan catabolites or vehicle (dimethyl sulfoxide) were added to the wells and the cells were cultured for 6 days at 37°C. Tryptophan catabolites (or vehicle controls) were replaced on day 3 in 100 µl of fresh RPMI 1640 culture medium. For detection of cytokine production by flow cytometry, the cells were stimulated on day 6 for 5 hours at 37°C with PMA (10 ng/ml) and ionomycin (1 µg/ml) in the presence of brefeldin A (GolgiPlug). Surface staining and intracellular staining were performed as described in the previous section. For FoxP3 staining, cells were harvested on day 6 and stained as described in the previous section. Notably, donor-to-donor variability was observed in the percentage of change in the Th1 population in response to 3-HAA, with some subjects showing increases and others decreases (or no change) in the fraction of Th1 cells.

Statistical analysis

The Mann-Whitney U test was used for group comparisons. In the Options Cohort (Study C), mixed-effects longitudinal statistical models were used to test the impact of peripheral memory T cell counts, and viral load on CD8+ T cell activation levels (%Ki67) during the 3- to 12-month time period after the estimated date of HIV infection. These models were run in the SAS System 9.2 and specified random effects for time and the individual. The Spearman rank correlation test was used to determine correlations between variables, with R_s being the Spearman correlation coefficient. P values of <0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/2/32/32ra36/DC1
Fig. S1. Characterization of IOD up-regulation in mDCs from HIV+ subjects.
Fig. S2. IOD activity on mDCs in vitro with combined IL-17 and/or IL-17a-IL-6-TNFα.

REFERENCES AND NOTES


E. Traub, Epidemiology of lymphocytic choriomeningitis in a mouse stock observed for four years. J. Exp. Med. 69, 801–817 (1939).


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