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Neuromodulatory Activities of CD4⁺CD25⁺ Regulatory T Cells in a Murine Model of HIV-1-Associated Neurodegeneration

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HIV-1-associated neurocognitive disorders are intrinsically linked to microglial immune activation, persistent viral infection, and inflammation. In the era of antiretroviral therapy, more subtle cognitive impairments occur without adaptive immune compromise. We posit that adaptive immunity is neuroprotective, serving in both the elimination of infected cells through CD8⁺ cytotoxic T cell activities and the regulation of neuroinflammatory responses of activated microglia. For the latter, little is known. Thus, we studied the neuromodulatory effects of CD4⁺ regulatory T cells (Treg; CD4⁺CD25⁺) or effector T cells in HIV-1-associated neurodegeneration. A newly developed HIV-1 encephalitis mouse model was used wherein murine bone marrow-derived macrophages are infected with a full-length HIV-1Env/vesicular stomatitis viral pseudotype and injected into basal ganglia of syngeneic immunocompetent mice. Adoptive transfer of CD3-activated Treg attenuated astroglial and microglia inflammation with concomitant neuroprotection. Moreover, Treg-mediated anti-inflammatory activities and neuroprotection were associated with up-regulation of brain-derived neurotrophic factor and glial cell-derived neurotrophic factor expression and down-regulation of proinflammatory cytokines, oxidative stress, and viral replication. Effector T cells showed contrary effects. These results, taken together, demonstrate the importance of Treg in disease control and raise the possibility of their utility for therapeutic strategies. The Journal of Immunology, 2009, 182: 3855–3865.

A spectrum of neurological dysfunctions is associated with advanced HIV-1 infection and termed HIV-1-associated neurocognitive disorders (HAND) (1). In the era of antiretroviral therapy and increased patient survival, nervous system impairment is more subtle with low level infection and focal neuroinflammation more closely correlated with mild neuropsychological signs and symptoms. The pathological correlate of HAND is encephalitis. Before the widespread use of antiretroviral drugs, encephalitis was characterized by the presence of multinucleated giant cells, profound viral replication, astrogliosis, microgliosis, myelin pallor, and neuronal dropout with severe compromise of dendritic arbor (2–4). HIV encephalitis (HIVE) remains prevalent, although attenuated by effective drug treatments. Patients show significant CNS lymphocytic infiltrates as a consequence of disease or immune reconstitution syndrome. Moreover, HIVE depends on the continuous flux of activated leukocytes toward the brain parenchyma rather than simply autonomous HIV infection and inflammation per se. CD4⁺ T cells as well as CD8⁺ T lymphocytes accumulate in brains of patients with progressive HIV-1 infection and AIDS (5–7). Prior work by C. Petito and colleagues examined T lymphocyte subsets in the CA1, CA3, and CA4 regions of the hippocampus of AIDS patients with and without HIVE and showed that hippocampal activated/memory CD45RO⁺ T lymphocytes were significantly increased in diseased hippocampal subregions (8). This led to the notion that perineuronal location of CD4⁺ cells provides the potential for lymphocyte-mediated neuronal injury or trans-receptor-mediated neuronal infection. The presence of activated microglia and brain macrophages with lower levels of virus remains a central pathological feature of disease. Increased inflammation can occur as a consequence of secreted viral and cellular proteins from activated or infected mononuclear phagocytes (MP; perivascular brain macrophages and microglia) (9) and include proinflammatory cytokines, chemokines, and arachidonic acid and its metabolites NO, quinolinic acid, and glutamate as well as HIV-1 proteins such as Tat, Nef, and gp120 (10–15).

Host immune surveillance against persistent viral infection includes CD8⁺ CTL and humoral and innate secretory responses (16–19). However, the mechanisms by which the virus escapes clearance remain unknown. Included in these responses are attempts to purge the infected host of latently infected cells (16, 17). Nonetheless, all immune responses, CD8⁺ T cells are among the most effective and were previously investigated in our prior reports in rodent models of neuroAIDS (18–20). We posit that in addition to CTL, CD4⁺CD25⁺ regulatory T cells (Treg) as well as effector T cells (Teff) play an important role in HAND control. Treg, a subset of CD4⁺ T cells, are now well recognized for their...
immune modulatory function and play pivotal roles in maintaining immunological tolerance. Their principal role is to attenuate T cell-mediated immunity and suppress autoreactive T cells (21–23). Teff promote inflammatory responses and speed recognition and immunity (24). We now report that Treg modulate immune responses in the brain and lead to neuronal protection in murine HIVE. Neurroprotection was found to be mediated by attenuating HIV-1-induced microglia activation and enhancing of neurotrophic factors. These results support the importance of Treg in the control of HIV-1-associated neurodegeneration in the antiretroviral era and when adaptive immune responses remain operative.

Materials and Methods

**Animals, infection of bone marrow–derived macrophages (BMM), and induction of HIV**

Four- to 6-wk-old male C57BL/6J mice (The Jackson Laboratory) were maintained in accordance with guidelines for the care of laboratory animals from the National Institutes of Health and with approval of the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (Omaha, NE). BMM were derived after a 7-day culture of bone marrow cells with macrophage CSF (M-CSF; a generous gift from Wyeth) and were infected as previously described (20). Briefly, vesicular stomatitis virus (VSV)-pseudotyped HIV-1 (HIV-1/VSV) was used to infect BMM at a concentration of 1 pg of HIV-1 p24 per cell for 24 h. After a continuous 5-day culture, >90% of BMM were virus positive according to HIV-1 p24 immunochemical tests (Dako-Cytomation). Reverse transcriptase activity as a function of [H]deoxythymidine triphosphate from BMM culture supernatants confirmed the extent of infection as previously described (25). To induce HIVE, HIV-1/VSV-infected BMM (1 × 10^6 cells/5 μl/mouse) were delivered by intracerebral (i.c.) injection into the basal ganglia of 4-wk-old C57BL/6J mice using stereotactic coordinates as previously described (26). Mice injected i.c. with PBS served as sham-injected controls.

**Isolation, activation, and transfer of Treg and Teff**

From pooled splenic and lymph node CD3^+ CD4^+ T cells enriched from negative selection columns (R&D System), Treg-enriched CD4^+CD25^+ and naive CD4^+CD25^- T cells were prepared by positive and negative selection for CD25^+ T cells, respectively, using PE-anti-CD25 (BD Pharmingen) magnetic beads conjugated to anti-PE mAb and passage over autoMACS columns (Miltenyi Biotech) as previously described (27). By flow cytometric analyses, T cells were shown to be >95% enriched for each T cell subset. Isolated CD4^+CD25^- Treg and CD4^+CD25^+ T cells were activated by culture in the presence of 0.5 μg/ml anti-CD3 (145-2C11; BD Pharmingen) and 100 U/ml mouse rIL-2 (R&D Systems). Three days later, 1.0 × 10^5 activated Treg or Teff (anti-CD3 stimulated CD4^+CD25^+ T cells) were harvested and adoptively transferred i.v. to HIVE mice.

**BMM and Treg/Teff cocultivations**

BMM were seeded at 1 × 10^6/well in 6-well plates containing a 1:1 ratio mixture of BMM and T cell medium. BMM and HIV-1/VSV-infected BMM were cocultivated with Treg or Teff for 6 days. Supernatants were collected as conditioned medium (CM). BMM viability was measured using the LIVE/DEAD viability cytotoxicity kit (Invitrogen) after removal of the cocultured Treg and Teff. Cell viability was measured by MTT assay (28).

**Measures of oxidative stress**

To assess hydrogen peroxide (H_2O_2) production from uninfected or infected BMM, cells were plated at 1 × 10^3/0.2 ml tissue culture medium/well in a 96-well fluorometer plate and stimulated for 24 h with 200 ng/ml mouse rTNF-α (R&D Systems) as previously described (27). The medium was removed and replaced with Krebs-Ringer buffer (Sigma-Aldrich) containing 10 μM PMA, 0.1 U/ml HRP, and 50 μM Amplex Red (Sigma-Aldrich). BMM cultured in the absence of TNF-α or PMA served as baseline controls. Fluorescence intensity was measured at 563 nm (excitation)/587 nm (emission) 90 min after the addition of Amplex Red using a microplate spectrophotometer (μQuant; BioTek Instruments) interfaced with analysis software (KC Junior; BioTek Instruments).

**Isolation and characterization of primary mouse neurons**

Eighteen-day-old embryonic fetuses were harvested from terminally anaesthetized pregnant C57Bl/6J mice. Cerebral cortices were dissected and digested using 0.25% trypsin (Invitrogen). Cortical digests were seeded at a density of 1.5 × 10^5 cells/well in 24-well plates containing poly-D-lysine-coated cover slips and cultured in neurobasal medium.
FIGURE 2. Treg attenuate chronic neuroinflammatory responses in HIVE mice. A, Flow cytometric analysis of Treg and Teff subsets from naive C57BL/6 mice showing percentage distribution of the following CD4 T cell phenotypes: CD4 CD25+ (red), CD4 CD25− (green), CD4 FoxP3+ (blue), or CD4 FoxP3− (yellow). B, Quantitative PCR of mRNA encoding FoxP3, TGF-β, IL-10, IL-2, and IFN-γ from CD3-activated Treg (green bars) and Teff (red bars). Mean ± SEM of mRNA levels was determined for triplicate cell samples and normalized to GAPDH. Significant differences in relative expression of mRNA from Treg compared with Teff were determined by Student’s t test, *p < 0.05. C, Treg inhibition of anti-CD3-mediated proliferation of 1 x 10⁶ Teff was assessed. Cells were cocultured for 72 h and pulsed with [3H]thymidine for the final 18 h of culture, harvested onto filters, and counted by beta scintillation spectrometry. D, To induce HIVE, HIV-1/VSV-infected BMM were stereotactically injected i.c. into the basal ganglia of syngeneic C57BL/6J mice. Sham controls were injected i.c. with PBS. Treg or Teff (1 x 10⁶) were adoptively transferred into HIVE mice 1 day postinfection. Serial sections of brain tissue that comprise the injection area were obtained on day 7 postinfection and analyzed by immunohistochemical and Western blot assays for p24, Iba1, Mac-1, GFAP, CD4, and FoxP3 Ags. Brains were collected at day 7 after i.v. injections. Representative brain sections from PBS and the HIV-1/VSV, HIV-1/VSV/Teff, and HIV-1/VSV/Treg groups showing expression of HIV-1 p24 (red) Iba1 (green), Mac-1 (green), GFAP (green), and FoxP3 (red). Where indicated, nuclei were stained with DAPI (scale bars, 50 μm; original magnification, ×400). Cellular colocalizations of intracellular HIV-1 p24 and membrane Mac-1 expression are shown in magnified inserts. E, Digital image quantification of fluorescence intensity in the stained area was analyzed under ×400 magnification using NIH Image J software. Eight fields in four sections in each experiment were subjected to quantitative analysis. Bar graphs represent mean of area stained positive intensities in a field of view (open bars, PBS; gray bars, HIV-1/VSV; speckled bars, HIV-1/VSV/Teff; or black bars, HIV-1/VSV/Treg). F, Representative Western blot analysis of Iba1, GFAP, TNF-α and β-actin levels from brains of mice treated with PBS, HIV-1/VSV, HIV-1/VSV/Teff, or HIV-1/VSV/Treg (Treg). G, Densitometric quantification of Western blots for Iba1, GFAP, and TNF-α levels in mice treated with PBS (open bars), HIV-1/VSV (gray bars), HIV-1/VSV/Teff (speckled bars), or HIV-1/VSV/Treg (black bars). Levels were normalized to β-actin levels and mean densities ± SEM were determined from four mice per group. E and G, Compared with PBS: *p < 0.05; **p < 0.01; ***p < 0.001; and compared with HIV/VSV group: #p < 0.05; and ###p < 0.001.
Supplemented with 2% B27, 1% penicillin/streptomycin, 0.2% FBS, and 0.5 mM l-glutamine (Invitrogen). After 10–14 days, neuron-enriched cultures contained >90% microtubule-associated protein (MAP)-2-positive cells with <2% glial fibrillary acidic protein (GFAP)-positive cells as determined by immunocytochemistry. Mature neurons were treated with CM collected from 24-h cocultures of HIV-1/VSV-infected BMM in the presence or absence of Teff or Treg.

**Immunohistochemistry**

Brain tissues were derived from perfused mice and processed as previously described (20). Murine microglia were detected with rabbit polyclonal Abs to Iba1 (ionizing calcium-binding adaptor molecule 1) (1/500; Wako) or Mac-1 (CD11b; 1/500; Serotec). Astrocytes were visualized with anti-rabbit GFAP Ab (1/1,000; DakoCytomation). Anti-HIV-1 p24 Abs (1/10; DakoCytomation) were used to identify HIV-1-infected cells. Putative Treg were identified by dual staining with anti-CD4 (1/100; DakoCytomation) and anti-Forkhead box P3 (FoxP3) (1/100; ProMab Biotechnologies) Abs (27). Abs to neuronal nuclei protein (NeuN) (1/100) and MAP-2 (1/1,000; Chemicon) were used to identify neurons, and mouse cross-reactive rabbit GFAP (1/1000; DakoCytomation), rabbit polyclonal anti-caspase-3 (1/1000; Cell Signaling), rabbit polyclonal anti-GFAP (1/1000; DakoCytomation), rabbit polyclonal anti-Iba1 (1/500; Wako), chicken monoclonal anti-human glial cell line derived neurotrophic factor (BDNF) (1/1000; Promega) were used for growth factor expression. Primary Abs were visualized with Alexa Fluor 488 (green)- and Alexa Fluor 594 (red)-conjugated secondary Abs (Invitrogen; Molecular Probes). Images were obtained by an Optronics digital camera fixed to Nikon Eclipse E800 (Nikon Instruments) using MagnaFire 2.0 software (Optronics). Fluorescence intensity in the stained area of serial brain sections encompassing the i.c. injection sites was analyzed under ×400 magnification using NIH Image J software. Bar graphs showed mean of fluorescence intensity per field of view. C and D, Western blot analysis of BDNF expression (C) and densitometric quantification of BDNF Western blots (D) from lysates of brain tissues from mice treated with PBS, HIV-1/VSV, HIV-1/VSV/Teff, or HIV-1/VSV/Treg. BDNF levels were normalized to β-actin expression. Values are expressed as mean ± SEM for four mice per group and were significant compared with the PBS group: *, p < 0.001; **, p < 0.01; ***, p < 0.001, and compared with HIV/VSV group: ##, p < 0.01; and ###, p < 0.001.

**FIGURE 3.** Treg are neuroprotective in HIVE mice. Mice were stereotactically injected into the basal ganglia with HIV-1/VSV-infected syngeneic BMM or with PBS alone as sham controls. After 1 day, Treg or Teff (1 × 10^6) were adoptively transferred into HIVE mice. Serial sections of brain tissue that encompassed the injection area were obtained on day 7 postinfection and analyzed by immunohistochemistry. A, Serial sections of brains from PBS control, HIV-1/VSV, HIV-1/VSV/Teff, and HIV-1/VSV/Treg were stained for MAP2 (green), NeuN (green), BDNF (red) and GDNF (red) and visualized by confocal laser-scanning microscopy. Images are shown at 400 original magnification and the scale bars equal 50 μm. B, Stained sections from mice (eight fields in four sections in each mouse) treated with PBS (open bars), HIV-1/VSV (gray bars), HIV-1/VSV/Teff (speckled bars), or HIV-1/VSV/Treg (black bars) were digitally analyzed using NIH Image J software. Bar graphs showed mean of fluorescence intensity per field of view. C and D, Western blot analysis of BDNF expression (C) and densitometric quantification of BDNF Western blots (D) from lysates of brain tissues from mice treated with PBS, HIV-1/VSV, HIV-1/VSV/Teff, or HIV-1/VSV/Treg. BDNF levels were normalized to β-actin expression. Values are expressed as mean ± SEM for four mice per group and were significant compared with the PBS group: *, p < 0.001; **, p < 0.01; ***, p < 0.001, and compared with HIV/VSV group: ##, p < 0.01; and ###, p < 0.001.
BDNF, and biotin-conjugated anti-TNF-\(\alpha\). Primary Abs and \(\beta\)-actin were detected with HRP-conjugated goat anti-mouse (1/10,000), goat anti-rabbit (1/10,000), goat anti-chicken (1/10,000), and mouse anti-\(\beta\)-actin mAb (1/10,000, Sigma-Aldrich). Proteins were visualized with an ECL kit (Bio-Rad Laboratories).

Cytokine arrays

Equal volumes of cell culture supernatants were incubated with the pre-coated cytokine Ab array according to the manufacturer’s instructions (AAM-CYT-3-2; RayBiotech). Densitometric analysis of the array was performed using the NIH Image J software.

Statistical analyses

The results were expressed as mean \(\pm\) SEM for each group. Statistical significance between groups was analyzed by Student’s \(t\) test using Microsoft Excel. Differences were considered statistically significant at \(p \leq 0.05\).

Results

HIVE mice

HIVE was established using BMM infected with HIV-1/VSV-pseudotyped virus and injected i.c. into the basal ganglia of syngeneic C57BL/6J mice (Fig. 1) (20). This led to the induction of HIV-1 induced focal encephalitis along the injection track as shown by HIV-1 immunostained cells, robust astrogliosis and microgliosis, and T cell infiltrate as evidenced by positive staining for expression of HIV-1 p24, GFAP, Iba1, and CD3.

Treg and Teff modulate neural responses in HIVE mice

Treg have been shown to have a potential role in modulating the immune response to HIV infection (29, 30). In an effort to assess the role of Treg in a mouse model of HIVE, we isolated and then characterized Treg and Teff T cell subsets from naive mice (27). Flow cytometric analyses indicated that Tregs were >85% CD4\(^+\)CD25\(^+\)FoxP3\(^+\) and naive Teff were >95% CD4\(^+\)CD25\(^-\)FoxP3\(^-\) T cells (Fig. 2A). Three days after CD3 activation, >95% of CD4\(^+\) Teff showed CD25 up-regulation without concomitant FoxP3 expression (data not shown). Additionally, mRNA levels for FoxP3, TGF-\(\beta\), and IL-10 from Treg were significantly elevated over those from Teff, whereas expression of IL-2 and IFN-\(\gamma\) mRNA levels was diminished by activated Treg and increased in Teff (Fig. 2B). Treg suppressed the proliferative response of CD3-activated Teff in a dose-dependent fashion (Fig. 2C). Taken together, the T cells used in these studies showed appropriate Treg and Teff phenotypes.

To evaluate the roles of Treg and Teff in regulating neuroinflammatory responses in HIVE mice, \(1 \times 10^6\) anti-CD3-activated Treg or Teff were adoptively transferred to HIV-1/VSV-infected...
recipients 24 h after induction of HIVE. By 7 days postinfection, immunohistochemistry staining of tissues surrounding the injection tracks indicated that HIV-1/VSV or HIV-1/VSV/Teff-infected mice exhibited dense GFAP and Iba1 expression compared with PBS-sham controls (Fig. 2D). In contrast, both GFAP and Iba1 expression were reduced in HIV-1/VSV/Treg-injected mice. Quantitative measurement of GFAP and Iba1 intensities confirmed significant increases in expression by HIV-1/VSV- and HIV-1/VSV/Teff-treated mice compared with PBS controls and significant reductions in the HIV-1/VSV/Treg group compared with the HIV-1/VSV and HIV-1/VSV/Teff groups (Fig. 2E). Of notable importance was the significant reduction of HIV-1 p24 levels in HIV-1/VSV and HIV-1/VSV/Teff mice compared with sham control and HIV-1/VSV/Teff-treated mice (Fig. 2, D and E). Based on the observations that Treg attenuate the neuroinflammatory responses following HIV-1 infection, we evaluated the ingress of CD4+ T cells into the brain. We observed the presence of CD4+ cells in the injection site of mice from all treatment groups (Fig. 2D). CD4+ cells were significantly increased in the HIV-1/VSV- and HIV-1/VSV/Teff-treated groups (Fig. 2E); however, in contrast, the ingress of CD4+ cells was diminished to the levels of sham control in infected mice treated with Treg (Fig. 2, D and E). Interestingly, CD4+ FoxP3+ double-positive cells were present in only the HIV-1/VSV/Treg-treated group.

Of the microglial secretory factors known to influence secondary neuronal degeneration, TNF-α is implicated in affecting neuronal cell loss (2, 13, 14, 31, 32). Western blot analysis of brain lysates revealed that the expression of TNF-α was increased by 2.7 and 19.9% in mice treated with HIV-1/VSV or HIV-1/VSV/Teff compared with PBS sham control (Fig. 2E). These data indicate that Treg, but not Teff, are capable of attenuating HIV-1/VSV-induced glia activation to a neuroinflammatory phenotype.

**Treg-mediated neuroprotection in HIVE mice**

To evaluate the neuroprotective abilities of T cells for HIVE, we measured neuronal density in diseased animals where Treg were adoptively transferred. To determine a mechanism for these effects, expressions of BDNF, GDNF, MAP2, and NeuN were measured with or without T cell transfers. Evidence of neuronal dropout was observed by NeuN/MAP2 immunostaining (Fig. 3A). Densitometric analysis of neurons revealed that HIV-1/VSV-infected mice showed 40 and 75% reductions in MAP2 and NeuN staining, respectively, and Treg-treated HIVE mice showed MAP2 and NeuN staining reductions comparable to those of HIV-1/VSV/Treg mice (Fig. 3B). In contrast, infected mice treated with Treg exhibited no significant decrease in neuronal expression of MAP2 or NeuN with neuron levels comparable to those of sham control mice. Densitometric analysis of cellular expression of growth factors revealed that BDNF and GDNF expression was diminished by >40% in mice treated with HIV-1/VSV-infected BM or those mice treated with Teff, whereas levels of growth factor expression in infected mice treated with Treg were comparable to or exceeded that of sham-treated controls. Enhanced expression of BDNF in HIV-1/VSV/Treg-treated mice was confirmed by Western blot analysis (Fig. 3C and D). These data, taken together, indicate that Treg enhance neurotrophin secretion and protect neurons in HIVE mice.

**Treg induces cytotoxicity in HIV-1/VSV-infected BMM**

To elucidate mechanisms for Treg-induced neuroprotection, we investigated whether Treg affected cell death of infected BMM. In these experiments, BMM were infected with HIV-1/VSV for 24 h and Treg or Teff were added at a BMM:T cell ratio of 3:1. Cell viability was determined by the MTT assay after 72 h of treatment with T cell subsets and was normalized as the percentage of uninfected BMM control cultures. Compared with uninfected BMM, viabilities of infected BMM in the absence of T cells or presence of Treg was reduced by 37% of uninfected BMM controls and diminished by >20% compared with either of the other infected BMM groups. These results were confirmed by LIVE/DEAD (Invitrogen) cytotoxicity staining, which demonstrated that infected BMM cultured in the absence of Treg increased cytotoxicity to 14.6% ± 2.7 and 19.9% ± 2.7%, respectively, compared with the cytotoxicity of uninfected BMM (Fig. 4B). In contrast, coculture of infected BMM with Treg increased BMM cytotoxicity to 28.6% ± 3.9%, thus confirming that the previously recorded diminution of viable BMM was due to increased cytotoxicity.

**FIGURE 5.** Treg reduce HIV-1 viral replication in BMM. HIV-1/VSV-infected BMM were cocultured with Teff or Treg at a ratio of 3:1 (BMM vs Treg or BBB vs Teff). A, Supernatants from cultures of uninfected BMM (Control; closed triangles), HIV-1/VSV-infected BMM (closed squares), HIV-1/VSV/Teff (open circles), and HIV-1/VSV/Treg (open diamonds) were collected from day 1 to day 6 after the addition of T cells and assessed for reverse transcriptase (RT) activity. B, Virally infected BMM cultured in the absence or presence Teff or Treg or uninfected BMM (Control) were harvested at the end of day 6 and stained for expression of HIV-1 p24 and visualized by light microscopy at ×400 original magnification. Scale bars equal 50 μm. C, HIV-1 p24-positive BMM were counted and percentages of HIV-p24-positive BMM were determined. A and C, Means ± SEM were determined from three independent experiments. Compared with HIV-1/VSV BMM group by Student’s t test: #, p < 0.01; ##, p < 0.001.
Next, we assessed whether HIV-1-infected BMM cytotoxicity requires cell-cell contact between the infected cells and Treg. 

**FIGURE 6.** Treg inhibit ROS production and cytotoxicity in BMM. Anti-CD3-activated Treg or Teff were cocultured in the absence or presence of HIV-1/VSV-infected BMM for 24 h. After removal of T cells, ROS production was measured as a function of H$_2$O$_2$ accumulation using an Amplex Red assay. Uninfected BMM cultured alone served as ROS baseline controls. A. Compared with uninfected BMM controls (open bar), percentage increases of ROS as a function of H$_2$O$_2$ levels were determined for HIV-1/VSV-infected BMM cultured alone (gray bar) or cocultured in the presence of Teff (HIV-1/VSV/Teff, speckled bar), or Treg (HIV-1/VSV/Treg, black bar). B. Uninfected BMM cultured in the absence (left set) or presence of PMA (right set) were cocultured without TNF-α or T cells (open bars) in the presence of TNF-α (gray bars), with TNF-α and Treg (speckled bars), or with TNF-α and Treg (black bars). A and B. Mean ± SEM were determined for three experimental determinations and significant differences were determined by Student’s $t$ test; compared with control group: $**$, $p < 0.01$; $***$, $p < 0.001$; compared with HIV-1/VSV group: $###$, $p < 0.001$; $####$, $p < 0.001$. C. Uninfected BMM (Control) and HIV-1/VSV-infected BMM were cultured in the absence or presence of Teff or Treg for 24 h and supernatants were harvested. Collected supernatants were then subjected to cytokine array blots (RayBiotech). Ovals encompass replicate blots to detect expression of IL-2 (oval 1), IL-12p40/p70 (oval 2), MCP-1 (oval 3), and MCP-5 (oval 4). D. Densitometric analysis of cytokine array blots were achieved by digital image analysis with NIH Image J software, and mean densities ± SD were determined for replicate determinations. Mean densities ± SD of positive controls for each array of control BMM and HIV-1/VSV-infected BMM cultured in the absence or presence of Teff or Treg were 204.4 ± 9.6, 206.3 ± 6.0, 201.7 ± 14.8, and 202.5 ± 11.9, respectively.

Treg-treated HIVE mice exhibited a greater density of TUNEL$^+$ BMM compared with mice HIV-1/VSV and HIV-1/ VSV/Teff groups. This observation suggested that Treg-induced apoptosis of HIV-1/VSV-infected BMM confers neuronal protection in HIVE mice.

Treg reduce HIV-1 replication, reactive oxygen species (ROS), and cytotoxicity in BMM

To test whether Treg mediated the inhibition of HIV-1 replication in HIV-1/VSV-infected BMM, we infected BMM with HIV-1/ VSV for 24 h. After viral washout, Treg or Teff were applied and cocultured for 6 days. Supernatants, collected at different time points, were used for an HIV-1 reverse-transcriptase activity assay. Compared with reverse transcriptase activities in HIV-1/VSV-infected BMM, levels of progeny virion production were significantly increased by day 1 in the HIV-1/VSV/Teff group and continued to remain higher until both levels reached a plateau at day 4 (Fig. 5A). In contrast, levels of progeny virion in infected BMM cultures treated with Treg (HIV-1/VSV/Treg) never approached those of the other two infected groups and were significantly below those of HIV-1/VSV infected BMM by day 3 and at times thereafter. Furthermore, the numbers of multinucleated giant cells, a hallmark of HIV-1 infection, were significantly reduced in the HIV-1/VSV/Treg group (data not shown). Also, immunostaining...
suggested that Treg inhibited HIV-1 p24 protein expression in virally infected BMM (Fig. 5B). Percentages of HIV-1 p24-positive BMM indicated that coculture with Treg, but not Teff, significantly reduced the number of HIV-1-infected BMM compared with HIV-1/VSV-infected BMM controls (Fig. 5C).

Because oxidative stress is known to enhance neurotoxicity by increased levels of superoxide radicals and NO (27, 33), we evaluated the extent that Treg may affect ROS production as a mechanism of neuroprotective activity. We hypothesized that Treg also suppress virally infected BMM-induced toxicity through suppression of ROS production. To test this, we assessed ROS production in HIV-1/VSV-infected BMM cocultured for 24 h in the absence or presence of anti-CD3-activated Teff or Treg. Compared with uninfected BMM controls, HIV-1/VSV-infected BMM resulted in a 4.7-fold increase in H$_2$O$_2$ production; however, Treg treatment of HIV-1/VSV-infected BMM significantly decreased H$_2$O$_2$ production ($p < 0.001$), although not to baseline control levels (Fig. 6A). In contrast, Teff treatment of HIV-1/VSV-infected BMM failed to significantly affect H$_2$O$_2$ production. To test the effect of Treg on the ROS responses, uninfected BMM were activated for 24 h with PMA and TNF-α and cocultured in the absence or presence of Teff or Treg. Similarly as in infected BMM, coculture with Treg significantly suppressed the ROS response of activated uninfected BMM, whereas Teff yielded no significant effects on ROS responses (Fig. 6B). Additionally, we analyzed cytokine secretion by a membrane-based cytokine array. Array analysis showed increased expression of IL-2, IL-12, MCP-1, and MCP5, by HIV/VSV-infected BMM or infected BMM treated with Teff compared with uninfected BMM (Fig. 6, C and D). In contrast, treatment of infected BMM with Treg diminished IL-2, IL-12, MCP-1, and MCP5 to levels below those attained either after infection or after infection and culture in the presence of Teff.

Treg induce neuroprotective responses from HIV/VSV-infected BMM CM

To substantiate the protective capacity of Treg to attenuate neuronal toxicity, we measured neuronal cell death in primary neuronal cultures cultured for 24 h in the presence or absence of CM from uninfected BMM (control) or HIV-1/VSV-infected BMM cultured in the absence or presence of Teff or Treg. Expression of MAP-2 and NeuN by primary neurons confirmed the neuronal integrity of control CM-treated neurons. TUNEL staining showed more apoptotic neurons in cultures after treatment with CM from HIV-1/VSV BMM and HIV-1/VSV/Teff BMM compared with control CM, whereas treatment with CM from HIV-1/VSV/Treg BMM showed fewer TUNEL-positive neurons (Fig. 7A). Quantitation of apoptotic neurons confirmed that the percentages of apoptotic neurons were significantly increased after treatment of primary neurons with CM from HIV-1/VSV BMM and HIV-1/VSV/Teff BMM compared with control CM (Fig. 7B). In contrast, treatment of neurons with CM from HIV-1/VSV/Treg BMM significantly diminished percentages of apoptotic neurons to levels attained with control CM.

Discussion

HAND is a late complication of progressive viral infection (34). Significant productive HIV-1 replication occurs in brain MP (perivascular macrophages and microglia) during late stages of infection concomitant with severe immune suppression and high peripheral viral loads. Secretory viral and cellular products, including virotoxins, proinflammatory cytokines, chemokines, NO, and quinolinic acid, lead to multinucleated giant cell formation, astrogliosis and microgliosis, and neuronal loss (9, 35, 36). Increasing expression or prolonged exposure to those cellular and viral products in advanced HIV-1 infection and encephalitis results in neuronal dysfunction and injury (37, 38). Nonetheless, despite the widespread use of antiretroviral therapy, HIV-associated cognitive impairments still persist and can occur throughout the course of viral infection (39). Thus, the means to control such disease-related complications remain important. We reasoned that this can be accomplished by natural immune surveillance and most notably through Treg.

Treg control self-reactivity and immune activation. During progressive HIV disease, the cells mediate down-regulation of specific immune responses and help limit immune activation. These may prove beneficial to the host. The role of Treg in HIV-1-associated cognitive impairment remains poorly defined due to the lack of relevant models, the low frequency of Treg, and interpatient variations such as age, genetics, and disease stage. To overcome these obstacles, we used a HIVE animal model established in our laboratory for neuroAIDS (20, 40). We show that the adoptive transfer of CD3-activated Treg clearly confers neuroprotection in HIVE mice. This is manifested, in part, by attenuating microglia and astrocyte inflammation, promoting increased levels of BDNF and GDNF production, and controlling the infiltration of reactive T
cells. These observations raise the question as to how Treg modulate microglia and astrocyte activation and control ongoing neurotoxic and viral replication in the brain.

Treg are involved in modulating the magnitude of host cellular immunity during pathologic processes, including cancer (21, 22) and infectious disease (41, 42), as well as autoimmune disease (23, 43). Furthermore, the immunosuppressive activity of Treg has been implicated in the inability of mice to mount an effective immune response to vaccination (44). Recent evidence implicates Treg in the control of HIV-1 and SIV-1 immunity and viral infections (24, 45–53). It is possible that progressive depletion of CD4+ T cells (54, 55) and viral persistence (56–58) seen during AIDS could be ameliorated by Treg depletion from peripheral blood, affecting increased anti-HIV CD4+ T cell responses (24, 45, 46, 59). Indeed, Treg maintains suppressive activities for HIV-specific CD4+ and CD8+ T cell responses (30, 50). Whether Treg can affect the disease course is in doubt, however. Prior studies reveal decreased numbers of Treg in patients who are chronically infected with HIV (24, 29, 45, 46, 59). This observation suggests that Treg, as a conventional T cell subset, are progressively lost during the course of HIV-1 infection. In contrast, other studies report increased frequency of Treg in lymphoid tissue from HIV-1-seropositive patients and macaques infected with SIV (48, 49, 51, 53, 60). Levels of FoxP3 mRNA that encode a critical Treg-associated translation factor are increased during antiretroviral therapy (47, 59). These observations led to the hypothesis that Treg could impact HIV-1 infection by suppressing immune activation or effector anti-HIV-1 specific T cell responses. Nonetheless, interest in the potential role of Treg in HIV-1-mediated disease is growing.

In our studies, the importance of Treg in the pathogenesis of HIV-infection is underscored by the diminution of the CD4+ T cell inflammatory response in the brain and the suppression of viral replication in HIV-infected CD4+ T cells. As an important component of the immune surveillance system, Treg control autoimmunity and reaction to self-Ag. This is evident from an abundant amount of evidence demonstrating that Treg modulate inflammatory responses in graft-vs-host-disease (61) and autoimmune disorders including arthritis (62), diabetes (63), and experimental autoimmune encephalitis (64, 65). Accumulating evidence indicates that Treg may control immunity to HIV infection; however, only limited studies of Treg in chronic immune activation, neurodegeneration, and neuronal injury exist. Because AIDS is associated with the loss of CD4+ T cells and progressive immune dysfunction, impairment or depletion of Treg by HIV-1 infection can contribute to immune hyperactivation and has been suggested to be a reliable predictor of AIDS progression (66). Removal of Treg from the cultures of peripheral or lymphoid leukocytes from HIV-infected patients or SIV-infected macaques enhances virus-specific immune responses (30, 48, 52). In addition, laboratory tests of HIV-specific T cell responses with CD4+ and CD8+ cells from most HIV-infected people are consistently abrogated by Treg (30). Furthermore, HIV-infected individuals without detectable numbers of Treg display significantly higher levels of plasma viremia, lower frequencies of CD4- T cell counts, and lower CD4/CD8 T cell ratios, all strong prognostic indicators in HIV disease progression (67). Such Treg-mediated suppression has been shown in vitro to be cell contact dependent and function in a cytokine-independent fashion. These findings suggest that Treg in the periphery, by suppressing virus-specific immunity, may contribute to uncontrolled viral replication and therefore have a detrimental role in HIV infection. However, our studies indicate that in the brain, Treg-mediated diminution of the CD4+ T cell inflammatory response led to the suppression of viral replication in HIV-infected CD4+ T cells.

Previous reports show that in SIV infection, virus-specific T cells are critical for eliminating viral infection; however these T cells persistently accumulate in brains of SIV-infected monkeys even after elimination of the virus (68). Moreover, during HIV infection, activated proinflammatory Th1 T cells infiltrate the brain, induce MP inflammatory responses, and accelerate neuronal dysfunction and deficits in neural structural integrity. Treg, as immune regulators with suppressive activity, can significantly regulate immune responses (69). Based on those observations, we adoptively transferred CD3-activated Treg into HIVe mice. Surprisingly, Treg trafficked to the inflammatory hemisphere of the brain, diminished inflammatory CD4+ T cell infiltration, and reduced MP-mediated neuroinflammatory responses. Moreover, adoptive transfer of activated Treg, but not Teff, enhanced BDNF and GDNF expression, reduced TNF-α secretion, and supported neuroprotection from HIVe.

To better understand the mechanisms of Treg in HIV infection, a cellular model of MP activation and viral infection was developed. We hypothesized that Treg inhibit virally infected, BMM-induced inflammatory cytokine secretion and ROS production, which are linked to neuronal death (27, 33). Indeed, we show that Treg significantly inhibited ROS production of virally infected BMM, ameliorated neurotoxicity, and facilitated neuronal survival. We also demonstrate that Treg induced apoptosis to kill virally infected BMM and abrogate HIV-1 replication. Treg-mediated apoptosis may be through two distinct apoptotic pathways. One is extrinsic, whereby extracellular signals lead to increased expression and activation of caspase-8 and rapid cleavage of caspase-3 (70). The other is intrinsic, in which signals such as cellular damage lead to the release of cytochrome c and the downstream activation of caspase-9, caspase-3, and other effector caspases (71). We observed that Treg treatment enhanced BMM caspase-3 expression, suggesting that Treg-mediated BMM apoptosis may be through a classical death receptor-initiated signaling cascade. However the exact pathway by which Treg initiate apoptosis in virally infected BMM remains enigmatic. Taken together, this study demonstrates that Treg modulated immune responses and reduced HIV-1 levels in a murine HIVe model. This was accomplished in part through an apoptotic mechanism(s) that affected infected BMM and was specifically associated with activated Treg, but not activated Teff. Based on the notion that Treg depletion could be a target for AIDS therapy to bolster the peripheral immune response, our results raise the question as to the effects of such cell depletion on the progression of neuroAIDS.

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Disclosures
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