Macrophage IL-12p70 Signaling Prevents HSV-1–Induced CNS Autoimmunity Triggered by Autoaggressive CD4⁺ Tregs

Kevin R. Mott,¹ David Gate,²,³ Mandana Zandian,¹ Sariab J. Allen,¹ Naveen Kumar Rajasagi,⁴ Nico van Rooijen,⁵ Shuang Chen,⁶ Moshe Arditi,⁶,⁷ Barry T. Rouse,⁴ Richard A. Flavell,⁶,⁸,⁹ Terrence Town,⁺²,⁵,⁷ and Homayon Ghiasi⁺¹

PURPOSE. CD4⁺CD25⁻FoxP3⁺ naturally occurring regulatory T cells (Tregs) maintain self-tolerance and function to suppress overly exuberant immune responses. However, it is unclear whether innate immune cells modulate Treg function. Here the authors examined the role of innate immunity in lymphoepithelial homeostasis.

METHODS. The involvement of B cells, dendritic cells (DCs), macrophages, natural killer (NK) cells, and T cells in central nervous system (CNS) demyelination in different strains of mice infected ocularly with herpes simplex virus type 1 (HSV-1) was investigated.

RESULTS. The authors found that depletion of macrophages, but not DCs, B cells, NK cells, CD4⁺ T cells, or CD8⁺ T cells, induced CNS demyelination irrespective of virus or mouse strain. As with macrophage depletion, mice deficient in interleukin (IL)-12p70 or IL-12p40 showed CNS demyelination after HSV-1 infection, whereas demyelination was undetectable in HSV-1–infected, IL-23p19–deficient, or Epstein-Barr virus–induced gene 3-deficient mice. Demyelination could be rescued in macrophage-depleted mice after the injection of IL-12p70 DNA and in IL-12p35⁻⁻ or IL-12p40⁻⁻ mice after injection with IL-12p35 or IL-12p40 DNA or with recombinant viruses expressing IL-12p35 or IL-12p40. Using FoxP3⁻, CD4⁻, CD8⁻, or CD25-depletion and gene-deficient mouse approaches, the authors demonstrated that HSV-1-induced demyelination was blocked in the absence of CD4, CD25, or FoxP3 in macrophage-depleted mice. Flow cytometry showed an elevation of CD4⁺CD25⁺FoxP3⁺ T cells in the spleens of infected macrophage-depleted mice, and adoptive transfer of CD4⁺CD25⁺FoxP3⁺ T cells to infected macrophage-depleted severe combined immunodeficient mice induced CNS demyelination.

CONCLUSIONS. The authors demonstrated that macrophage IL-12p70 signaling plays an important role in maintaining immune homeostasis in the CNS by preventing the development of autoimmune CD4⁺ Tregs. (Invest Ophthalmol Vis Sci. 2011; 52:2321–2333) DOI:10.1167/iovs.10-6536

Resistance and susceptibility to autoimmune diseases in human and experimental animal models is caused by both specific and nonspecific immunity.¹,² The adaptive immune system can respond to a variety of infections and maintains tolerance by suppressing self-reactive lymphocytes. Induction of self-tolerance depends on the highly regulated process of positive and negative selection of developing T cells by various cell types.³,⁴ Macrophages, dendritic cells (DCs), natural killer (NK) cells, and B cells are involved in the induction of both autoimmunity and tolerance.⁵–⁷ Although these cells facilitate the elimination of autoreactive lymphocytes in the thymus, it remains unclear which cell type is required to protect the central nervous system (CNS) from spontaneous autoimmunity and to control the release of self-reactive T cells into the CNS. Although a multitude of factors may cause autoimmunity and inflammation in the CNS, infectious agents such as neurotropic viruses are potent stimuli in this scenario.

Among the neurotropic viruses, viral infection caused by herpes simplex virus (HSV) is the most prevalent in the United States. It is estimated that 70% to 90% of the adult American population has antibodies to HSV-1, HSV-2, or both.⁸ A key hallmark of HSV infection is the ability of the virus to establish latent infection of sensory neurons, resulting in periods of recurrent disease.⁹ Herpesviruses (including HSV-1, HSV-2, human cytomegalovirus, Epstein-Barr virus, human herpesvirus [HHV]-6, and HHV-8) have been shown to trigger the autoimmune disease multiple sclerosis (MS), which is hallmark by CNS infiltration of immune cells that cause demyelination of brain and spinal cord (SC) axons.¹⁰ Many non-herperviruses have also been implicated in MS,¹¹–¹⁷ suggesting that the induction of demyelinating disease is not restricted to herpesviruses.

One of the major diseases associated with degradation of the myelin sheath is MS.¹ Visual disturbances are initial manifestations of MS, and optic neuropathy caused by demyelination of the optic nerve is a common cause of visual and neurologic dysfunction in young adults with MS.¹⁸,¹⁹ Optic
neuropathy can be used as an early prognostic factor during the subsequent course of MS. We have previously demonstrated that infection of different strains of mice with a recombinant HSV-1-expressing murine IL-2 (HSV-IL-2) resulted in demyelination in the brains, SCs, and optic nerves (ONs) of infected mice. By contrast, mice infected with wild-type viruses or with similarly constructed recombinant HSV-1 viruses expressing IFN-γ or IL-4 did not experience CNS demyelination. To determine what factors mediate self-tolerance and protection against autoimmunity in HSV-1-infected mice, we probed for the involvement of B cells, DCs, macrophages, NK cells, and T cells. Our results demonstrate that macrophages play a critical and indispensable role in orchestrating self-tolerance and prevention of autoimmunity. We also show that, similar to the results obtained using our macrophage depletion approach, IL-12p35−/− and IL-12p40−/− mice exhibited demyelination after ocular HSV-1 infection with wild-type viruses. In macrophage-depleted IL-12p35−/− or IL-12p40−/− mice, demyelination occurred with the expansion of CD4+CD25+ T cells. Add-back of IL-12p35 to IL-12p35−/− or IL-12p40 to IL-12p40−/− mice rescued demyelination in infected animals, and CNS injury was absent when CD4+, CD25+, or FoxP3+ cells were depleted. Further, adoptive transfer of CD4+CD25+ T cells to infected macrophage-depleted severe combined immunodeficient (SCID) mice induced CNS demyelination. We demonstrated that CD4+CD25+ FoxP3+ Tregs have an autoregressive function that is inhibited by macrophage IL-12p70 signaling, resulting in the prevention of HSV-1-induced CNS injury.

**Materials and Methods**

**Viruses, Cells, and Mice**

Plaque-purified HSV-1 strains McKrae (wild-type) or KOS and HSV-1 recombinant viruses expressing IL-12p35 and IL-12p40 were grown in rabbit skin cell monolayers in minimal essential medium (MEM) containing 5% fetal calf serum, as previously described. McKrae virus is resistant at an infectious dose of 2 × 10^3 plaque-forming units (PFU)/eye, whereas KOS, DM (the parental virus for HSV-1 IL-12p35 and HSV-1 IL-12p40 viruses, which lacks both the LAT and γδT45 transcripts), HSV-1 IL-25p, and HSV-1 IL-12p40 viruses are attenuated strains. Female BALB/c, BALB/c-IL-12p35−/−, BALB/c-IL-12p40−/−, BALB/c-CD19−/− (B-cell deficient), BALB/c-SCID, BALB/c-EBI3−/−, C57BL/6, C57BL/6-CD4−/−, C57BL/6-CD8−/−, C57BL/6-IL-12p35−/−, C57BL/6-IL-12p40−/−, C57BL/c-IL-23p19−/−, C57BL/6/FoxP3+Tg, and C57BL/6-IL-12ra−−/− (CD25-deficient) mice aged 6 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6/FoxP3+Tg was a gift from Alexander Y. Rudensky. Female hemizygous C.FVB-Tg (Itgax-DTR/GFP) 57 Lan/J mice on a BALB/c background and C57BL/6/FoxP3+Tg were bred at Cedars Sinai Medical Center, whereas C57BL/6-IL-23p19−/− were bred at the University of Tennessee at Knoxville.

**Ocular Infection**

Mice were infected ocularly with 2 × 10^5 PFU McKrae, KOS, HSV-1 IL-12p35, HSV-1 IL-12p40, or DM virus. Each virus was suspended in 5 μL tissued culture media and administered as an eye drop. All mice on a BALB/c background were infected with KOS, HSV-1 IL-12p35, HSV-1 IL-12p40, or DM virus, whereas all C57BL/6 mice were infected with McKrae or DM virus. In this study to show that demyelination is not HSV-1 strain specific, we have used wild-type virulent HSV-1 strain McKrae, wild-type avirulent HSV-1 strain KOS, and highly avirulent HSV-1 strain DM that lacks both the LAT and the γδT45 transcripts as challenge viruses. No ocular pathology was detected in ocularly infected mice by day 14 after infection.

**Macrophage Depletion**

Liposome encapsulation of dichloromethylene diphosphonate (Cl2MDP) was performed as previously described. To deplete macrophages, each mouse received 100 μL of the mixture intraperitoneally and subcutaneously. Macrophage depletion was carried out on days −5, −2, +1, +4, +7, and +10 relative to ocular infection with HSV-1.

**Depletion of DCs and FoxP3**

Female BALB/c-DTR mice were depleted of their DCs using 100 ng diphtheria toxin (DT) in 100 μL PBS administered intraperitoneally, as described previously with minor modifications. Briefly, the first depletion was performed 24 hours before ocular infection, followed by four additional depletions on days +1, +4, +7, and +10 relative to ocular infection. Control mice were treated with PBS. FoxP3+Tg mice were depleted of their FoxP3 by treatment with DT, as described previously. Briefly, the mice were administered DT 72 and 24 hours before ocular infection, followed by five additional treatments on days +1, +3, +5, +7, and +9 after infection.

**Depletion of CD4+, CD8+, or CD25+ T Cells**

Each mouse received an intraperitoneal injection of 100 μg purified GK1.5 (anti-CD4), 2.43 (anti-CD8), or PC61.5.3 (anti-CD25) monoclonal antibodies (NCCc, Minneapolis, MN) in 100 μL PBS 5 and 2 days before ocular challenge. The injections were repeated on days +1, +4, +7, and +10 relative to ocular infection. Mock-depleted control mice were injected similarly with the irrelevant mAb of the same isotype.

**Depletion of NK Cells with Anti–Asialo GM1**

One milligram of rabbit anti–asialo GM1 antibody (Wako Chemicals, Dallas, TX) was dissolved in 1 mL PBS, and each mouse received multiple intraperitoneal injections of 100 μg antibody in 100 μL PBS, as described previously. The first depletion was performed 5 days before ocular infection, followed by five additional depletions on days −2, +1, +4, +7, and +10 relative to the start of ocular infection. Control mice were treated with an equal concentration of freeze-dried normal rabbit serum in PBS.

**DNA Immunization**

The complete open reading frame for IL-12p35 and IL-12p40 was cloned into the Vir1055 expression vector, whereas the complete open reading frame for IL-12p70 was purchased from InvivoGen (San Diego, CA). Plasmid DNA encoding each gene was purified on a cesium chloride gradient. In each experiment, five mice per group were inoculated intramuscularly into each quadriceps with 100 μg each cesium chloride purified DNA in a total volume of 50 μL using a 27-gauge needle 7 days before ocular infection. As a negative control, we used mock-vaccinated mice that were similarly injected with vector DNA alone.

**Isolation of CNS and Spleen Cells for Flow Cytometry**

Brains and spleens from macrophage- or mock-depleted mice were harvested on days 8 and 11 postinfection (PI) with KOS. Single-cell suspensions from individual mouse brains and spleens were prepared as described previously. Monoclonal antibodies used for flow cytometry were purchased from BD Biosciences (San Diego, CA) and eBiosciences (San Diego, CA). Cell surface and intracellular staining of single-cell suspensions of mononuclear cells (MNCs) and splenocytes from individual mice was accomplished according to the manufacturer's instructions. Antibodies included anti-CD3-PE-Cy7 (clone 145-2C11), anti-CD4-FITC (clone 13T4), anti-CD8a-PE (clone 53–6.7), anti-CD25-PE-CY7 (clone PC61.5.3), and anti-FoxP3-Pacific blue (clone FJK-16s). Penta-color flow cytometric analyses of MNCs or spleen cells were performed using a FACScan (BD Biosciences). Percentages of cells stained with mAbs were calculated by forward or side scatter gating of MNCs or splenocyte preparations. Controls included nonrelevant isotype-matched antibody, no primary antibody, or no secondary antibody alone. A minimum of 1 × 10^4 events was acquired based on a live cell gate.
Adoptive Transfer
Donor untreated BALB/c mice were killed, spleens were pooled, and single-cell suspensions were prepared. CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>-</sup> T cells from pooled suspensions were isolated magnetically as described by the manufacturer (Miltenyi Biotec, Auburn, CA). Each recipient SCID mouse was injected once intraperitoneally with 1 × 10<sup>4</sup> CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>-</sup> T cells in 300 μL MEM, and a subset of SCID mice received 1 × 10<sup>6</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells. Recipient SCID mice were ocularly infected 4 hours after transfer with KOS.

Tissue Preparation
ONs, brains, and SCs of experimental and control mice were removed at necropsy on days 7, 10, and 14 PI, embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Sakura, Torrance, CA) for cryosectioning, and stored at −80°C.

Demyelination Morphometry
Transverse sections of brains, SCs, and ONs, each 10 μm thick (spaced 50 μm apart), were sectioned using a cryostat (CM3050S; Leica, Wetzlar, Germany), air dried overnight, and fixed in acetone for 3 minutes at 25°C. The presence or absence of demyelination in infected mice was evaluated using Luxol fast blue (LFB) staining of formalin-fixed sections of ONs, as we described previously.<sup>20</sup> Demyelination in each section was confirmed by monitoring adjacent sections.

Immunohistochemistry and Image Analyses
Transverse sections of SCs, 10 μm thick (spaced 50 μm apart), were sectioned and mounted on slides (Superfrost Plus Gold; Fisher Scientific), then air dried for 10 minutes and stored at −80°C. For microscopic analyses, we applied marking pen (PAP Pen; Invitrogen, Carlsbad, CA) around the tissue and preblocked in serum-free protein block (DAKO Cytomation) for 30 minutes at 25°C. We then diluted primary antibody in serum-free protein block and incubated slides overnight at 4°C. After three rinses for 5 minutes each in PBS, we incubated slides for 1 hour at 25°C with appropriate Alexa Fluor 488–, Alexa Fluor 594–, or Alexa Fluor 647–conjugated secondary antibodies (Invitrogen-Molecular Probes). After an additional three rinses for 5 minutes each with PBS at 25°C, we air dried slides in the dark and mounted them with antifade reagent (Prolong Gold with DAPI; Invitrogen-Molecular Probes). Fluorophores were imaged in separate channels (ApoTome-equipped Axio Imager Z1; Carl Zeiss Microimaging, Thornwood, NY). We variously used the following antibodies for immunohistochemistry: mouse HSV-1 (FITC-conjugated, 1:100; GenWay, San Francisco, CA), rabbit GFAP (1:1000; Dako), rat CD11b (1:200; Serotec, Raleigh, NC), and rat F4/80 (1:50; Serotec). Three images each of white matter and gray matter were captured for three sections through each SC, and we obtained a threshold optical density that best distinguished staining from background. For HSV-1, GFAP, CD11b, and F4/80 burden analyses, data are reported as the percentage of labeled area captured (positive pixels) divided by the full area captured (total pixels). Threshold optical densities and burden analyses were measured using ImageJ software, release 1.40g (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Statistical Analysis
To analyze the presence or absence of demyelination in experimental and control groups, Fisher’s exact tests were performed (Instat; Graph-Pad, San Diego, CA). Statistical analyses for immunohistochemistry photomicrographs were performed using data analysis software (Excel; Microsoft, Redmond, WA). For single comparisons of the means, we used t-tests for independent samples to assess significance. An examiner blinded to sample identities performed all analyses, and code was not broken until analyses were completed. For all analyses, we set alpha levels at 0.05.

RESULTS
Role of Innate and Adaptive Immunity in Protection from CNS Demyelination in Infected Mice
To assess the impact of innate and adaptive immunity in protection from CNS demyelination in HSV-1–infected mice, female BALB/c mice were depleted of macrophages, DCs, NK cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or CD4<sup>+</sup> plus CD8<sup>+</sup> T cells, as described in Materials and Methods. Mice were ocularly infected with the avirulent HSV-1 KOS strain. Demyelination in ON, SC, and brain was analyzed using day 14 PI tissue stained with LFB. Representative photomicrographs of ON sections from infected or control mice are shown in Figures 1 and 2.
Conspicuous demyelination was present in ONs of infected macrophage-depleted mice (Fig. 1A, arrows) but not in either mock depleted (Fig. 1B) or uninfected macrophage-depleted mice (Fig. 1C). A similar pattern of results was observed in brain and SC after macrophage depletion and infection (Figs. 1D, 1F, arrows). Demyelination was not detected in mock-depleted brains (Fig. 1E) or SCs of infected mice (Fig. 1G).

In contrast to macrophage depletion results described in Figure 1, mice that were depleted of NK cells, DCs, CD4^+ T cells, CD8^+ T cells, or both CD4^+ and CD8^+ T cells did not show evidence of ON demyelination (Fig. 2). Similarly, demyelination was undetectable in brains or SCs of treated mice (data not shown). Furthermore, to determine whether B cells play any role in CNS demyelination, BALB/c-CD19^−/− mice were infected with the HSV-1 KOS strain as described. No demyelination was detected in ONs, brains, or SCs of mock-depleted mice (Fig. 3). Furthermore, mice that were depleted of their macrophages and ocularly infected with DM virus also showed CNS demyelination (Fig. 3; see arrows). As expected, no demyelination was detected in ONs, brains, or SCs of mock-depleted mice (data not shown). These data suggest that demyelination induced in the absence of macrophages is not specific to individual mouse or virus strains.

**Demyelination Time Course in Optic Nerves of Macrophage Depleted and Infected Mice**

To determine the time course of demyelination in the CNS of macrophage-depleted mice, we depleted female BALB/c mice of macrophages, infected them with KOS, and harvested ONs at 8 and 11 days PI. On day 8 PI, demyelination was absent in macrophage- or mock-depleted mice (Figs. 4A, 4C). However, a different pattern of results was evident on day 11 PI in macrophage-depleted mice, which manifested prominent ON demyelination (Fig. 4B; see arrows). By contrast, demyelination was not detected in mock-depleted mice (Fig. 4D). Similar results were obtained with SCs and brains (data not shown). Thus, our results show that demyelination is copious in infected macrophage-depleted mice as early as day 11 PI.

**Increased Astrocyte Infection in Macrophage-Depleted Mice**

To investigate potential differences in HSV-1 infectivity between macrophage- and mock-depleted mice, we performed...
Figure 3. Detection of demyelination in CNS of macrophage-depleted C57BL/6 mice. C57BL/6 mice were macrophage or mock-depleted and infected ocularly with the virulent HSV-1 strain McKrae or the avirulent DM virus that lacks the LAT and \( \gamma/\delta \) transcripts. Representative ON, brain, and SC sections on day 14 PI from infected mice are shown. Arrow: area of demyelination selected for inlay (20×).

immunostaining on the same SC samples used to assess demyelination (Fig. 1). Immunostaining for HSV-1 and GFAP (a well-established maker of astrocytes) showed a high degree of colocalization, confirming that most infected cells in SCs of both macrophage- and mock-depleted mice were astrocytes. We observed markedly increased HSV-1 infection of GFAP\(^+\) astrocytes in SCs of macrophage-depleted mice compared with mock-depleted mice (Fig. 5A). Quantitative image analyses revealed significant increases in HSV-1 and GFAP burden in SC white matter (\( P < 0.001 \)) and gray matter (\( P < 0.001 \)) of macrophage-depleted versus mock-depleted mice (Figs. 5B, 5C). We observed significant decreases in signal intensity for the macrophage cell surface markers CD11b (\( P < 0.01 \) in white matter; \( P < 0.001 \) in gray matter) and F4/80 antigen (\( P < 0.01 \) in white matter and gray matter) in macrophage-depleted versus mock-depleted mice (Figs. 5B, 5C), providing confirmatory evidence that macrophage depletion was successful.

**Enumeration of Regulatory T Cells in HSV-1-Infected Mice Depleted of Macrophages**

We sought to determine whether the frequency of regulatory T cells (Tregs) might be altered in macrophage-depleted HSV-1-infected mice. Macrophage- and mock-depleted mice infected with HSV-1 were killed on day 8 PI, and splenocytes were stained and analyzed by flow cytometry for the presence of CD3, CD4, CD8, CD25, and Foxp3 antigens, as described in Materials and Methods. Figure 6A shows a representative histogram of overlapping macrophage-depleted versus mock-depleted cells that are CD3\(^+\)CD4\(^+\)CD25\(^+\)FoxP3\(^+\) or CD3\(^+\)CD8\(^+\)CD25\(^+\)FoxP3\(^+\). Compared with CD3\(^+\)CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells from mock-depleted mice, macrophage-depleted mice contained a higher percentage of CD3\(^+\)CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells (2.06% vs. 0.62%, respectively). In contrast, the level of CD3\(^+\)CD8\(^+\)CD25\(^+\)FoxP3\(^+\) T cells was the same between mock-depleted and macrophage-depleted groups (Fig. 6A). Figure 6B shows the average number of CD3\(^+\)CD4\(^+\)CD25\(^+\)FoxP3\(^+\) and CD3\(^+\)CD8\(^+\)CD25\(^+\)FoxP3\(^+\) T cells from mock-depleted and macrophage-depleted groups. Quantitation analyses of results from Figure 6A revealed increased expansion of a CD3\(^+\)CD4\(^+\)CD25\(^+\)Foxp3\(^+\) (Treg) population of cells in spleens of macrophage-depleted mice compared with mock-depleted mice (Fig. 6B). These CD4\(^+\) Tregs composed approximately 42% of the T-cell population in spleens of macrophage-depleted mice compared with \(<18\%\) of the T-cell population in spleens of mock-depleted mice (Fig. 6B). The differences between CD4\(^+\) Treg numbers in macrophage- and mock-depleted mice were statistically significant (\( P < 0.05 \)). Abundance of CD3\(^+\)CD8\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs in spleens of macrophage-depleted mice was slightly higher than in mock-depleted animals, but these differences did not reach statistical significance (Fig. 6B; \( P = 0.11 \)). These results raise the possibility that, in the absence of macrophages, CD4\(^+\) Tregs expand and may contribute to virus-induced CNS demyelination.

Figure 4. Time course of appearance of demyelination in macrophage-depleted mice. BALB/c mice were macrophage or mock-depleted and infected ocularly with the avirulent HSV-1 strain KOS. ONs were obtained on days 8 and 11 PI, fixed, sectioned, and stained with LFB. Representative photomicrographs of ONs are shown. Arrow: area of demyelination selected for inlay (20×).
**CD4⁺ Tregs Mediate HSV-1–Induced CNS Demyelination in the Absence of Macrophages**

Both CD4⁺ and CD8⁺ T cells play key roles in effecting demyelination in animal models of MS. 13–15 To directly address whether T lymphocytes were responsible for HSV-1–induced demyelination in the absence of macrophages, we codepleted BALB/c mice of macrophages and CD4⁺/CD8⁺ T cells before infection with KOS, as described in Materials and Methods. Interestingly, infected mice that were codepleted of macrophages and CD4⁺ T cells did not show signs of demyelination in their ONs (Fig. 7A), but mice that were depleted of CD8⁺ T cells showed evidence of demyelination, as indicated by a rarefied LFB staining pattern (Fig. 7C, arrows). To confirm these results, we depleted CD4⁻/⁻ or CD8⁻/⁻ mice of macrophages and ocularly infected them with KOS and assessed demyelination in the CNS on day 14 PI. Similar to the aforementioned codepletion results, we did not detect demyelination in ONs of CD4⁻/⁻ mice (Fig. 7B), whereas CD8⁻/⁻ mice did develop ON demyelination (Fig. 7D, arrows).

To assess whether CD25⁺ T cells were driving CNS demyelination in macrophage-depleted animals, BALB/c mice were codepleted of CD25 and macrophages, as described in Materials and Methods. Similar to CD4 depletion and CD4⁺/CD8⁺ mice, animals depleted of both CD25 and macrophages showed no signs of demyelination in their ONs (Fig. 7E). This result was confirmed when CD25⁻/⁻ mice were depleted of macro-
CD4⁺/CD25⁺ T cells contribute to CNS demyelination in the absence of macrophages. Because FoxP3 is a bona fide marker for Tregs, we depleted FoxP3DTR mice of FoxP3⁺ cells and macrophages and infected them with HSV-1 strain McKrae, as described in Materials and Methods. Demyelination was not detectable in ONs, brains, or SCs of double-depleted and infected mice (Fig. 9, left). However, demyelination was detected in the CNS of mice that were macrophage depleted but not depleted of their FoxP3 (Fig. 9, left). These results suggest that CD4⁺ Tregs drive CNS demyelination in the absence of macrophages.

Role of IL-12 Family Members in CNS Demyelination

Our results suggested that, in the absence of macrophages, HSV-1 infection causes CNS demyelination. One of the main functions associated with macrophages is IL-12 production.
IL-12 family members are heterodimeric complexes composed of an α-subunit (p35, p19, or p28) and a β-subunit (p40 or Epstein-Barr virus–induced gene 3 [EBI3]). The various IL-12 family subunits bind to each other to form full-length IL-12p70, IL-12, IL-12p80, IL-23, IL-27, or IL-35. To determine which of the particular IL-12 family members, when deficient, aggravated CNS demyelination in our system, we infected IL-12p35−/−, IL-12p40−/−, IL-23p19−/−, or IL-35EBI3−/− mice with wild-type HSV-1 and monitored CNS demyelination on day 14 PI. After infection, both IL-12p35−/− and IL-12p40−/− mice demonstrated ON demyelination. By contrast, demyelination was undetectable in ONs of infected IL-23p19−/− (Fig. 10A; IL-23p19-KO) or IL-35EBI3−/− (Fig. 10A; EBI3-KO) mice. Thus, the absence of IL-12p35 or IL-12p40 enabled CNS demyelination in HSV-infected mice.

To determine whether it was possible to rescue demyelination in IL-12p35−/− or IL-12p40−/− mice, we forced expression of the same IL-12 subunit by injecting IL-12p35−/− mice with dbl-IL-12p35 recombinant virus or by infecting IL-12p40−/− mice with dbl-IL-12p40 recombinant virus. Strikingly, demyelination was not detected in the ONs of IL-12p35−/− mice infected with dbl-IL-12p35 virus (Fig. 10B, dbl-IL-12p35) or in IL-12p40−/− mice infected with dbl-IL-12p40 virus (Fig. 10B, dbl-IL-12p40). To confirm these results, we injected IL-12p35−/− mice with IL-12p35 DNA or IL-12p40−/− mice with IL-12p40 DNA and ocularly infected animals with wild-type HSV-1, as described in Materials and Methods. Similar to results obtained with recombinant viruses, injection of IL-12p35−/− mice with IL-12p35 DNA (Fig. 10B, IL-12p35 DNA), or IL-12p40−/− mice with IL-12p40 DNA (Fig. 10B, IL-12p40 DNA) blocked demyelination in ONs of infected mice. However, when we administered IL-12p35 DNA or recombinant virus to IL-12p40−/− mice or IL-12p40 DNA or recombinant virus to IL-12p35−/− mice, CNS demyelination was not abrogated (data not shown). These patterns of demyelination were also observed in brains and SCs of infected mice (data not shown). These results suggest that the absence of IL-12p35 or IL-12p40, but not IL-23p19 or IL-35EBI3 genes, leads to CNS demyelination in HSV-1-infected mice.
CNS Demyelination in the Absence of Macrophages

Our depletion studies showed that macrophages are an essential cellular component that regulates tolerance to mitigate against HSV immunopathology. In addition, our results with IL-12p35−/− and IL-12p40−/− mice show that the absence of IL-12 signaling may be the main contributing factor to HSV-1-induced demyelination and may raise the possibility that the IL-12p70 macrophage response arm is required to mitigate against HSV-1-induced autoimmunity and demyelination. Thus, we hypothesized that macrophage IL-12p70 signaling may play a protective role against HSV-1-induced demyelination. It has previously been shown that DNA vaccine-encoded immunogen is produced by DCs and macrophages residing at the site of inoculation, with subsequent elicitation of immune response either by directly transfected antigen-presenting cells (APC) or by cross-presentation of antigen expressed from transfected somatic cells by APCs. To test our hypothesis, we injected IL-12p70 DNA into macrophage-depleted mice to determine whether it might compensate for the absence of macrophage immunity and thus prevent demyelination in infected mice. We macrophage-depleted BALB/c or C57BL/6 mice and immunized them with IL-12p70 DNA heterodimer (containing both IL-12p35 and IL-12p40 subunits) 7 days before ocular infection. BALB/c mice were infected with KOS, whereas C57BL/6 mice were infected with the McKrae HSV-1 strain. Demyelination in ONs, brains, and SCs of infected mice was measured on day 14 PI. Demyelination was not observed in the CNS of BALB/c or C57BL/6 mice injected with IL-12p70 DNA (Fig. 11). However, mice injected with vector DNA displayed CNS demyelination (Fig. 11). Similar results were obtained with C57BL/6 mice injected with vector DNA, and no toxicity was detected in IL-12p70 DNA injected mice (data not shown). Thus, our results suggest that demyelination in the CNS of macrophage-depleted mice is due to the absence of IL-12p70 macrophage signaling.

**Astrocyte Infection Is Reduced in Macrophage-Depleted Mice Expressing IL-12p70**

Our data described in Figure 5 suggest that astrocytes are rendered susceptible to HSV-1 infection in the absence of macrophages. To investigate potential differences in HSV-1 infectivity in macrophage-depleted mice injected with IL-12p70 DNA versus vector DNA, we performed immunostaining on the same SC samples used to determine demyelination in Figure 10. Qualitatively, we observed conspicuous reduction in HSV-1–infected GFAP+ astrocytes in the SCs of mice injected with IL-12p70 DNA versus vector DNA (Fig. 12A). Quantitative image analyses revealed statistically significant reductions in both HSV-1 and GFAP burden in SC white matter (P < 0.001) and gray matter (P < 0.001) when comparing IL-12p70 DNA injection with vector-injected mice (Fig. 12B). These data show that the expression of IL-12p70 in macrophage-depleted mice functionally compensates for macrophage deficiency, thereby preventing CNS demyelination. These data further validate IL-12p70 activity in macrophages as protective against virus-induced autoimmunity.

**DISCUSSION**

Naturally occurring Tregs have been identified by constitutive expression of CD4 and CD25 (the IL-2 receptor α chain). CD4+CD25+ T cells specifically express the forkhead/winged helix transcription factor gene P3 (FoxP3). The development and function of Treg is critically dependent on this transcriptional repressor. Mice and humans lacking FoxP3 die of severe autoimmune diseases, whereas the transduction of FoxP3 in naïve CD4+ T cells is sufficient to convert these cells to Treg. CD4+CD25+ Treg are critical immune regulators in the context of autoimmunity, allergy, and infectious disease and also are known for limiting tissue damage and inflammation associated with both innate and adaptive immune responses. There is growing interest in the identification of Tregs in various pathologic conditions, and recent studies indicate that CD4+CD25+ T cells with regulatory function can be detected in inflamed tissues. Overall, CD4+CD25+ T cells have been tied to both suppressive and pathogenic roles, and they may prevent the host from mounting an immune response to autoantigens.
such as tumor antigens. In line with our results, it was previously shown that Tregs are essential for establishing and maintaining persistent infection by Leishmania major, Helicobacter pylori, Listeria monocytogenes, and Mycobacterium tuberculosis. In a series of experiments outlined below, we have shown that the pathogenic role of CD4+CD25+FoxP3+ T cells is associated with the absence of IL-12p70 macrophage function. On the whole, results described in this study are not contradictory with the positive role that Tregs play to control autoimmunity in the intact or functional immune system. In fact, we only note an autoimmune Treg phenotype when IL-12p70 macrophages function is ablated.

In this study, we demonstrate that macrophage depletion in various mouse strains causes CNS demyelination in response to HSV-1 infection. The demyelinated lesions involve periventricular white matter, brain stem, and spinal cord white matter. Our results appear to be specific to macrophages because the absence of B cells, NK cells, DCs, CD4+ T cells, CD8+ T cells, or both CD4+ and CD8+ T cells did not induce autoimmune disease in HSV-1-infected mice that were macrophage sufficient. Macrophages exhibit a wide variety of innate immune functions, including phagocytosis, IL-12 production, and antigen presentation. IL-12 stimulates the proliferation and cytotoxicity of T cells and promotes Th1 responses by eliciting IFN-γ and TNF-α production. Using a variety of approaches, we have demonstrated that HSV-1-induced demyelination is caused by IL-12–dependent macrophage function. IL-12p70 is a heterodimeric glycoprotein produced by a variety of immunocompetent cells, including macrophages, monocytes, and DCs, as well as neutrophils and B cells. However, we have shown that DCs, B cells, and NK cells are not able to substitute for macrophage-derived IL-12p70. Our results also show that other APCs were not capable of compensating for the function of macrophages to eliminate autoreactive T cells after HSV-1 infection. Thus, our results are consistent with the notion that anti-inflammatory macrophage responses are critically important to enable CNS repair.

Our data also show that both IL-12p35−/− and IL-12p40−/− mice mimic macrophage-depleted wild-type mice on development of CNS demyelination after ocular HSV-1 infection. Demyelination was reversed when IL-12p35−/− mice were injected with IL-12p35 DNA or infected with an HSV-1 recombinant virus expressing the IL-12p35 gene. Similar results were obtained when IL-12p40−/− mice were immunized with IL-12p40 DNA or an IL-12p40-expressing virus. However, demyelination was not blocked when IL-12p35−/− mice were infected with an IL-12p40-expressing virus or when IL-12p40−/− mice were infected with IL-12p35–expressing HSV-1. These data suggest that both IL-12p35 and p40 subunits are required for protection from HSV-1–induced CNS demyelination, thereby supporting a role for macrophage-derived IL-12p70 in protection from autoimmunity. Although CNS demyelination in macrophage-depleted mice was blocked by IL-12p70 DNA injection, IL-12p35 or p40 DNA alone or together did not block CNS demyelination in wild-type macrophage-depleted mice, indicating that the blockade of autoimmunity is dependent on the IL-12p70 heterodimer.
EBI3−/− mice (necessary for IL-27 or IL-35 after heterodimerization with IL-27p28 or IL-12p35, respectively). Previous reports have indicated that IL-12 is involved in demyelination in the experimental autoimmune encephalomyelitis (EAE) animal model of MS. However, it was later shown that IL-12p35 was not required for the development of EAE. Using IL-23p19−/− mice, it was demonstrated that IL-23, but not IL-12, plays a critical role in the induction of EAE. Our results demonstrate that macrophages, by producing IL-12p70, but not IL-23, IL-27, or IL-35, play a central role in protection from virus-induced immunopathology.

T cells are considered to play a critical role in the induction of demyelination. In the present report, we have shown that demyelination is eliminated in macrophage-depleted mice by the codepletion of CD25, CD4, or FoxP3, but not CD8. We confirmed our depletion studies by adoptive transfer of CD4+ CD25+ to recipient SCID mice that were depleted of macrophages. Overall, our results suggest that a functional interplay exists whereby macrophages use IL-12p70 to counterbalance autoaggressive CD4+ CD25+ FoxP3+ T cells. It was previously shown that IL-12 transgenic mice develop neuroinflammatory disorders and demyelinating disease. However, in this study, we are supplementing the absence of IL-12 function of macrophages by using HSV-1 recombinant viruses or plasmid DNA expressing IL-12p70 (essentially an add-back assay). In line with the protective role of IL-12p70 we have shown in this study, recent reports have ruled out the IL-12/T3-1 axis as responsible for the development and progression of inflammatory and autoimmune diseases while implicating the IL-23/T3-17 axis as mediator of autoimmunity.61,62

There is disagreement in the literature regarding HSV-1 infection of astrocytes in vivo and in vitro. It has previously been shown that infection of astrocytes with HSV-1 does not promote GFAP synthesis, and astrocytes from rat, human, and mouse cultures are resistant to HSV infection. However, more recent reports have indicated that HSV-1 does infect astrocytes both in vivo and in vitro and that the virus productively replicates in these cells. Additionally, Itoyama et al. demonstrated that the loss of astrocytes precedes CNS demyelination, which contrasts strikingly with the reactive astrocitosis seen in other demyelinating lesions, such as acute EAE, progressive multifocal leukoencephalopathy, or acute MS. In this report, we demonstrate that the depletion of macrophages followed by infection with HSV-1 leads to significant upregulation of HSV-1 and GFAP antigens in the CNS. This suggests that a relationship exists between astrocytes and macrophages that protects against CNS demyelination. These data are supported by a recent report showing that loss or disruption of astrocyte function exacerbates autoimmune disease in the CNS. We propose that the activation of astrocytes in the absence of macrophage IL-12p70 supports autoreactive CD4+ Tregs, which induce CNS demyelination. Strikingly, both astrocytic activation and HSV-1 infection can be reversed by the expression of IL-12p70 DNA.

In summary, though Tregs have a well-known role in the preservation of immune tolerance and suppression of overly exuberant chronic immune responses and autoimmunity, our results suggest that the generation of naturally occurring Tregs in the absence of macrophage IL-12p70 can lead to virally induced immunopathology. Our study also suggests that this mechanism is likely to be nonredundant. Thus, preventing autoinflammation induced by CD4+ Tregs during viral infection represents an important pharmacotherapeutic target for CNS disease brought on by HSV-1 infection.

References

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