Use of cytokine immunotherapy to block CNS demyelination induced by a recombinant HSV-1 expressing IL-2

Mandana Zandian[1], **Kevin R. Mott**[1], **Sariah J Allen**[1], **Oana Dumitrascu**[1,2], **Jane Z Kuo**[2], and **Homayon Ghiasi**[1,*]

[1]Center for Neurobiology and Vaccine Development, Ophthalmology Research, Department of Surgery and Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA

[2]Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA

Abstract

We previously have described a model of MS in which constitutive expression of murine IL-2 by HSV-1 (HSV-IL-2) causes CNS demyelination in different strains of mice (Zandian et al 2009, IOVS, 50:3275). In the current study, we investigated whether this HSV-IL-2-induced demyelination can be blocked using recombinant viruses expressing different cytokines or by injection of plasmid DNA. We have found that co-infection of HSV-IL-2-infected mice with recombinant viruses expressing IL-12p35, IL-12p40, or IL-12p35 + IL-12p40 did not block the CNS demyelination, and that co-infection with a recombinant virus expressing IFN-γ exacerbated it. In contrast, co-infection with a recombinant virus expressing IL-4 reduced demyelination, while co-infection of HSV-IL-2 infected mice with a recombinant HSV-1 expressing the IL-12 heterodimer (HSV-IL-12p70) blocked the CNS demyelination in a dose-dependent manner. Similarly, injection of IL-12p70 DNA blocked HSV-IL-2-induced CNS demyelination in a dose-dependent manner and injection of IL-35 DNA significantly reduced CNS demyelination. Injection of mice with IL-12p35 DNA, IL-12p40 DNA, IL-12p35 + IL-12p40 DNA, or IL-23 DNA did not have any effect on HSV-IL-2-induced demyelination, while injection of IL-27 DNA increased the severity of the CNS demyelination in the HSV-IL-2 infected mice. This study demonstrates for the first time that IL-12p70 can block HSV-IL-2-induced CNS demyelination and that IL-35 can also reduce this demyelination, whereas IFN-γ and IL-27 exacerbated the demyelination in the CNS of the HSV-IL-2-infected mice. Our results suggest a potential role for IL-12p70 and IL-35 signaling in the inhibition of HSV-IL-2-induced immunopathology by preventing development of autoaggressive T cells.

Keywords

Recombinant virus; IL-12; IL-35; ocular; multiple sclerosis

*Corresponding author. Center for Neurobiology and Vaccine Development - D2024, Cedars-Sinai Burns and Allen Research Institute, 8700 Beverly Blvd., Los Angeles, CA 90048, PHONE: (310) 423-0593, FAX: (310) 423-0302, ghiasih@cshs.org.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Demyelinating diseases constitute a spectrum of immunopathologic syndromes in which, myelin, the fatty covering of nerve cell fibers in the brain, optic nerve, and spinal cord is destroyed 1. While the causes and pathogenesis of demyelination are unknown, one hypothesis is that autoimmunity to antigens of the CNS is triggered by environmental factors, such as viral infections, in genetically susceptible individuals and that the activated immune response leads to myelin destruction. One of the major diseases associated with degradation of the myelin sheath is multiple sclerosis (MS) 2, 3. An early manifestation of MS is visual disturbances and optic neuropathy due to demyelination of the optic nerve. This is a common cause of visual and neurological dysfunction in young adults diagnosed with MS 4–8. Thus, the occurrence of optic neuropathy can be used as a prognostic indicator early in the course of MS 4–8.

Epidemiologic studies have implicated interleukin (IL)-2 in the pathology of MS and patients with MS have elevated levels of IL-2 in their cerebrospinal fluid and sera 9–12. IL-2-deficient mice are more resistant to experimental autoimmune encephalitis (EAE) than their heterozygote and wild-type counterparts 13. To explore the possibility that IL-2 plays a role in the pathology of MS in conjunction with viral infection, we constructed a recombinant herpes simplex virus type 1 (HSV-1) that expresses murine IL-2 constitutively 14 as well as a panel of control recombinant viruses that express murine IL-4, interferon (IFN)-γ, IL-12p35, IL-12p40, or IL-12p70 15–18. Ocular infection of different strains of mice (*i.e.*, BALB/c, C57BL/6, SJL/6, and 129SVE) with the HSV-IL-2 virus results in demyelination of the optic nerves, the spinal cords, and brains of the infected mice as determined by histologic examination of tissues obtained at necropsy 19, 20. The HSV-IL-2-infected mice also develop optic neuropathy as determined by changes in the visual-evoked cortical potentials (VECPs) 20. Demyelination in mice is not detectable after infection with wild-type HSV-1 alone, HSV-IL-4 or HSV-IFN-γ viruses, which are identical to HSV-IL-2 except that they express IL-4 or IFN-γ instead of IL-2 19, 20.

We have previously found that both CD8+ and CD4+ T cells contribute to HSV-IL-2-induced CNS demyelination with CD8+ T cells being the primary inducers of demyelination (manuscript in preparation). In the adoptive transfer studies, all of the transferred T cells were positive for expression of FoxP3, irrespective of their CD25 status, and depletion of FoxP3 blocked CNS demyelination by HSV-IL-2. Furthermore, we have shown previously that HSV-IL-2 induced a T_H0 response in infected mice 19, which suggested that the presence of a T_H0 response may be a contributing factor to HSV-IL-2-induced demyelination. It is known that IL-12 favors the production of T_H1 cells through its ability to drive the differentiation of T_H0 cells into T_H1 cells 21–23, whereas IL-4 favors a T_H2 response 24, 25. Thus, it is possible that by manipulating the cytokine environment such that it can push the T_H0 responses toward a T_H1 and/or T_H2, demyelination may be prevented. We therefore undertook the current studies to determine whether IL-4, IFN-γ, IL-12p35, IL-12p40, IL-12p70, IL-23, IL-27, or IL-35 modulate or exacerbate the CNS demyelination that occurs in mice that are infected ocularly with HSV-IL-2.

RESULTS

Demyelination in HSV-IL-2-infected mice is not blocked by co-infection with HSV-IL-4 virus

We have shown previously that ocular infection of mice with HSV-IL-2 recombinant virus induces demyelination of CNS in different strains of mouse, including BALB/c and C57BL/6 mice [20]. Other investigators have reported that a recombinant HSV-1 expressing IL-4 protected rhesus monkeys [26] and mice [27, 28] from autoimmune encephalomyelitis. Thus, to determine if HSV-IL-2-induced CNS demyelination can be blocked by IL-4, BALB/c mice were infected ocularly with equal amounts (2×10^5 per eye) of HSV-IL-2 and HSV-IL-4, or with HSV-IL-2 alone or HSV-IL-4 alone. At day 14 post infection, mice were sacrificed and the brain, spinal cord, and optic nerves collected, post-fixed and stained with the myelin stain, LFB. Representative photomicrographs of the LFB-stained sections are shown in Figure 1A. The results indicated that demyelination was not inhibited in the spinal cord or optic nerves of the mice that were co-infected with HSV-IL-2 and HSV-IL-4 at a 1:1 ratio (Fig. 1A, Left panels). Similarly, demyelination was also detected in brains of HSV-IL-2 infected mice (not shown). No demyelination was detected in the CNS of mice infected with HSV-IL-4 alone (Fig. 1A, Right Panels).

To determine if higher levels of HSV-IL-4 were capable of affecting HSV-IL-2-induced CNS demyelination, groups of BALB/c mice were subjected to the same experimental protocol except that they were co-infected with HSV-IL-4 at a dose (2×10^7 PFU/eye) that was 100-fold higher than the dose of HSV-IL-2 (2×10^5 PFU/eye) used. Demyelination was not blocked in the brains, spinal cords, or optic nerves of the mice that were co-infected with HSV-IL-2 and HSV-IL-4 at a 1:100 ratio (Fig. 1A, Middle panels). Similar results were obtained when female C57BL/6 were co-infected with HSV-IL-2 and HSV-IL-4 recombinant viruses (data not shown). Collectively, these results indicate that IL-4 does not protect against IL-2-induced CNS demyelination in this mouse model.

Demyelination in HSV-IL-2-infected mice is not blocked following co-infection with HSV-IFN-γ virus

The roles of IFN-γ in MS remain controversial and a clinical trial of rIFN-γ for MS was discontinued prematurely due to worsening of disease [29]. To evaluate the effects of HSV-IFN-γ on CNS demyelination induced by HSV-IL-2, BALB/c or C57BL/6 mice were infected ocularly with 2×10^5 per eye of HSV-IL-2 and 2×10^5 per eye of HSV-IFN-γ, or HSV-IFN-γ alone. At day 14 post infection, the mice were sacrificed and the brains, spinal cords, and optic nerves were stained with LFB. Representative photomicrographs of the stained sections of spinal cords and optic nerves are shown in Figure 1B. Demyelination was not inhibited in the spinal cords or optic nerves of the mice co-infected with the HSV-IL-2 plus HSV-IFN-γ at a 1:1 ratio (Fig. 1B, Left Panels, 1:1). Similarly, demyelination was also detected in brains of HSV-IL-2 infected mice (not shown). To determine if higher doses of HSV-IFN-γ were able to block HSV-IL-2-induced CNS demyelination additional mice were subjected to the same experimental protocol except that they were co-infected with 100-fold higher dose of HSV-IFN-γ (2×10^7 PFU/eye) relative to HSV-IL-2 (2×10^5 PFU/eye). Demyelination was not blocked in the brain, spinal cords, or optic nerves of the mice co-infected with a 1 to 100 ratio of HSV-IL-2 to HSV-IFN-γ (Fig. 1A, Middle panels). These

results suggest that HSV-IFN-γ, even at a 100-fold higher dose, does not block HSV-IL-2-induced CNS demyelination in either mouse strain tested.

Demyelination in HSV-IL-2-infected mice is not blocked by co-infection with HSV-IL-12p35, HSV-IL-12p40, or HSV-IL-12p35 + HSV-IL-12p40

In preliminary studies using the HSV-1 recombinant viruses expressing murine IL-12p35 and IL-12p40 that we have constructed 30, we observed that infection of macrophages with HSV-IL-2, but not wild-type HSV-1 or recombinant HSV-1 expressing IL-4 or IFN-γ, altered the ratio of the IL-12p35 and IL-12p40 transcripts (unpublished observation). We therefore tested the possibility that co-infection of HSV-IL-2-infected mice with HSV-IL-12p35, HSV-IL-12p40, or HSV-IL-12p35 + HSV-IL12p40 may prevent demyelination. As a control some mice were co-infected with HSV-IL-2 and wild-type (wt) virus. BALB/c mice were infected with HSV-IL-2 + HSV-IL-12p35, HSV-IL-2 + HSV-IL-12p40, HSV-IL-2 + HSV-IL-12p35 + HSV-IL12p40, or HSV-IL-2 + wt at 2×10^5 PFU/eye of each virus. As shown in Figure 1C, at day 14 post infection, demyelination was observed in the spinal cords and optic nerves of all mice infected ocularly with HSV-IL-2 + HSV-IL-12p35, HSV-IL-2 + HSV-IL-12p40, HSV-IL-2 + HSV-IL-12p35 + HSV-IL12p40, or HSV-IL-2 + wt and similar results were obtained on analysis of the brains of the infected mice (data not shown). None of these recombinant viruses (HSV-IL-12p35, HSV-IL-12p40, or HSV-IL-12p35 + HSV-IL12p40) protected the mice from HSV-IL-2-induced demyelination, even when a higher dose (2×10^7 PFU/eye) of the recombinant viruses were used (data not shown). Moreover, multiple intraperitoneal injections of mice with the recombinant viruses did not inhibit CNS demyelination from HSV-IL-2 infection (data not shown). The biological activity characteristic of the IL-12 heterodimer cannot be detected in the absence of either subunit 33, 34 as the individual subunits do not exhibit IL-12 heterodimer activity by themselves 35, 36. Thus, our observation of lack of positive effect by the individual subunits is consistent with the literature. The absence of a protective effect by these recombinant viruses against HSV-IL-2-induced CNS demyelination suggests that these viruses do not promote the formation of the IL-12 heterodimer *in vivo*.

Demyelination in HSV-IL-2-infected mice can be blocked by co-infection with the IL-12 heterodimer in a dose-dependent manner

In contrast to HSV-IL-12p35 or HSV-IL-12p40, a recombinant HSV-1 that expresses both IL-12p35 and IL-12p40 in-frame as a heterodimer (HSV-IL-12p70, also called M002) has biological activity *in vivo* 18, 37. Therefore, we used an HSV-IL-12p70 recombinant virus to determine whether co-infection of mice with this virus would prevent HSV-IL-2-induced demyelination in infected mice. BALB/c mice were infected with HSV-IL-2 and HSV-IL-12p70 at a 1:1, 1:10, or 1:100 ratio and demyelination in the optic nerves and spinal cords of the infected mice was evaluated on day 14 post infection. As expected, infection of the mice with HSV-IL-12p70 alone did not induce demyelination (Fig. 2, IL-12p70, right panels at 2×10^7 PFU/eye). Demyelination was observed in the optic nerves and spinal cords of all BALB/c mice infected with HSV-IL-2 and HSV-IL-12p70 at a 1:1 ratio (Fig. 2, 1:1 column, Table 1), while only 1 mouse showed demyelination in its spinal cord but not brain or optic nerve (Table 1). In marked contrast, none of the mice infected with a 1:100 ratio of HSV-IL-2 to HSV-IL12p70 showed any signs of demyelination (Fig. 2, 1:100 column,

Table 1). These results suggest that the IL-12 heterodimer can inhibit HSV-IL-2-induced CNS demyelination in ocularly infected mice in a dose-dependent manner, and high enough concentrations can block disease pathology in its entirety.

Demyelination in HSV-IL-2-infected mice can also be blocked by injection of IL-12p70 DNA

To further explore the possibility that IL-12p70 can block HSV-IL-2 induced demyelination, we treated HSV-IL-2-infected mice with IL-12p70 DNA. It has previously been shown that DNA vaccine–encoded immunogen is produced by dendritic cells and macrophages residing at the site of inoculation with subsequent elicitation of immune response either by directly transfected antigen-presenting cells (APC) or via cross presentation of antigen expressed from transfected somatic cells by APCs 31. Thus, BALB/c mice were injected with IL-12p70 DNA or vector DNA 1X, 2X, or 3X and infected with HSV-IL-2 4h after the first, second, or third injection. Demyelination in the optic nerves, spinal cords, and brains of infected mice were measured on day 14 post-infection and the data are summarized in Table 1. Eighty percent (4 out of 5) mice injected once with IL-12p70 DNA were protected from HSV-IL-2-induced CNS demyelination and similar results were obtained when the mice were injected twice and infected with HSV-IL-2 (Table 1). Demyelination was not observed in the optic nerves, spinal cords, or brains of the BALB/c mice that had been injected with IL-12p70 DNA three times and infected with HSV-IL-2 (Table 1). As expected mice injected with vector DNA and infected with HSV-IL-2 exhibited demyelination in their optic nerves, spinal cords, and brains (not shown). These experiments also were carried out using C57BL/6 mice and similar results were obtained (data not shown). Collectively, these results suggest that IL-12p70 DNA can inhibit HSV-IL-2-induced CNS demyelination in ocularly infected mice in a dose-dependent manner as does HSV-IL-12p70 virus.

Roles of other IL-12 family members in blocking CNS demyelination by HSV-IL-2

To determine if any other members of the IL-12 family can alter demyelination, BALB/c mice were injected with IL-23 DNA, IL-27 DNA, or IL-35 DNA, either once or three times before ocular infection with HSV-IL-2. Demyelination in the optical nerves, spinal cord, and brain of infected mice was evaluated on day 14 post-infection. Demyelination was detected in the optical nerves, spinal cords, and brain of all BALB/c mice injected with IL-23 DNA (Fig. 3 and Table 2, IL-23) and IL-27 DNA (Fig. 3 and Table 2, IL-27,). In contrast, demyelination was detected in the spinal cord and brain but not optic nerves of one of the mice injected once with IL-35 DNA, while after three injections demyelination was only detected in the spinal cord of the injected mice (Fig. 3 and Table 2 IL-35). Thus, our results suggest that IL-35 DNA reduced the severity of demyelination in injected mice, whereas neither IL-23 nor IL-27 blocked HSV-IL-2-induced CNS demyelination in infected mice.

Quantitation of demyelination severity in infected mice

We observed that the severity of the HSV-IL-2-induced demyelination appeared to be greater in the presence of HSV-IFN-γ (Fig. 1B) than in the presence of HSV-IL-4 (Fig. 1A) or parental virus. To determine if more plaques are present in the CNS of HSV-IL-2 + HSV-IFN-γ-infected mice as compared with HSV-IL-2 alone, HSV-IL-2 + HSV-IL-4, or HSV-IL-2 + wt HSV-1, we counted the number and size of the observed plaques in the spinal cords of infected mice. The data are shown as the area of demyeli nation per total stained

sections in Figure 4. The area of demyelination in HSV-IL-2 + wt HSV-1-infected mice was similar to mice that were infected with HSV-IL-2 alone (at a 1:1 ratio) (Fig. 4). Also, the level of demyelination was not increased when mice were infected with a 100-fold higher dose of wt HSV-1 (ratio of wt to HSV-IL-2 = 1:100) (Fig. 4). However, the area of demyelination in HSV-IL-2 + HSV-IL-4-infected mice was significantly lower than mice infected with HSV-IL-2 alone (at a 1:1 ratio) (Fig. 4). The level of demyelination was not altered when mice were infected with a 100-fold higher dose of HSV-IL-4 (Fig. 4). In contrast to co-infection with HSV-IL-4 recombinant virus, the area of demyelination was greater in the CNS of mice co-infected with HSV-IL-2 + HSV-IFN-γ (at a 1:1 ratio) than in the CNS of mice infected with HSV-IL-2 alone, HSV-IL-2 + HSV-IL-4, or HSV-IL-2 + wt HSV-1 (Fig. 4). However, the area of demyelination in HSV-IL-2 + HSV-IFN-γ infected mice was not increased when mice were infected with a 100-fold higher dose of HSV-IFN-γ (ratio of HSV-IFN-γ to HSV-IL-2 = 1:100). Furthermore, co-infection of HSV-IL-2 infected mice with HSV-IL-12p70 virus reduced severity of demyelination in co-infected mice (at a 1:1 ratio) than in the CNS of mice infected with HSV-IL-2 alone (Fig. 4), while no demyelination was detected in HSV-IL-2 + HSV-IL-12p70 infected mice when mice were infected with a 100-fold higher dose of HSV-IL-12p70 (Fig. 4). Thus, our results suggest that co-infection of HSV-IL-2-infected mice with HSV-IFN-γ increases CNS demyelination in a dose-independent manner, IL-4 affect the IL-2-induced demyelination by decreasing its severity, wt HSV-1 does not affect the HSV-IL-2-induced demyelination, neither increasing nor decreasing its severity, while HSV-IL-12p70 affected the HSV-IL-2-induced demyelination by decreasing its severity in a dose-dependent manner.

HSV-IL-2 infection of mice that were injected with IL-12, IL-23, IL-27, or IL-35 DNA also produced different patterns of demyelination (Fig. 3). We quantitated the area of demyelination (the number and size of observed plaques in the spinal cords) in mice injected either once or three times with each of these cytokine DNAs. As shown in Figure 5 (one injection), mice injected once with either IL-12, IL-23, or IL-35 DNA had significantly smaller areas of demyelination than mice injected with vector control or mice injected with IL-27. A greater area of demyelination was detected in the CNS of mice injected with IL-27 once than other groups (Fig. 5, One Injection). No demyelination was detected in the CNS of mice injected three times with IL-12 DNA, and no changes were detected in the level of demyelination in mice injected three times with either IL-23 or IL-23 (Fig. 5, Three injections). Similarly, a smaller area of demyelination was detected in the CNS of mice injected three times with IL-35 DNA (Fig. 5). Thus, our results suggest that injection of mice with IL-27 increased the severity of CNS demyelination, whereas IL-12, IL-23 and IL-35 reduced the severity of CNS demyelination with IL-12 and IL-35 playing a more important role in reducing or eliminating demyelination, respectively. IL-12 reduced the severity of demyelination in a dose-dependent manner, while reduction in demyelination in IL-23 and IL-35 injected mice was dose-independent as was the increase in the severity of demyelination in IL-27 DNA injected mice.

Our Co-infection results with HSV-IL-12p70 and DNA injection with IL-12 DNA, IL-23 DNA, and IL-35 DNA showed significant reduction or elimination of HSV-IL-2 induced CNS demyelination in infected mice. Since IL-12p35 and/or IL-12p40 are components of IL-12p70, IL-23, IL-27, and IL-35, to determine if IL-12p35 or IL-12p40 individually or in

combination would alter the severity of HSV-IL-2 induced demyelination, mice were co-infected with HSV-IL-2 + HSV-IL12p35, HSV-IL-2 + HSV-IL12p40, or HSV-IL-2 + HSV-IL12p35 + HSV-IL-12p40. Similarly, some mice were injected with vector DNA, IL-12p35 DNA, IL-12p40 DNA, or IL-12p35 + IL-12p40 DNA and injected mice were ocularly infected with HSV-IL-2. We counted the number and size of the observed plaques in the spinal cords of infected mice. The data are shown as the area of demyelination per total stained sections in Figure 6. The area of demyelination in HSV-IL-2 + HSV-IL-12p35, HSV-IL-2 + HSV-IL12p40, or HSV-IL-2 + HSV-IL-12p35 + HSV-IL-12p40 infected mice was similar to mice that were infected with HSV-IL-2 alone (Fig. 6, Left Panel). Similarly, no increase in demyelination was observed in mice injected with IL-12p35 DNA, IL-12p40 DNA, or IL-12p35 + IL-12p40 DNA and infected with HSV-IL-2 compared with vector DNA (not shown). Thus, our results suggest that in contrast to heterodimer forms of IL-12, IL-23, or IL-35, the individual forms of IL-12p35 or IL-12p40 do not affect the HSV-IL-2-induced demyelination, neither increasing nor decreasing its severity.

DISCUSSION

We have reported previously that HSV-IL-2 causes demyelination in periventricular white matter, brain stem, and spinal cord white matter infected mice, whereas wild-type HSV-1 strain McKrae, HSV-1 strain KOS, parental dLAT2903, or recombinants HSV-1 expressing IL-4 or IFN-γ do not 19, 20. To further confirm the importance of IL-2 in our HSV-IL-2 model of MS, we looked at the possibility of blocking MS-like symptoms in HSV-IL-2 infected mice by co-infection of HSV-IL-2 infected mice with recombinant viruses expressing T_H1 or T_H2 cytokines. The results of these co-infection experiments were then confirmed by injecting mice with plasmid DNAs expressing the genes of interest.

In contrast to IL-2 9–12, the levels of IL-4 have been reported to be lower than normal in the sera of patients with MS 32, 33. Recombinant IL-4 (rIL-4) has been shown to diminish demyelination and improve the clinical course of mice with EAE, apparently by influencing the development of the T cells to favor protective, rather than harmful, effects 27. When fed to mice, retinoids, which increase the levels of IL-4 and TGF-α, also improve the course of EAE 34. It also has been shown that CNS gene therapy with HSV-1 vectors expressing IL-4 protected rhesus monkeys 26 and mice 29, 30 from autoimmune encephalomyelitis, suggesting that vectors containing anti-inflammatory cytokine genes may have a therapeutic potential for MS. Similar to these previous reports, we significantly reduced but could not block HSV-IL-2 CNS demyelination in mice co-infected with HSV-IL-4, independent of dose of HSV-IL-4 relative to that of HSV-IL-2.

In MS, a clinical trial of rIFN-γ was discontinued prematurely due to worsening of disease 29. Similarly, in this study we have shown that the CNS demyelination observed in HSV-IL-2-infected mice was exacerbated on co-infection with HSV-IFN-γ recombinant virus in dose-independent manner even though mice infected with HSV-IFN-γ recombinant virus alone do not develop CNS demyelination 19, 20. It has been reported that in EAE induced by $CD8^+$ T cells, administration of anti–IFN-γ antibody reduces the severity of the disease, whereas in EAE induced by $CD4^+$ T cells, administration of anti–IFN-γ actually aggravates the disease 35. In our model, both $CD4^+$ and $CD8^+$ T cells contribute to CNS demyelination

with CD8^{+} T cells playing a more prominent role then the CD4^{+} T cells (Manuscript in preparation). Thus, the greater demyelination we observed in the presence of HSV-IFN-γ recombinant virus could be due in a shift in the T_H1 arm of the immune response in the infected mice.

Previously, we have shown that the infection of mice with HSV-IL-2 induces a $T_H1 + T_H2$ response 19. IL-12 is known to play a major role in regulating the balance of the T_H1 and T_H2 responses 36, 37. It has been reported that IL-12 is a critical cytokine in the pathogenesis of EAE 38 and subsequent studies showed that the IL-12p40 component of IL-12 is involved in EAE-induced CNS pathology but this effect is mediated by the binding of the IL-12p40 to IL-23p19 rather than its binding to IL-12p35 39, 40. Thus, an imbalance of IL-12p40 and IL-12p35 in the HSV-IL-2-infected mice may be responsible for a shift to a $T_H1 + T_H2$ pattern of cytokine responses as we reported previously 19. Recently, we have shown that HSV-IL-2 infection of macrophages alters the balance of IL-12p35 and IL12p40 transcripts, which could alter the development of the T_H1 response in the HSV-IL-2 infected mice. This possibility is supported by our current results that showed that co-infection of mice with a recombinant HSV-1 expressing IL-12p70 blocked HSV-IL-2-induced demyelination in a dose-dependent manner. At a 1:1 or 1:10 ratio of HSV-IL-2 to HSV-IL-12p70, 80% of the co-infected mice were protected from demyelination, and at a ratio of 1:100, none of the infected mice developed CNS demyelination. Similarly, when mice were injected either one or twice with IL-12p70 DNA and then infected with HSV-IL-2, 80% of the mice were protected from CNS demyelination, while 100% of the mice injected three times with IL-12p70 DNA were protected from demyelination. However, co-infection of mice with HSV-IL-2 + HSV-IL-12p35, HSV-IL-2 + IL-12p40, or HSV-IL-2 + HSV-IL-12p35 + IL-12p40 did not block demyelination. Similarly injection of IL-12p35 DNA alone, IL-12p40 DNA alone, or a mixture did not block demyelination in HSV-IL-2 infected mice.

The IL-12 family members are heterodimeric complexes, composed of an α-subunit (p35, p19, or p28) and a β-subunit {p40 and Epstein–Barr virus-induced gene 3 (EBI3)} 41. The p40 subunit covalently binds to the p35 and p19 chains to form IL-12p70 and IL-23, respectively, while EBI3 binds noncovalently to p28 and p35 to form IL-27 and IL-35, respectively 42. In this study, we have shown that in contrast to IL-12p70, injection of mice with IL-23 or IL-27 did not protect injected mice from HSV-IL-2-induced demyelination, while IL-35 significantly reduced CNS demyelination. In this study, we found that injection of mice with IL-27, but not IL-23 or IL-35, exacerbated the severity of demyelination in HSV-IL-2 infected mice. IL-27 is a pleiotropic cytokine with divergent effects. It has been reported that IL-27 negatively regulates T_H2 responses following intestinal nematode infection 43 and asthma 44 and that IL-27 neutralization protects mice against lethal septic peritonitis by enhancing the neutrophil function 45. IL-27 also was shown to inhibit peripheral conversion of T cells into inducible Tregs 46. The IL-27 inhibitory effect on T_H2 and cytokine production is due to downregulation of GATA-3 and upregulation of T-bet expression 47. Thus, in contrast to other members of IL-12 family, IL-27 seems to have a negative effect on T_H-development and cytokine production. Therefore, the higher demyelination in IL-27 injected mice could be due to a shift in T_H1/T_H2 responses. Some studies suggested that the IL-23/T_H17 pathway plays a crucial role in the development of

autoimmunity 48. However, our results rule out the involvement of IL-23 in HSV-IL-2-induced demyelination. In this study, we have shown that IL-35 blocks demyelination in optic nerves and brain of HSV-IL-2-infected mice and significantly reduces demyelination in SC of infected mice. Previously it was shown that IL-35 displays immunosuppressive properties 49, 50.

The results indicate that: (1) The development of the demyelinated lesions observed after infection of mice with HSV-IL-2 is blocked in a dose-dependent manner on co-infection with IL-12p70 recombinant virus or injection of IL-12p70 DNA. Demyelination also was significantly reduced on injection of IL-35 DNA injection; (2) The HSV-IL-2-induced demyelination was reduced by co-infection with recombinant virus expressing IL-4 or injection of IL-23 DNA; (3) The HSV-IL-2-induced demyelination was not altered by co-infection with recombinant viruses expressing IL-12p35, IL-12p40 or both; and (4) The HSV-IL-2-induced demyelination was enhanced by co-infection with IFN-γ recombinant virus or injection of IL-27 DNA. In summary, our results suggest a potential role for IL-12p70 and IL-35 in the inhibition of HSV-IL-2-induced immunopathology.

MATERIALS AND METHODS

Mice, viruses, and cells

Six-week old female BALB/c or C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were handled in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research under an approved IACUC protocol.

Plaque-purified HSV-1 strains, McKrae or KOS (wild type) and HSV-1 recombinant viruses expressing IL-2, IL-4, IFN-γ, IL-12p35, IL-12p40, or IL-12p70 (HSV-IL-2, HSV-IL-4, HSV-IFN-γ, HSV-IL-12p35, HSV-IL-12p40, and HSV-IL-12p70) were grown in rabbit skin (RS) cell monolayers in minimal essential medium (MEM) containing 5% fetal calf serum (FCS), as described previously 14, 15, 17. McKrae and HSV-IFN-γ viruses are virulent at an infectious dose of 2×10^5 plaque forming units (PFU)/eye, whereas the other viruses used in this study are attenuated. Previously we have shown that in tissue culture, the replication of the HSV-IL-2 was two-logs lower than the wild-type virus at a low MOI 14, 15, 17. Addition of recombinant anti-IL-2 polyclonal antibody markedly enhanced HSV-IL-2 replication in tissue culture. In the 7-day period after ocular infection of BALB/c mice, the replication of HSV-IL-2 was significantly lower than that of wild-type virus in tear cultures, whole eyes, and brain, but was equivalent to wild-type replication in the trigeminal ganglia.

Ocular infection

Mice were infected ocularly with 2×10^5 PFU of McKrae, KOS, recombinant HSV-IL-2, HSV-IL-4, HSV-IFN-γ, HSV-IL-12p35, or HSV-IL-12p40 per eye. Some mice were co-infected with 2×10^5 PFU/eye of HSV-IL-2 and 2×10^5, 2×10^6, or 2×10^7 PFU of HSV-IL-4, HSV-IFN-γ, HSV-IL-12p70, HSV-IL-12p35, HSV-12p40, or HSV-IL-12p35 + HSV-IL-12p40 virus. Each virus was suspended in 5 μl of tissue culture media and administered as an eye drop.

Preparation of optic nerve, spinal cord, and brain for pathologic analysis

The optic nerves, spinal cords, and brains of the experimental and control mice were removed at necropsy on day 14 post-infection. They were then snap-frozen in an isopentane-liquid nitrogen bath and stored at −80°C. Transverse sections of each tissue, 8–10 μm thick, were cut, air-dried overnight, and fixed in acetone for 3 min at 25°C 51. Demyelination in each section was confirmed by monitoring adjacent sections.

Analysis of demyelination using Luxol Fast Blue (LFB) staining

The presence or absence of demyelination in the optic nerves, spinal cords, and brains of infected mice was evaluated using LFB staining of formalin-fixed sections as we described previously 19. The number of plaques, size of plaques, and shape of plaques on multiple fields were evaluated by investigators, who were blinded to the treatment groups, using serial sections of CNS tissues. The amount of myelin loss in the stained sections of spinal cords, optic nerves, and brains also was measured using an image analysis system coupled to a microscope. The area of demylinated lesion (clear) to the blue (normal) were measured using 150 random sections from brain, spinal cords, and optic nerves of each animal. The percentage of myelin loss was calculated by dividing the lesion size into the total area for each section.

DNA injection

The complete open-reading frames (ORF) for IL-12p70, IL-23, IL-27, and IL-35 (pORF-mIL12, pORF-mIL23, pORF-mIL27, pORF-mIL35) were purchased from InvivoGen (San Diego, CA), while the ORF for IL-12p35 and IL-12p40 was inserted into pVR1055 as we described previously 52. Plasmids DNA were purified using a cesium chloride gradient. In each experiment, five mice per group were injected intramuscularly (in the quadriceps) using a 27 gauge needle with 100 μg of cesium chloride-purified DNA in a total volume of 50 μl of PBS once (4 h before ocular infection), twice (7 days and 4 h before ocular infection), or three times (14, days, 7 days and 4 hr before ocular infection). As a negative control, we used mock-treated mice that were similarly injected with vector DNA alone.

Statistical analysis

Fisher's exact tests were performed using the computer program Instat (GraphPad, San Diego) to compare demyelination in infected mice and in control groups. Results were considered statistically significant when the P value was <0.05.

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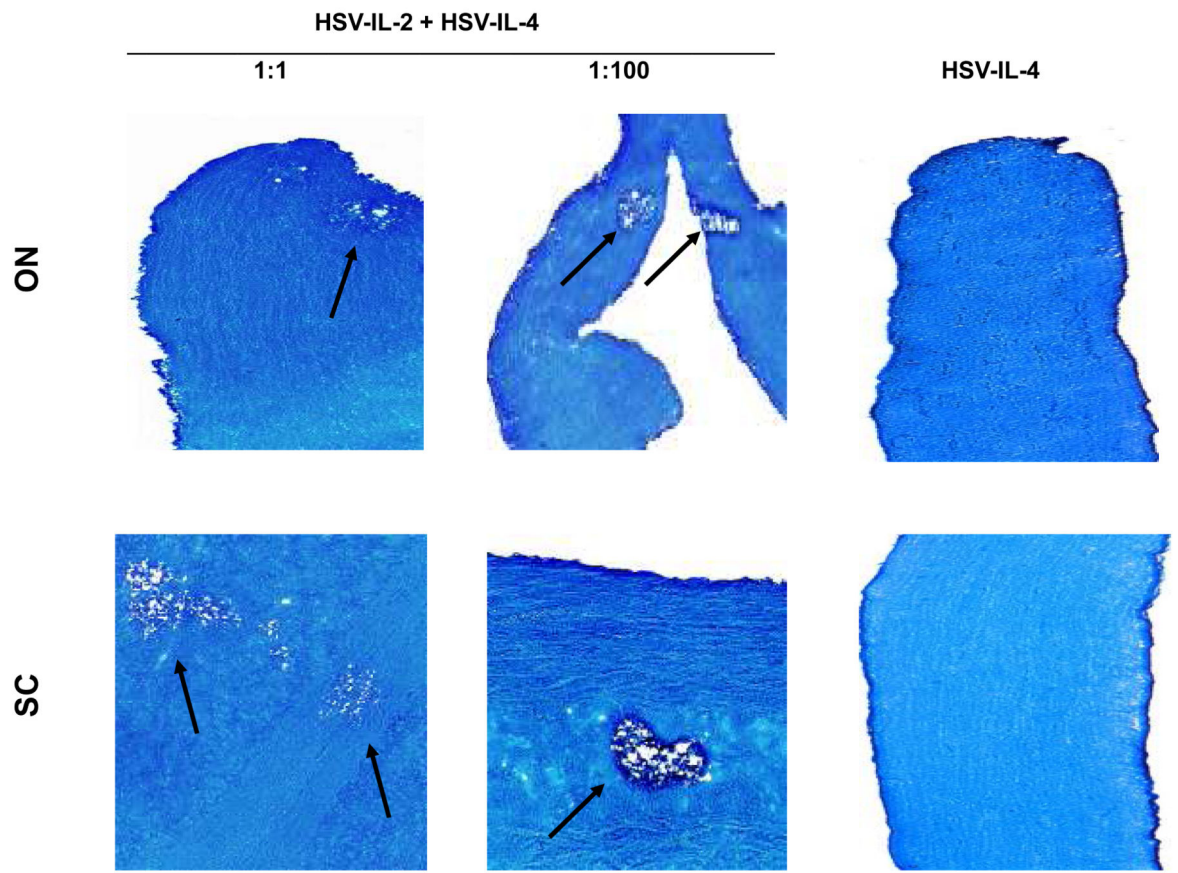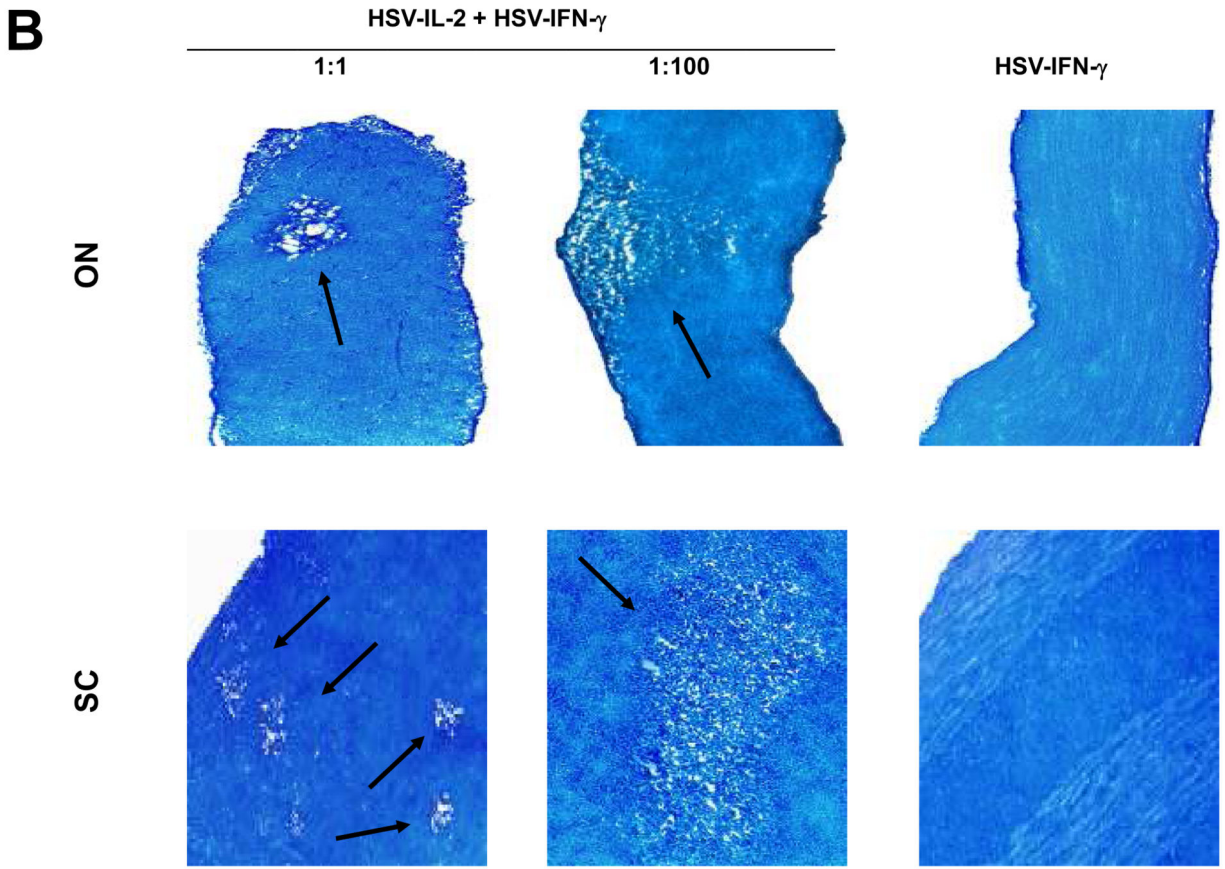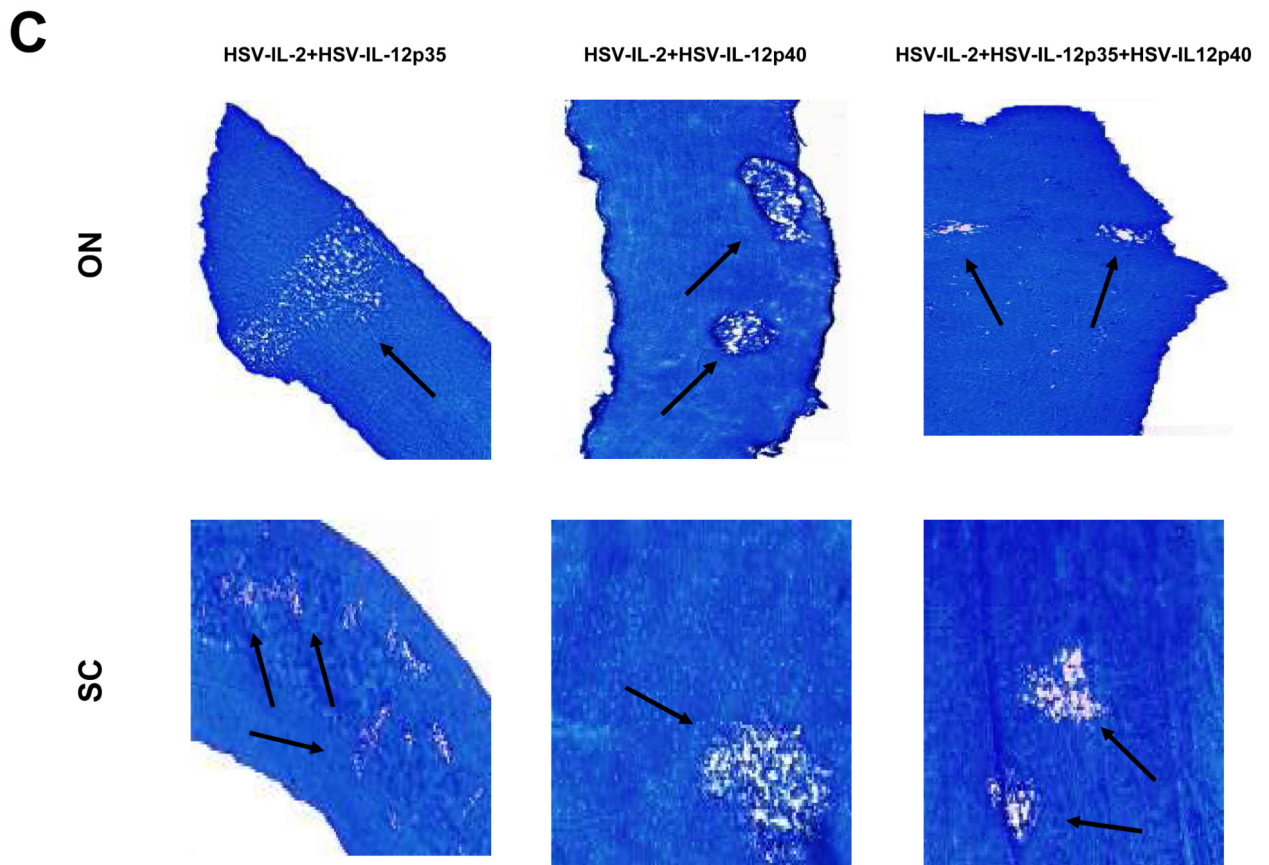
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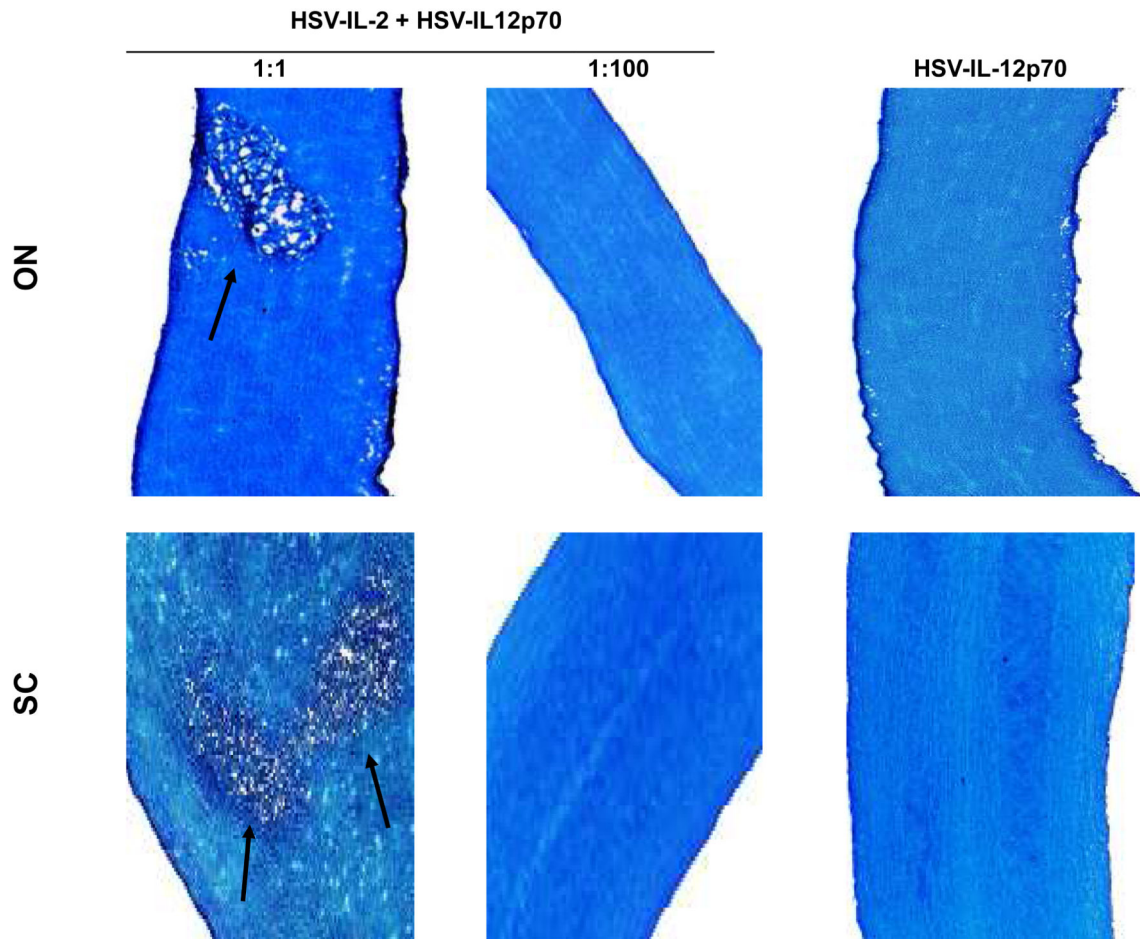HSV-IL-2+HSV-IL-12p35   HSV-IL-2+HSV-IL-12p40   HSV-IL-2+HSV-IL-12p35+HSV-IL12p40

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**Fig. 1. Effects of IL-4, IFN-γ, IL-12p35, and IL-12p40 on HSV-IL-2-induced CNS demyelination**
Mice were co-infected ocularly with HSV-IL-2 + HSV-IL-4 or HSV-IL-2 + HSV-IFN-γ at
1:1 or 1:100 ratios as described in Materials and Methods. In addition, some mice were co-
infected ocularly with HSV-IL-2 + HSV-IL-12p35, HSV-IL-2 + HSV-IL-12p40, or HSV-
IL-2 + HSV-IL-12p35 + HSV-IL-12p40 as a 1:1 ratio as described in Materials and
Methods. Control mice were infected with 2×10^7 PFU/eye of HSV-IL-4 or HSV-IFN-γ. On
day 14 post infection, optic nerves and spinal cords were collected, fixed, sectioned, and
stained with Luxol fast blue (LFB). Representative photomicrographs are shown. Arrows
indicate areas of demyelination. 10X direct magnification. Panels: (A) Effects of IL-4 on
HSV-IL-2-induced CNS demyelination; (B) Effects of IFN-γ on HSV-IL-2-induced CNS
demyelination; and (C) Effects of IL-12p35 and IL-12p40 on HSV-IL-2-induced CNS
demyelination.

**Fig. 2. Blocking of demyelination in HSV-IL-2 infected mice by IL-12p70 HSV-1 recombinant virus**

BALB/c mice were co-infected with HSV-IL-2 + HSV-IL-12p70 at 1:1, 1:10, or 1:100 ratios of HSV-IL-2 to HSV-IL-12p70. On day 14 post-infection, optic nerves and spinal cords were collected, fixed, sectioned, and stained with LFB. Representative photomicrographs are shown. Arrows indicate areas of demyelination. 10X direct magnification.
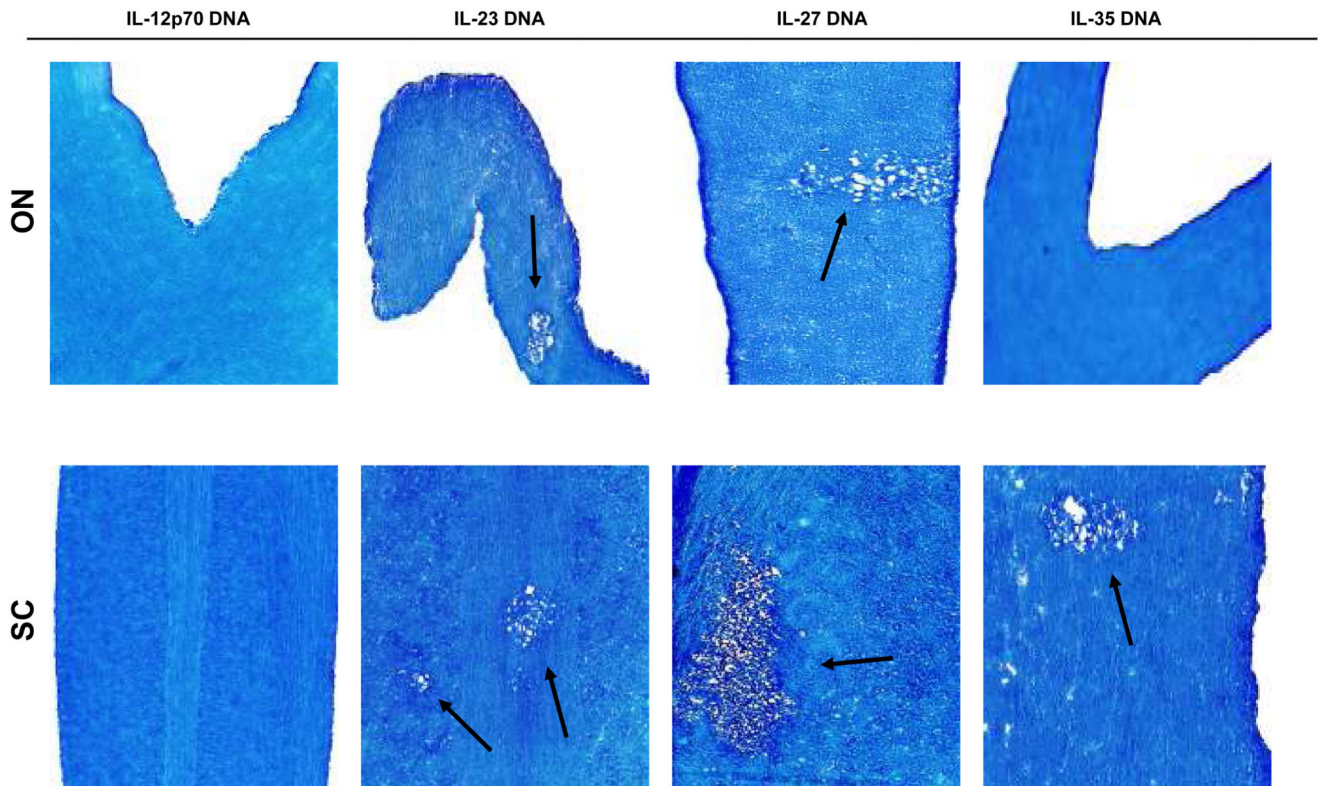
Fig. 3. Effects of IL-12 family members on HSV-IL-2-induced demyelination
BALB/c mice were injected intramuscularly 3X with IL-12p70 DNA, IL-23 DNA, IL-27
DNA, IL-35 DNA as described in Materials and Methods. Four hours after the third DNA
injection, mice were infected ocularly with HSV-IL-2. On day 14 post infection, optic
nerves and spinal cords were collected, fixed, sectioned, and stained with LFB.
Representative photomicrographs are shown. Arrows indicate areas of demyelination. 10X
direct magnification.
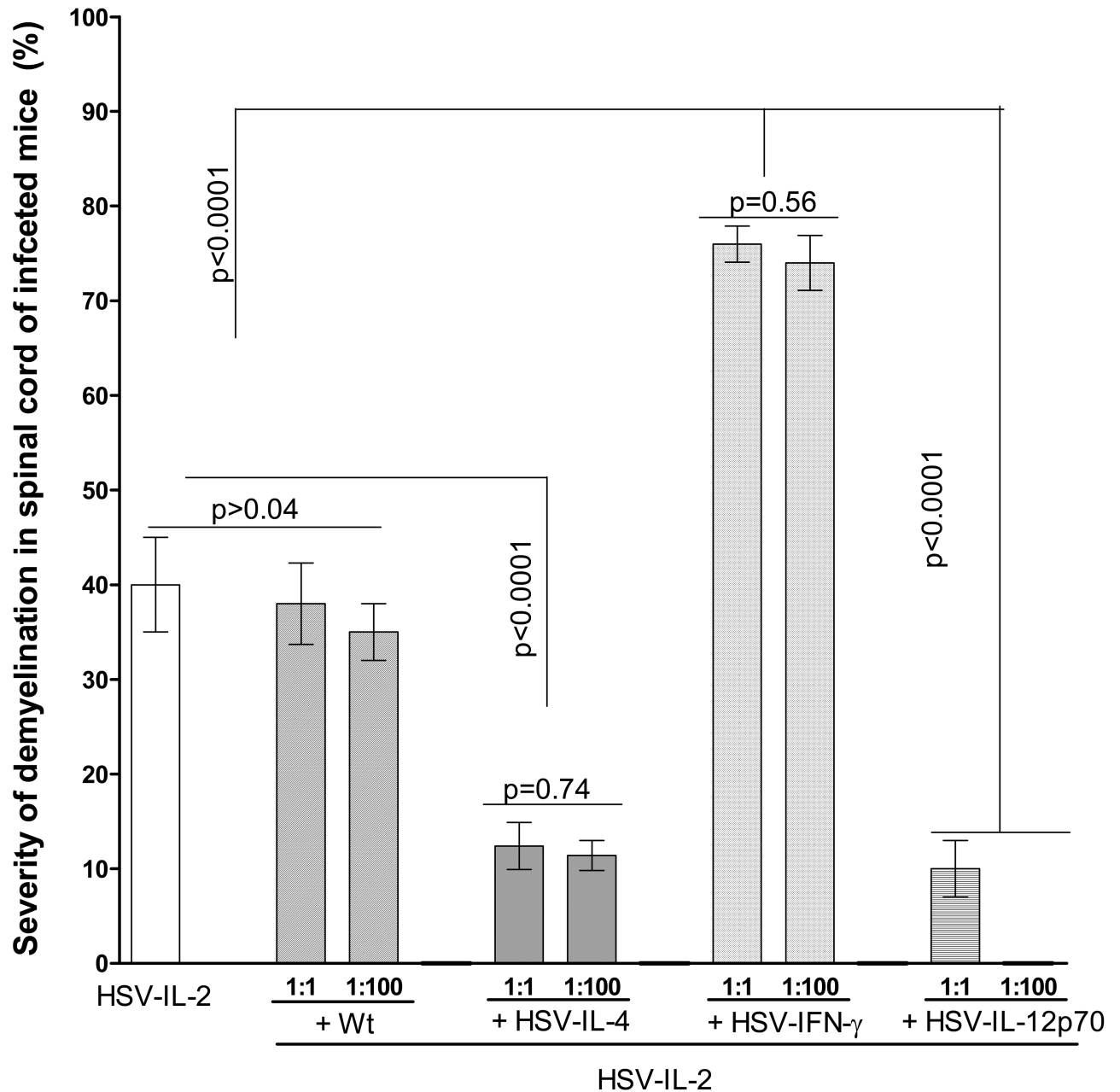
Fig. 4. Severity of CNS demyelination in mice co-infected with HSV-IL-2 and recombinant viruses expressing different cytokine genes
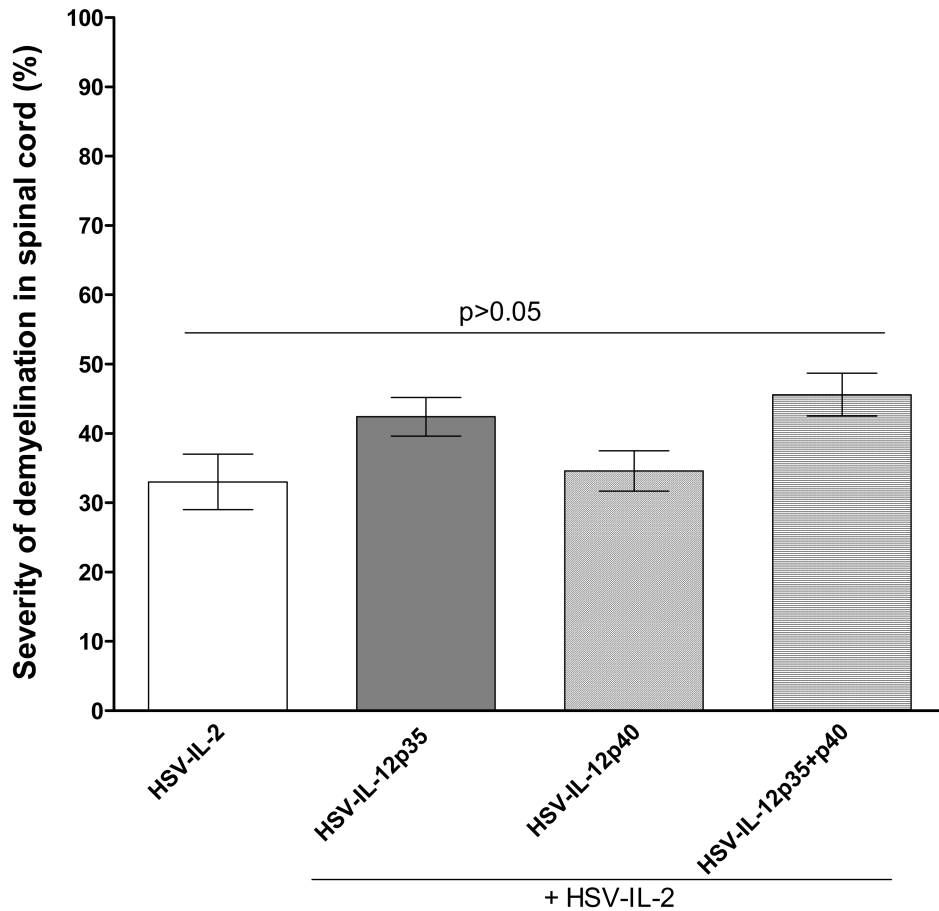
The spinal cords of each of the 5 animals in each treatment group described in Figures 1, 2, 3, and 4 were sectioned, every fourth section was stained, and the size of the demyelination plaques in the entire sections of the spinal cords was determined as described in Materials and Methods. Data are presented as the percentage of the demyelination area in each section per total sections stained using a total of 150 sections from 5 mice per group.
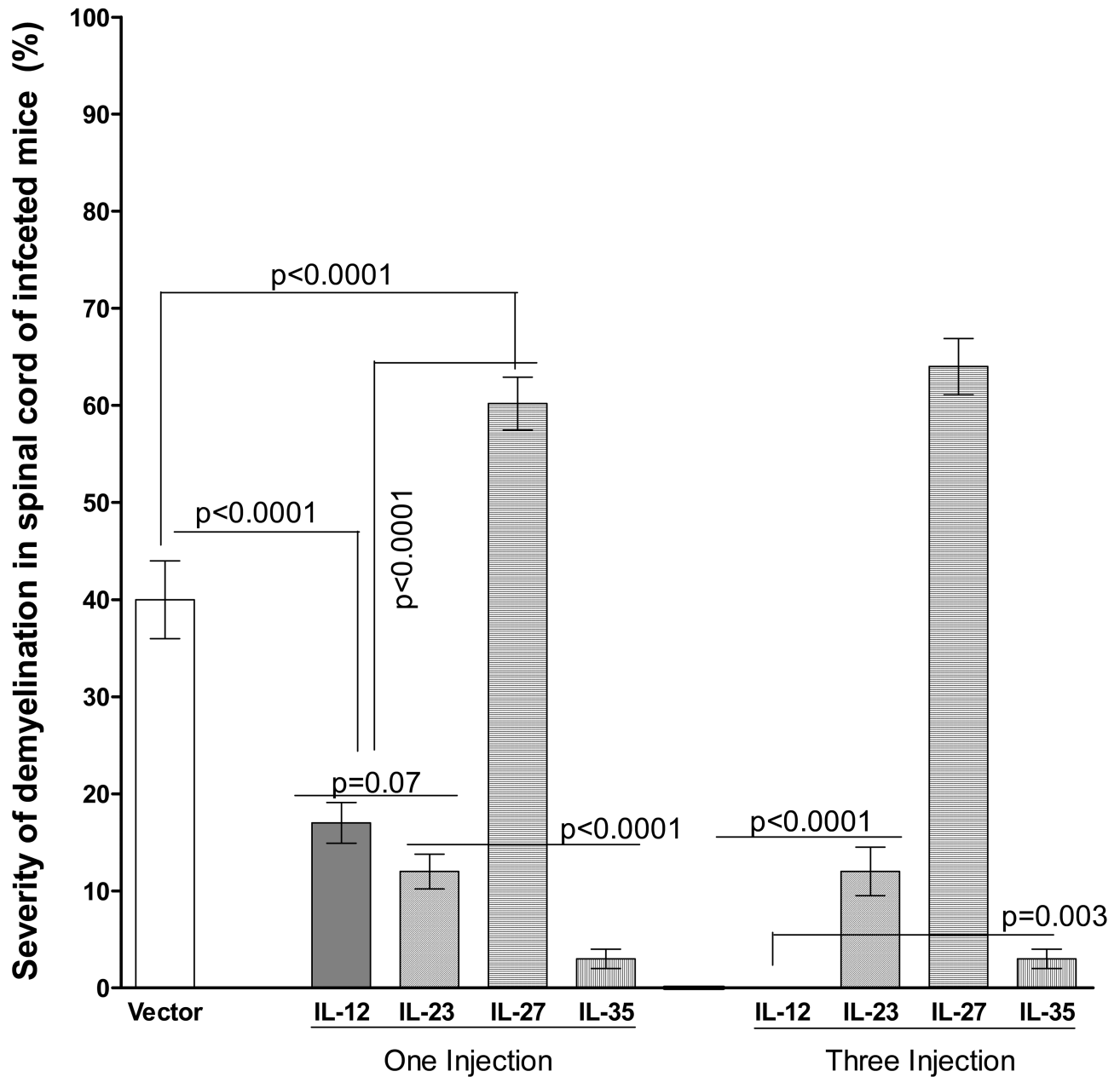
Fig. 5. Severity of CNS demyelination in mice injected with IL-12 family DNAs and infected with HSV-IL-2

The spinal cords of each of the 5 animals in each treatment group described in Figure 5 were sectioned every fourth section was stained, and the size of the demyelination plaques in the entire sections of the spinal cords was determined as described in Materials and Methods. Data are presented as the percentage of the demyelination area in each section per total sections stained using a total of 150 sections from 5 mice per group.

Fig. 6. Severity of CNS demyelination in mice infected with recombinant viruses expressing IL-12 subunits or injected with their DNAs and infected with HSV-IL-2

The spinal cords of each of the 5 animals in each treatment group co-infected with HSV-IL-2 + HSV-IL-12p35, HSV-IL-2 + HSV-IL-12p40, or HSV-IL-2 + HSV-IL-12p35 + HSV-IL-12p40 were sectioned every fourth section was stained, and the size of the demyelination plaques in the entire sections of the spinal cords was determined as described in Materials and Methods. Data are presented as the percentage of the demyelination area in each section per total sections stained using a total of 150 sections from 5 mice per group.

Table 1

Role of IL-12 in protection from CNS demyelination by HSV-IL-2 recombinant virus.[a]

| | Mice with CNS demyelination/Total number of mice in treated group | | | | | | |
| | HSV-IL-2 + HSV-IL-12p70 | | | IL-12p70 DNA immunization | | |
CNS	1:1	1:10	1:100	1X	2X	3X
Brain	5/5 (100%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	1/5 (20%)	0/5 (0%)
Optic nerve	3/5 (60%)	1/5 (20%)	0/5 (0%)	1/5 (20%)	1/5 (20%)	0/5 (0%)
Spinal cord	5/5 (100%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	1/5 (20%)	0/5 (0%)

[a] Mice were co-infected with 1:1,1:10, or 1:100 ratio of HSV-IL-2 to HSV-IL-12p70, while some mice were immunized 1X, 2X, or 3X and ocularly infected with 2×10^5 PFU/eye of HSV-IL-2. The presence of demyelination in the optic nerves, spinal cords, and brains of 5 mice per group was assessed on day 14 post infection.

Table 2

Role of IL-12 family in protection from CNS demyelination by HSV-IL-2 recombinant virus.[a]

| CNS | Mice with CNS demyelination/Total number of mice in the treated group | | | | | | | |
| | 1 Immunization | | | | 3 Immunizations | | | |
	IL-23	IL-27	IL-35		IL-23	IL-27	IL-35
Brain	5/5 (100%)	5/5 (100%)	1/5 (20%)		5/5 (100%)	5/5 (100%)	0/5 (0%)
Optic nerve	5/5 (100%)	5/5 (100%)	0/5 (0%)		5/5 (100%)	5/5 (100%)	0/5 (0%)
Spinal cord	5/5 (100%)	5/5 (100%)	1/5 (20%)		5/5 (100%)	5/5 (100%)	1/5 (20%)

[a] Mice were injected 1X or 3X with IL-23, IL-27, or IL-35 DNA as described in Materials and Methods. Injected mice were ocularly infected with 2×10^5 PFU/eye of HSV-IL-2 4 hr after the 1X or the 3X injection. Presence of demyelination in the optic nerves, spinal cords, and brains of 5 mice per group was assessed on day 14 post infection.