Type 2 Innate Lymphoid Cells Induce CNS Demyelination in an HSV-IL-2 Mouse Model of Multiple Sclerosis
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SUMMARY
We previously reported that infection of different mouse strains with a recombinant HSV-1 expressing IL-2 (HSV-IL-2) caused CNS demyelination. Histologic examination of infected IL-2rα−/−, IL-2rβ−/−, and IL-2rγ−/− mice showed demyelination in the CNS of IL-2rα−/− and IL-2rβ−/− mice but not in the CNS of IL-2rγ−/− infected mice. No demyelination was detected in mice infected with control virus. IL-2rγ−/− mice that lack type 2 innate lymphoid cells (ILC2s) and ILCs, play important roles in host defense and inflammation. We next infected ILC1−/−, ILC2−/−, and ILC3−/− mice with HSV-IL-2 or wild-type (WT) HSV-1. In contrast to ILC1−/− and ILC3−/− mice, no demyelination was detected in the CNS of ILC2−/−-infected mice. However, transfer of ILC2s from WT mice to ILC2−/− mice restored demyelination in infected recipient mice. CNS demyelination correlated with downregulation of CCL5 and CXCL10. This study demonstrates that ILC2s contribute to HSV-IL-2-induced CNS demyelination in a mouse model of multiple sclerosis.

INTRODUCTION
Degradation of the myelin sheath in the brain, optic nerve (ON), and spinal cord has been associated with a number of diseases, with multiple sclerosis (MS) being the most common syndrome of CNS inflammatory demyelination (Hunter et al., 1997; Martin et al., 1992; Noseworthy et al., 2000; Sospedra and Martin, 2005). The World Health Organization estimates that over 2.5 million people suffer from MS globally and according to the National MS Society, approximately 400,000 Americans have MS. The economic impact of MS in the US is estimated to be more than $28 billion per year, and available therapies are generally not effective for MS. Epidemiologic studies have implicated environmental and genetic factors in the development of MS, and it has been suggested that infectious agents (Hafler, 2004; Hemmer et al., 2002), particularly certain viruses, may be involved in this process (Challoner et al., 1995; Friedman et al., 1999). However, this concept remains controversial (Boman et al., 2000; Martin et al., 1997; Mirandola et al., 1999) and, if an infectious agent is involved, it may not be sufficient to initiate the disease.

Several lines of evidence suggest that the cytokine IL-2 is involved in demyelination during MS progression. First, the number of IL-2-secreting cells and amount of IL-2 in the sera of MS patients are elevated (Gallo et al., 1989; Lu et al., 1993; Trotter et al., 1989), and second, levels of soluble IL-2 receptor (sIL-2r) are increased in both the sera (Bansil et al., 1991; Gallo et al., 1989; Greenberg et al., 1988; Hartung et al., 1990; Traugott, 1987) and CSF of MS patients (Adachi et al., 1989; Kittur et al., 1990). In addition, supernatants from MS patients' T lymphocytes cause damage to myelin and glial cells in vitro (Selma et al., 1988a, b), suggesting that MS T lymphocytes are pre-activated in vivo to produce demyelination factors. In these studies, the percentage of MS patient T lymphocytes that express the IL-2r correlates with the degree of supernatant-induced demyelination in vitro. The presence of IL-2 is also associated with disease state in MS mouse models (McCombe et al., 1998; Pettiet et al., 2000; Yang et al., 2002). To dissect the role of IL-2 in the context of viral infection during MS, we constructed a recombinant herpes simplex virus type 1 (HSV-1) that constitutively expresses mouse IL-2 (HSV-IL-2) and constructed similar recombinant viruses expressing mouse IL-4, IFN-γ, IL-12p35, or IL12p40 genes for use as controls (Ghiasi et al., 2002b; Osorio and Ghiasi, 2003; Osorio et al., 2003; Zandian et al., 2009). Mice that were ocularly infected with HSV-IL-2 developed optic neuropathy as determined by changes in visual-evoked cortical potentials (VECPs) (Zandian et al., 2009) and pathologic changes in the ON and CNS (Osorio et al., 2003; Zandian et al., 2009), whereas...
recombinant HSV viruses expressing IL-4, IFN-γ, IL-12p35, IL-12p40, or IL-12p70 did not induce optic neuropathy or CNS pathology (Glisi et al., 2001, 2002a). Similarly, delivery of IL-2 into the brains of mice using Alzet osmotic mini-pumps prior to ocular infection with wild-type (WT) HSV-1 produced eye disease and CNS pathology whereas WT HSV-1 alone did not, nor did injection of IL-2 DNA, IL-2 protein, or IL-2 peptides into other mouse strains prior to infection with wt HSV-1 (Mott et al., 2013).

IL-2 is a pleiotropic cytokine that plays a major role in regulating the adaptive immune response (Waldmann, 2006). IL-2 signals through its heterotrimeric receptor consisting of α (IL-2rα, CD25), β (IL-2rβ, CD122), and γ (IL-2rγ, CD132) chains (Minami et al., 1993; Waldmann, 2006). IL-2rα is the low-affinity IL-2 expressed on activated T and B lymphocytes. IL-2rα alone is not a signaling receptor and pairs with IL-2rβ chain (expressed constitutively on a subset of CD8+ spleen T cells, on NK cells, and at lower levels on a small population of resting B cells) or IL-2rγ (expressed constitutively at low levels on most lymphocytes, myeloid cells, and embryonic thymocytes) (Minami et al., 1993; Waldmann, 2006). In addition to IL-2, IL-2rγ is also the receptor for IL-4, IL-7, IL-9, IL-15, and IL-21 (Waldmann, 2006).

Our published studies showing that elevated IL-2 levels, together with an environmental factor—viral infection—can initiate CNS demyelination are consistent with other published studies. Indeed, published studies on the function of IL-2 in CNS demyelination suggest that its atypical activation is linked to pathogenesis. Whether IL-2 can directly, or by binding to individual, or combinations of its receptors, contribute to CNS demyelination is not known. To determine the role of IL-2rs in CNS demyelination we ocularly infected IL-2rγ−/−, IL-2rβ−/−, and IL-2rγ−/− mice with HSV-IL-2 recombinant virus and control WT virus. No demyelination was detected in IL-2rγ−/−-infected mice, implicating a role for innate lymphoid cells (ILCs) in CNS demyelination. Ocular infection of ILC1−/−, ILC2−/−, and ILC3−/− mice with HSV-IL-2 suggested that ILC2s play a role in HSV-IL-2-induced CNS demyelination. Adoptive transfer of bone-marrow-derived ILC2s from WT mice to ILC2−/− mice restored demyelination in recipient mice. Our results demonstrate that ILC2s do play a significant role in determining the outcomes of CNS demyelination following ocular HSV-IL-2 infection in mice.

RESULTS

Role of IL-2rs in HSV-IL-2-Induced Demyelination in Infected Mice

IL-2 binds to the IL-2 receptor (IL-2r), which has three forms: α (i.e. IL-2rα or CD25), β (i.e. IL-2rβ or CD122), and γ (i.e. IL-2rγ or CD132). A functional IL-2 receptor is generated by homo- or heterogeneous combinations of these forms having different affinities on different cell types (Minami et al., 1993; Waldmann, 2006). To determine the possible involvement of IL-2rs in regulating HSV-IL-2-induced demyelination, we infected IL-2rα−/−, IL-2rβ−/−, and IL-2rγ−/− mice ocularly with HSV-IL-2 as we described previously (Osorio et al., 2005; Zandian et al., 2009; Mott et al., 2015). Control mice were similarly infected with parental virus. We previously reported that CNS demyelination is observed after day 10 postinfection (PI) with HSV-IL-2 virus (Zandian et al., 2009). Thus, at day 14 PI, mice in the current study were sacrificed and brain, spinal cord, and ON were collected, postfixed, and stained with the myelin stain, Luxol Fast Blue (LFB), as we described previously (Osorio et al., 2005; Zandian et al., 2009). Representative photomicrographs of brain, spinal cord, and ON sections from mice infected with HSV-IL-2 or parental virus are shown in Figure 1.

Demyelination was observed in brain, spinal cord, and ON of IL-2rα−/− and IL-2rβ−/− mice infected with HSV-IL-2 (Figure 1, HSV-IL-2 panels), whereas no demyelination was detected in brain, spinal cord, and ON of IL-2rα−/− and IL-2rβ−/− mice infected with parental HSV (Figure 1, Parental panels). In contrast, no demyelination was detected in brain, spinal cord, and ON of IL-2rγ−/− mice infected with HSV-IL-2 (Figure 1A, HSV-IL-2 panels). Thus, our results suggest that the absence of IL-2rγ blocks HSV-IL-2-induced CNS demyelination in ocularly infected mice, whereas the absence of IL-2rα and IL-2rβ does not.

Role of ILCs in HSV-IL-2-Induced Demyelination in Infected Mice

The above results suggest that the absence of IL-2γ, but not the absence of IL-2α and IL-2β, blocked HSV-IL-2-induced CNS demyelination. Previously it was reported that CD132−/− mice lack ILC2s (Wong et al., 2012; Yokota et al., 1999). IL-2 is a critical regulator of ILC2 function (Roediger et al., 2015). In addition to ILC2s, CD132−/− mice also are lacking NK cells (Cao et al., 1995). ILCs are known to play important roles in host defense, metabolic homeostasis, and tissue repair. Increasing evidence that ILCs contribute to inflammation is stimulating interest in developing therapeutic strategies to target specific ILC populations (Abt et al., 2016a, 2016b; McKenzie et al., 2014; Seillet et al., 2016; Rafei-Shamsabadi et al., 2018; Zook and
Three major ILC members are ILC1s, ILC2s, and ILC3s (Spits et al., 2013). To determine the contribution of ILC2s to CNS demyelination and if ILC1s or ILC3s also have a role in HSV-IL-2-induced CNS demyelination, we ocularly infected ILC1<sup>−/−</sup>, ILC2<sup>−/−</sup>, and ILC3<sup>−/−</sup> mice with HSV-IL-2 as in Figure 1.

Control mice were similarly infected with parental virus. At day 14 PI, mice were sacrificed and brain, spinal cord, and ON were collected, post-fixed, and stained with the myelin stain, LFB, as described earlier. Photomicrographs of brain, spinal cord, and ON sections from mice infected with HSV-IL-2 or parental virus are shown in Figure 2. Demyelination was detected in brain, spinal cord, and ON of ILC1<sup>−/−</sup> and ILC3<sup>−/−</sup> mice but not in ILC2<sup>−/−</sup> mice (Figure 2, HSV-IL-2 panels). As expected, no demyelination was detected in brain, spinal cord, and ON of ILC1<sup>−/−</sup>, ILC2<sup>−/−</sup>, and ILC3<sup>−/−</sup> mice infected with parental virus (Figure 2, parental virus). These results suggest that the absence of demyelination in IL-2r<sup>g</sup><sup>−/−</sup> mice is due to the absence of ILC2s and not natural killer (NK) cells.

**Role of ILC2s in HSV-IL-2-Induced CNS Demyelination**

Because the above results suggested that ILC2s are involved in CNS demyelination (Figure 2), ILC2s were isolated from bone marrow of naive WT mice, and the cells were injected intravenously into ILC2<sup>−/−</sup> recipient mice as described in Transparent Methods. Two weeks after adoptive transfer, all ILC2<sup>−/−</sup> recipient mice were infected ocularly with HSV-IL-2. Fourteen days after infection, the mice were sacrificed and brain, spinal cord, and ON were collected, post-fixed, and stained with LFB. Representative photomicrographs are shown in Figure 3. We found that adoptive transfer of ILC2s caused demyelination in brain, spinal cord, and ON of ILC2<sup>−/−</sup> recipient mice (Figure 3). Patterns of demyelination in the brain, SC, and ON of ILC2<sup>−/−</sup> recipient mice was similar to that of WT mice. The results of these studies provide further evidence that ILC2s contribute to CNS demyelination.

**Virus-Expressed IL-2 Binds to the Cell Surface of ILC2s In Vitro**

To determine if IL-2 expressed by HSV-IL-2 binds to ILC2s, isolated ILC2s were infected with HSV-IL-2 or parental virus or mock infected as described in Transparent Methods. The cells were stained with anti-IL-2 antibody and analyzed using confocal microscopy. The results verified that IL-2 expressed by HSV-IL-2 was detected on the surface of infected ILC2s, whereas no IL-2 expression was detected in parental- or mock-infected cells (Figure 4). Similar to our previous studies (Ghiasi et al., 2002b; Osorio et al., 2005), this result shows that IL-2 is expressed in infected ILC2s and also binds to the surface of purified ILC2s.
Identifying Genes that may Contribute to CNS Demyelination in WT Mice

HSV-IL-2 infection of WT mice causes CNS demyelination, whereas infection of mice with parental virus does not (Dumitrascu et al., 2014; Mott et al., 2013; Zandian et al., 2009, 2011a, 2011b). Based on MS and experimental autoimmune encephalomyelitis (EAE) published studies, we investigated the roles of selected Pdcd1 (PD1), CSF2 (GM-CSF), IL-5, IL-6, IFNgr1, CCL5 (RANTES), CXCL10, Tnfrsf9 (4-1BB/CD137), HIF1a, CSF2rb, Havcr2 (Tim-3), and CTLA4 genes in CNS demyelination in vivo. On day 14 PI, RNAs were isolated as previously described (Mott et al., 2007a, 2007b) from brains of WT mice infected with HSV-IL-2 or parental virus. The results are presented in Figure 5 as “fold increase” in WT mice infected with HSV-IL-2 or parental virus compared with the baseline mRNA levels in brains of WT uninfected naive mice. Levels of PD1 (Figure 5A; p < 0.05), GM-CSF (Figure 5B; p < 0.05), CCL5 (Figure 5F; p < 0.01), CCXL10 (Figure 5G; p < 0.01), 4-1BB (Figure 5H; p < 0.05), and CTLA4 (Figure 5L; p < 0.001) were significantly lower in HSV-IL-2-infected mice than in parental-virus-infected mice. In contrast, levels of IL-5 (Figure 5C; p > 0.05), IL-6 (Figure 5D; p > 0.05), IFNgr1 (Figure 5E; p > 0.05), HIF1a (Figure 5I; p > 0.05), CSF2rb (Figure 5J; p > 0.05), and Tim-3 (Figure 5K; p > 0.05) were similar in HSV-IL-2-infected and parental-infected mice.

Figure 2. Role of ILCs in HSV-IL-2-Induced CNS Demyelination
Female ILC1−/−, ILC2−/−, and ILC3−/− mice were infected ocularly with HSV-IL-2 or parental virus as described in Transparent Methods. On day 14 PI, brain, spinal cord, and ON were collected, fixed, sectioned, and stained with LFB. Representative photomicrographs are shown. Arrows indicate areas of demyelination. ×10 objective lens was used.

Figure 3. Demyelination in ILC2−/− Mice Following Adoptive Transfer of ILC2s
Bone-marrow-derived ILC2s were isolated from naive WT mice and transferred IV into recipient ILC2−/− mice. Fourteen days postadoptive transfer, recipient ILC2−/− mice were infected ocularly with HSV-IL-2. On day 14 PI, brain, spinal cord, and ON were collected, fixed, sectioned, and stained with LFB. Representative photomicrographs are shown. Arrows indicate areas of demyelination. ×10 objective lens was used.
Identifying Genes that may Contribute to CNS Demyelination in ILC2−/− Mice

The above results suggest that the absence of ILC2 in ILC2-deficient mice blocked CNS demyelination following infection of ILC2−/− mice with HSV-IL-2 virus, whereas restoring ILC2 by transferring ILC2 to ILC2-deficient mice restored CNS demyelination in HSV-IL-2-infected mice (Figures 2 and 3). To determine the contribution of ILC2 to CNS demyelination, we compared gene expression in mouse brains from ILC2-deficient and ILC2-restored mice following infection with HSV-IL-2 virus as in Figure 5. RNAs were isolated from brains of virus-infected ILC2-deficient and ILC2-restored mice, and relative levels of the same 12 transcripts shown in Figure 5 were determined by RT-qPCR. “Fold increase” in ILC2-deficient and ILC2-restored mice infected with HSV-IL-2 virus was compared with baseline mRNA levels in brains of uninfected naive ILC2-deficient mice (Figure 6). Levels of CCL5 (Figure 6F; p < 0.01) and CXCL10 (Figure 6G; p < 0.01) were significantly lower in ILC2-transferred mice, which is consistent with results obtained with WT mice infected with HSV-IL-2. However, we did not detect significant differences in PD1 (Figure 6A, p > 0.05), GM-CSF (Figure 6B, p > 0.05), IL-5 (Figure 6C, p > 0.05), IL-6 (Figure 6D, p > 0.05), IFNγ1 (Figure 6E, p > 0.05), 4-1BB (Figure 6H, p > 0.05), HIF1α (Figure 6I, p > 0.05), CSF2rb (Figure 6J, p > 0.05), Tim-3 (Figure 6K, p > 0.05), and CTLA4 (Figure 6L, p > 0.05) mRNA levels between ILC2-deficient and ILC2-transferred mice. Thus, similar to WT mice, upregulation of CCL5 and CXCL10 correlates with CNS demyelination upon infection. However, results for PD1, GM-CSF, 4-1BB, and CTLA4 differed between WT and ILC2−/− infected mice.

DISCUSSION

Epidemiologic studies have implicated both environmental and genetic factors in MS (Hunter et al., 1997; Noseworthy et al., 2000). MS is an autoimmune disease, perhaps initiated by a viral infection, that attacks
Figure 5. Identifying Genes that may Contribute to CNS Demyelination in WT Mice

Effects of viral IL-2 on gene expression in the brain of HSV-1-infected WT mice were determined on day 14 PI. RNA isolated from brains of WT mice infected with HSV-IL-2 or parental virus was used to measure the expression of Pdcd1 (PD1), CSF2 (GM-CSF), IL-5, IL-6, IFNγr1, CCL5 (RANTES), CXCL10, Tnfrsf9 (4-1BB/CD137), HIF1α, CSF2rb, Havcr2 (Tim-3), and CTLA4 in virus-infected brains. qRT-PCR was performed using total RNA as described in the Transparent Methods.
and degrades the myelin sheath (Martin et al., 1992). Numerous viruses have been proposed as causative agents. Various herpes viruses, including HSV-1, HSV-2, HCMV, EBV, HHV-6, and HHV-7, have been implicated as the trigger for an autoimmune response leading to MS (Daibata et al., 2000; Ferrante et al., 2000; Knox et al., 2000), although other studies have disputed these findings (Nicoll et al., 1992; Taus et al., 2000). EAE is the primary experimental animal model for MS (Zamvil and Steinman, 1990). However, outcomes in the EAE model are influenced by the species and strain of experimental animals, materials used for immunization, and the type of adjuvant employed (Martin et al., 1992). In addition to the EAE model of MS, numerous other animal models for MS that have been developed generally use either the viral model (Bureau et al., 1998) or the direct autoimmune model (Cua et al., 1999) to initiate disease. Because IL-2 has been implicated in MS (Gallo et al., 1988, 1989; Lu et al., 1993; Trotter et al., 1989) and viruses have been implicated in initiating MS (Daibata et al., 2000; Ferrante et al., 2000; Knox et al., 2000), we explored the effects of infecting mice with a recombinant HSV-1 that expresses murine interleukin-2 (HSV-IL-2) (Ghiassi et al., 2002b). In contrast to other models of demyelination, this HSV-IL-2 model of CNS demyelination incorporates both a viral (i.e., HSV-1) and an immune component (i.e., IL-2). Using our HSV-IL-2 model of CNS demyelination we have made the following nine observations: (1) Ocular infection of female BALB/c, C57BL/6, SJL/6, and 129SVE mice with HSV-IL-2 results in demyelination in the brain, spinal cord, and ONs of infected mice (Osorio et al., 2005; Zandian et al., 2009). (2) Ocular infection with parental or WT viruses, or with similarly constructed recombinant HSV-1 expressing either IFN-γ (HSV-IFN-γ) or IL-4 (HSV-IL-4) does not induce CNS demyelination (Osorio et al., 2005). (3) Similar to the MS condition, female mice were more susceptible to HSV-IL-2-induced demyelination than were male mice (Zandian et al., 2009). (4) We detected CNS demyelination after delivering IL-2 into the mouse brain using osmotic mini-pumps or by injecting mice with rIL-2 protein, IL-2 DNA, or IL-2 synthetic peptides prior to infection with the WT HSV-1 strains McKrae and KOS (Mott et al., 2013). (5) A single mutation in the IL-2 open reading frame (T27A) completely blocked CNS demyelination in this model (Mott et al., 2013). (6) CD4+ and CD8+ T cells are both involved in HSV-IL-2-induced CNS demyelination, whereas macrophages are protective (Mott et al., 2011; Zandian et al., 2011a); (7) DCs, NK cells, and B cells play no role in demyelination (Zandian et al., 2011a). (8) HSV-IL-2-induced CNS demyelination was blocked by co-infecting mice with a recombinant HSV-1 expressing IL-12p70 (HSV-IL-12p70) or injecting with IL-12p70 DNA (Mott et al., 2011). Lastly, (9) a comparison of MOG35-55, MBP35-47, and PLP190-209 models of EAE with our HSV-IL-2-induced MS model (Dumitrascu et al., 2014) showed that our HSV-IL-2 model was similar to the MOG model and both differed from the MBP and PLP models.

ILC1s, ILC2s, and ILC3s have been shown to play important roles in host defense, metabolic homeostasis, tissue repair, and can contribute to inflammation (Abt et al., 2016a, 2016b; McKenzie et al., 2014; Seillet et al., 2016; Zook and Kee, 2016). Previous studies found that IL-2 signaling pathway plays an important role in regulating ILC2s (Minami et al., 1993; Roediger et al., 2015; Waldmann, 2006). In the current study, we evaluated the role of IL-2 receptors (IL-2Rα, IL-2Rβ, and IL-2Rγ) and types 1, 2, and 3 ILCs in HSV-IL-2-induced CNS demyelination. We detected demyelination plaques in brain, spinal cord, and ONs of mice infected with HSV-IL-2 expressing IL-2Rα and IL-2Rβ but not expressing IL-2Rγ. As expected, no demyelination was detected in the CNS of mice infected with control virus. In addition to be a receptor for IL-2, IL-2Rγ is involved in generating ILC2s and NK cells (Wallrapp et al., 2018; Wong et al., 2012) and is a receptor for IL-4, IL-7, IL-9, IL-15, and IL-21 (Waldmann, 2006). Thus, the absence of demyelination in IL-2Rγ−/− mice could be due to the absence of IL-2Rγ binding to IL-2, the absence of ILC2s, the absence of NK cells, or the effect of IL-2Rγ absence on IL-4, IL-7, IL-9, IL-15, and IL-21 functions. Thus, we looked at demyelination in brain, spinal cord, and ONs of ILC1−/−, ILC2−/−, and ILC3−/− mice infected with HSV-IL-2 or control virus. We did not find demyelination in the CNS of ILC2−/− mice infected ocularly with HSV-IL-2 virus, although demyelination was observed in ILC1−/− and ILC3−/−-infected mice. These results showed that the absence of ILC2s in IL-2Rγ−/− mice, not its binding to IL-2 or its effect on IL-4, IL-7, IL-9, IL-15, and IL-21 functions, contributed to HSV-IL-2-induced demyelination. To show that ILC2s specifically contribute to CNS...
Figure 6. Determining Genes that may Contribute to CNS Demyelination in ILC2-/- Mice
LC2-deficient mice or ILC2-deficient mice that received ILC2 from WT mice were infected with HSV1-IL-2. On day 14 PI, RNA was isolated from brains of ILC2-deficient mice or ILC2-deficient mice that received ILC2 from WT as described in Figure 3. qRT-PCR was performed using total RNA, and expression of respective genes in naive ILC2-deficient mice was used as a baseline to estimate relative expression of each transcript in brains of virus-infected mice. GAPDH expression
Figure 6. Continued

was used to normalize the relative expression of each transcript. Each point represents the mean ± SEM from three brains for each group of mice. p value was determined using a one-way ANOVA test.

Panels: (A) PD1 transcript; (B) CSF2 (GM-CSF) transcript; (C) IL-5 transcript; (D) IL-6 transcript; (E) IFNγ1 transcript; (F) CCL5 (RANTES) transcript; (G) CXCL10 transcript; (H) Tnfrsf9 (4-1BB/CD137) transcript; (I) Hif1α transcript; (J) CSF2rb transcript; (K) Havcr2 (Tim-3) transcript; and (L) CTLA4 transcript.

demyelination in WT mice, we next performed adoptive transfer of BM-derived ILC2s to ILC2−/− mice and infected the reconstituted mice with HSV-IL-2 virus. As expected, we observed demyelination in the brains, spinal cords, and ONs of recipient ILC2−/− mice. Thus, CNS demyelination in HSV-IL-2-infected mice is due to the presence of ILC2s, not ILC1s or ILC3s. Quantification of the number, size, and shape of plaques in the CNS of IL-2rα−/−, IL2rg−/−, ILC1−/−, ILC3−/−, or ILC2−/− mice that received ILC2 from WT mice were similar. Thus, this side by side comparison of CNS demyelination in different knockout mice did not show exacerbation of disease.

ILC2s include both a “natural” subset that is present during homeostasis and an “inflammatory” subset that is generated during an immune response (Sonnenberg and Hepworth, 2019). Although ILCs are found in limited numbers, they play important roles in protection and pathogenicity (Cording et al., 2016; Ebbo et al., 2017; Hams et al., 2014; Lee et al., 2015; Moral et al., 2020; Rauber et al., 2017; Salimi et al., 2013; Spits and Di Santo, 2011; Tait Wojno and Artis, 2016; Zook and Kee, 2016). Consistent with our study, ILC2s have been implicated in the development of allergy, asthma, dermatitis, and fibrosis (Cording et al., 2016; Ebbo et al., 2017; Hams et al., 2014; Salimi et al., 2013; Spits and Di Santo, 2011; Tait Wojno and Artis, 2016; Zook and Kee, 2016). ILC2s can induce airway hyper-reactivity independent of type 2 TH2 cells and adaptive immunity following lung infection with influenza A virus (Chang et al., 2011). ILC2s, which are the dominant ILCs in the lung, contribute to inflammation (Hurrell et al., 2019; Rigas et al., 2017) and are also the predominant ILC population in human and mouse brain (Cardoso et al., 2017; Klose et al., 2017; Wallrapp et al., 2017). Mucosal neurons regulate Tα2 inflammation by releasing neuromeric U (NMU) that directly activates population in human and mouse brain (Cardoso et al., 2017; Klose et al., 2017; Wallrapp et al., 2017). In this study we have shown that IL-2 expressed by HSV-IL-2 binds to ILC2s. Thus, similar to NMU, the binding of overexpressed IL-2 to the surface of ILC2s, combined with viral infection, may activate ILC2s leading to CNS demyelination. IL-2-deficient CD4+ T cells are known to more effectively control influenza A virus infection in the lung than do T cells that produce IL-2 (McKinstry et al., 2019). Thus, a combination of IL-2 expression and viral infection may induce pathogenesis in the CNS or lung of mice infected with HSV-IL-2 and influenza viruses, respectively.

Many factors have been implicated in protecting against or inducing MS and EAE with contradictory results depending on which model of CNS demyelination is used (Beck et al., 2003; Galli et al., 2019; Hafler, 2004; Haines et al., 1996; Hemmer et al., 2002; Hunter et al., 1997; Martin et al., 1992; Noseworthy et al., 2000; Zamvil and Steinman, 1990). Thus, we looked at the involvement of PD1, GM-CSF, IL-5, IL-6, IFNγ1, CCL5 (RANTES), CXCL10 (IP-10), 4-1BB/CD137, HIF1α, CSF2rb, Tim-3, and CTLA4 in demyelination or protection from demyelination in the brain of infected mice. Demyelination in both WT and ILC2−/− mice that received adoptive transfer of ILC2 from WT mice correlated with suppression of RANTES (also known as CCL5) and IP-10 (Interferon gamma-induced protein also known as C-X-C motif chemokine 10 (CXCL10). Our results suggest that in the presence of ILC2s, suppression of CCL5 and CXCL10 by a combination of IL-2 and viral infection correlated with demyelination. However, levels of both CCL5 and CXCL10 increased significantly in mice that were infected with parental virus or in ILC2−/− mice that were infected with HSV-IL-2. Previously we reported no expression of CCL5 or CXCL10 in ILC1 or ILC3 cells with and without infection with WT HSV-1, whereas both CCL5 and CXCL10 were significantly upregulated in HSV-1-infected ILC2 (Hirose et al., 2019). Similar to this study and our previous report, CXCL10 is elevated following infection with hepatitis C virus and HIV (Falconer et al., 2010; Lagging et al., 2006). In contrast to our study, CXCL10+ cells were recently shown to be pathogenic in the CNS of EAE mice (Giladi et al., 2020). CCL5 plays a primary role in the inflammatory immune response and has been implicated in EAE and MS (Gonzalez-Amaro and Sanchez-Madrid, 2002; Jee et al., 2002). Overall, the role of CCL5 and CXCL10 in inflammatory autoimmunity, particularly in neuroinflammation, is controversial (Karim, 2020; Lalor and Segal, 2013; Mills Ko et al., 2014; Muller et al., 2010). Both CCL5 and CXCL10 are chemotactic for T cells and play an active role in recruiting leukocytes into inflammatory sites. We previously showed that CD4+ and CD8+ T cells are both involved in HSV-IL-2-induced CNS demyelination (Zandian et al., 2011a). In this study we showed that the presence of ILC2s is required for CNS demyelination. Similarly, previous studies
showed that ILC2s in different tissues and in response to the local environment, selectively express distinct cytokine patterns for cell activation (Ricardo-Gonzalez et al., 2018). ILC2s have been shown to express MHCII, CD80, CD86, and OX40L and also to act as APCs for antigen presentation to T cells (Halim et al., 2018; Mazi et al., 2015; Oliphant et al., 2014). Thus, specific ILC2s activation may contribute to protection or disease as previously reported (Cording et al., 2016; Ebbo et al., 2017; Hams et al., 2014; Lee et al., 2015; Rauber et al., 2017; Salmi et al., 2013; Spits and Di Santo, 2011; Tait Wojno and Artis, 2016; Zook and Kee, 2016). Our current study suggests communication between ILC2s and T cells via IL-2 produced by HSV-IL-2 and virus infection function as a critical enhancer of T cell autoreactivity. However, the absence of ILC2s can block the effects of HSV-IL-2 on CNS pathology due to elevated expression of CCL5 and CXCL10, which are defensive molecules produced by neurons to recruit protective T cells. In contrast, suppression of CCL5 and CXCL10 in the presence of ILC2 by HSV-IL-2 may alter the T cell phenotype, and these pathogenic T cells may cause CNS demyelination.

Previously we have shown that FoxP3+ T cells contribute to CNS demyelination and depletion of FoxP3 blocks CNS demyelination by HSV-IL-2 (Zandian et al., 2011a). Our previous studies also have shown that HSV-IL-2-induced CNS demyelination was associated with T cells having a CD62LhiCD45RBloFoxP3hi, whereas the absence of demyelination was associated with CD62LloCD45RBhiFoxP3lo (Osorio et al., 2005; Zandian et al., 2011a).

In summary, the results presented here are in line with our previously studies (Dumitrascu et al., 2014; Mott et al., 2011, 2013; Osorio et al., 2005; Zandian et al., 2005a) and suggest that a combination of HSV-1 infection and overexpression of IL-2 is responsible for dysregulation of ILC2 and reduced expression of chemotactic cytokines CCL5 and CXCL10, leading to enhanced recruitment of pathogenic T cells (i.e., CD62LhiCD45RBloFoxP3hi) and thus CNS demyelination.

Limitations of the Study
As our studies were done using a mouse model, it is currently not known if these results are applicable to humans. Future studies using ILC2s isolated from MS patients or cadavers should be done to validate our findings.

Resource Availability
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Homayon Ghiasi (ghiasih@cshs.org).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
This study did not generate datasets or analyze codes.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101549.

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AUTHOR CONTRIBUTIONS
SH and HG conceived the studies. SH, PSJ, and UJ performed experiments and generated primary data, including developing methodology, validation, and data curation. SH and HG performed formal analysis and visualization. SH, SW, and JU assisted in animal colony maintenance and performing some mouse
experiments. SH performed and analyzed transcriptional profiling data. SH, KT, QA, and HG contributed to writing the manuscript. All authors contributed to reviewing and editing the final manuscript. HG was responsible for project supervision, administration, and funding acquisition.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Type 2 Innate Lymphoid Cells Induce CNS Demyelination in an HSV-IL-2 Mouse Model of Multiple Sclerosis

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# Key Resources Table

| REAGENT or RESOURCE | SOURCR | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Biotin-conjugated anti-mouse CD3e (145-2C11) | BioLegend | Cat#100304 |
| Biotin-conjugated anti-mouse CD5 (53-7.3) | BioLegend | Cat#100604 |
| Biotin-conjugated anti-mouse CD45R (RA3-6B2) | BioLegend | Cat#103204 |
| Biotin-conjugated anti-mouse Gr-1 (RB6-8C5) | BioLegend | Cat#108404 |
| Biotin-conjugated anti-mouse CD11c (N418) | BioLegend | Cat#117304 |
| Biotin-conjugated anti-mouse CD11b (M1/70) | BioLegend | Cat#101204 |
| Biotin-conjugated anti-mouse Ter119 (TER-119) | BioLegend | Cat#116204 |
| Biotin-conjugated anti-mouse FcεRIa (MAR-1) | BioLegend | Cat#134304 |
| Biotin-conjugated anti-mouse TCR-gd (eBioGL3) | ThermoFisher Scientific | Cat#13-5711-82 |
| PECy7-conjugated anti-mouse CD127 (A7R34) | BioLegend | Cat#135014 |
| APCCy7-conjugated anti-mouse CD45 (30-F11) | BioLegend | Cat#103116 |
| PE-conjugated anti-mouse ST2 (DIH9) | BioLegend | Cat#145303 |
| anti-mouse CD16/32 (93) | BioLegend | Cat#101302 |
| Alexa Fluor 488-conjugated anti-mouse IL-2 (JES6-5H4) | BioLegend | Cat#503813 |

**Bacterial and Virus Strains**

| LAT2903 | Homayon Ghiasi, Cedars-Sinai Medical Center (Perng et al., 1994) | N/A |
| HSV-IL-2 | Homayon Ghiasi, Cedars-Sinai Medical Center (Ghiasi et al., 2002) | N/A |

**Chemicals, Peptides, and Recombinant Proteins**

| Minimum Essential Medium (MEM) | Corning | Cat#10-010-CV |
| Fetal Bovine Serum (FBS) | Omega Biosciences | Cat#FB-02 |
| Mouse recombinant IL-33 | BioLegend | Cat#580508 |
| Streptavidin-FITC | BioLegend | Cat#405202 |
| Prolong Gold Antifade Mountant with DAPI | ThermoFisher Scientific | Cat#P36931 |
| O.C.T. compound | Sakura Finetek | Cat#4583 |
| Trizol reagent | ThermoFisher Scientific | Cat#15596026 |

**Critical Commercial Assays**

| High-Capacity cDNA Reverse Transcription Kit | ThermoFisher Scientific | Cat#4368813 |
| Experimental Models: Cell Lines |  |
|--------------------------------|-----------------------------------|
| Rabbit skin (RS) cells         | Homayon Ghiasi, Cedars-Sinai Medical Center (Perng et al., 1994) | N/A |

| Experimental Models: Organisms/Strains |  |
|---------------------------------------|-----------------------------------------------|
| Mouse: C57BL/6                         | Jackson Laboratory                            | Stock#000664 |
| Mouse: IL-2rα<sup>-/-</sup>            | Jackson Laboratory                            | Stock#002952 |
| Mouse: IL-2rβ<sup>-/-</sup>            | Jackson Laboratory                            | Stock#002816 |
| Mouse: IL-2rγ<sup>-/-</sup>            | Jackson Laboratory                            | Stock#003174 |
| Mouse: ILC1<sup>-/-</sup>              | Jackson Laboratory                            | Stock#004648 |
| Mouse: ILC2<sup>-/-</sup>              | Dr. ANJ Mackenzie (MRC Laboratory of Molecular Biology, United Kingdom) | N/A |
| Mouse: ILC3<sup>-/-</sup>              | Jackson Laboratory                            | Stock#007571 |

| Oligonucleotides |  |
|------------------|-----------------------------------------------|
| TaqMan Gene Expression Assay, see Materials and methods | ThermoFisher Scientific | Cat#4331182 |

| Software and Algorithms |  |
|-------------------------|-----------------------------------------------|
| Graphpad Prism (ver. 4) | Graphpad                                     | N/A |
| Connect Data Analysis Apps | ThermoFisher Scientific | https://www.thermofisher.com/us/en/ |
Experimental Model and Subject Details

**Cells and Virus.** Rabbit skin (RS) cells were generated in our laboratory, prepared, grown in minimal essential medium (MEM) media plus 5% FBS and used as described previously (Perng et al., 1994). Plaque-purified HSV-1 recombinant virus expressing IL-2 (HSV-IL-2) and parental virus for HSV-IL-2 (LAT2903) were grown in RS cell monolayers in MEM containing 5% fetal calf serum, as we described previously (Ghiasi et al., 2002a; Ghiasi et al., 2001, 2002b).

**Mice.** Inbred IL-2rα−/−, IL-2rβ−/−, IL-2rγ−/−, ILC1−/−, and ILC3−/− mice were obtained from the Jackson Laboratory (Bar Harbor, ME), ILC2−/− mice were a gift from Andrew McKenzie (MRC Laboratory of Molecular Biology, Cambridge Biomedical Campus, United Kingdom) and described previously (Halim et al., 2018; Rafei-Shamsabadi et al., 2018). All mice have a B6 background and were bred in-house. WT C57BL/6 mice were used as a control. Only female mice (6 to 8-wk-old) were used in the study due to more resistance of male mice to CNS demyelination (Zandian et al., 2009). All animal procedures were performed in strict accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH *Guide for the Care and Use of Laboratory Animals* (ISBN 0-309-05377-3). The animal research protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center (Protocol #6134).

**Ocular Infection.** Female mice were infected ocularly with 2 X 10⁵ PFU per eye of HSV-IL-2 or parental virus, in 2μl of tissue culture media as an eye drop without corneal scarification as we have described previously (Hirose et al., 2019; Mott et al., 2015). The presence of infectious virus in the eye of ocularly infected mice on days 1-5 post infection and the presence of viral DNA on day 14 post infection were monitored by PCR. Infectious virus was detected in all infected mice (not shown).
Analysis of demyelination using Luxol Fast Blue (LFB) staining. The presence or absence of demyelination in ON, SC, and brains of infected mice was evaluated using LFB staining of formalin-fixed sections of ON, SC, and brain as we described previously (Osorio et al., 2005). Every 4th section of ON, SC, and brain was stained with LFB. 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 brains, SCs and ONs was measured using the NIH Image J software analysis system. The areas of demyelination (clear-white) to normal tissue (blue) were quantified using 150 random sections from the brain and SCs or 30 sections from ONs of each animal. Demyelination in each section was confirmed by monitoring adjacent sections. The percentage of myelin loss was calculated by dividing the lesion size into the total area for each section.

Isolation of type 2 ILCs. C57BL/6 mice were intraperitoneally treated with recombinant mouse (rm)IL-33 (IP; 1µg/mouse, BioLegend, San Diego, CA) for three days. On the fourth day, BM cells were collected from the femur and tibia and resuspended in phosphate buffered saline (PBS) solution containing 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid (EDTA). BM ILC2s were stained based on the lack of expression of classical lineage markers (CD3e, CD5, CD45R, Gr-1, CD11c, CD11b, Ter119, TCRγδ, and FcεRI) and positive expression of CD45, ST2, and CD127 as previously described (Hirose et al., 2019; Hurrell et al., 2019; Rigas et al., 2017). The following mouse antibodies were used: biotinylated anti-mouse lineage CD3e (145-2C11), CD5 (53-7.3), CD45R (RA3-3B2), Gr-1 (RB6-8C5), CD11c (N418), CD11b (M1/70), Ter119 (TER-119), FcεRia (MAR-1) (BioLegend) and TCR-gad (eBioGL3) (eBioscience). Streptavidin-FITC, PE-Cy7 anti-mouse CD127 (A7R34), APCCy7 anti-mouse CD45 (30-F11) were purchased from BioLegend. ILC2s were FACS purified using BD FACS ARIA III (BD Biosciences, San Jose, CA) with purity of >95%.

Immunostaining of ILC2. 5 X 10^4 ILC2 cells per tube were seeded in 5ml polystyrene round-bottom tube (Corning, Corning, NY) and infected with 10 PFU/cell of HSV-IL-2, parental virus or mock infected for 24 h. The cells were washed with staining buffer (cold PBS supplemented with 2% fetal calf serum and 0.05% sodium azide), and preincubated with anti-mouse CD16/32 (BioLegend) for 15 min., then incubated with anti-mouse IL-2 antibody conjugated with Alexa Fluor 488 (BioLegend) for 30 min.,
then washed once with staining buffer. Cells were fixed with 4 % paraformaldehyde in PBS for 10 min. at
room temperature and then washed with staining buffer. Cells were mounted onto slide glass with
Prolong Gold Antifade Mountant (ThermoFisher Scientific, Waltham, MA). The fluorophores were imaged
by confocal microscopy using a Leica SP5-X confocal microscope, image acquisition and data analysis
system (Leica Microsystems, Buffalo Grove, IL).

**Adoptive transfer of ILC2s.** To confirm the effect of ILC2s on CNS demyelination, ILC2s were
isolated from bone-marrow of WT mice as described above and previously (Hirose et al., 2019). Each
recipient ILC2⁻/⁻ mouse was injected intravenously (IV) once with 1 X 10⁵ ILC2s in MEM (100 µl). Mice
were then infected ocularly with HSV-IL-2 virus two wk after transfer of the ILC2s.

**RNA Extraction, cDNA Synthesis and TaqMan RT-PCR.** Brains were collected from naive mice
and mice that survived ocular infection on day 14 PI and individual brains were embedded in O.C.T.
compound (Sakura Finetek, Tokyo, Japan), quickly frozen with dry ice, and stored at -80°C until
sectioning. Brains were sectioned with Microm HM550 cryostat microtome (ThermoFisher) at 9 um
thickness, -18°C and 20 to 40 sections were collected in a 2ml sample tube. 0.5 ml Trizol reagent
(ThermoFisher) was added and samples were stored at -80°C until processing. Tissue processing, total
RNA extraction, and RNA yield were performed as we have described previously (Mott et al., 2007a;
Mott et al., 2007b). Following RNA extraction, 1000 ng of total RNA was reverse-transcribed using
random hexamer primers and recombinant Molony Murine Leukemia Virus Reverse Transcriptase from
the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the
manufacturer's recommendations. The levels of various RNAs were evaluated using commercially
available TaqMan Gene Expression Assays (Thermo Fisher Scientific) with optimized primer and probe
concentrations. Primer probe sets consisted of two unlabeled PCR primers and the FAM™ dye-labeled
TaqMan MGB probe formulated into a single mixture. All cellular amplicons also included an intron-exon
junction to eliminate signal from genomic DNA contamination. The assays used in this study were as
follows: 1) PD-1 (programmed death 1) ABI assay I.D. Mm00435532_m1 – Amplicon size 65 bp; 2) GM-
CSF (Csf2 – colony stimulating factor 2 (granulocyte-macrophage)) ABI Mm01290062_m1 – Amplicon
size 125 bp; 3) IL-5 (interleukin 5) ABI Mm00439646_m1 – Amplicon size 62 bp; 4) IL-6 (interleukin 6)
ABI Mm00446190_m1 – Amplicon size 78 bp; 5) IFNgr1 (interferon gamma receptor 1)

Mm00599890_m1 – Amplicon size 85 bp; 6) RANTES (CCL5 – chemokine (C-C motif) ligand 5) ABI

Mm01302427_m1 - Amplicon size 103 bp; 7) CXCL10 (chemokine (C-X-C motif) ligand 10) ABI

Mm00445235_m1 – Amplicon size 59 bp; 8) 4-1BB (Tnfrsf9 – tumor necrosis factor receptor superfamily, member 9) ABI

Mm00441899_m1 – Amplicon size 71 bp; 9) Hif1a (hypoxia inducible factor 1, alpha subunit) ABI

Mm00468869_m1 – Amplicon size 75 bp; 10) Csf2rb (colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)) ABI

Mm00655745_m1 – Amplicon size 125 bp; 11) TIM3 (Havcr2 – hepatitis A virus cellular receptor 2) ABI

Mm00454540_m1 – Amplicon size 98 bp; 12) CTLA4 (cytotoxic T-lymphocyte-associated protein 4) ABI

Mm00486849_m1 – Amplicon size 71 bp and 13) GAPDH used for normalization of transcripts, ABI Mm999999.15_G1 – Amplicon size 107 bp.

Quantitative real-time RT-PCR (qRT-PCR) was performed using QuantStudio 5 System (Thermo Fisher Scientific, Waltham, MA) in 384-well plates as described previously (Mott et al., 2007a; Mott et al., 2007b). The threshold cycle (CT) values, which represent the PCR cycles at which there is a noticeable increase in the reporter fluorescence above baseline, were determined using Applied Biosystems qPCR software (Thermo Fisher Scientific).

Statistical analyses. Student’s t test and ANOVA were performed using the computer program Prism (GraphPad, San Diego, CA). Results were considered statistically significant when the "P" value was <0.05.