Distinct roles for IP-10/CXCL10 in three animal models, Theiler's virus infection, EAE, and MHV infection, for multiple sclerosis: implication of differing roles for IP-10

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Theiler's murine encephalomyelitis virus (TMEV) causes demyelination with inflammation of the central nervous system (CNS) in mice and is used as an animal model for multiple sclerosis (MS). Interferon-γ inducible protein-10 kDa (IP-10) is a CXC chemokine and a chemoattractant for CXCR3+ T cells. IP-10 mRNA is expressed in the CNS during TMEV infection. However, administration of anti-IP-10 serum caused no difference in clinical signs, inflammation, demyelination, virus persistence or anti-virus antibody response in TMEV infection, while levels of virus specific and auto reactive lymphoproliferation increased. This likely reflects a difference in the pathogenesis of TMEV infection from that of two other animal models for MS, mouse hepatitis virus infection and experimental allergic encephalomyelitis (EAE), where blocking of IP-10 resulted in clinical and histological improvement with suppression of antigen specific lymphoproliferation. In this review, we compare and contrast the roles of IP-10 between the three animal models for MS, and discuss the relevance to MS patients with different clinical courses.

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Introduction

Chemokines and chemokine receptors: differential chemokine receptor expression between Th1 and Th2 cells

Chemokines, chemotactic cytokines, are a group of small (~8–14 kDa) structurally related molecules that selectively attract leukocyte subsets into tissues; some chemokines act specifically toward neutrophils and others toward monocytes or T cells.¹ Chemokines are subdivided into four groups depending on the positions of conserved cysteine residues within the protein. The groupings are termed the CXC (α-chemokine), CC (β-chemokine), C (γ-chemokine), and CX₃C (δ-chemokine) chemokines.¹ Functionally, chemokines can be divided into two categories. Inflammatory chemokines are induced or strongly upregulated in peripheral tissues by inflammation, while immune (system) chemokines or constitutive chemokines fulfill housekeeping functions and may be involved in constitutive leukocyte trafficking.² Chemokines bind to proteoglycans on the surface of endothelial cells of blood vessels. Leukocytes interact with these chemokines through receptors; this triggers integrin activation, leading to firm adhesion.³,⁴ Chemokines produced by a variety of tissue cells drive leukocyte migration through tissue to the target microenvironment.

Selected chemokine receptors are expressed predominantly on leukocyte cell types, including Th1 and Th2 cells. In humans, Th1 cells have been demonstrated to express CXCR3 (CD183, receptor for IP-10) and CCR5 (CD195, receptor for MIP-1α, MIP-1β, and RANTES), whereas Th2 cells express CCR3, CCR4 and CCR8. Although the differential expression of chemokine receptors on Th subsets has also been suggested for mice,⁵ it is not well defined compared with those of human subsets.⁶,⁷

Theiler's murine encephalomyelitis virus infection: three distinct CNS diseases

Theiler’s murine encephalomyelitis virus (TMEV) belongs to the family Picornaviridae, the genus Cardiovirus. TMEV subgroups have been demonstrated to cause biologically distinct clinical diseases in mice.⁸,⁹ The TO subgroup of TMEV, including Daniels (DA) and BeAn viruses, causes a biphasic disease in mice.¹⁰ During the acute phase, one week after infection, virus infects neurons in the gray matter of the brain and causes an intense mononuclear cell (MNC) infiltration (acute polioencephalomyelitis). Although inflammation in the gray matter subsides about two weeks after infection in susceptible mouse strains, such as SJL/J mice, the mice remain persistently infected. Infected mice usually develop a spastic paralysis one month after infection.

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(chronic phase). During the chronic phase, virus infects glial cells and macrophages in the white matter of the spinal cord. Neurpathology during the chronic phase of TMEV infection is similar to that of multiple sclerosis (MS) where demyelination is accompanied by perivascular and meningeal infiltration composed of CD4$^+$ and CD8$^+$ T cells and macrophages. In contrast to DA or BeAn viruses, GDVII virus (GDVII subgroup) is a highly neuroviral strain and causes an acute fatal polioencephalomyelitis, in which active viral replication in neurons is accompanied by neuronal apoptosis and microglial proliferation with minimal T cell infiltration. H101 virus is a variant of DA virus and causes pachymeningitis, which leads to communicating hydrocephalus in surviving mice. H101 virus can neither infect neuronal cells nor cause MNC infiltration in the central nervous system (CNS) parenchyma; infiltrates are restricted to meninges. Although we do not know the precise mechanisms in which activated T cells are recruited and remain in the CNS in TMEV infection, the role of chemokines has recently been investigated.

**Chemokine expression is not necessarily associated with TMEV-induced disease**

Hoffman et al. first demonstrated chemokine expression in the CNS of susceptible SJL/J mice infected with the BeAn strain of TMEV. They showed that MIP-1$^\alpha$, MIP-1$^\beta$, RANTES, MCP-1, C10 and IP-10 mRNAs were specifically expressed in the spinal cord, but not in the spleen or lymph nodes of infected mice. In addition, Murray et al. demonstrated that IP-10, RANTES and MCP-1 mRNA were expressed predominantly in the brains in both resistant B10.M and susceptible B10 mice, five days after DA virus infection (acute phase). All chemokine expression subsided by day 21, but was re-expressed on day 45 (chronic phase) in only the spinal cord of susceptible mice. These studies suggest that there is coordinated regulation and regionally restricted expression of chemokines in the biphasic disease induced by TO subgroup viruses of TMEV.

On the other hand, Ransohoff et al. compared the levels of mRNAs encoding chemokines MCP-1, RANTES and IP-10 in susceptible PL/J and resistant C57BL/6 mice that possessed or lacked either CD4$^+$ or CD8$^+$ cells (CD4$^+$ or CD8$^+$ mice) during the chronic phase of DA virus infection. In this study, chemokine expression did not correlate with a susceptible or resistant genotype, demyelination or CD4$^+$ and CD8$^+$ T cell infiltration. Therefore, neither CD4$^+$ nor CD8$^+$ T cells appeared to regulate the expression of these chemokines. Here, the authors suggested a fundamental difference with regard to chemokine induction between TMEV infection and experimental allergic encephalomyelitis (EAE), an experimental autoimmune animal model for MS. In EAE, the cytokine products of leukocytes could regulate chemokine expression. In TMEV infection, chemokine expression might be independent of the adaptive immune response; chemokine expression could be driven by innate CNS immune responses to infection, including those of astrocytes and microglia. In support of the idea that the astrocytes, rather than T cells, are potential sources of chemokines during TMEV infection, one study found that in vitro treatment of astrocytes, oligodendrocytes and microglia with either live or UV-inactivated TMEV led to the expression of mRNAs for several chemokines including IP-10.

Previously, we compared cytokine and chemokine mRNA expression between three distinct TMEV infections: DA virus, GDVII virus and H101 virus infections. Using an RNase protection assay (RPA), we detected the same pattern of chemokine (RANTES, MCP-1, IP-10, MIP-1$^\alpha$, MIP-1$^\beta$ and MIP-2) mRNA expression in the CNS during all three infections. Since the difference in the number and distribution of MNC infiltrates is a main pathological feature distinguishing the three TMEV infections, similar chemokine patterns suggest that resident cells rather than MNC infiltrate produce chemokines. In addition, chemokine expression was identical during the acute and chronic phases of DA virus infection. Therefore, chemokine expression appears not to be a main determinant driving disease progression in DA virus infection. This conclusion is concordant with those reported by Ransohoff et al.

**Role of IP-10/CXCL10 in MS and TMEV infection**

**Upregulation of IP-10 and its receptor CXCR3 in MS**

Among the chemokines that were detected in TMEV infection, IP-10 is of particular interest. IP-10, interferon (IFN)-$\gamma$ inducible protein 10 kDa/CXC chemokine ligand (CXCL) 10/cytokine responsive gene 2 (Crg-2), is a non-ELR (glutamic acid–leucine–arginine) CXC chemokine. IP-10 is inducible by IFN-$\gamma$ and is a potent chemoattractant for activated T cells and NK cells by binding to the CXCR3 receptor. CXCR3 has been shown to be differentially expressed on lymphocytes and is expressed at much higher levels on Th1 cells than on Th2 cells.

The role of IP-10 has been investigated in MS. Within demyelinating lesions or in the surrounding parenchyma, IP-10 has been detected in astrocytes, which were identified by immunohistochemistry with glial fibrillary acidic protein (GFAP) or morphologically. Macrophages and T cells are also potential sources of IP-10. Simpson et al. reported that macrophages expressed IP-10 within plaques. In vitro, IP-10 was expressed by CD14$^+$ monocytes after stimulation, but not by unstimulated monocytes, while no significant difference in the percentage of IP-10$^+$ monocytes was seen between controls and MS patients. Myelin specific CD8$^+$ T cell lines derived from MS patients were shown to synthesize IP-10 and chemoattract myelin specific CD4$^+$ T cell lines.

A significant increase in IP-10 levels was also detected in the cerebrospinal fluid (CSF). Franciotta et al. demonstrated that serum and CSF IP-10 levels were significantly higher in acute MS patients versus patients with stable MS. However, IP-10 levels did not correlate with clinical signs. In addition, neither methylprednisolone nor IFN-$\gamma$a therapy altered IP-10 levels. Sørensen et
Here, we investigated the role of IP-10. Neutralization of IP-10 does not alter the clinical or pathological outcome of TMEV infection.

Expression of the receptor for IP-10, CXCR3, has also been demonstrated in the CNS of MS patients. CXCR3+ cells were found to comprise 5-25% of perivascular MNCs in MS lesions, and CXCR3+ cells were closely related to IP-10 expression. Balashov et al. also showed a few CXCR3+ lymphocytes were present in the brain. On the other hand, Simpson et al. found CXCR3 expression not only in T cells, but also in astrocytes within the MS plaque.

An increase in the percentage of CXCR3+ cells in the CSF compared with that in peripheral blood has been reported in MS. Here, CXCR3 is proposed to mediate recruitment and/or retention of MNCs in the CNS. The enrichment of CXCR3+ cells in the CSF seemed to be independent of underlying CNS pathology. Teleshova et al. found that percentages of CXCR3+ T cells in the CSF were higher in MS than in other noninflammatory neurological diseases, but similar to levels in inflammatory neurological diseases, suggesting the changes observed in MS were not MS-specific, but reflect CNS inflammation.

On the other hand, another group found no differences in percentages of CXCR3+ T cells either in the CSF or in the blood between MS and noninflammatory neurological diseases. This is in agreement with reports in which no alteration was found in percentages of CXCR3+ T cells in the CSF compared with that in peripheral blood has been reported in MS. Here, CXCR3 is proposed to mediate recruitment and/or retention of MNCs in the CNS. The enrichment of CXCR3+ cells in the CSF seemed to be independent of underlying CNS pathology. Teleshova et al. found that percentages of CXCR3+ T cells in the CSF were higher in MS than in other noninflammatory neurological diseases, but similar to levels in inflammatory neurological diseases, suggesting the changes observed in MS were not MS-specific, but reflect CNS inflammation.

Neutralization of IP-10 does not alter the clinical or pathological outcome of TMEV infection

Here, we investigated the role of IP-10 in vivo during TMEV infection using anti-IP-10 serum. Anti-IP-10 sera can neutralize IP-10 and block its association with CXCR3 on Th1 cells and NK cells. Both of these cell types have been suggested to play important roles in TMEV infection. Th1 cells are one of the candidate effector cells that cause demyelination by delayed type hypersensitivity (DTH) responses and epitope spreading during the chronic phase of TMEV infection. NK cells, on the other hand, have been shown to protect from polioencephalomyelitis during the acute phase of TMEV infection in resistant C57BL/10 mice.

Neutralization of IP-10 enhances TMEV-specific and autoreactive lymphoproliferation, but not virus-specific antibody responses

We also compared humoral and cellular immune responses to TMEV between mice treated with NRS or anti-IP-10 serum during the acute or chronic phase of infection. Using an enzyme-linked immunosorbent assay (ELISA), we titrated serum anti-TMEV antibodies, as described previously.

By day 35, all groups of mice developed significant TMEV antibody responses. Mean TMEV antibody titers (log~10) during the acute phase were 9.6 ± 0.5 with NRS injections and 10.6 ± 0.5 with anti-IP-10 serum injections, while those during the chronic phase were 10.6 ± 0.5 with NRS injections and 10 ± 0.7 with anti-IP-10 serum injections. There was no statistical difference between the groups (P > 0.05, ANOVA).
On day 35, MNCs were isolated from spleens of infected mice. MNCs were stimulated with irradiated TMEV-infected spleen cells (TMEV-APC). Since TMEV-infected mice develop autoreactive immune responses in the spleen,

MNCs were also stimulated with irradiated uninfected syngenic spleen cells (uninfected APC). Cells were cultured for five days.

Mice injected with anti-IP-10 serum either during acute phase or chronic phase showed higher lymphoproliferation against TMEV infected APC. This suggests that administration of anti-IP-10 serum, to some extent, can induce immunomodulation in vivo in TMEV infection.

**Role of IP-10/CXCL10 in MHV infection and EAE**

**Neutralization of IP-10 reduces MNC infiltration in MHV-induced demyelinating disease**

In the study described in the previous section, we did not find significant differences in clinical signs, neuropathology, virus persistence or anti-viral antibody responses between control mice and mice administered anti-IP-10 serum. This is in contrast to other animal models of MS, where neutralization of IP-10 has been shown to suppress clinical disease (Table 1).

**IP-10 modulates EAE clinically and histologically**

In EAE, Ransohoff et al.

IP-10 mRNA expression in the liver in this EAE model was...
also reported by Ransohoff et al. but not by others. The amount of chemokine mRNA had diminished to control levels by day 21 despite the presence of inflammation. Godiska et al. also demonstrated expression of IP-10 mRNA in spinal cords during the acute, remission and relapsing phases not only in actively induced EAE but also in adoptive transfer EAE induced by a PLP specific T cell line. When the PLP specific T cell line was stimulated in vitro with PLP peptide, no IP-10 mRNA was detected, but MIP-1a, MIP-1b, TCA3 and RANTES mRNAs were found. In other studies, protection from PLP-induced EAE by treatment with an altered peptide ligand was accompanied by reduced levels of IP-10 and its receptor CXCR3.

IP-10 or its mRNA expression in the CNS were also detected in C57BL/6 and Sv129 X C57BL/6 mice sensitized with myelin oligodendrocyte glycoprotein (MOG) peptide, Lewis rats sensitized with myelin basic protein (MBP), and macaque monkeys inoculated with human brain white matter. The role of IP-10 in EAE has also been investigated by modulating IP-10 in vivo. Wojcik et al. showed that intrathecal infusion of antisense oligonucleotides against IP-10 reduced clinical signs in Lewis rats with MBP-induced EAE without concomitant alteration of inflammation in the spinal cord. Similarly, Fife et al. found that administration of anti-IP-10 antibody decreased clinical and histological disease in SJL/J mice receiving PLP specific T cells.

DNA immunization has been used not only as a way of targeted delivery of gene products, but also as a way to elicit an immune response against the gene product encoded by the construct. Wildbaum et al. reported that administration of plasmid DNA encoding IP-10 suppressed EAE with production of anti-IP-10 antibodies and alteration of myelin antigen specific lymphoproliferation from Th1 to Th2. The suppression was seen not only when the naked DNA was given prior to induction of EAE, but also six to seven days after EAE induction, or 15–17 days after EAE induction (effector phase). Neutralization of IP-10 by anti-IP-10 antibodies produced by DNA vaccination seemed to play the pivotal role, since these antibodies inhibited the development of disease when passively transferred to other EAE animals.

### Discussion

Distinct roles of IP-10 and CXCR3+ T cells could contribute to differences in efficacy of IP-10 neutralization among animal models for MS

Table 1 illustrates the differences in the effect of IP-10 blocking studies between three animal models for MS. While blocking of IP-10 resulted in clinical or histological improvement with suppression of antigen specific lymphoproliferation in both EAE and MHV models, TMEV infected mice showed no alteration in CNS disease, clinically or histologically, and increased levels of lymphoproliferation. The failure to ameliorate clinical and pathological disease by anti-IP-10 serum treatment in TMEV infection compared with EAE and MHV infection could be due to a difference in the role CXCR3+ cells play, particularly Th1 cells and NK cells, in each model. In EAE, CD4+ Th1 cells are known to initiate demyelinating disease, while the role of Th1 cells in TMEV infection is still controversial. Further, a relative lack of NK cells in SJL/J mice, which were used in our experiments, suggests that NK cells might not be the major target of IP-10 in models using this strain of mouse.

Another factor that could influence the efficacy of the anti-IP-10 treatment is where, in the body, the antiserum blocks the interaction between IP-10 and its receptor. In some EAE models, IP-10 expression has been demonstrated in secondary lymphoid organs, which would help in the recruitment and activation of encephalitogenic T cells to these sites. However, no IP-10 expression has been detected in lymphoid organs in TMEV infection. This might explain why neutralization of IP-10 resulted in an

<table>
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<th>Model</th>
<th>Treatment</th>
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<th>Lymphoproliferation*</th>
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<tr>
<td>Active EAE</td>
<td>intrathecal antisense</td>
<td>5–6 days after EAE induction</td>
<td>improve</td>
<td>no change</td>
<td>N.E.</td>
<td>N.E.</td>
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<tr>
<td>Passive EAE</td>
<td>anti-IP-10 antibody</td>
<td>days 0 and 2</td>
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<td>decrease</td>
<td>N.E.</td>
<td>no change or decrease**</td>
<td>37</td>
</tr>
<tr>
<td>Active EAE</td>
<td>DNA vaccine</td>
<td>before or after EAE induction</td>
<td>improve</td>
<td>decrease</td>
<td>N.E.</td>
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<td>anti-IP-10 antibody</td>
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<td>decrease</td>
<td>decrease</td>
<td>N.E.</td>
<td>33</td>
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<tr>
<td>MHV</td>
<td>IP-10−/− mice</td>
<td>entire period</td>
<td>N.E.</td>
<td>decrease</td>
<td>decrease</td>
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<td>46</td>
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<tr>
<td>TMEV</td>
<td>anti-IP-10 antibody</td>
<td>acute or chronic</td>
<td>no change</td>
<td>no change</td>
<td>no change</td>
<td>increase</td>
<td>Present study</td>
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N.E., not examined.
* Myelin antigen specific lymphoproliferation in EAE, and virus antigen specific lymphoproliferation in MHV or TMEV infection.
** No change in antigen-specific IFN-γ production. The authors also described that antigen (PLP)-specific lymphoproliferation was unchanged, although figure 6(C) of Fife et al. showed mild suppression of PLP-specific lymphoproliferation in anti-IP-10 treated group.
increase in antigen specific and autoreactive T cell responses in TMEV infection, but not in EAE.

Although we do not know exactly where IP-10 and its receptor interact in the CNS, the difference in the location of the interaction among animal models for MS might be important in using anti-IP-10 as a therapeutic agent. Theoretically, anti-IP-10 antibody could block IP-10 not only on endothelial cells but also within the brain parenchyma, if there is a disruption of the blood–brain barrier around regions producing IP-10. IP-10 has been shown to bind not only to its receptor CXCR3 but also to heparan sulfate proteoglycans (HSPG). In the CNS, the majority of the proteoglycans contain either chondroitin sulfate or heparan sulfate side chains. Some are constituents of the extracellular matrix and others are bound to the cell surface. Therefore, while CXCR3+ encephalitogenic T cells can bind to IP-10 displayed by HSPG on endothelial cells during transendothelial migration into the CNS, CXCR3+ T cells might also bind to IP-10 presented by HSPG on neural cells and extracellular matrix, helping encephalitogenic T cells remain in the CNS. In this scenario, i.p. injection of anti-IP-10 antibody could effectively block the chemokine presented on endothelial cells and prevent activation of integrin on encephalitogenic cells, thereby blocking invasion of MNCs into the CNS. It may not be, however, effective enough to block the interaction between IP-10 and CXCR3+ T cells in the CNS parenchyma. Although breakdown of the blood–brain barrier has been reported in inflammatory lesions in the CNS, it may not be significant enough for anti-chemokine antibody to penetrate and function in the CNS parenchyma. Here, the differences in the preservation of blood–brain barrier between animal models could also influence the efficacy of treatment with the antibody.

Roles of IP-10 and CXCR3+ T cells may differ among MS patients with different clinical courses

The difference in efficacy of IP-10 modulation among animal models for MS was discussed above. This difference may reflect the potentially different roles of IP-10 in individual patients with MS, since MS has been suggested to be multifactorial and has clinical and histological subtypes. One confounding factor is that different strains of mice, C57BL/6, BALB/c and SJL/J, have been shown to differ in IP-10 mRNA expression in spleens, even when immunologically naïve. In addition, one model of EAE involves no CNS expression of IP-10. In IFN-γ knockout BALB/c mice, IP-10 was not expressed in the CNS when EAE was induced with two subcutaneous injections of bovine MBP. Thus, the production of IP-10 in the CNS and its role in EAE development has been proposed to differ among various subtypes of EAE.

The spectrum of clinical disease of MS is diverse. The National Multiple Sclerosis Society (USA) has defined four clinical courses of MS by an international consensus among clinicians involved in MS clinical research and care: RR, primary progressive (PP), secondary progressive (SP), and progressive relapsing (PR). Since the clinical, epidemiological and pathological findings in PP-MS are notably different from those described for RR-MS, the question arises whether PP-MS and RR-MS are two distinct disease entities. Using an ELISA, Scarpini et al. demonstrated that IP-10 levels were significantly elevated in CSF and sera from RR and SP, but not from PP-MS patients. Similarly, Jalonen et al. detected IP-10 mRNA from peripheral blood MNCs in one of 11 patients with SP-MS and two of 17 patients with RR-MS, but none of the seven patients with PP-MS, using RPA. In contrast, using ELISA, Mahad et al. demonstrated that the concentration of IP-10 was significantly greater in patients with RR-MS compared with SP-MS. These studies suggest that roles of IP-10 may differ among MS patients with different clinical courses. On the other hand, one study found no differences in IP-10 levels between MS patients with different clinical courses, probably due to the fact that IP-10 expression in patients of one clinical form was highly variable.

The same situation is apparent in the analysis of the number of CXCR3+ cells in the blood from MS patients. Sørensen and Sellebjerg showed that IFN-β1b treatment did not alter the percentage of CXCR3+ cells in peripheral blood MNCs from patients with SP-MS, while the same authors reported that IFN-β1a suppressed the expression of CXCR3 on T cells in RR-MS. On the other hand, Martínez-Cáceres et al. found a significant increase in the percentage of CXCR3 in CD14+ macrophages in peripheral blood by flow cytometry with no differences between RR-, SP- and PP-MS. The above studies suggest that the role of IP-10 in RR-MS might be different from those in the progressive forms of MS, although conflicting results make it difficult to draw strong conclusions at this time. The characteristic clinical course of TMEV induced demyelination is a gradual, nearly continuously worsening baseline without distinct relapses. Thus, TMEV infection is an animal
model for the progressive forms of MS. This is different from the other animal models for MS; most of their clinical courses are either acute monophasic or RR with a few exceptions. Therefore, the distinct outcome seen in neutralization of IP-10 in TMEV infection might be due to a common unique immunological pathomechanism in progressive forms of demyelinating diseases. Thus, the clinical course as well as the genetic background of each patient with MS should be considered for the clinical application of neutralization of IP-10 in humans.

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