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Neutralization of the Chemokine CXCL10 Reduces Inflammatory Cell Invasion and Demyelination and Improves Neurological Function in a Viral Model of Multiple Sclerosis[1]

Michael T. Liu,* Hans S. Keirstead,†‡ and Thomas E. Lane[2*†]

Intracerebral infection of mice with mouse hepatitis virus (MHV) results in an acute encephalomyelitis followed by a chronic demyelinating disease with clinical and histological similarities with the human demyelinating disease multiple sclerosis (MS). Following MHV infection, chemokines including CXC chemokine ligand (CXCL)10 (IFN inducible protein 10 kDa), CXCL9 (monokine induced by IFN-γ), and CC chemokine ligand 5 (RANTES) are expressed during both acute and chronic stages of disease suggesting a role for these molecules in disease exacerbation. Previous studies have shown that during the acute phase of infection, T lymphocytes are recruited into the CNS by the chemokines CXCL10 and CXCL9. In the present study, MHV-infected mice with established demyelination were treated with antisera against these two chemokines, and disease severity was assessed. Treatment with anti-CXCL10 reduced $CD4^+$ T lymphocyte and macrophage invasion, diminished expression of IFN-γ and CC chemokine ligand 5, inhibited progression of demyelination, and increased remyelination. Anti-CXCL10 treatment also resulted in an impediment of clinical disease progression that was characterized by a dramatic improvement in neurological function. Treatment with antisera against CXCL9 was without effect, demonstrating a critical role for CXCL10 in inflammatory demyelination in this model. These findings document a novel therapeutic strategy using Ab-mediated neutralization of a key chemokine as a possible treatment for chronic human inflammatory demyelinating diseases such as MS. ***The Journal of Immunology,*** **2001, 167: 4091–4097.**

Multiple sclerosis (MS)[3] is a demyelinating disease characterized by chronic inflammation and progressive immune-mediated destruction of the myelin sheath (1). The inflammatory process is thought to be mediated in part by T lymphocytes and macrophages that are recruited to the CNS in response to chemotactic signals such as chemokines. Two chemokines that have been implicated in the pathogenesis of MS are CXC chemokine ligand (CXCL)10 (IFN-γ inducible protein 10 kDa) and CXCL9 (monokine induced by IFN-γ) (2–5). These chemokines operate by binding to the receptor CXCR3 on T lymphocytes and NK cells (6–8). A recent study has shown that CXCL10 and CXCL9 are present within the cerebral spinal fluid of MS patients during clinical attacks (3). In addition, postmortem analyses of MS brains reveal increased expression of CXCL10 and CXCL9 by astrocytes and CXCR3-positive T lymphocytes within inflammatory lesions undergoing demyelination (3–5). These observations have led to the hypothesis that CXCL10 and/or CXCL9 are key chemoattractants for the inflammatory cells that contribute to demyelination. The availability of blocking Abs for particular chemokines provides an opportunity to test their role in contributing to inflammatory cell invasion and myelin destruction in animal models of MS.

Intracerebral infection of mice with mouse hepatitis virus (MHV), a (+)-strand RNA virus that is a member of the *Coronaviridae* family, triggers a vigorous immune response involving both T lymphocytes and macrophages (9–12). CXCL10 and CXCL9 have important roles in attracting leukocytes into the CNS during the acute stage of disease. Indeed, neutralization of either CXCL10 or CXCL9 activity through administration of specific antisera results in increased viral titers within the brain that correlates with decreased T lymphocyte (both $CD4^+$ and $CD8^+$ subsets) infiltration into the CNS and diminished IFN-γ expression (11, 12).

Following the acute phase of infection, virus persists within white matter tracts, and mice often develop a chronic demyelinating disease in which there is continued leukocyte infiltration into the spinal cord, extensive myelin stripping, and neurological impairment including partial-to-complete hindlimb paralysis (13, 14). Chronic CXCL10 expression is associated with demyelinating lesions in MHV-infected mice suggesting that CXCL10 contributes to the pathogenesis of demyelination by attracting leukocytes into the CNS. Given the important role of CXCL10 during the acute inflammatory stage of disease, the present study evaluated whether administration of anti-CXCL10 antisera to persistently infected mice with established inflammation and demyelination could alter the course of disease.

We demonstrate that treatment with anti-CXCL10 antisera during the chronic phase of MHV-induced CNS disease prevents progression of demyelination within spinal cord white matter tracts and improves neurological function in persistently infected mice. In contrast, administration of anti-CXCL9 antisera was ineffective. These results document the feasibility of treating demyelinating diseases with Abs to chemoattractant molecules and also provides

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[3] Abbreviations used in this paper: MS, multiple sclerosis; MHV, mouse hepatitis virus; NRS, normal rabbit serum; p.i., postinfection; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; LFB, Luxol fast blue.

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strong evidence that CXCL10 is a key chemoattractant during chronic demyelination in this model.

Materials and Methods

Virus and mice

MHV strain J2.2-V.1 was provided by J. Fleming (University of Wisconsin, Madison, WI) (15). Age-matched (5- to 7-wk old) male C57BL/6 mice (H-2^b background) were used for all studies (National Cancer Institute, Bethesda, MD). Following anesthetization by inhalation of methoxyflurane (Pitman-Moore, Washington Crossing, NJ), mice were injected intracranially with 1000 PFU of MHV suspended in 30 μl of sterile saline. Control (sham) animals were injected with 30 μl of sterile saline alone. Animals were sacrificed at days 7, 12, 21, and 28 postinfection (p.i.), at which point brains and spinal cords were removed for analysis in studies described. For determination of viral burden within the CNS, one-half of each brain was used for plaque assay on the DBT astrocytoma cell line as previously described (assay sensitivity is ~100 PFU/g tissue (2.0 $\log_{10}$); Refs. 10–12).

Ab preparation and treatment of mice

Rabbit antisera specific to either CXCL10 or CXCL9 have been previously described (11, 12, 16). These reagents effectively neutralize activity in vivo and do not cross-react with each other or other known chemokines (11, 12, 16). Mice were injected i.p. with 0.5 ml of Ab (~0.5 mg/ml) on days 12, 14, 16, and 19 p.i. and sacrificed at days 21 and 28 p.i. Clinical scoring was as follows: 0, no abnormality; 1, limp tail; 2, waddling gait and partial hindlimb weakness; 3, complete hindlimb paralysis; 4, death (10). Treatments were coded and clinical scores determined independently by three investigators. Treatment with increasing amounts of either anti-CXCL9 or anti-CXCL10 (>0.5 ml) did not result in improved clinical or histologic disease. A control group was treated with 0.5 ml of normal rabbit serum (NRS).

Mononuclear cell isolation and flow cytometry

A single cell suspension was obtained from brains of mice treated with anti-CXCL10, anti-CXCL9, or NRS at days 21 and 28 p.i., and FACS analysis was performed as previously described (10). Briefly, brains were removed and homogenized by grinding the tissue followed by mincing with a razor blade to obtain a single cell suspension. Cell suspensions were transferred to 15-ml conical tubes and Percoll (Pharmacia, Uppsala, Sweden) was added for a final concentration of 30%. One milliliter of 70% Percoll was underlaid, and the cells were centrifuged at 1300 × *g* for 30 min at 4°C. Cells were removed from the interface and washed twice. FITC-conjugated rat anti-mouse CD4, CD8 (BD PharMingen, San Diego, CA), and F4/80 (Serotec, Oxford, England) were used to detect infiltrating $CD4^+$ and $CD8^+$ T lymphocytes as well as macrophages, respectively. As a control, an isotype-matched FITC Ab was used. Cells were incubated with Abs for 30 min at 4°C, washed, fixed in 1% paraformaldehyde, and analyzed on a FACStar (BD Biosciences, Mountain View, CA). Data are presented as the percentage of positive cells within the gated population. Data represent between three and six mice per treatment group examined in two separate experiments.

$CD4^+$ and $CD8^+$ T lymphocyte enrichment

Mononuclear cells were obtained from the CNS of experimental mice at days 21 and 28 p.i. according to the method described above, and a single cell suspension was obtained and pooled (three mice per group at each time point). Cells were resuspended in 90 μl of degassed, Ca^{2+} and Mg^{+2}-free PBS supplemented with 0.5% BSA (MACS buffer) and stained with magnetic Abs specific for either CD4 or CD8 (Miltenyi Biotec, Auburn, CA) at a 1/10 dilution for 15 min at 4°C. Cells were washed, centrifuged at 300 × *g* for 10 min, and resuspended in 500 μl of MACS buffer. The cell suspension was then applied to a MACS MS^+ separation column (Miltenyi Biotec), washed three times, and eluted in 1 ml of MACS buffer. Flow cytometry confirmed >95% purity of T lymphocyte fractions. Total RNA was isolated from the T cell subsets and subjected to RT-PCR to evaluate CXCR3 expression.

RT-PCR

Total RNA was extracted from brains of $CD4^+$ and $CD8^+$ T lymphocyte subsets at defined times p.i. using TRIzol reagent (Life Technologies, Carlsbad, CA) and reverse transcribed with the AMV reverse transcriptase system (Promega, Madison, WI). PCR amplification was performed on resulting cDNA with primers specific for either IFN-γ (Clontech Laboratories, South San Francisco, CA); CXCR3: forward 5′-GCGGCCG CAACTCTTCCATTGTGGGCAG, reverse 5′-GAATTCAAGGCCCCTCGA TAGAAGTT; CC chemokine ligand (CCL)5: forward 5′-GTCGACGCGGG TACCATGAAGATCTCT, reverse 5′-TCTAGAAACCCTCTATCCTAGCT CATCTC; or L32: forward 5′-AACGCTCAGCTCCTTGACAT, reverse 5′-A ACCCAGAGGCATTGACAAC. PCR amplification was performed on an automated PerkinElmer (Norwalk, CT) model 480 DNA thermocycler with the following profile: step 1, initial denaturation at 94°C for 45 s; step 2, annealing at 60°C for 45 s; and step 3, extension at 72°C for 2 min. Steps 1–3 were repeated 29 times for a total of 30 cycles. For cDNA generated from $CD4^+$ or $CD8^+$ T lymphocyte subsets, a total of 35 reaction cycles were used. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Sequence analysis of the PCR amplicons confirmed primer specificity.

ELISA

Brains were removed from MHV-infected mice that were administered anti-CXCL10, anti-CXCL9, or NRS at 21 days p.i. and homogenized (PowerGen 125; Fisher Scientific, Pittsburgh, PA) in PBS and centrifuged at 1300 × *g* for 10 min. Following collection of the supernatant, CCL5 (RANTES) and IFN-γ protein levels in brain were determined using the Quantikine M mouse RANTES and IFN-γ immunoassay kits according to manufacturers specifications (R&D Systems, Minneapolis, MN) (10–12).

Histology

Spinal cords were removed at 21 and 28 days p.i. and fixed by immersion overnight in 10% normal buffered formalin after which portions of tissue were embedded in paraffin or TAAB resin (Electron Microscopy Services, Fort Washington, PA). The severity of demyelination was determined by three methods: 1) Luxol fast blue (LFB) staining of spinal cords (8 μm sections) and analysis by light microscopy. Demyelination was scored as follows: 0, no demyelination: 1, mild inflammation accompanied by loss of myelin integrity; 2, moderate inflammation with increasing myelin damage; 3, numerous inflammatory lesions accompanied by significant increase in myelin stripping; and 4, intense areas of inflammation accompanied by numerous phagocytic cells engulfing myelin debris (10). Slides containing stained spinal cord sections were blinded and scored by three investigators. Scores were averaged and presented as average ± SEM. The data presented represent a minimum of six spinal cord sections per mouse with three to six mice per time point. 2) To determine the extent of demyelination and remyelination, the lesion-containing length of spinal cord was cut into 1-mm transverse blocks and processed so as to preserve the craniocaudal sequence and orientation. The tissue blocks were rinsed in 0.1 M phosphate buffer, pH 7.4, for 30 min, postfixed in 2% OsO_4, dehydrated in ascending alcohols, and embedded in TAAB resin. Thin sections (1 μM) were cut from each block, stained with alkaline toluidine blue, and examined by light microscopy. 3) Electron microscopic analysis. Blocks were trimmed then cut at 100 nm, mounted on copper grids, uranyl acetate and lead citrate stained, and viewed under a Hitachi (Tokyo, Japan) EM 600 electron microscope at 75 kV. In adult animals there is a relationship between axon circumference and myelin sheath thickness (number of lamellae) expressed by the g-ratio (axon diameter: total fiber diameter); in remyelination this relationship changes such that myelin sheaths are abnormally thin for the axons they surround (17). An abnormally thin myelin sheath, relative to axonal diameter, was the criterion for oligodendrocyte remyelination. Absence of a myelin sheath was the criterion for demyelination. In all cases, slides were blinded and read independently by three investigators.

Statistical analysis

Statistically significant differences between groups of mice were determined by *t* test using Sigma-Stat 2.0 software (Jandel, Chicago, IL), and *p* values of ≤ 0.05 were considered significant.

Results

Treatment with anti-CXCL10, but not anti-CXCL9, reduces neurologic disease

Intracranial infection of C57BL/6 mice with MHV results in an acute encephalomyelitis with virus replicating primarily within glial cells. Plaque assays document increased viral titers in the brain at day 7 p.i. (3.9 ± 0.2 $\log_{10}$ PFU/g tissue; $n = 4$), but by day 12 p.i., infectious virus is not detected (<2 Log_{10} PFU/g tissue; $n = 4$). The majority of animals survive the acute stage of

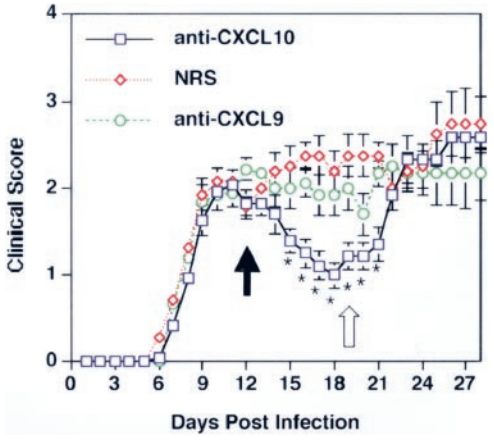

FIGURE 1. Clinical disease is decreased in anti-CXCL10-treated mice. Mice were infected intracranially with 1000 PFU of MHV and treated i.p. with anti-CXCL10, anti-CXCL9, or NRS at days 12, 14, 16, and 19 p.i. Three days following the first injection (15 days p.i.) mice treated with anti-CXCL10 displayed a significant decrease in clinical disease that lasted until 21 days p.i. Treatment was stopped at day 19 p.i. for all groups. By 23 days p.i., mice formerly treated with anti-CXCL10 began to display an increase in the severity of clinical symptoms and eventually developed comparable levels of clinical disease as mice formerly treated with either anti-CXCL9 or NRS. Dark arrow indicates start treatment date (day 12 p.i.), open arrow indicates final treatment date (day 19 p.i.) (anti-CXCL10, $n = 12$; anti-CXCL9, $n = 9$; NRS, $n = 12$). $*$, $p < 0.001$ when compared with both anti-CXCL9- and NRS-treated mice. The clinical data are presented as mean ± SEM and represent the results of three separate experiments.

disease and develop a demyelinating disease characterized by persistence of viral RNA and Ag within white matter tracts accompanied by mononuclear cell infiltration and myelin destruction (10, 13, 15, 18–20). By day 12 p.i., animals developed clinical disease characterized by awkward movement that progressed to partial-to-complete hindlimb paralysis (Fig. 1). T lymphocytes and activated macrophage/microglia (as determined by F4/80 Ag expression) are present within the CNS at this time, and demyelinating lesions are readily detected (Table I).

CXCL10 is associated with demyelinating lesions in mice persistently infected with MHV (18). Although CXCL9 mRNA is not detected in MHV-infected mice past day 12 p.i., it does not preclude the possibility that CXCL9 protein is present in sufficient amounts to contribute to disease (11). Therefore, to determine the role of CXCL10 and CXCL9 in contributing to demyelination in mice persistently infected with MHV, mice were treated with anti-CXCL10, anti-CXCL9, or NRS beginning on day 12 p.i., which represents a time in which demyelination is established and neurological deficits such as hindlimb paralysis are evident (Fig. 1, Table I). Treatment with anti-CXCL10 resulted in a significant reduction in clinical disease severity beginning at 15 days p.i. that lasted until day 21 p.i. ($p < 0.001$) (Fig. 1). During this time, mice treated with either anti-CXCL9 or NRS exhibited hindlimb paralysis and limited mobility, whereas anti-CXCL10-treated mice displayed almost complete restoration of motor skills. Removal of anti-CXCL10 treatment (day 20 p.i.) resulted in an increase in clinical disease severity such that by day 28 there were no differences between any experimental groups (Fig. 1).

Treatment with anti-CXCL10 reduces T lymphocyte and macrophage infiltration into the CNS

Correlating with the decrease in clinical disease in mice treated with anti-CXCL10 were FACS data demonstrating a significant reduction in $CD4^+$ T lymphocytes (58 and 55.2% reduction, $p < 0.02$) and macrophage/microglia cells (54.5 and 50% reduction, $p < 0.01$) present within the CNS of these animals at day 21 p.i. as compared with T lymphocyte and macrophage/microglia levels in anti-CXCL9- and NRS-treated mice, respectively (Table I). Treatment with CXCL9 antisera did not significantly reduce either $CD4^+$ T lymphocyte or macrophage/microglial cell entry into the CNS of infected mice (Table I). Although anti-CXCL10 and anti-CXCL9 treatment resulted in an ~25% decrease in $CD8^+$ T lymphocyte levels as compared with NRS-treated mice, this difference was not significant (Table I). Activated T lymphocytes (both $CD4^+$ and $CD8^+$ subsets) infiltrating into the CNS of MHV-infected mice express CXCR3, the receptor for CXCL10 and CXCL9 (12). In an attempt to determine whether reduced T lymphocyte infiltration into the CNS of anti-CXCL10-treated mice correlated with reduced expression of CXCR3, RT-PCR was performed on RNA isolated from either $CD4^+$ or $CD8^+$ T lymphocyte subsets at defined times p.i. Such analysis revealed a pronounced decrease in the level of CXCR3 mRNA expression in $CD4^+$ T lymphocytes isolated from mice treated with anti-CXCL10 at day 21 p.i. when compared with mice treated with NRS (Fig. 2*A*). In contrast, expression of CXCR3 mRNA in $CD8^+$ T lymphocytes was comparable in mice treated with either anti-CXCL10 or NRS at the same time point, supporting FACS data demonstrating similar levels of $CD8^+$ T lymphocyte infiltration at 21 days p.i. (Fig. 2*A*). Interestingly, CXCR3 mRNA expression in $CD4^+$ T lymphocytes isolated from NRS-treated mice at 21 days p.i. was markedly increased when compared with CXCR3 mRNA expression in $CD8^+$ T lymphocytes isolated from either anti-

Table I. *Cellular infiltration and demyelination is reduced in anti-CXCL10-treated mice*[a]

Treatment	Day p.i.	*n*	Demyelination	Percent Infiltration		
				CD4	CD8	F480
Sham	12	2	0	0.32 ± 0.13[b]	0.45 ± 0.1	6.57 ± 0.7
No treatment	12	2	2.3 ± 0.2	16.4 ± 1.0	20.7 ± 2.3	26.7 ± 1.7
NRS	21	6	2.8 ± 0.1	18.1 ± 2.5	10.2 ± 2.0	28.9 ± 2.7
	28	5	2.3 ± 0.5	11.1 ± 0.8	4.70 ± 1.7	16.3 ± 0.4
Anti-CXCL9	21	4	3.3 ± 0.1	19.3 ± 2.3	7.40 ± 2.4	31.9 ± 4.5
	28	3	2.8 ± 0.4	12.4 ± 2.7	1.30 ± 1.5	ND
Anti-CXCL10	21	6	0.8 ± 0.3[c]	8.10 ± 1.9[d]	7.50 ± 2.1	14.5 ± 1.9[e]
	28	5	3.0 ± 0.5	14.8 ± 1.3	6.90 ± 1.7	19.1 ± 0.1

[a] Data represent results of two independent experiments.
[b] Data presented as mean ± SEM.
[c] $p < 0.001$ when compared to both NRS- and anti-CXCL9-treated mice.
[d] $p < 0.02$ when compared to both NRS- and anti-CXCL9-treated mice.
[e] $p < 0.01$ when compared to both NRS- and anti-CXCL9-treated mice.

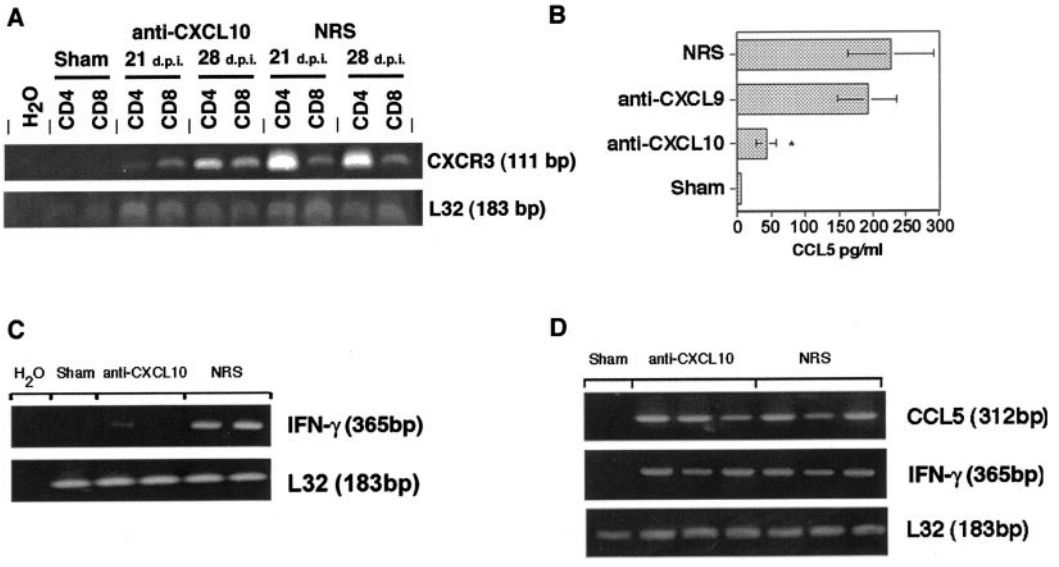

FIGURE 2. *A,* CXCR3 mRNA expression. CXCR3 mRNA expression in $CD4^+$ and $CD8^+$ T lymphocytes isolated from the CNS of mice treated with either anti-CXCL10 or NRS at 21 and 28 days p.i. $CD4^+$ T lymphocytes obtained from mice treated with anti-CXCL10 display a reduced expression of CXCR3 mRNA transcripts when compared with control mice treated with NRS at 21 days p.i. In contrast, comparable levels of CXCR3 mRNA expression in $CD8^+$ T lymphocytes are found in mice treated with anti-CXCL10 and NRS at 21 and 28 days p.i., supporting FACS data showing similar levels of $CD8^+$ T lymphocyte infiltration into the CNS at all times examined. Corresponding to the return of inflammatory cells into the CNS at 28 days p.i., $CD4^+$ T lymphocytes isolated from mice formerly treated with anti-CXCL10 display increased expression of CXCR3 mRNA when compared with those observed at 21 days p.i. *Top panel,* CXCR3 (111 bp); *bottom panel,* L32 (183 bp). The results presented represent enriched lymphocytes pooled (three mice per group) at each time point. *B,* CCL5 protein levels at 21 days p.i. CCL5 protein levels from brains of mice at 21 days p.i were determined by ELISA. Mice treated with anti-CXCL10 displayed significantly decreased levels of CCL5 when compared with either NRS- or anti-CXCL9-treated mice (21 days p.i.). $*$, $p < 0.05$ (anti-CXCL10: $n = 3$; anti-CXCL9: $n = 3$; and NRS: $n = 3$). *C,* IFN-γ mRNA levels at 21 days p.i. IFN-γ protein was not detected by ELISA; therefore, RT-PCR analysis was used to evaluate IFN-γ expression at day 21 p.i. Mice treated with anti-CXCL10 show a marked decrease in the levels of IFN-γ mRNA transcripts in the CNS when compared with NRS-treated mice. *Top panel,* IFN-γ (365 bp); *bottom panel,* L32 control (183 bp). Each lane represents an individual mouse. *D,* CCL5 and IFN-γ mRNA levels at 28 days p.i. RT-PCR analysis of CCL5 and IFN-γ expression within the CNS of MHV-infected mice at 28 days p.i. Total RNA was extracted from the brains of MHV-infected mice formerly treated with either anti-CXCL10 or NRS at 28 days p.i. and subjected to RT-PCR. Mice formerly treated with anti-CXCL10 and NRS show comparable levels of both CCL5 and IFN-γ mRNA transcripts in the CNS. *Top panel,* CCL5 (312 bp); *middle panel,* IFN-γ (365 bp); *bottom panel,* L32 control (183 bp). Each lane represents an individual mouse.

CXCL10- or NRS-treated mice, suggesting differential expression of this chemokine receptor on T lymphocyte subsets during the chronic stage of MHV infection. Removal of anti-CXCL10 treatment (day 20 p.i.) resulted in an increase in the severity of clinical disease symptoms that correlated with the return of inflammatory $CD4^+$ T lymphocytes and activated macrophage/microglia into the CNS (Fig. 1 and Table I). Corresponding with the return of inflammation within the CNS of mice formerly treated with anti-CXCL10 is the increased expression of CXCR3 mRNA in the $CD4^+$ T lymphocyte subset at 28 days p.i. as compared with levels at 21 days p.i. (Fig. 2*A*).

As both $CD4^+$ and $CD8^+$ T lymphocytes are important in the elimination of MHV from the CNS during acute disease, the dramatic decrease in levels of T lymphocytes present within the CNS of mice treated with anti-CXCL10 suggested the possibility of viral recrudescence. To test for this, brain and spinal cord samples from mice treated with anti-CXCL9, anti-CXCL10, or NRS at days 21 and 28 p.i. were examined for the presence of virus by plaque assay. Replicating virus was not detected in any experimental group at these time points (data not shown).

Treatment with anti-CXCL10 reduces IFN-γ and CCL5 expression

Activated T lymphocytes obtained from the CNS of MHV-infected mice during chronic disease express IFN-γ (21, 22). In addition, CCL5 expression is associated with demyelinating lesions in persistently infected mice, and expression of this chemokine has been shown to contribute to demyelination by attracting macrophages into the CNS (10). Analysis of CCL5 and IFN-γ expression at day 21 p.i. revealed decreased levels (as assessed by ELISA and RT-PCR) in mice treated with anti-CXCL10 when compared with control animals (Fig. 2, *B* and *C*). These data support FACS analysis demonstrating the pronounced decrease in T lymphocyte entry into the CNS as this population of cells is thought to produce or influence the expression of CCL5 and IFN-γ in MHV-infected mice (10, 12, 23). Moreover, the decreased macrophage infiltration into the CNS of anti-CXCL10-treated mice can be explained as a result of lowered levels of CCL5 (10). Analysis of IFN-γ and CCL5 mRNA levels within the CNS at day 28 p.i. revealed comparable levels for both transcripts in mice formerly treated with anti-CXCL10 and NRS (Fig. 2*D*). The increased expression of both IFN-γ and CCL5 correlated with the return of T lymphocytes and macrophages into the CNS as well as clinical disease.

Treatment with anti-CXCL10 reduces the extent of myelin destruction and results in myelin repair

The fact that anti-CXCL10 treatment resulted in reduced clinical disease severity and leukocyte infiltration into the CNS suggested that demyelination may be affected as T lymphocytes and macrophages are considered important contributors to demyelination in MHV-infected mice (10, 23, 24). Indeed, LFB staining of spinal cords indicated that mice treated with anti-CXCL10 exhibited a marked reduction in the numbers of inflammatory foci present within white matter tracts; this was accompanied by a significant

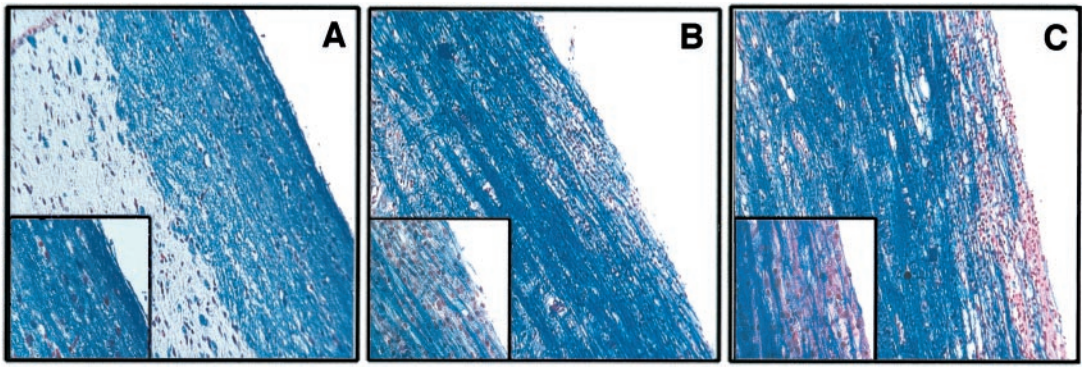

FIGURE 3. LFB staining of spinal cords. Demyelination is reduced in mice treated with anti-CXCL10 as compared with NRS- or anti-CXCL9-treated mice at day 21 p.i. Representative spinal cord sections from mice treated with anti-CXCL10 21 days p.i. (*A*), mice treated with anti-CXCL9 21 days p.i. (*B*), or mice treated with NRS 21 days p.i. (*C*). Sections were stained with LFB to assess the severity of demyelination. Numerous inflammatory foci and areas of myelin stripping are present within the white matter tracts of NRS- or anti-CXCL9-treated mice, whereas limited numbers of inflammatory cells are detected in white matter tracts of anti-CXCL10-treated mice, and only minimal damage is detected. (magnification, ×100, inset ×400).

reduction in the severity of demyelination ($p < 0.001$; 0.8 ± 0.3, $n = 6$) when compared with either NRS- (2.8 ± 0.1, $n = 6$) or anti-CXCL9 (3.3 ± 0.1, $n = 4$)-treated mice (Fig. 3 and Table I). Furthermore, additional analysis of demyelination by toluidine blue staining of spinal cord sections revealed that mice treated with NRS displayed numerous inflammatory foci and robust demyelination throughout the ventral, lateral, and dorsal columns (Fig. 4, *B* and *D*). In contrast, demyelination was limited to the ventral column in mice treated with anti-CXCL10 supporting the observation that progression of disease is impeded (Fig. 4, *A* and *C*). Removal of anti-CXCL10 treatment correlated with a marked increase in the severity of demyelination such that there were no appreciable differences between any of the experimental groups by day 28 p.i. (Table I).

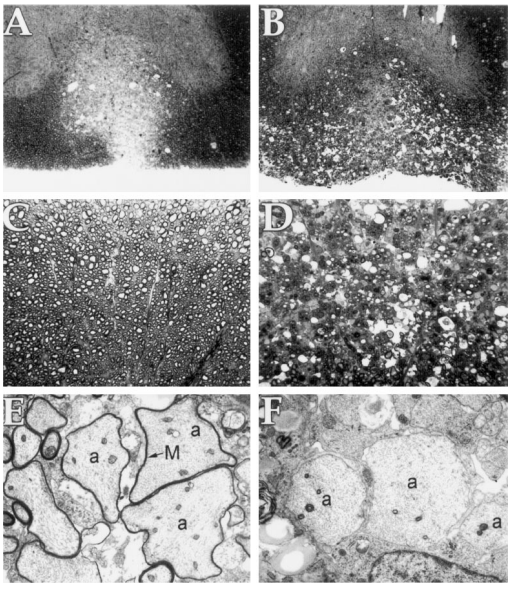

FIGURE 4. Toluidine blue-stained photomicrographs and electron micrographs of anti-CXCL10-treated mice (*A, C,* and *E*) and NRS-treated mice (*B, D,* and *F*). *A,* Toluidine blue-stained transverse section of an anti-CXCL10-treated mouse, showing that the region of demyelination is well defined and limited to the ventral column. *B,* Toluidine blue-stained transverse section of an NRS-treated mouse, showing that the lesion extends throughout the ventral and lateral columns. *C,* Higher magnification of a toluidine blue-stained transverse section of the lateral column in an anti-CXCL10-treated mouse, showing normal myelination. *D,* Higher magnification of a toluidine blue-stained transverse section of the lateral column in an NRS-treated mouse, showing extensive demyelination, vacuolization, and debris-laden macrophages. *E,* Electron micrograph of an anti-CXCL10-treated mouse showing axons within the ventral column with thin myelin sheaths (denoted by M and arrow) surrounding axon (a) characteristic of remyelination. *F,* Electron micrograph of an NRS-treated mouse, showing axons (a) within the ventral column with no evidence of remyelination. ×200 for *A* and *B*; ×600 for *C* and *D*; ×8000 for *E* and *F*.

Because anti-CXCL10 treatment attenuated disease progression, the possibility arose that treatment was promoting myelin repair. We evaluated electron micrographs from treated and control animals for profiles suggestive of myelin repair. An abnormally thin myelin sheath, relative to axonal diameter, was the criterion for oligodendrocyte remyelination (17). As illustrated in Fig. 4, 80.1 ± 5.1% ($n = 4$ spinal cords) of the axons within the ventral column lesion in anti-CXCL10-treated mice were surrounded by a thin myelin sheath, characteristic of remyelination (Fig. 4*E*). In contrast, only 4 ± 0.71% ($n = 3$ spinal cords) of the axons within the ventral column lesion in NRS-treated mice showed evidence of remyelination, the majority being demyelinated (Fig. 4*F*). The relative failure of remyelination in control mice may be due to the ongoing demyelination, a conclusion that is supported by the increased number of T lymphocytes and macrophages, as well as the increased levels of CCL5 and IFN-γ in this treatment group.

Discussion

The immune response to MHV infection of the CNS contributes to viral clearance (10–12, 25–28) as well as demyelination (10, 23, 24, 29). An orchestrated expression of chemokines occurs within the brains and spinal cords of MHV-infected mice; this precedes and accompanies leukocyte infiltration indicating an important role for these molecules in contributing to host defense and disease development by attracting leukocytes into the CNS following MHV infection (18). Indeed, recent studies from our laboratory have shown that administration of anti-CXCL9 or anti-CXCL10 antisera to MHV-infected mice during acute disease results in increased viral titers within the CNS that correlates with diminished T lymphocyte infiltration and IFN-γ expression (11, 12). These studies indicate that both CXCL9 and CXCL10 serve as important sentinel molecules in host defense following MHV infection and contribute to a protective Th1-mediated response. During chronic disease, CXCL10 expression is primarily associated with demyelinating lesions in MHV-infected mice suggesting an important role in contributing to disease by attracting leukocytes into the

CNS (18). In addition, recent studies have indicated that T lymphocytes are important contributors to demyelination in MHV-infected mice (10, 23). Therefore, the present study was undertaken to determine whether chronic expression of CXCL10 contributes to the pathogenesis of MHV-induced demyelination by attracting T lymphocytes into the CNS.

The data presented clearly indicate that administration of anti-CXCL10 to MHV-infected mice with established demyelination resulted in 1) an improvement in clinical disease, 2) diminished infiltration of T lymphocytes and activated macrophage/microglia into the CNS, 3) reduced expression of IFN-γ and CCL5, 4) suppression of ongoing demyelination, and 5) a dramatically increased number of remyelinated axons within lesions. These beneficial effects were fully reversed when anti-CXCL10 treatment was discontinued. Specifically, cessation of anti-CXCL10 delivery resulted in 1) the restoration of clinical disease, 2) the return of T lymphocytes and macrophage/microglia within the CNS, 3) comparable levels of IFN-γ and CCL5 mRNA expression, and 4) robust demyelination. Taken together, the results presented provide the first clear evidence that CXCL10 is an important contributor to the pathogenesis of demyelination in the MHV model system by regulating inflammatory cell infiltration into the CNS during the chronic phase of disease. In addition, these studies illustrate the delicate balance that exits between chemokine expression as it relates to host defense and disease development. Specifically, early expression of CXCL10 is beneficial as it serves to attract antiviral T lymphocytes into the CNS that participate in elimination of virus (12). Paradoxically, persistent infection of mice leads to chronic expression of CXCL10 that results in T lymphocyte infiltration into the CNS. Once present, activated T lymphocytes influence the expression of CCL5, which has been shown to contribute to disease pathogenesis, through attraction of additional T lymphocytes and macrophages, that ultimately results in myelin destruction (10, 23). Importantly, the current study supports and extends earlier work demonstrating that Ab-mediated targeting of CXCL10 results in a modulation in the severity of neuroinflammation and disease development (12).

Neutralization of CXCL10 activity resulted in a dramatic improvement in the clinical status of treated animals (Fig. 1). Within 3 days of treatment, mice treated with anti-CXCL10 displayed a significant improvement in clinical disease as compared with mice treated with either anti-CXCL9 or NRS. Upon cessation of anti-CXCL10 treatment at day 20 p.i., anti-CXCL10 animals continued to display only minor signs of clinical disease as compared with the other experimental groups. However, by day 22 p.i., mice formerly treated with anti-CXCL10 began to show signs of worsening disease, e.g., limited mobility and paralysis such that at day 28 p.i. there were no differences in clinical disease severity between experimental groups. Continued treatment of mice with anti-CXCL10 antisera until day 28 p.i. prolongs the reduction in clinical and histologic disease only slightly (data not shown). The limited period that anti-CXCL10 treatment has on MHV-induced CNS disease most likely reflects accelerated decay of the rabbit polyclonal antisera resulting from degradation and/or elimination of the reagent by the host immune response rather than a transient role for CXCL10 in contributing to chronic demyelination. Furthermore, previous studies have shown almost exclusive expression of CXCL10 within and around demyelinating lesions of mice persistently infected with MHV as late as 35 days p.i., indicating a prominent role for CXCL10 in contributing to demyelination throughout the chronic stage of disease (18). Therefore, we believe that CXCL10 does not influence leukocyte infiltration and demyelination for only a limited time but throughout the course of disease.

Anti-CXCL10 treatment had a pronounced effect upon $CD4^+$ T lymphocyte recruitment (55% reduction at day 21 p.i. as compared with control mice), whereas $CD8^+$ T lymphocyte infiltration (26% reduction at day 21 p.i.) into the CNS was not significantly affected (Table I). The marked reduction in $CD4^+$ T lymphocyte infiltration in mice treated with anti-CXCL10 correlated with an inhibition in the progression of demyelination. These data support and extend previous studies indicating an important role for this population of cells in amplifying the severity of demyelination in MHV-infected mice (10, 23). We have previously proposed that $CD4^+$ T lymphocytes may accomplish this by producing and/or influencing the expression of CCL5 that serves to attract macrophages into the CNS (10). The data presented in this report support these earlier observations in that the reduction in $CD4^+$ T lymphocyte infiltration into the CNS of anti-CXCL10-treated mice correlated with reduced expression of CCL5, which was accompanied by a dramatic decrease in macrophage/microglia levels (Table I). Correspondingly, removal of anti-CXCL10 treatment resulted in the return of $CD4^+$ T lymphocytes, increased expression of CCL5, and increased numbers of macrophage/microglia as well as robust demyelination. Collectively, these data indicate that CXCL10 promotes demyelination by attracting $CD4^+$ T lymphocytes into the CNS that, in turn, accelerate disease through CCL5 expression and macrophage infiltration.

Although $CD8^+$ T lymphocyte infiltration into the CNS was reduced in mice treated with anti-CXCL10, the effect was not significant when compared with mice treated with either anti-CXCL9 or NRS (Table I). These data were somewhat surprising in light of our previous study that indicated that neutralization of CXCL10 resulted in $>$75% decrease in both $CD4^+$ and $CD8^+$ T lymphocyte entry into the CNS during acute disease (12). One possible mechanism contributing to this observation is that $CD8^+$ T lymphocytes do not express similar levels of CXCR3 as compared with $CD4^+$ T lymphocytes during the chronic stage of MHV infection. This theory is supported by the decreased level of CXCR3 mRNA expression observed in $CD8^+$ T lymphocytes as compared with expression in the $CD4^+$ T lymphocyte subset at both 21 and 28 days p.i. (Fig. 2*A*). In addition, it is also possible that $CD8^+$ lymphocytes may be responding to different chemotactic signals during the chronic stage of disease.

An important observation was the pronounced suppression of ongoing demyelination accompanied by improved neurological function in anti-CXCL10-treated mice. The gross increase in remyelination suggests that diminished effector cell recruitment, e.g., $CD4^+$ T lymphocyte and macrophages, into the CNS halts disease progression and repair may be initiated. However, the mechanism(s) by which repair is undertaken are currently unknown at this time. It is possible that lowered numbers of $CD4^+$ T lymphocytes and macrophages result in decreased levels of potentially cytotoxic factors that allow for increased expression of myelin-encoding genes (30, 31). Alternatively, oligodendrocyte progenitor cells, which are present within the adult CNS and have been shown to participate in remyelination, may be attracted to damaged areas within the spinal cord in the absence of CXCL10 activity (32–34). Although we do not know whether myelin repair in the anti-CXCL10-treated mice is associated with functional recovery from conduction block, other studies have demonstrated that conduction velocity and frequency response properties of axons are restored to near normal values following remyelination (35).

In summary, the data presented offer new insight into the functional significance of CXCL10 expression during MHV-induced

demyelinating disease. Extrapolation of these data to human neuroinflammatory diseases such as MS indicate that therapies designed at targeting CXCL10 should be considered as a viable treatment strategy for reducing the severity of neuroinflammation and demyelination.

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