

Antibody Targeting of the CC Chemokine Ligand 5 Results in Diminished Leukocyte Infiltration into the Central Nervous System and Reduced Neurologic Disease in a Viral Model of Multiple Sclerosis¹

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Intracerebral infection of mice with mouse hepatitis virus, a member of the *Coronaviridae* family, reproducibly results in an acute encephalomyelitis that progresses to a chronic demyelinating disease. The ensuing neuropathology during the chronic stage of disease is primarily immune mediated and similar to that of the human demyelinating disease multiple sclerosis. Secretion of chemokines within the CNS signals the infiltration of leukocytes, which results in destruction of white matter and neurological impairment. The CC chemokine ligand (CCL)5 is localized in white matter tracts undergoing demyelination, suggesting that this chemokine participates in the pathogenesis of disease by attracting inflammatory cells into the CNS. In this study, we administer a mAb directed against CCL5 to mice with established mouse hepatitis virus-induced demyelination and impaired motor skills. Anti-CCL5 treatment decreased T cell accumulation within the CNS based, in part, on viral Ag specificity, indicating the ability to differentially target select populations of T cells. In addition, administration of anti-CCL5 improved neurological function and significantly ($p \leq 0.005$) reduced the severity of demyelination and macrophage accumulation within the CNS. These results demonstrate that the severity of CNS disease can be reduced through the use of a neutralizing mAb directed against CCL5 in a viral model of demyelination. *The Journal of Immunology*, 2004, 172: 4018–4025.

Multiple sclerosis (MS)⁵ is a human neuroinflammatory disease that is characterized by demyelinated axons and often associated with chronic inflammation of the CNS (1–3). Although the immunopathology of MS lesions is complex and varied, trafficking and accumulation of immune cells within the CNS are thought to play an important role in the establishment and progression of disease (1, 4). In many instances, the pathology associated with MS is thought to be mediated by the presence of T cells and activated macrophages, all of which are presumably responding to chemotactic signals derived within the CNS (4). Although the mechanisms regulating leukocyte entry into the CNS are not well understood, emerging evidence points to an important role for chemokines in participating in this process (5,

6). Numerous chemokines have been detected within the CNS of MS patients as well as within active plaque lesions, suggesting that these molecules contribute to demyelination by attracting targeted populations of leukocytes into the CNS (7–9). One chemotactic factor considered important to leukocyte trafficking is the CC chemokine ligand (CCL)5, which has been shown to be expressed within the CNS of MS patients (7, 9). For example, samples of cerebral spinal fluid isolated from MS patients undergoing clinical relapse contain significantly higher levels of CCL5 compared with control populations (9). CCL5 transcripts are also expressed in lesions of active demyelination in samples of postmortem brain tissue from MS patients, and peripheral T cells isolated from MS patients exhibited enhanced migration in response to CCL5 (8, 10, 11). These observations have led to the hypothesis that selective neutralization of CCL5 signaling may reduce the severity of neuroinflammation and disease (12, 13).

Infection of the CNS of susceptible mice with mouse hepatitis virus (MHV), a positive-strand RNA virus and a member of the *Coronaviridae* family, reproducibly results in an acute encephalomyelitis followed by a demyelinating disease that is similar to the human demyelinating disease MS (14–16). MHV infection initiates a robust cell-mediated response in which both CD4⁺ and CD8⁺ T cells are essential in controlling viral replication and spread (17–21). However, viral clearance is incomplete, and viral RNA and protein can persist within white matter tracts. These areas of viral persistence are often associated with demyelinating lesions, and recent studies have indicated an important role for both T cells and macrophages in contributing to myelin destruction (17, 22–25). CCR5 and its primary ligand CCL5 are important contributors to the trafficking of leukocytes into the CNS of MHV-infected mice (17, 25–27). In support of this is the demonstration that administration of neutralizing antisera to CCL5 during the acute stage of disease resulted in decreased T cell and macrophage

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⁵ Abbreviations used in this paper: MS, multiple sclerosis; MHV, mouse hepatitis virus; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; p.i., postinfection; i.c., intracranial(ly); S, surface glycoprotein; M, transmembrane protein; RPA, RNase protection assay; LFB, Luxol fast blue.

infiltration, which correlated with significantly reduced levels of demyelination in the CNS (17). During the chronic stage of disease, continued infiltration of T lymphocytes and macrophages into the CNS results in extensive myelin destruction and neurological impairment. T cells infiltrating into the CNS of MHV-infected mice express CCL5, which presumably serves to attract activated leukocytes and activated macrophages (17).

The current study evaluates the functional contributions of CCL5 in participating in inflammation and demyelination in mice persistently infected with MHV. In this study, we demonstrate that treatment with a neutralizing mAb specific for mouse CCL5 results in 1) improved neurological function, 2) decreased infiltration of T cells and macrophages into the CNS, and 3) a significant ($p \leq 0.005$) reduction in demyelination. Additionally, anti-CCL5 treatment selectively targeted T cell subsets based, in part, on their viral Ag specificity, indicating an ability to differentially target T cells during chronic disease in this model. Taken together, these results further implicate CCL5 as an active participant in the maintenance of a chronic immune-mediated demyelinating disease and provide further evidence that targeting chemokines may offer an efficacious way of combating human neuroinflammatory diseases such as MS.

Materials and Methods

Anti-CCL5 mAb

Hybridoma cell lines producing mAb against mouse CCL5 were created by immunizing BALB/c mice with a peptide corresponding to an epitope (KKWVQEYINYLEMS) previously shown to produce neutralizing Abs to CCL5 (17, 28, 29). Splens from immunized mice were removed and fused with SP2/0 myeloma cells using polyethylene glycol (30). Hybridoma cell lines that produced Abs against CCL5 were selected by ELISA and cloned twice by limiting dilution. This selection resulted in 13 positive clones for CCL5. Anti-CCL5 hybridoma clones were then selected based on their ability to recognize full-length CCL5 protein via ELISA and their viability in culture. Clone R6G9 was chosen and produces a mAb that is an IgG1 isotype, κ L chain. Abs were isolated and purified from culture supernatant by affinity chromatography on protein G-Sepharose columns and filter sterilized for use in vivo. R6G9 showed reactivity to recombinant mouse CCL5 (rCCL5; Cell Sciences, Norwood, MA) to a dilution of 1/156,000 via ELISA. The R6G9 anti-CCL5 mAb does not cross-react with other mouse CC chemokines such as monocyte chemoattractant protein-1/CCL2 or macrophage-inflammatory protein-1 α /CCL3 or the mouse CXC chemokines IFN- γ -inducible protein-10/CXC chemokine ligand (CXCL)10 or monokine induced by interferon- γ /CXCL9 as determined by ELISA (28, 29).

Chemotaxis assay

C57BL/6 mice were injected i.p. with MHV strain JHM (kindly provided by S. Stohlman (Keck School of Medicine, University of Southern California, Los Angeles, CA)), and splenocytes were isolated at day 7 postinfection (p.i.). Enriched populations of CD4⁺ and CD8⁺ T cells were obtained using a magnetic Ab to either CD4 or CD8 Ag, respectively (Miltenyi Biotec, Auburn, CA). Enriched cultures were expanded in the presence of 5 μ M peptide corresponding to the immunodominant CD4 epitope present within the transmembrane (M) protein spanning residues 133–147 (M133–147) or the immunodominant CD8 epitope present within the surface (S) glycoprotein at residues 510–518 (S510–518) for 6 days at 37°C (31, 32). Live cells were isolated using Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). Activated macrophages were obtained from C57BL/6 mice injected i.p. with 1 ml of thioglycolate. For chemotaxis assays, 5 \times 10⁵ T cells or macrophages were placed in the top chamber of a Transwell plate (6.5 mm; 5- μ m pore size; Corning, Corning, NY). The bottom well contained either 100 ng/ml rCCL5 alone or 100 ng/ml rCCL5 that had been preincubated for 30 min with either 50 or 200 μ g/ml anti-CCL5 mAb. Cells were placed at 37°C for 3 h, and migration was determined by counting the number of cells in the bottom well in five random high-power fields (\times 100) for each sample (33).

Virus and mice

MHV strain J2.2V-1 was kindly provided by Dr. J. Fleming (University of Wisconsin, Madison, WI) and was used for all intracranial (i.c.) infections (34). Age-matched (5–7 wk old) C57BL/6 mice (H-2^b background) were used for all experiments (National Cancer Institute, Bethesda, MD). Fol-

lowing anesthetization by inhalation of methoxyflurane (Pitman-Moore, Washington Crossing, NJ), mice were injected i.c. with 1000 PFU of MHV suspended in 30 μ l of sterile saline (35). Control (sham) animals were injected with 30 μ l of sterile saline alone. Animals were sacrificed at defined time points, and brains and spinal cords were removed for analysis in studies described. One-half of each brain at each time point was either stored at -80°C for RNA isolation or used for FACS analysis. Immune splenocytes were obtained from C57BL/6 mice injected i.p. with MHV-JHM at day 7 p.i. and used for analysis of virus-specific T cells as described below.

Ab administration

Beginning day 12 p.i., MHV-infected C57BL/6 mice were treated via i.p. injection with 250 μ g of either anti-CCL5 Ab (R6G9) or an IgG1 isotype-matched control Ab (Sigma-Aldrich, St. Louis, MO) suspended in 500 μ l of sterile PBS. Mice received treatment on days 12, 14, 16, 18, and 20 p.i. for a total of five injections.

Clinical disease

Clinical disease was assessed using a previously described scale (17). Briefly, clinical scores can be defined as follows: 1, limp tail; 2, waddling gait and partial hindlimb paralysis; 3, complete hindlimb paralysis; and 4, death. Clinical scores are presented as mean \pm SEM.

Mononuclear cell isolation and intracellular cytokine staining

Cells were obtained from the brains of infected mice at days 21 and 28 p.i., and a single-cell suspension was obtained using a previously described method (36, 37). Intracellular staining for IFN- γ was performed using a total of 10⁶ cells stimulated separately for 6 h with either the CD4 or CD8 epitopes M133–147 and S510–518, respectively, and stained for intracellular IFN- γ using PE-conjugated anti-IFN- γ (1:50; XMG1.2; BD Pharmingen, San Diego, CA) for 1 h at 4°C (35–40). Additional Abs used for immunophenotyping cells in these studies include allophycocyanin-conjugated rat anti-mouse CD4 (BD Pharmingen), allophycocyanin-conjugated rat anti-mouse CD8 (BD Pharmingen), FITC-conjugated rat anti-mouse F4/80 (Serotec, Oxford, U.K.), and PE-conjugated rat anti-mouse CD45 (BD Pharmingen) (36, 37). In all cases, isotype-matched FITC-conjugated or PE-conjugated Ab was used. Cells were incubated with Abs for 1 h at 4°C, washed, and fixed in 1% paraformaldehyde. Following fixation, cells were analyzed using a FACStar flow cytometer (BD Biosciences, Mountain View, CA). Data are presented as the percentage of positive cells within the gated population. Total numbers of cells were calculated by multiplying the percentage of positive cells by the total number of isolated cells.

RNase protection assay (RPA)

Total RNA was obtained from brains of mice at days 21 and 28 p.i. using TRIzol reagent (Invitrogen, Carlsbad, CA). CCL5 and CXCL10 transcripts were analyzed using the mCK-5 multitemplate probe set (BD Pharmingen). RPA analysis was performed with 15 μ g of total RNA using a previously described protocol (17, 41). A probe for L32 was included to verify consistency in RNA loading and assay performance. For quantification of signal intensity, autoradiographs were scanned and chemokine transcript signals were normalized as the ratio of band intensity to the L32 control (17, 41). Analysis was performed using NIH Image 1.61 software.

Histology

Spinal cords were removed at days 21 and 28 p.i. and fixed by immersion overnight in 10% normal buffered formalin before paraffin embedding. The severity of demyelination was determined by Luxol fast blue (LFB) staining of spinal cords and analyzed via light microscopy. LFB-stained spinal cord sections were coded and read blind by three investigators. 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 (17). An average of five spinal cords was scored per group at each time point. Scores were averaged and presented as mean \pm SEM.

Statistical analysis

Statistically significant differences between groups of mice were determined by Student's *t* test, and values of $p \leq 0.05$ were considered significant.

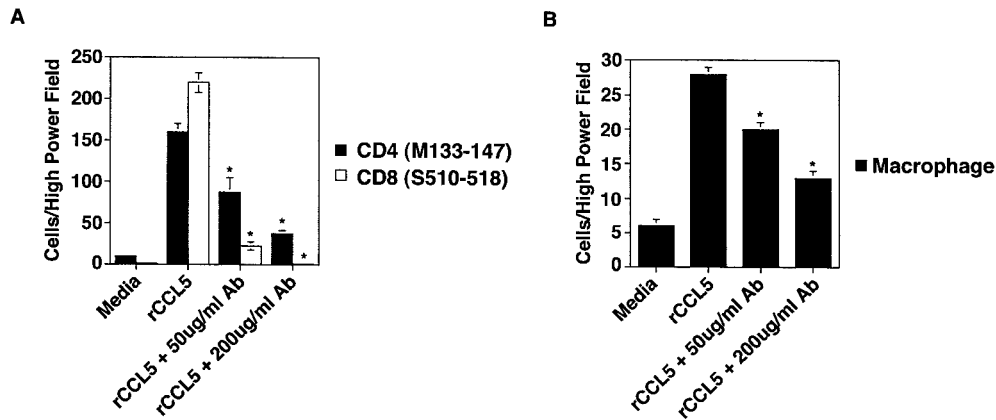


FIGURE 1. CCL5 induced chemotaxis of macrophages and virus-specific T cells. CD4⁺ and CD8⁺ T cells were obtained from the spleens of mice infected i.p. with MHV (day 7 p.i.) and expanded for 6 days in the presence of either M133–147 or S510–518 peptides, respectively. Activated macrophages were obtained from the peritoneum of thioglycolate-injected mice. *A*, Recombinant mouse CCL5 (rCCL5) at 100 ng/ml exerted a potent chemotactic effect upon both CD4⁺ and CD8⁺ T cells. Inclusion of a mouse anti-mouse CCL5 mAb resulted in a dose-dependent decrease in the ability of T cells to respond. *B*, Thioglycolate-elicited macrophages migrated in response to rCCL5, and this was reduced following inclusion of the anti-CCL5 mAb. Results presented are representative of two independent experiments. *, $p \leq 0.01$.

Results

Anti-CCL5 mAb reduces CCL5-induced migration of activated macrophage and virus-specific T cells

CCL5 is predominantly expressed within the brain and spinal cord following MHV infection, suggesting a role in both host defense and disease by attracting inflammatory cells into the CNS (17, 41). Indeed, administration of rabbit polyclonal antisera specific for mouse CCL5 immediately following MHV infection of the CNS resulted in diminished T cell and macrophage infiltration into the CNS, delayed clearance of virus from the brain, and a reduction in the severity of demyelination (17). These data indicate that CCL5 contributes to both T cell and macrophage migration and accumulation within the CNS of MHV-infected mice during acute disease. To more accurately assess the importance of CCL5 with regard to T cell migration, M133–147-specific CD4⁺ and S510–518-specific CD8⁺ T cells were obtained from the spleens of mice infected i.p. with MHV. The ability of these cells to respond to CCL5 signaling as well as the ability of a mouse anti-mouse CCL5 mAb to block migration was measured using an *in vitro* chemotaxis assay. Exposure to 100 ng/ml recombinant mouse CCL5 (rCCL5) resulted in a marked increase in chemotaxis for both CD4⁺ and CD8⁺ T cells (Fig. 1A). Preincubation of increasing concentrations of anti-CCL5 mAb with rCCL5 resulted in a pronounced decrease ($p \leq 0.01$) in the ability of T cells to migrate (Fig. 1A). In addition, thioglycolate-elicited macrophages were also able to migrate in response to rCCL5, although the effect was not as pronounced as for T cells. Macrophage chemotaxis was also significantly reduced ($p < 0.01$) following inclusion of the anti-CCL5 mAb (Fig. 1B).

Treatment of mice with an anti-CCL5 neutralizing mAb reduces clinical disease severity and T cell trafficking into the CNS

The question of whether CCL5 expression amplifies the severity of demyelination during chronic disease (>12 days p.i.) in persistently infected mice is important and remains to be answered. Therefore, mice were infected with MHV and were separated into two groups at day 12 p.i. with equivalent clinical disease. Each group was treated with 250 μ g of either anti-CCL5 or an isotype-matched control Ab administered every other day via i.p. injection until day 20 p.i. This treatment resulted in a significant ($p \leq 0.05$) improvement in clinical disease beginning at day 14 and lasting

through day 21 (Fig. 2). Removal of anti-CCL5 treatment at day 20 resulted in a gradual worsening of clinical disease in mice; however, the severity of disease never reached that of mice treated with the isotype control Ab (Fig. 2). Treatment of mice with doses >250 μ g did not further diminish clinical disease (data not shown).

The reduction in clinical disease severity observed in mice treated with the anti-CCL5 mAb led us to investigate T cell infiltration into the CNS, because CCL5 signaling may be important in regulating trafficking of these cells during chronic disease. Isolation of mononuclear cells from the brains of anti-CCL5-treated and control-treated mice at day 21 p.i. showed an ~80% reduction ($p \leq 0.0001$) in the accumulation of total CD4⁺ and CD8⁺ T cells in mice treated with anti-CCL5 as compared with control-treated mice at day 21 p.i. (Table I). To investigate the effect of anti-CCL5 treatment on the ability of virus-specific T cells to migrate and accumulate within the CNS, numbers of CD4⁺ and CD8⁺ T cells

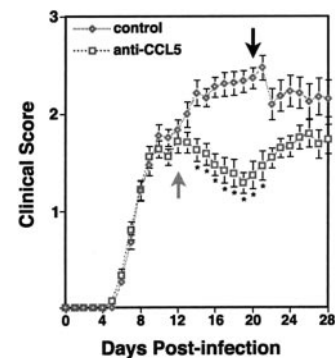


FIGURE 2. Clinical disease is decreased in anti-CCL5-treated mice. Mice were i.c. infected with 1000 PFU of MHV and treated i.p. with anti-CCL5 or an isotype-matched control Ab on days 12, 14, 16, 18, and 20, and clinical disease severity was determined at the indicated times. Upward gray arrow indicates the day treatment began (day 12 p.i.), and downward black arrow indicates the day treatment ended (day 20 p.i.). Treatment with anti-CCL5 mAb resulted in a significant reduction (*, $p \leq 0.05$) in clinical disease beginning 2 days posttreatment and lasting until 2 days following removal of Ab treatment. Data for clinical disease are presented as the mean \pm SEM. The data presented represent the results of three separate experiments with a minimum of eight mice per experiment.

Table I. *Anti-CCL5 treatment alters T cell accumulation within the CNS of MHV-infected mice*

Treatment	Day	n	CD4 ^a	CD4 (M133–147)	CD8	CD8 (S510–518)
Isotype control	21	9	$1.33 \times 10^5 \pm 1.7 \times 10^4$	$1.2 \times 10^4 \pm 1.7 \times 10^3$	$7.7 \times 10^4 \pm 1.0 \times 10^4$	$1.6 \times 10^4 \pm 3.1 \times 10^3$
Anti-CCL5	21	8	$3.0 \times 10^4 \pm 4.2 \times 10^3$ ^b	$3.2 \times 10^3 \pm 5.4 \times 10^2$ ^c	$1.6 \times 10^4 \pm 2.4 \times 10^3$ ^b	$6.2 \times 10^3 \pm 1.7 \times 10^3$ ^d
Isotype control	28	11	$6.3 \times 10^4 \pm 8.5 \times 10^3$	$8.3 \times 10^3 \pm 1.7 \times 10^3$	$3.8 \times 10^4 \pm 3.0 \times 10^3$	$1.2 \times 10^4 \pm 1.5 \times 10^3$
Anti-CCL5	28	8	$8.9 \times 10^4 \pm 1.6 \times 10^4$	$8.7 \times 10^3 \pm 7.6 \times 10^2$	$4.9 \times 10^4 \pm 8.6 \times 10^3$	$1.0 \times 10^4 \pm 1.4 \times 10^3$

^a Flow data are presented as total number of cells within the gated population \pm SEM. Data are from two separate experiments.

^b $p \leq 0.0001$ when compared with control-treated mice at day 21 p.i.

^c $p \leq 0.0002$ when compared with control-treated mice at day 21 p.i.

^d $p \leq 0.01$ when compared with control-treated mice at day 21 p.i.

specific for the immunodominant epitopes M133–147 and S510–518, respectively, were examined via intracellular IFN- γ staining. We focused on T cells specific for the immunodominant epitopes, because these cells are predominant during chronic disease in comparison with cells specific for other epitopes (24, 42). In addition, previous studies have shown that adoptive transfer of either M133–147-specific CD4⁺ T cells or S510–518-specific CD8⁺ T cells into MHV-infected mice results in demyelination (24, 26, 27, 43, 44). Therefore, existing evidence indicates that these T cell populations are important in contributing to disease. Treatment with anti-CCL5 mAb resulted in a modulation in the migration patterns of Ag-specific T cells. Examination of total numbers of T cells specific for these peptides revealed a 73% decrease ($p \leq 0.0002$) in the numbers of M133–147-specific CD4⁺ T cells in anti-CCL5-treated mice compared with control-treated at 21 days p.i. (Table I and Fig. 3). Additionally, a \sim 60% reduction in the number of S510–518-specific CD8⁺ T cells was detected at 21 days p.i.

Analysis of Ag-specific T cells present within the CNS at day 21 p.i. revealed no difference in the frequency of M133–147-specific T cells in anti-CCL5-treated mice as compared with control-treated mice (Fig. 3). In contrast, at day 21 p.i., there is a \sim 1.6-fold increase in the frequency of S510–518-specific CD8⁺ T cells present within the CNS of anti-CCL5-treated mice (27.9%) as compared with control-treated mice (17.2%), indicating that anti-

CCL5 treatment did not have as pronounced of an effect on CD8⁺ T cells specific for this epitope as compared with either CD8⁺ T cells responding to different viral Ags or activated non-virus-specific cells (Table I and Fig. 3). Collectively, these data argue that CCL5 exhibits an ability to target select populations of T cells for trafficking into the CNS during chronic disease. The reduction in T cell entry into the CNS was not sustained when anti-CCL5 mAb treatment was halted as demonstrated by the equivalent or increased numbers of CD4⁺ and CD8⁺ T cells recovered from the CNS of control-treated mice at day 28 p.i. (Table I and Fig. 3).

Reduced CCL5 chemokine mRNA expression following anti-CCL5 treatment

CCL5 and CXCL10 are prominently expressed within the CNS of mice persistently infected with MHV, and previous studies by our laboratory indicate these chemokines contribute to demyelination by attracting T cells and macrophages into the CNS (17, 41). To determine whether anti-CCL5 treatment altered the expression profile of either chemokine within the CNS, mRNA transcript levels were determined. Mice treated with anti-CCL5 showed a significant reduction ($p \leq 0.05$) in mRNA transcripts for CCL5 compared with control-treated mice on day 21 p.i. Treatment with anti-CCL5 mAb resulted in a slight decrease in CXCL10 mRNA expression; however, this difference was not significant as compared with control-treated mice (Fig. 4). Ab treatment was stopped

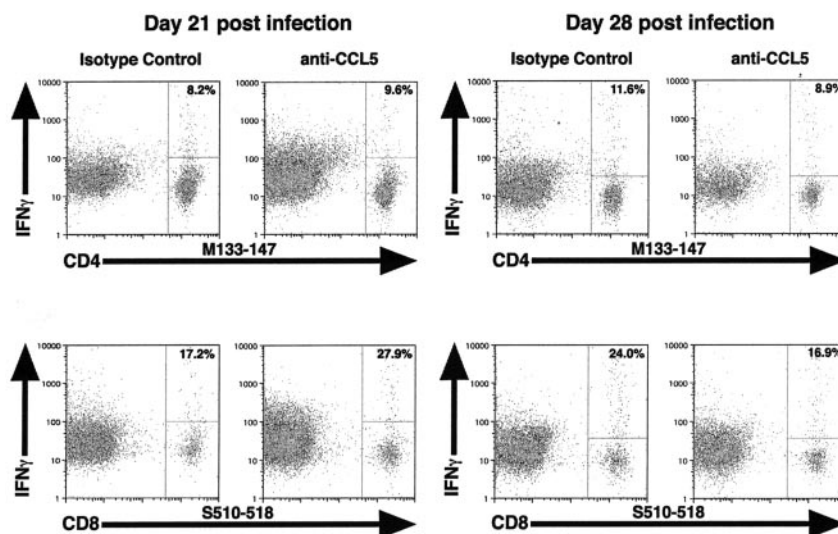


FIGURE 3. Infiltration of M133–147-specific CD4⁺ and S510–518-specific CD8⁺ T cells into the CNS is reduced following anti-CCL5 mAb treatment. Infiltration of virus-specific CD4⁺ and CD8⁺ T cells into the CNS of anti-CCL5 mAb and isotype-matched control Ab-treated mice was determined by intracellular IFN- γ staining following stimulation with M133–147 and S510–518 peptides, respectively, at days 21 and 28 p.i. The average frequency of both CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺ cells within the total CD4⁺ or CD8⁺ population is indicated in the *upper right-hand* of the dot blots shown. Total numbers of both M133–147- and S510–518-specific CD4⁺ and CD8⁺ T cells, respectively, are decreased in comparison with numbers present in control mice (Table I). By day 28 p.i., both the frequency and total numbers of Ag-specific CD4⁺ and CD8⁺ T cells are similar (Table I). Dot plots shown are from representative mice from a total of two separate experiments.

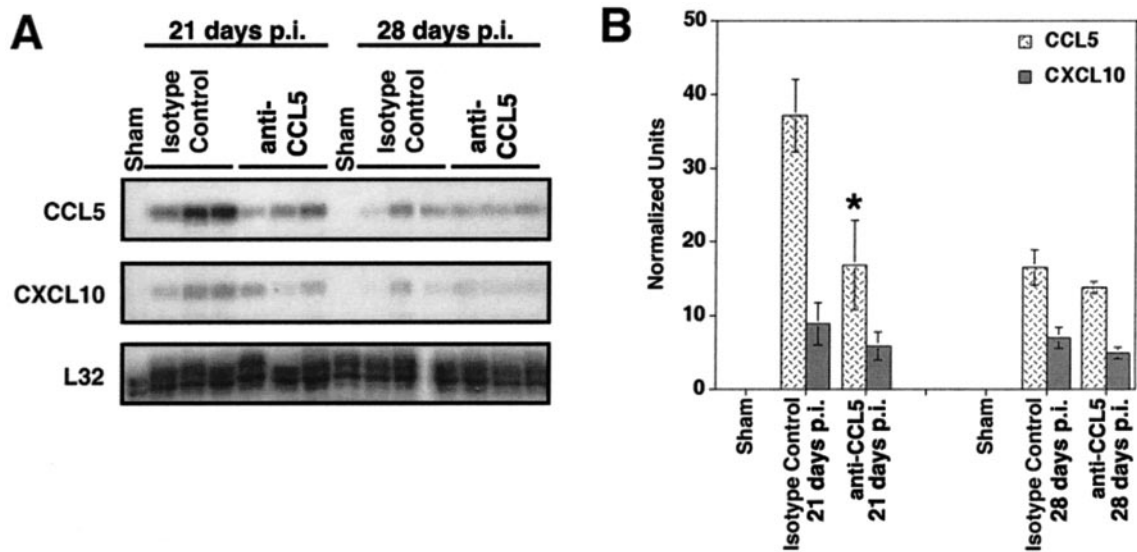


FIGURE 4. Reduced CCL5 mRNA expression within the CNS of anti-CCL5 mAb-treated mice. *A*, Total RNA was isolated from the brains of MHV-infected mice treated with anti-CCL5 mAb or control Ab at days 21 and 28 p.i. and subjected to RPA analysis to assess *CCL5* and *CXCL10* gene expression. Each lane represents an individual mouse. *B*, Quantitative analysis of CCL5 and CXCL10 transcript expressed. Densitometric analysis of each lane representing a brain sample from an individual mouse was performed on the scanned autoradiograph *A*. *, $p \leq 0.05$ when compared with mice treated with an isotype-matched control Ab. Data are from two separate experiments and are presented as the mean \pm SEM.

at day 20 p.i., and expression of CCL5 and CXCL10 mRNA levels were analyzed at day 28 p.i. Comparable levels of CCL5 transcripts are present within the CNS of mice formerly treated with the anti-CCL5 mAb as compared with control-treated mice (Fig. 4). Additionally, the difference in CXCL10 transcript levels between the two experimental groups of mice was not significant at this time (Fig. 4).

CCL5 contributes to macrophage entry and demyelination

CCL5 is a macrophage chemoattractant, and infiltration of these cells into the CNS is associated with demyelination during MHV-induced CNS disease (23, 25, 35). Therefore the presence of activated macrophages (F480⁺CD45^{high}) within the CNS of anti-CCL5-treated mice was investigated. Administration of anti-CCL5 mAb resulted in >60% ($p \leq 0.005$) reduction in the number of activated macrophages in the CNS at day 21 p.i. compared with control-treated mice (Table II). Analysis of the severity of demyelination in anti-CCL5-treated and control-treated mice was performed via microscopic analysis of spinal cords stained with LFB. The reduction in macrophage infiltration in anti-CCL5-treated mice correlated with a significant ($p \leq 0.005$) reduction in the severity of demyelination when compared with control-treated mice (Table II and Fig. 5). When anti-CCL5 mAb treatment was halted at day 20 p.i., entry of activated macrophages into the CNS was no longer blocked as evidenced by the equivalent numbers of macrophages present within the CNS of mice formerly treated with

either anti-CCL5 or control Ab (Table II and Fig. 5). When macrophage entry was no longer restrained, the difference in inflammation and demyelination was less obvious between the two groups. Removal of anti-CCL5 treatment resulted in an increase in the severity of demyelination as evaluated at day 28 p.i., which was comparable with that of control mice (Table II, Fig. 5).

Discussion

MHV infection of the CNS results in a rapid and robust expression of chemokines that first precedes and then accompanies the entry of T lymphocytes and activated macrophages into the CNS (41). Recent studies have demonstrated a role for specific chemokines in directing both the migration and accumulation of T cells and macrophages into the CNS of MHV-infected mice (17, 35, 45, 46). For example, early expression of CXCL10 and CXCL9 is important in promoting viral clearance from the brain by attracting CXCR3-positive T cells into the CNS (45, 46). Conversely, chronic expression of CXCL10 in mice persistently infected with MHV contributes to myelin destruction by enhancing T cell accumulation within the CNS (17). During acute disease, CCL5 is prominently expressed within the CNS, and Ab-mediated neutralization resulted in delayed viral clearance accompanied by reduced T cell and macrophage infiltration as well as diminished demyelination (17). Although we have shown that CCL5 expression during acute disease regulates leukocyte entry into the CNS and the course of disease, the functional role of CCL5 during chronic disease (≥ 12

Table II. *Macrophage infiltration and demyelination is reduced following treatment with anti-CCL5 mAb*

Treatment	Day	CD45 ^{high} F480 ^a	Demyelination
Isotype control	21	$4.9 \times 10^4 \pm 7.1 \times 10^3$ (7) ^b	3.4 ± 0.1 (12)
Anti-CCL5	21	$1.9 \times 10^4 \pm 5.3 \times 10^3$ (7) ^c	2.4 ± 0.1 (10) ^c
Isotype control	28	$2.1 \times 10^5 \pm 8.6 \times 10^4$ (7)	3.2 ± 0.1 (11)
Anti-CCL5	28	$2.6 \times 10^5 \pm 1.1 \times 10^5$ (6)	3.3 ± 0.1 (12)

^a Flow data are presented as total number of cells within the gated population \pm SEM. Data are from two separate experiments.

^b Parentheses indicate the number of mice examined.

^c $p \leq 0.005$ when compared with control-treated mice at day 21.

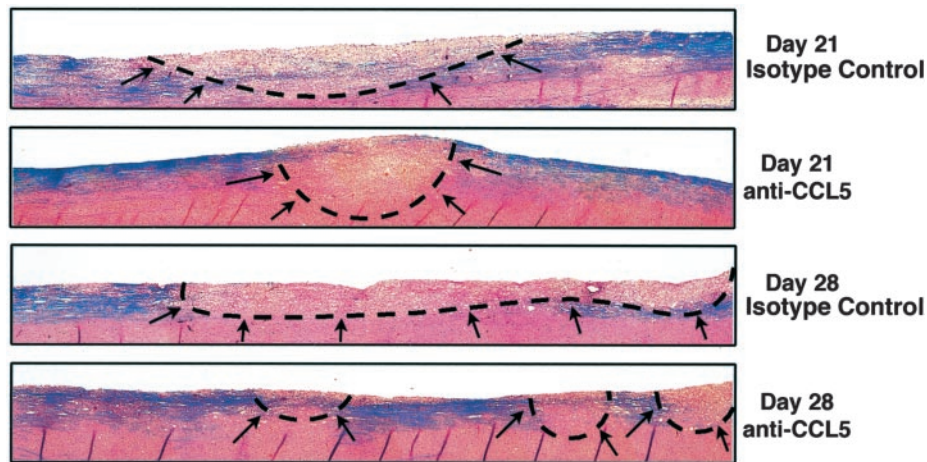


FIGURE 5. The severity of demyelination is reduced in anti-CCL5 mAb-treated mice. Representative LFB staining of spinal cords removed from anti-CCL5 mAb-treated or isotype-matched control Ab-treated mice at day 21 or 28 p.i. At day 21 p.i., control-treated mice display extensive demyelination along the entire spinal cord, whereas anti-CCL5 mAb-treated mice display only focal demyelinating lesions. Ab treatment was stopped in both groups at day 20 p.i., and by day 28 p.i., control-treated mice exhibited a similar level of demyelination as seen at day 21 p.i., whereas anti-CCL5-treated mice had increased numbers of demyelinating lesions as compared with day 21 p.i. Dashed lines and arrows outline areas of demyelination in spinal cords shown. Spinal cords presented are representative of an average of five individual mice per group from two separate experiments.

days p.i.) was not characterized. The importance of this is demonstrated by the fact that there are distinct chemokine gene expression profiles that exist within the CNS of MHV-infected mice during acute and chronic disease, and this may have a pronounced effect on leukocyte trafficking into the CNS (17, 41). Moreover, the activation state of T cells, e.g., effector vs memory will also be affected depending on the stage of disease, and this influences chemokine receptor expression on the cell surface, thereby dictating the ability of these cells to respond to chemokine signaling (47, 48). Finally, CCL5 has been implicated in contributing to disease in MS patients by attracting T cells and macrophages into the CNS (8, 9). These observations highlight the relevance of attaching a functional role to CCL5 expression during chronic disease in mice persistently infected with MHV.

The results presented clearly demonstrate that mAb-mediated neutralization of CCL5 in mice with an established demyelinating disease results in a dramatic improvement in neurological function as early as 2 days following the first treatment. Furthermore, the results presented demonstrate that neutralization of CCL5 following the induction of established demyelinating disease dramatically affects T cell accumulation within the CNS. Examination of the Ag specificity of CD4⁺ and CD8⁺ T cells within the CNS of anti-CCL5-treated mice revealed a 73% reduction in M133–147-specific CD4⁺ T cells and a 60% reduction in S510–518-specific CD8⁺ T cells (Table I and Fig. 3). These data indicate that M133–147-specific CD4⁺ T cells are more responsive to CCL5-induced chemotaxis as compared with S510–518-specific CD8⁺ T cells during chronic disease. We have previously demonstrated increased expression for CCR1 and CCR5 transcripts in both M133–147- and S510–518-specific CD4⁺ and CD8⁺ T cells, respectively (26, 27). Whether the altered trafficking pattern of these particular Ag-specific T cell subsets is a result of differential expression of CCL5-specific receptors such as CCR1 and/or CCR5 at this stage of disease is not yet known and is the focus of ongoing study. Collectively, these data support the notion that both CD4⁺ and CD8⁺ T cells can contribute to demyelination, and that chemokine signaling regulates the migration of T cells into the CNS based, in part, on their Ag specificity (17, 24, 26, 27, 35, 44, 49, 50).

Treatment with anti-CCL5 resulted in reduced mRNA expression of CCL5 during the treatment period. Treatment with the anti-

CCL5 mAb also resulted in a slight reduction in CXCL10 mRNA expression as compared with control-treated mice; however, this difference was not significant. The reduction in CCL5 expression is likely attributed to the diminished entry of both CD4⁺ and CD8⁺ T cells, because we have previously shown that infiltrating T cells are capable of expressing this chemokine. In addition, activated T cells are capable of producing cytokines that can directly induce CCL5 gene expression by both T cells and glia, and this may also contribute to reduced expression of this chemokine in anti-CCL5 mAb-treated mice. Although numerous cytokines as well as chemokines have been shown to modulate CCL5 gene expression by influencing promoter activity, there is no direct evidence that CCL5 is capable of autoregulation in T cells or other cell types (51). The fact that CXCL10 mRNA levels are not dramatically reduced in anti-CCL5 mAb-treated mice as compared with control-treated mice most likely reflects the fact that astrocytes, and not infiltrating T cells or macrophages, are the predominant cellular source for this chemokine in mice persistently infected with MHV (41). MHV infection of astrocytes results in robust expression of CXCL10 that is most likely controlled by type I IFN expression and not by cytokines and/or chemokines derived from infiltrating T cells such as IFN- γ and CCL5, respectively. Moreover, MHV infection of the CNS of RAG1^{-/-} mice (lacking T and B cells) results in robust expression of CXCL10 by astrocytes, which further supports the fact that expression of this chemokine is not dependent on factors generated from infiltrating T cells (52).

The data presented support previous studies implicating macrophages as important in contributing to white matter destruction during chronic disease (17, 23, 25, 35). In addition, these results further support an important role for CCL5 in promoting macrophage migration and accumulation within the CNS following MHV infection. Ab-mediated targeting of CCL5 during both acute and chronic disease affected the ability of macrophages to traffic into the CNS, indicating that the stage of disease does not affect the ability of CCL5 to promote macrophage chemotaxis. Inflammatory macrophages most likely respond to CCL5 signaling through expression of CCR5. This is supported by the fact that macrophage accumulation within the CNS is reduced in MHV-infected CCR5^{-/-} mice, and this correlates with reduced demyelination (25).

Although treatment with anti-CCL5 did reduce the severity of clinical disease and resulted in an attenuation of demyelination, the severity of myelin destruction remained greater than that observed following Ab targeting of CXCL10, suggesting differential roles for CCL5 and CXCL10 in participating in disease (35). Anti-CXCL10 treatment of MHV-infected mice had a more pronounced effect on CD4⁺ T cells as compared with CD8⁺ T cells, indicating that chronic expression of CXCL10 exerts a more potent chemotactic effect on CD4⁺ T cells than CD8⁺ T cells. Taken together, these data argue that chronic expression of both CCL5 and CXCL10 work synergistically in attracting inflammatory cells such as T cells and macrophages into the CNS. Whether the responding T cell populations are expressing both CXCR3 and CCR5 and whether a signaling hierarchy exists with regard to either CXCL10 or CCL5, respectively, in this model remain to be determined. One possibility is that the difference in response to these chemotactic signals reflects differential expression of specific chemokine receptors on the responding T cells. Although CD8⁺ T cells can clearly contribute to myelin destruction, our data argue that CD4⁺ T cells are ultimately of greater importance in amplifying the severity of neuropathology observed in this model. The downstream effect of diminished T cell entry into the CNS is reduced expression of CCL5, which, when elevated, serves to attract activated macrophages into the CNS, where they actively participate in demyelination. Finally, these data further support the feasibility of Ab-mediated targeting of select chemokines in the treatment of neuroinflammatory diseases such as MS.

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