Journal of NeuroVirology, 12: 251–261, 2006

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280600873819

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healthcare

Anti-CCL2 treatment inhibits Theiler's murine encephalomyelitis virus-induced demyelinating disease

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Theiler's murine encephalomyelitis virus induces a demyelinating disease (TMEV-IDD) of the central nervous system (CNS) in susceptible mouse strains with accompanying histopathology characterized by mononuclear cell infiltrates. In susceptible strains of mice such as SJL, virus establishes a persistent infection in macrophages, induces a CNS infiltration by macrophages, T cells, and B cells, which results in chronic-progressive paralysis. In the present report the authors have investigated the functional role of CCL2 (monocyte chemotactic protein-1) in the induction and progression of demyelinating disease. Treatment of infected mice at day 0, 14, or 28 with anti-CCL2 resulted in a significant decrease in the clinical disease progression. Further analysis of anti-CCL2–treated mice revealed decreased CNS inflammation and mononuclear cell infiltration with an accompanying change in inflammatory cytokine responses. There was an overall decrease in the absolute numbers of CNS-infiltrating $CD4^+$ T cells, macrophages, and B cells. Finally, anti-CCL2 treatment resulted in decreased viral load in the CNS. These data directly demonstrate a role for CCL2 in the pathogenesis of TMEV-IDD. *Journal of NeuroVirology* (2006) **12,** 251–261.

Keywords: cell trafficking; chemokines; demyelinating disease; multiple sclerosis; neuroimmunology; Theiler's virus

Introduction

Intracerebral infection of susceptible inbred strains of mice with Theiler's murine encephalomyelitis (TMEV)[1] leads to a chronic, immune-mediated demyelinating disease (TMEV-IDD) (Miller *et al*, 1994). Because of the suspected viral etiology, chronic pathology, and primary demyelination accompanied by mononuclear cell infiltration seen in multiple sclerosis (MS), TMEV-induced demyelinating disease is considered to be an excellent experimental model of MS (Miller and Karpus, 1994). Virus is rapidly cleared from the peripheral circulation; however, TMEV persists within the central nervous system (CNS) of susceptible mice for their lifetime (Clatch *et al*, 1990), serving as a reservoir for chronic activation of virus-specific T cells (Lipton *et al*,

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This work was supported by The National Multiple Sclerosis Society grant RG 3056-A-2 and NIH grant P01 NS023349 (WJK).

Received 16 March 2006; revised 21 April 2006; accepted 7 June 2006.

[1]*Abbreviations* BHK-21, baby hamster kidney cell line 21; BSA, bovine serum albumin; CCL2, monocyte chemotactic protein-1; CCL3, macrophage inflammatory protein-1α; CCL4, macrophage inflammatory protein-1β; CCL5, RANTES; CNS, central nervous system; CXCL1, macrophage inflammatory protein-2; CXCL10, gamma interferon inducible protein-10; H&E, hematoxylin and eosin; NRS, normal rabbit serum; TMEV, Theiler's murine encephalomyelitis virus; TMEV-IDD, Theiler's murine encephalomyelitis virus–induced demyelinating disease.

1995). Although TMEV has been shown to preferentially reside in CNS macrophages, the role of TMEV infection in the chronic activation of macrophages to secrete soluble proinflammatory cytokines is unknown. In addition to infecting macrophages, TMEV has been shown to infect glial cells and serve as a reservoir for persistent viral infection (Brahic *et al*, 1981; Rodriguez *et al*, 1983; Zheng *et al*, 2001). As a result of CNS TMEV infection, increasing virus-specific delayed type hypersensitivity (DTH) as been shown to correlate with development of increasing disease severity, supporting a role for Th1-mediated CNS inflammation (Clatch *et al*, 1986). Virus replication occurs in the spinal cords (Ozden *et al*, 1991; Yamada *et al*, 1990) and focal inflammation, consisting primarily of T cells and macrophages, is limited to the spinal cord white matter (Lipton, 1975). From 2 to 5 months following infection, extensive demyelination occurs, leading to clinical paralysis (Dal Canto and Lipton, 1975). The resulting course of paralysis is chronic-progressive in nature and differs from the relapsing-remitting form seen in experimental autoimmune encephalomyelitis (EAE) (Miller and Karpus, 1994).

One of the major characteristics of TMEV-IDD includes CNS mononuclear cell infiltration over the disease course. Immune responses originate in peripheral lymphoid tissue and once activated, the specific lymphocytes leave the organized lymphoid tissue and accumulate in the CNS. A three-step model has been proposed to explain how leukocytes in flow conditions enter target tissue types (Butcher, 1991). Chemokines are thought to play a central role in the migration and accumulation of cells in specific tissue sites. Chemokines are potent chemoattractant factors that are divided into four highly conserved gene families: C-x-C, C-C, C, and C-x_3-C, designated by the position of the first two cysteines (Murphy *et al*, 2000).

The expression of chemokines in a variety of mouse models of virus-induced demyelinating disease has been recently described. We showed that both CCL2 and CCL3 protein expression in the CNS correlated with the development of TMEV-IDD in SJL mice (Hoffman *et al*, 1999). In a separate study TMEV-IDD was induced using different strains of virus: DA, GDVII, and H101. CCL5, CCL2, CXCL10, CCL4, CCL3, and CXCL1 mRNA expression was detected in both the brain and spinal cord during all three infections (Theil *et al*, 2000). Murray *et al* (2000) examined spinal cords during the acute and chronic phases of TMEV infection in mice susceptible (B10.M, H-2^f) and resistant (B10, H-2^b) to virus-induced demyelination. In this model TMEV infection resulted in robust expression of mRNA for CXCL10, CCL5, and CCL2, but not CXCL1, in brains and spinal cords in both strains of mice within 5 days. During the chronic, demyelinating phase of infection, there was a resurgence in CXCL10, CCL5, and CCL2 mRNA in spinal cords of susceptible B10.M mice. More recently we demonstrated that overexpression of CCL2 in the CNS resulted in enhanced macrophage accumulation as well as an accelerated induction of disease (Bennett *et al*, 2003). These reports have demonstrated the expression of chemokines during TMEV-IDD. In the present report we determined the function of CCL2 in the development and progression of TMEV-IDD using an *in vivo* antibody treatment approach.

Results

Anti-CCL2 treatment ameliorates clinical disease severity

Our previous work demonstrated that CCL2 was expressed in the CNS of TMEV-infected SJL mice (Hoffman *et al*, 1999). In order to determine the functional role of this chemokine in the development of and progression of TMEV-IDD, we employed an *in vivo* chemokine neutralization approach similar to what we have reported previously (Kennedy *et al*, 1998). At day 0 SJL mice were infected with 3×10^6 plaque-forming units (pfu) of TMEV and given an intravenous (i.v.) injection of either anti-CCL2 or normal rabbit serum (NRS) as a control and monitored for the development of TMEV-IDD clinical disease. The results shown in Figure 1A demonstrate that treatment of mice with anti-CCL2 at the time of infection resulted in a significantly reduced course of disease severity ($P < .05$, days 84 to 97 post infection). Anti-CCL2 treatment also significantly reduced the incidence of disease (3/10) compared to NRS control-treated mice (10/10). In a second set of experiments, we treated TMEV-infected mice with either anti-CCL2 or NRS 14 days after infection and monitored the mice for development of TMEV-IDD clinical disease. The results shown in Figure 1B demonstrate that anti-CCL2 treatment resulted in significantly reduced clinical disease development ($P < .05$, days 58 to 86 post infection). Although there was a significant decrease in disease severity, there was no difference in disease incidence between anti-CCL2–treated (10/10) and NRS control–treated mice (10/10) in this experiment. In the third set of experiments, we treated mice at day 28 post infection with either anti-CCL2 or NRS. The results shown in Figure 1C demonstrate that treatment with anti-CCL2 resulted in significantly decreased clinical disease progression ($P < .05$, days 60 to 81 post infection). Treatment with anti-CCL2 did not result in a decrease in disease incidence when compared to NRS-treated control mice. The results of these three experiments suggested that CCL2 was an important component of TMEV-IDD pathogenesis and that neutralization of the chemokine during a broad time period of disease induction affected the outcome of disease progression. Because the greatest effect on clinical disease was seen when mice were treated 14 days after TMEV infection, we concentrated our efforts on understanding the mechanism of anti-CCL2 treatment at day 14 post infection.

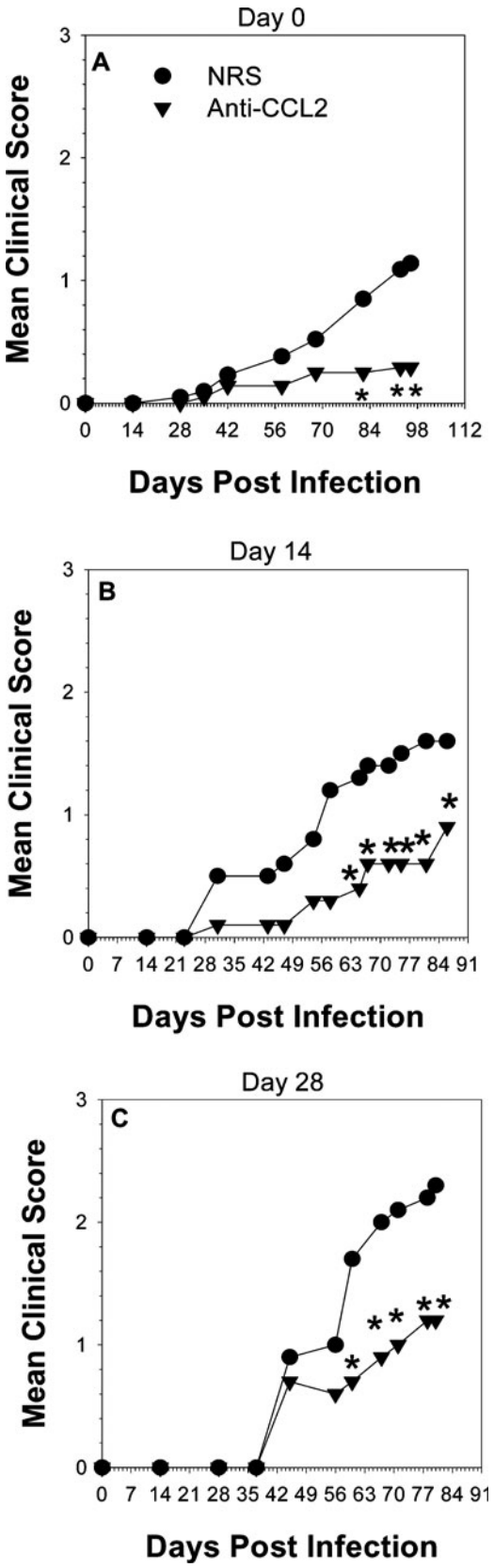

Figure 1 Anti-CCL2 treatment reduced clinical TMEV-IDD severity. Groups of 10 mice were infected with 3×10^6 pfu BeAn TMEV and subsequently administered anti-CCL2 or NRS i.p. Clinical disease was determined as specified in Materials and Methods. The data show the mean clinical disease score per timepoint. (**A**) Treatment with anti-CCL2 the same day as TMEV infection resulted in significantly decreased clinical disease progression ($P < .05$, days 84 to 97 post infection). (**B**) Treatment with anti-CCL2 at day 14 post infection resulted in a significant clinical disease decrease from days 58 to 86 post infection ($P < .05$) when compared to NRS-treated mice. (**C**) Treatment with anti-CCL2 at 28 days post infection significantly decreased clinical disease progression ($P < .05$, days 60 to 81 post infection).

Anti-CCL2 treatment inhibits histological TMEV-induced CNS inflammation

Because anti-CCL2 inhibited the progression of clinical TMEV-IDD, we wanted to evaluate CNS inflammation and immunologic parameters in the treated mice. As shown in Figure 2A, control mice treated with NRS at day 14 post infection showed extensive perivascular mononuclear cell infiltration. In contrast, anti-CCL2–treated mice show a reduced amount of perivascular inflammation (Figure 2B). These histological data were quantified and there is a statistically significant decrease in the number of perivascular lesions in the CNS of anti-CCL2–treated mice compared to NRS-treated controls (Figure 2C). A small subset of mice (three per group) was analyzed for demyelination by toludine blue staining of thin Epon sections of lumbar spinal cord at approximately 60 days post infection. Using a qualitative assessment, NRS-treated mice demonstrated extensive spinal cord demyelination (+ + +, ++, + + +) compared to anti-CCL2–treated mice that showed reduced spinal cord demyelination (++, +/−, +). The histologic results suggested that CCL2 regulates CNS inflammation and subsequent demyelination.

Anti-CCL2 treatment decreases CNS mononuclear cell infiltration

The histologic data suggested that anti-CCL2 treatment resulted in fewer inflammatory cells in the CNS. To directly determine if this was the case, we enumerated the overall mononuclear cell infiltration in the CNS of treated and control mice by simple microscopic cell counting. Mice were infected with TMEV and treated with either anti-CCL2 or NRS as a control at day 14 post infection. CNS and splenic tissue was harvested at day 56 post infection. The results in Figure 3A demonstrate that anti-CCL2 treatment resulted in a significantly decreased number of mononuclear cells in the CNS. In contrast, there was no significant decrease in the mononuclear cell number in the spleen of anti-CCL2-compared to NRS-treated mice (Figure 3B). These data indicate an organ-specific effect of anti-CCL2 treatment.

Our next experiment was to analyze the CNS of anti-CCL2– and NRS-treated mice for individual mononuclear cell subsets by flow cytometry. Mice were infected with TMEV and treated with either anti-CCL2 or NRS as a control at day 14 post infection.

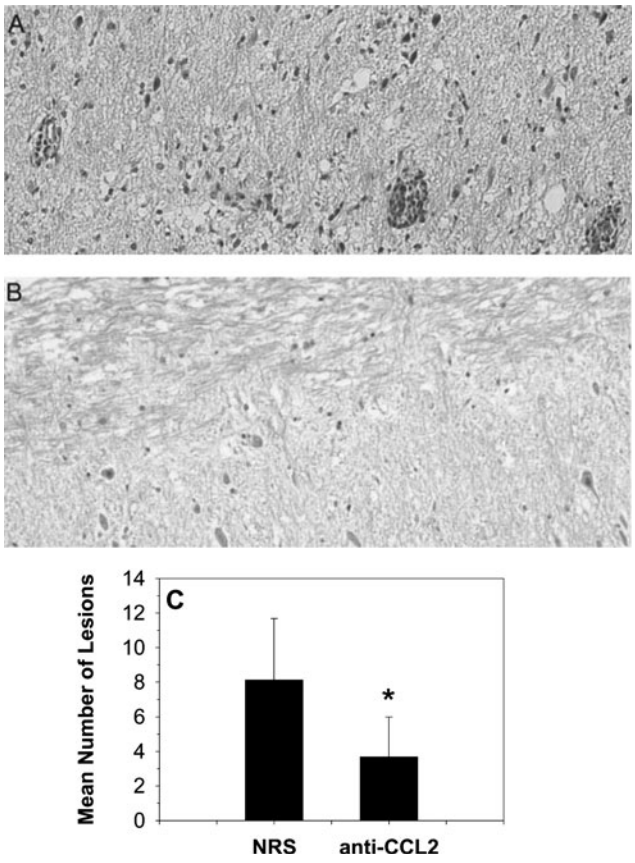

Figure 2 Anti-CCL2 treatment resulted in reduced CNS inflammation in TMEV-infected mice. Representative mice from Figure 1B were killed and the CNS was prepared for standard H&E histology. (**A**) A representative section from the spinal cord of NRS-treated mice shows three prominent perivascular mononuclear cuffs (100× magnification). (**B**) A representative section from the spinal cord of anti-CCL2–treated mice shows a lack of perivascular mononuclear cuffs (100× magnification). (**C**) The histological infiltration of the spinal cord was examined from multiple sections of spinal cord from each treatment group and quantified according to the scoring system described in Materials and Methods. Anti-CCL2–treated mice showed a significantly decreased histological score when compared to NRS-treated mice ($^*P < .05$).

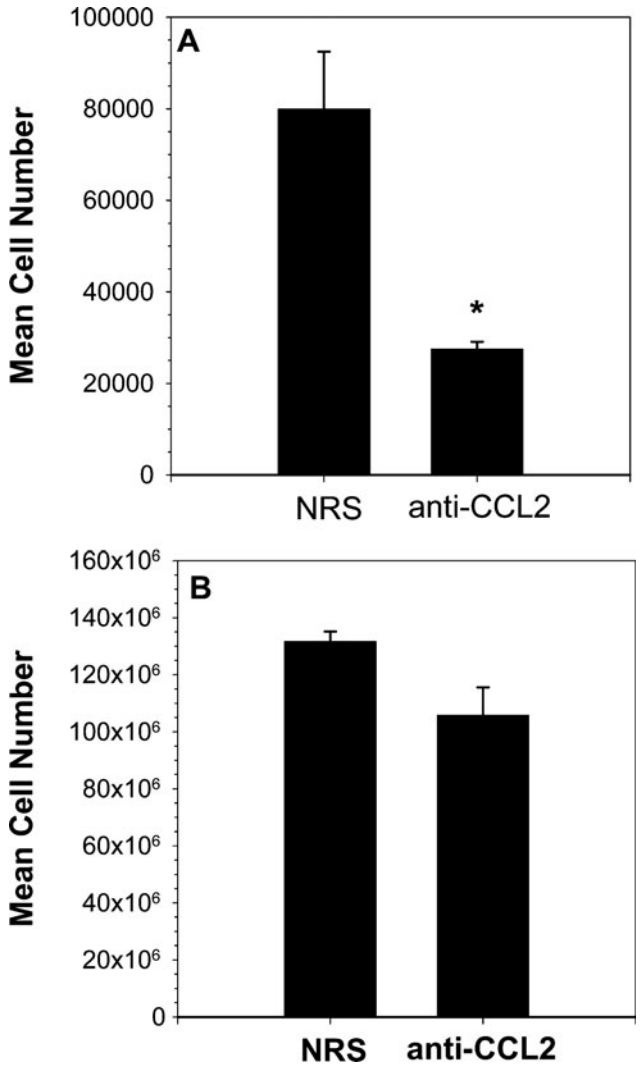

Figure 3 Anti-CCL2 treatment resulted in reduced CNS accumulation of mononuclear cells. Groups of 10 mice were infected with 3×10^6 pfu TMEV and subsequently administered anti-CCL2 or NRS i.p. 14 days following infection. Six weeks following antibody treatment, at a time when clinical disease in the anti-CCL2 was decreased compared to the NRS-treated controls, mononuclear cells were harvested from pooled spinal cords using Percoll gradients (**A**) or spleen (**B**) and enumerated by light microscopy. There was a significant decrease in the number of mononuclear cells in the CNS of anti-CCL2–treated mice compared to NRS-treated control mice ($^*P < .05$). There was no difference in the numbers of splenic mononuclear cells between the two groups.

CNS and splenic tissue were harvested at day 56 post infection. As shown in Figure 4A, we were unable to identify any significant differences in the proportions of CNS-infiltrating mononuclear cells between the anti-CCL2–treated and the control groups. The proportion of $CD45^+$ lymphocytes in the CNS was identical for NRS-treated control mice (37%) and anti-CCL2–treated mice (32%). The proportion of $CD45^{high}CD11b^+$ monocytes was also not different between the NRS-treated control group (5%) and the anti-CCL2–treated group (8%). Although there appeared to be a difference in the $CD45^{low}CD11b^+$ microglia population between the NRS-treated control (39%) and the anti-CCL2–treated (49%) groups, this was not statistically significant as the percentage was found to vary over a number of different experimental replicates. There were also no apparent significant differences in the proportions of either $CD4^+$ or $CD8^+$ T cells between the NRS-treated (24%, 8%) and anti-CCL2–treated (18%, 9%) groups. Finally, there was no difference in the percentage of $CD19^+$ B cells or NK cells between either group (data not shown). However, when we used a quantitative flow cytometric method (Keeney *et al*, 1998) of counting the absolute numbers of macrophages ($CD45^{hi}CD11b^+$), microglia ($CD45^{lo}CD11b^+$), $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and natural killer (NK) cells ($DX\text{-}5^+$)

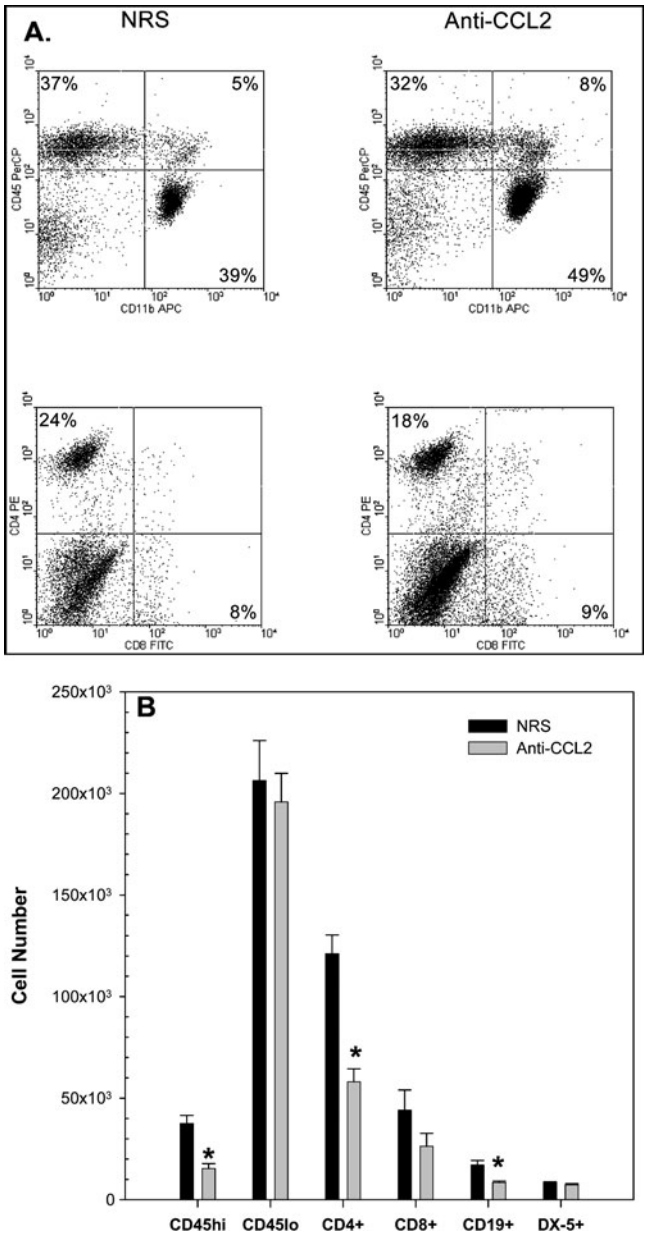

Figure 4 CCL2 regulation of mononuclear cell subset accumulation in the CNS. Groups of 10 mice were infected with 3×10^6 pfu TMEV and subsequently administered anti-CCL2 or NRS i.p. 14 days following infection. Six weeks following antibody treatment, at a time when clinical disease in the anti-CCL2 was decreased compared to the NRS-treated controls, mononuclear cells were harvested from pooled spinal cords from each group using Percoll gradients, stained with specific monoclonal antibodies, and immunophenotypically analyzed by flow cytometric analysis. The data was collected using Becton Dickinson Cell Quest software and analyzed using Win Midi. (**A**) There were no significant differences in mononuclear cell subset proportions between anti-CCL2– and NRS-treated mice. (**B**) Anti-CCL2 treatment reduced the number of CNS-infiltrating macrophages and $CD4^+$ T cells. Absolute numbers of CNS-infiltrating macrophages ($CD45^{hi}CD11b^+$), $CD4^+$ T cells, $CD8^+$ T cells, and resident microglia ($CD45^{lo}CD11b^+$) were determined by flow cytometry in four individual mice. There is a significant difference in the mean number of $CD45^{hi}CD11b^+$ macrophages and $CD4^+$ T cells between the anti-CCL2– and NRS-treated groups ($^*P < .05$).

in the CNS of anti-CCL2–treated and NRS-control mice, we found a statistically significant reduction of macrophages, $CD4^+$ T cell, and B cell numbers in the anti-CCL2–treated group (Figure 4B). When the data from both Figures 3 and 4 are considered together, it is clear that although there are no differences between controls and anti-CCL2–treated mice in terms of proportions of CNS-infiltrating T cells and macrophages, there are dramatic differences in the total number of CNS-infiltrating $CD4^+$ T cells, macrophages, and B cells.

Anti-CCL2 does not alter peripheral T-cell immune responses but decreases CNS inflammatory response

We (Karpus *et al*, 1997, 1998) and others (Gu *et al*, 2000) have previously demonstrated that CCL2 can promote development of Th2 responses and regulate Th1 responses. Therefore, we assessed the possibility of whether anti-CCL2 treatment resulted in an alteration of the TMEV-specific immune response and whether that effect directly contributed to the observed amelioration of clinical disease development and progression. Splenic TMEV-specific T-cell responses were determined from mice treated with either anti-CCL2 or NRS 14 days post infection. We chose to examine $VP2_{70-86}$–specifc responses as this is the dominant TMEV $CD4^+$ T cell epitope in SJL mice (Gerety *et al*, 1994). In both of the following recall response assays, the same number of input cells were used to elicit proliferative and cytokine responses. The splenic recall T-cell proliferation results are shown in Figure 5A and demonstrate no significant differences between anti-CCL2– and NRS-treated mice. Viral antigen-specific recall Th1 responses were also measured in the cervical lymph node (CLN) and CNS of anti-CCL2–treated and control mice. The results in Figure 5B demonstrate that there were no differences in the frequency of Th1 cells in either the CLN or CNS of anti-CCL2–treated mice when compared to controls. To further determine if there were differences in $CD4^+$ T-cell activation between control and anti-CCL2–treated groups that might explain the differences in clinical disease progression, we isolated mononuclear cells from the CNS of both groups 45 days after disease induction and assessed CD25 and CD44 expression. There was no difference in the percentage of $CD4^+CD25^+$ or $CD4^+CD44^+$ T cells in the CNS of NRS- or anti-CCL2–treated mice (data not shown). Similarly, there was no difference in the peripheral anti-TMEV-specific immunoglobulin response (data not shown).

Anti-CCL2 treatment inhibits CNS TMEV load

To determine whether disease reduction in the anti-CCL2–treated mice might be a result of lower viral titers in the CNS we performed plaque assays for the presence of replication-competent virus. Mice were

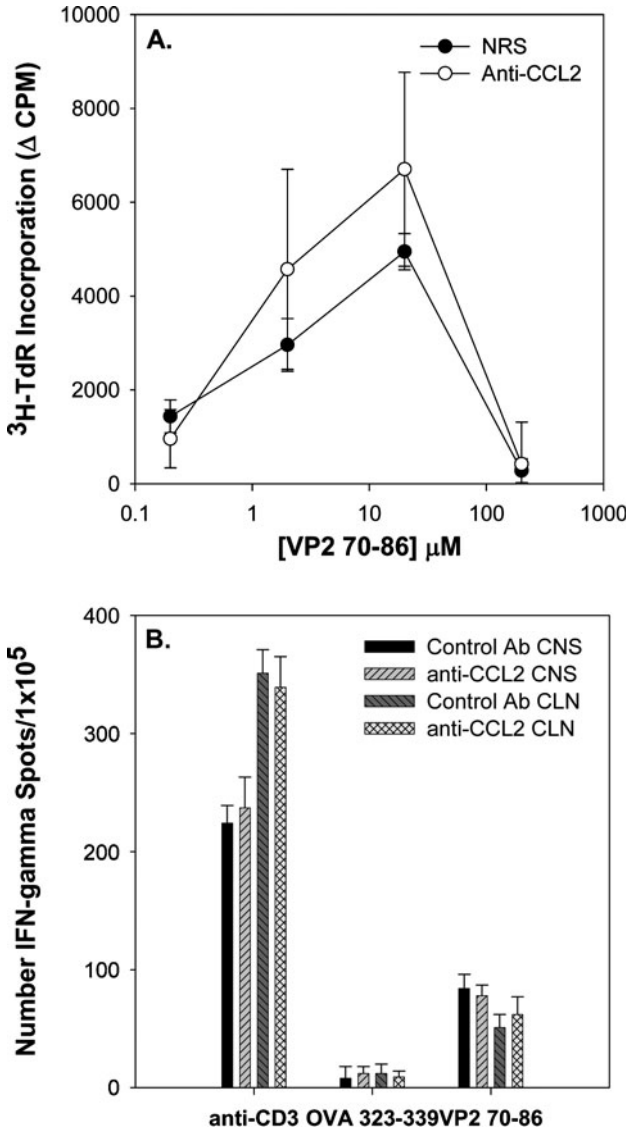

Figure 5 Anti-CCL2 treatment and TMEV-specific T cell responses. Groups of 12 mice were infected with 3×10^6 pfu TMEV and subsequently administered anti-CCL2 or NRS i.p. 14 days following infection. Between 5 and 6 weeks following antibody treatment, at a time when clinical disease in the anti-CCL2 was decreased compared to the NRS-treated controls, mononuclear cells were harvested from the spleens of four mice in each group, pooled, and analyzed for TMEV $VP2_{70-86}$–specific T-cell proliferation. The remaining mice were monitored for clinical disease progression to insure the efficacy of anti-CCL2 treatment. (**A**) There is no difference in antigen-specific splenic T cell proliferation. The data are shown as ΔCPM where the background CPM were subtracted from the antigen-stimulated CPM. The background CPM for the splenocytes from the anti-CCL2-treated mice was 1026 ± 213, whereas the background CPM for the splenocytes from the NRS-treated control mice was 862 ± 371. (**B**) There were no significant differences in the frequency of Th1 responses between anti-CCL2– and NRS-treated mice. The data represent CNS T-cell ELISPOT analysis and are shown as the mean number of IFN-γ–producing cells/10^5 total input cells. OVA323-339 peptide served as a negative antigen control.

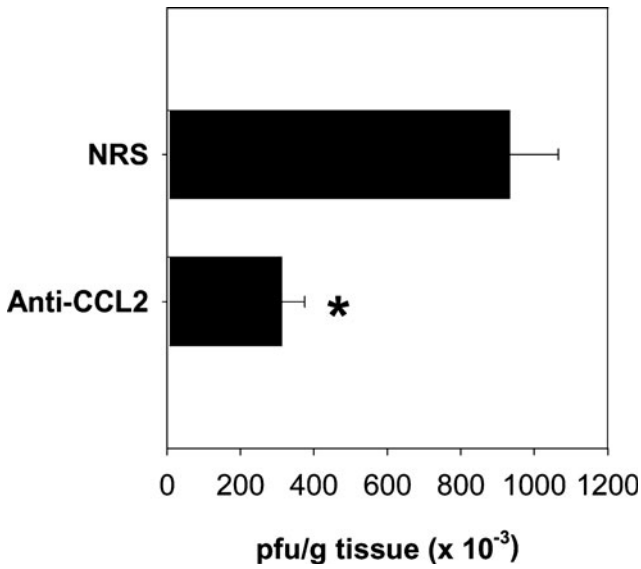

Figure 6 Anti-CCL2 treatment reduced TMEV replication in the CNS. Mice were infected with 3×10^6 pfu TMEV at day 0 and treated with either anti-CCL2 or control NRS on day 14. Approximately 4 weeks later when the NRS-treated mice showed normal TMEV-IDD progression and the anti-CCL2–treated mice showed decreased clinical disease severity. Spinal cords were harvested and analyzed for viral load using a plaque assay. The data show a significant decrease in the presence of TMEV in the spinal cords of anti-CCL2–treated mice when compared to NRS-treated control mice ($^* P < .05$).

infected with TMEV at day 0 and treated with anti-CCL2 or NRS as a control on day 14. Approximately 4 weeks later when the NRS-treated mice showed normal TMEV-IDD progression and anti-CCL2–treated mice showed decreased clinical disease, spinal cords were harvested, pooled, and assayed for the presence of replication competent virus. When individual spinal cords from either NRS- or anti-CCL2–treated mice were examined for the presence of live TMEV, 5/5 of the spinal cords from anti-CCL2–treated mice showed significantly decreased levels of viable virus. The average results from each group are shown in Figure 6 and demonstrate that anti-CCL2 treatment resulted in a significant decrease (~70% decrease) in replication-competent TMEV present in the CNS. These data demonstrate that anti-CCL2 treatment inhibited the ability of replication-competent virus to persist in the CNS.

Discussion

In the present report we hypothesized that CCL2 expression in the CNS played a significant functional role in the development and/or progression of TMEV-IDD based on our earlier demonstrations that expression of this chemokine in TMEV-infected CNS was up-regulated after infection (Hoffman *et al*, 1999) and

that forced expression of CCL2 in the CNS resulted in early-onset disease (Bennett *et al*, 2003). CCL2 is produced by a number of cell types in the CNS that include astrocytes, microglia, as well as infiltrating macrophages and lymphocytes. Others have also reported CNS CCL2 expression following TMEV infection (Murray *et al*, 2000; Theil *et al*, 2000); however, the significance of these expression patterns with respect to disease development and/or progression had not been addressed. We chose to treat mice with antichemokine antibodies and assess the resulting clinical disease severity based on our success with this approach in the past at identifying distinct roles for chemokines during autoimmune CNS demyelinating disease (Fife *et al*, 2001a; Karpus *et al*, 1995; Kennedy *et al*, 1998). Our present results demonstrate that CCL2 expression in TMEV-IDD plays a major role in the development of clinical TMEV-IDD (Figure 1). Treatment with anti-CCL2 at either day 0, 14, or day 28 relative to TMEV infection all resulted in significant clinical disease reductions. However, treatment at day 14 resulted in the most profound decrease in clinical disease (Figure 1). Therefore, we chose to concentrate on this treatment regimen for the examination of the mechanism of disease reduction.

Three major possibilities exist to explain our findings: (1) anti-CCL2 treatment decreased the accumulation of inflammatory mononuclear cells in the CNS, thereby resulting in less tissue damage and reduced clinical disease severity; 2) anti-CCL2 treatment resulted in a decreased TMEV-specific immune response, thereby resulting in less tissue damage and reduced clinical disease severity; or 3) anti-CCL2 treatment resulted in a decreased inflammatory cytokine response in the CNS by cells other than $VP2_{70-86}$–specific T cells. From our present data we conclude that CCL2 regulates clinical disease development and/or progression by controlling the accumulation of inflammatory cytokine-producing mononuclear cells in the CNS. In support of this idea we have demonstrated that anti-CCL2 treatment inhibited the amount of CNS inflammation as assessed by the number of perivascular lesions (Figure 2) and the inhibition of overall mononuclear cell accumulation in the CNS (Figure 3). Furthermore, there was a decreased CNS infiltration of $CD4^+$ T cells (Figure 4B) in anti-CCL2–treated mice. Expression of inflammatory cytokines in the CNS has been shown to correlate with development of TMEV-IDD clinical disease (Begolka *et al*, 1998). We observed that there was no change in the frequency of interferon gamma (IFN-γ)-producing cells in the CNS of treated and control mice (Figure 5B). This observation, together with the fact that where there was no change in the proportion of CNS-infiltrating mononuclear cells in the CNS (Figure 4A), supports that idea that anti-CCL2 treatment did not reduce clinical disease by affecting the proportions of CNS T-cell responses and is consistent with the idea that treatment decreased the absolute numbers of responding cells in the CNS (Figure 4B). Nevertheless, it is possible that anti-CCL2 treatment decreased the CNS accumulation of T cells specific for autoantigens and that the decreased disease could be a result of fewer autoreactive T cells in the CNS. Indeed, it has been demonstrated that autoreactive T cells arise as a result of TMEV infection of SJL mice (Katz-Levy *et al*, 2000). Furthermore, these T-cell responses appear to play a significant role in the progression of TMEV demyelinating disease (Neville *et al*, 2002). Moreover, we have previously demonstrated that disease-inducing T cells can have a different chemokine receptor expression pattern compared to other bystander CNS-infiltrating T cells in the EAE system (Fife *et al*, 2001b). T-cell populations can express CCR2 (Kuziel *et al*, 1997), the receptor for CCL2, and are attracted by its ligand, CCL2 (Lu *et al*, 1998). We have also observed that CCR2 is expressed by T cells (Fife *et al*, 2001b) and macrophages (unpublished). We are currently in the process of determining the chemokine receptor expression patterns of CNS-infiltrating autoreactive T cells in TMEV-infected mice and their responsiveness in terms of migration to CCL2.

Although CCR2 and CCL2 expression have been implicated in the generation of Th1 (Boring *et al*, 1997) and Th2 (Gu *et al*, 2000) responses, respectively, we found that treatment of TMEV-infected mice with anti-CCL2 did not result in a significant alteration in T-cell antigen–specific immune responses in either the periphery or CNS (Figure 5A and B) or the peripheral TMEV-specific immunoglobulin response (not shown). These data further support our idea that the mechanism of disease severity reduction in anti-CCL2–treated mice is related to the decreased accumulation of the absolute number of inflammatory $CD4^+$ T cells and macrophages in the CNS not to a deviation in the immune response.

Our data point toward anti-CCL2 treatment decreasing the CNS mononuclear cell infiltrate following TMEV infection and thereby decreasing chronic clinical paralytic disease. Three additional possibilities exist to explain why the decrease in the absolute CNS mononuclear cell infiltrate would result in a decrease in clinical disease. These include (1) a decrease in virus-specific T cells capable of expressing IFN-γ and subsequently activating macrophages; (2) a decrease in accumulation of macrophages which are the end stage effector cells in the demyelination process; and (3) a decrease in macrophages that are also one of the cell types in which TMEV replicates and establishes a persistent infection. Although, there is no apparent decrease in the frequency of the CNS viral-specific Th1 response in anti-CCL2–treated mice (Figure 5B), there is a decrease in the accumulation of CNS macrophages, thereby making all three possibilities viable additional explanations for a decrease in clinical disease. Although other cell types can be infected by TMEV (Aubert and Brahic, 1995; Jarousse *et al*, 1998), it is important to note that infiltrating monocytes/macrophages harbor the persistent

TMEV infection for the life of the mouse (Clatch *et al*, 1990; Levy *et al*, 1992). Therefore, inhibiting the CNS accumulation of macrophages could result in both the reduction of the effector cell involved in demyelination as well as the host cell for TMEV replication that would drive persistent *in situ* T-cell activation and subsequent macrophage activation.

Understanding the importance of the chemokine contribution to disease development and progression is important as new chemokine receptor-specific therapies are being developed for use in inflammatory diseases (Ransohoff and Bacon, 2000). A CCR1 antagonist has proven efficacious in reducing clinical disease and CNS inflammation in a rat model of EAE (Liang *et al*, 2000), whereas modified CCL5 (met-RANTES) has been shown to antagonize CCR1 and CCR3 (Elsner *et al*, 1997) as well as inhibit collagen-induced arthritis (Plater-Zyberk *et al*, 1997). Further understanding of the roles of chemokines in disease pathogenesis will allow for effective antagonist drug development.

Materials and methods

Mice
Five- to six-week-old SJL/J female mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were 6 to 7 weeks old at the initiation of the experiments. Animal care was provided according to Public Health Service Policy and approved by the Northwestern University Animal Care and Use Committee.

TMEV virus stocks
Virus was prepared as described previously (Miller *et al*, 1994). Briefly, confluent monolayers of BHK-21 cells (ATCC) were infected with the BeAn 8386 strain of TMEV for 72 h. Virus was precipitated with NaCl and polyethylene glycol (PEG) and then pelleted by centrifugation. Virus was further purified by ultracentrifugation on discontinuous 20% to 70% sucrose and Cs_2SO_4 gradients. Finally, virus was pelleted, resuspended in phosphate-buffered saline (PBS), and measured for optical absorbance at 260/280. Plaque assays of the supernatant were performed on BHK-21 cells.

TMEV intracerebral inoculation
Mice were anesthetized with methoxyflurane (Pitman Moore) and injected in the right cerebral hemisphere with 3×10^6 plaque-forming units (pfu) of TMEV (BeAn strain) in 30 μl of sterile Dubecco's modified Eagle medium (DMEM). Mice were examined 2 to 3 times per week for the first 3 weeks then daily until all infected animals were exhibiting neurological signs of TMEV-IDD. After signs of clinical disease, mice were examined biweekly. Clinical symptoms were scored as (1) waddling gait; (2) severe waddling gait and righting reflex impairment; or (3) hind limb paralysis with/without incontinence. Clinical data have been expressed as the mean clinical score at a particular timepoint.

Histology
Histological evaluation was performed on representative mice from each experimental group. Mice were anesthetized with methoxyflurane (Pitman-Moore) and perfused through the left ventricle with ~60 ml of PBS. Spinal cords were extruded by flushing the vertebral canal with PBS. The most caudal 1 cm of the lumbar spinal cord was fixed in a phosphate-buffered 10% formalin solution and embedded in paraffin. Twelve 10-μm sections from each animal were stained with hematoxylin and eosin and examined for the presence of mononuclear cell infiltration. Histological scores were determined using the following scale: 0, no mononuclear cell infiltration; 1, 1 to 5 perivascular lesions per section with parenchymal infiltration; 2, 5 to 10 perivascular lesions per section with parenchymal infiltration; and $3 > 10$ perivascular lesions per section with extensive parenchymal infiltration. The mean histological score $\pm$ SD was calculated for each group.

Isolation of CNS-infiltrating mononuclear cells and splenocytes
Mice were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL) and perfused through the left ventricle with ~60 ml of PBS. Spinal cords were extruded by flushing the vertebral canal with PBS and rinsed in PBS. Lymph nodes and spleens were removed from the same mice and placed in HBSS. Tissues were forced through 100-mesh stainless steel screens to yield a single cell suspension. Red blood cells in the spleen preparations were lysed by hypotonic shock in Tris-NH_4Cl (pH 7.3) and the cells were washed and resuspended in HBSS. CNS mononuclear cells were isolated by centrifugation ($500 \times g$) at 24°C on a 30% to 70% discontinuous Percoll (Pharmacia, Piscataway, NJ) gradient. Cells were collected from the interface, washed in Hank's balanced salt solution (HBSS), counted using a hemocytometer, and resuspended in isotonic buffered saline (IBS) (Baxter Diagnostics, McGaw Park, IL) containing 0.1% NaN_3 and 0.2% bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO) for flow cytometric analysis and in HBSS alone for functional assays.

Antibodies
Monoclonal antibodies to murine CD4 (RM4-5), CD8a (Ly-2), CD11b (M1/70), CD19 (1D3), CD45 (Ly-5), CD16/32 (2.4G2, anti-mouse Fc$R\gamma$II/III), DX-5, and isotype control antibodies were purchased from PharMingen (San Diego, CA). Polyclonal anti-CCL2 and anti-CCL3 was used as previously described (Kennedy *et al*, 1998). The polyclonal antibodies were titered by direct enzyme-linked immunosorbent assay (ELISA) and specificity was verified by

the failure to cross react with any other cytokine or chemokine tested (e.g., mIL-1α, mIL-2, IL-6, hIL-8, TNF, CCL5 [RANTES], hCCL3, CXCL1 [mMIP-2], CCL4 [MIP-1β], or CXCL10 [IP-10][1] as measured by either direct ELISA or the ability to neutralize *in vitro* chemokine-induced mononuclear cell migration (Lukacs *et al*, 1994; Strieter *et al*, 1992). At the time of the experiments the titer of the anti-chemokine antibodies was $>10^6$ (i.e., the dilution of antisera that gave a specific positive signal above control background). Each mouse was treated with two doses of 0.5 ml of either specific anti-chemokine antisera or control normal rabbit serum a day apart. We have determined that 0.5 ml of anti-CCL2 polyclonal antisera contained at least 500 μg of specific IgG.

Flow cytometry
Cells ($0.5–1 \times 10^6$) were incubated with anti-mouse FcRγII/III for 15 min at 4°C in to block Fc mediated binding. Cells were washed in IBS followed by incubation with specific antibodies CD4, CD8a, CD19, CD45, and CD11b at a predetermined optimal concentration for 15 min at 4°C. As a control, parallel populations of cells were incubated in the presence of isotype-matched control antibodies. Cells were washed and resuspended in 0.5 ml IBS. Data collection and analysis were performed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer using Cellquest software with 5×10^4 events/analysis.

In vitro *T-cell proliferation assays*
Spleen cells were obtained from mice at the peak of acute clinical disease for the control treated group. Tissues were forced through 100-mesh stainless steel screens to yield a single cell suspension. Red blood cells in the spleen preparations were lysed by hypotonic shock in Tris-NH_4Cl (pH 7.3) and the cells were washed and resuspended in HBSS. Cells were cultured in 96-well microtiter plates (Corning-Costar, Acton, MA) at 5×10^6 viable cells/ml in DMEM (GIBCO, Grand Island, NY) containing 5×10^{-5} M 2-mercaptoethanol (GIBCO), 2 mM L-glutamine (GIBCO), 100 μg/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), 0.1 M nonessential amino acids (GIBCO), and 5% fetal calf serum (FCS) (Hy-clone, Logan, UT) in the presence of 0, 0.5, 5, and 50 μM $VP2_{70-86}$. Ovalbumin peptide ($OVA_{323-339}$) was used as a nonspecific antigen control. Cells were incubated at 37°C in a humidified atmosphere containing 7.5% CO_2. The cells were pulsed with 1 μCi of ^{3}H-TdR (ICN Radiochemicals, Irvine, CA) after 72 h, harvested after 96 h, and ^{3}H-TdR uptake was detected using a Packard Topcount microplate scintillation counter (Packard Instruments, Meriden, CT). Results are presented as the mean ± SEM of triplicate wells.

Elispot assay
ELISPOT plates (Millipore, Ann Arbor, MI) were coated with 4 μg/ml capture anti-IFN-γ (R46A2, Pharmingen) for overnight at 4°C. Spleen cells or CNS mononuclear cells were plated with or without $VP2_{70-86}$ at a concentration of 50 μM. Plates were incubated at 37°C for 24 h and washed with PBS to remove cells. The detection antibody, anti-IFN-γ-biotin (XMG1.2; Pharmingen), was added at a concentration of 1 μg/ml and plates were incubated 3 h at 37°C. Bound secondary antibodies were visualized using anti-biotin-alkaline phosphatase and BCIP/NBT as a substrate (BioRad). Background staining and spot-forming cells were enumerated using an Immunospot (Cellular Tech) instrument.

Plaque assay
Specific CNS TMEV titers were determined by plating serial 10-fold dilutions of spinal cord and brains tissue in triplicate on confluent BHK cells. The plates were incubated at 37°C for 6 h after which the clarified tissue homogenate was removed from the cell layer by extensive washing. The cell monolayer was overlaid with methyl cellulose and incubated for an additional 3 days at 33°C. The methyl cellulose was removed by aspiration and the monolayer was stained with crystal violet for the visualization of plaque formation by microscopy. Both positive (a known titer of TMEV) and negative (tissue homogenate from un-infected mice) controls were included in each assay. The mean number of plaques was determined for each dilution and the titer of virus was calculated as plaques per g/wet tissue.

Statistical analysis
The percentage of mice showing clinical disease or mice showing CNS cytokine mRNA expression was compared by χ^2 using Fisher's exact probability. Single comparisons of two means were analyzed by the Student's t test. P values $\leq .05$ were considered significant. All of the experiments were performed at least three times to ensure reproducibility. The results shown are representative of similar experiments.

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