Journal of NeuroVirology, 9: 315–324, 2003

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280390201029

Taylor & Francis
Taylor & Francis Group

Adenovirus-mediated expression of CXCL10 in the central nervous system results in T-cell recruitment and limited neuropathology

Matthew J Trifilo and Thomas E Lane

Department of Molecular Biology and Biochemistry, University of California, Irvine, California, USA

In the present study, the authors evaluated the contributions of the CXC chemokine ligand (CXCL)10 to leukocyte recruitment into the central nervous system (CNS) and disease development. Instillation of a replication-deficient adenovirus that expresses CXCL10 (AdCXCL10) into the CNS of C57BL/6 mice resulted in a rapid (day 3) and prolonged (day 21) infiltration of both $CD4^+$ and $CD8^+$ T cells as compared to mice infected with an adenovirus vector containing β-galactosidase (Adβgal). Despite increased T-cell infiltration into the CNS of AdCXCL10-infected mice, production of proinflammatory chemokines normally associated with the recruitment of activated T cells into the CNS was muted and mice developed limited neuropathology. Therefore, these results indicate that T-cell infiltration in the absence of appropriate activation is not sufficient to induce pathology within the CNS and that additional signals other than CXCL10 are required for induction of an immune-mediated neurologic disease. *Journal of NeuroVirology* (2003) **9,** 315–324.

Keywords: chemokines; coronavirus; neuropathology; T cells

Introduction

CXCL10 (formerly known as interferon-inducible protein 10 kDa—IP-10) is a non-glutamic acid-leukine-arginine (ELR) CXC chemokine ligand that exerts a chemotactic effect on T cells of the Th1 subtype and on natural killer (NK) cells by binding to the chemokine receptor CXCR3 expressed on the surface of these cells (Bonecchi *et al*, 1998; Qin *et al*, 1998). In addition, recent studies indicate that CXCL10 exerts a diverse array of biological activities, including inducing antimicrobial effects (Cole *et al*, 2001), inhibiting angiogenesis (Angiolillo *et al*, 1995; Strieter *et al*, 1995), and contributing to T-cell effector responses (Dufour *et al*, 2002). Both type I and II interferons have been shown to induce CXCL10 gene expression in numerous cell types (Vanguri and Farber, 1990; Luster and Ravetch, 1987; Gattass *et al*, 1994). Expression of CXCL10 has been demonstrated in many inflammatory disease conditions, such as psoriasis (Gottlieb *et al*, 1988; Flier *et al*, 2001), rheumatoid arthritis (Patel *et al*, 2001), glomerular nephritis (Romagnani *et al*, 1999), diabetes (Shimada *et al*, 2001), and tissue-graft rejection (Hancock *et al*, 2000, 2001), and is thought to contribute to disease progression by attracting leukocytes into affected tissues in which CXCL10 is expressed.

CXCL10 is also considered to contribute to various neuroinflammatory pathologies, including stroke (Wang *et al*, 2000), Alzheimer's disease (Xia *et al*, 2000), viral encephalitis (Liu *et al*, 2000; Lahrtz *et al*, 1997), as well as the demyelinating disease multiple sclerosis (MS) (Sorensen *et al*, 1999; Simpson *et al*, 2000; Balashov *et al*, 1999). Astrocytes have been demonstrated to be the primary cellular source of CXCL10 surrounding active demyelinating lesions present in MS patients (Sorensen *et al*, 1999; Simpson *et al*, 2000; Balashov *et al*, 1999). Patients diagnosed with relapsing-remitting MS exhibited increased

Address correspondence to Thomas E Lane, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, CA 92697-3900, USA. E-mail: tlane@uci.edu

The authors gratefully acknowledge the Viral Vector Facility at University of California, Irvine, for help and guidance in construction and characterization of replication-deficient adenoviruses. This work was supported by NIH grant NS/AG41249 awarded to TEL.

Received 23 August 2002; revised 18 December 2002; accepted 6 January 2003.

levels of CXCL10 within the cerebrospinal fluid (CSF) during periods of clinical disease that correlated with increased numbers of CXCR3-expressing T cells (Sorensen *et al*, 1999). When clinical symptoms subsided, CSF levels of CXCL10 were reduced and T-cell infiltration was limited. These studies suggest that CXCL10 contributes to the pathology of demyelination by attracting T cells into the central nervous system (CNS), which then participate in white matter destruction. In support of this theory are recent studies from animal models of demyelination that have increased our understanding of the functional significance of CXCL10 expression within the CNS. Administration of neutralizing antibodies specific for CXCL10 to mice infected with mouse hepatitis virus (MHV) resulted in a marked improvement in neurologic disease, accompanied by an inhibition in the progression of demyelination and increased remyelination that correlated with reduced T-cell infiltration into the CNS (Liu *et al*, 2001b). Moreover, MHV infection of $CXCL10^{\leq/\leq}$ mice results in reduced T-cell infiltration into the CNS and only mild demyelination (Dufour *et al*, 2002). CXCL10 also contributes to neuroinflammation and disease in mice with the autoimmune demyelinating disease, experimental autoimmune encephalitis (EAE) (Fife *et al*, 2001). Collectively, these studies indicate that CXCL10 expression contributes to demyelination by attracting T cells into the brain and spinal cord.

In addition to promoting T-cell recruitment into the CNS, recent investigations have indicated a potential role for CXCL10 in enhancing T-cell effector functions, including proliferation and cytokine production in response to antigen (Dufour *et al*, 2002). These data give rise to the possibility that in addition to recruiting T cells into the CNS, CXCL10 may also directly contribute to T-cell activation, as characterized by cytokine and/or chemokine expression within the CNS, and this contributes to the development of neuropathology. Although numerous chemokines are expressed during neuroinflammatory disease conditions, there is increasing evidence that CXCL10 may be a key signaling molecule with regards to induction and maintenance of T-cell infiltration and neurologic damage. However, the majority of these studies were performed using either a neurotropic virus capable of replicating within the CNS or in an autoimmune model of demyelination in which a defined encephalitogen, e.g., myelin basic protein (MBP), is employed (Liu *et al*, 2001b; Fife *et al*, 2001). Therefore, the capacity in which CXCL10 contributes to neuropathology in the absence of these signals is not well defined.

With this in mind, the present study was undertaken to further investigate the contributions of CXCL10 in the development of neurologic disease. To this end, we have constructed a replication-deficient adenovirus that expresses murine CXCL10 and instilled this virus into the CNS of mice. The results presented indicate that adenovirus-mediated expression of CXCL10 within the CNS results in robust and prolonged infiltration of both $CD4^+$ and $CD8^+$ T cells. However, there was only limited grey and white matter pathology, suggesting that additional chemokines and/or populations of leukocytes are required for disease to occur.

Results

Characterization of a replication-deficient adenovirus vector that expresses CXCL10

A replication-deficient adenovirus expressing murine CXCL10 (AdCXCL10) was constructed as described in Materials and methods. *In vitro* infection of an astrocyte cell line with AdCXCL10 resulted in increased expression of CXCL10 protein within 24 hours post infection (p.i.) as compared to cells infected with the adenovirus vector containing μ-galactosidase (Adμgal) as detected by Western blot analysis (Figure 1**A**). These results indicated that CXCL10 expression was derived primarily by adenovirus vector containing the murine CXCL10 gene and was not in response to adenovirus infection of cells. In order to determine if the adenovirus-derived CXCL10 was able to attract activated lymphocytes, a chemotaxis assay was performed using lymphocytes obtained from the spleens of mice immunized with MHV. Previous studies have indicated that MHV infection results in increased expression of the CXCL10 receptor CXCR3 on T cells, therefore these cells will respond to CXCL10 signaling (Liu *et al*, 2000). Exposure of activated lymphocytes to supernatant from AdCXCL10-infected cells resulted in a significant increase ($P \leq .05$) in cell migration as compared to cells exposed to supernatant from Adμgal-infected cells (Figure 1**B**). Addition of anti-CXCL10 antibody to the AdCXCL10 supernatant resulted in a significant reduction ($P \leq .05$) in chemotaxis, indicating that CXCL10 was responsible for the increased leukocyte migration associated with AdCXCL10 supernatant.

Chemokine gene expression within the CNS of infected mice

Mice were injected intracranially with $1 \leq 10^7$ plaque-forming units (PFU) of either AdCXCL10 or Adμgal and were sacrificed at defined times p.i. There was no apparent clinical disease in mice infected with either virus as determined out to day 21 p.i. To confirm that instillation of AdCXCL10 into the brains of mice resulted in increased expression of CXCL10, chemokine gene expression was examined at days 3, 7, 14, and 21 p.i. As shown in Figure 2**A**, transcripts for CXCL10 are clearly detectable within the CNS of AdCXCL10-infected mice at all time points examined**.** Quantification of gene transcript levels revealed the highest expression of CXCL10 production at day 3 p.i., with decreasing levels at each subsequent time point (Figure 2**B**). Analysis of CXCL10 transcripts within the CNS of

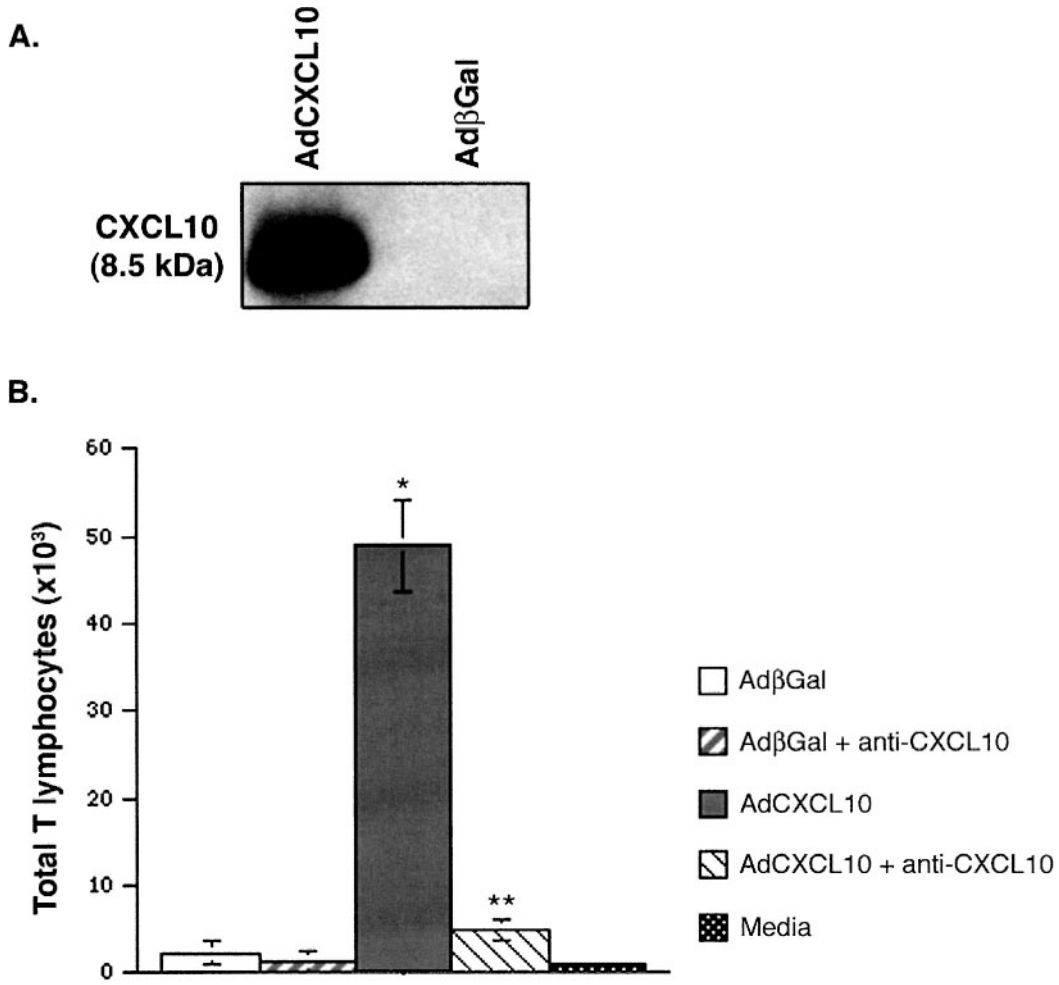

Figure 1 CXCL10 protein expression following AdCXCL10 infection of cell culture. (**A**) Western blot analysis revealed CXCL10 protein present in supernatants obtained from AdCXCL10-infected ASTL-1 cells, but not Adμgal-infected cells, following 24 h of infection. Recombinant mouse CXCL10 was included as a positive control (not shown). (**B**) Effects of CXCL10 expression on leukocyte migration. Leukocyte migration is significantly increased (* $P \leq .05$) following exposure to supernatants obtained from AdCXCL10-infected ASTL-1 cells collected 24 h p.i. as compared to supernatants from either Adμgal-infected ASTL-1 cells or medium alone. Anti-CXCL10 antibody significantly reduced (** $P \leq .05$) AdCXCL10-induced migration. Data presented represent the results of three separate experiments, each run in triplicate.

Adμgal-infected mice revealed reduced expression at all time points analyzed as compared to AdCXCL10-infected mice, suggesting only limited chemokine production was induced in response to the viral vector (Figure 2**A**, **B**). Transcripts for the chemokine CXCL9 (Mig—monokine induced by interferon [IFN]-μ) were also detected within the brains of mice infected with AdCXCL10 at all time points analyzed, but remained undetectable following Adμgal infection (Figure 2**A**, **B**). Although CXCL9 production has been previously shown to be influenced by IFN-μ (Farber, 1997), little to no IFN-μ was detectable within the CNS of AdCXCL10-infected mice at all time points analyzed (data not shown). Transcripts for the CC chemokine ligands (CCLs) CCL5 (CCL5/RANTES—regulated on activation, normal T cell expressed and secreted) and CCL2 (MCP-1—monocyte chemoattractant protein-1) were also present in the CNS of both groups of infected mice (Figure 2**C**). Analysis of transcript levels revealed a significant increase in CCL2 expression within the CNS of AdCXCL10-infected mice as compared to Adμgal infection at all time points analyzed (Figure 2**D**). Comparable levels of CCL5 transcripts were found in the brains of both AdCXCL10- and Adμgal-infected mice (Figure 2**D**). These data indicate differential expression of chemokine genes occurs within the CNS and may be the result of increased inflammation within the CNS of AdCXCL10-infected mice.

AdCXCL10-infected mice exhibit increased T-cell infiltration into the CNS

In order to determine if increased expression of CXCL10 within the CNS of AdCXCL10-infected mice correlated with increased T-cell infiltration, mononuclear cells were immunophenotyped by flow cytometry. Such analysis revealed an overall increase

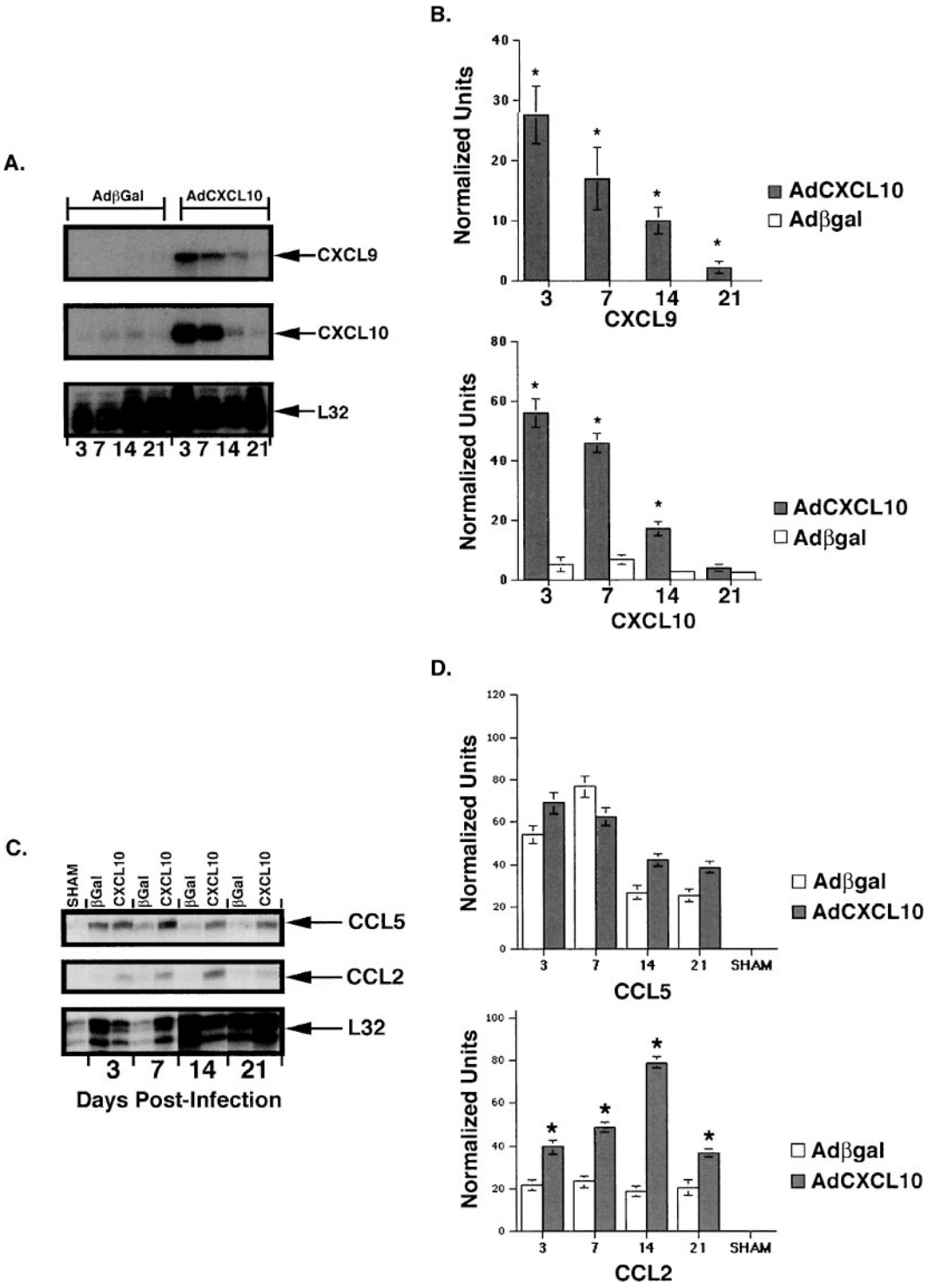

Figure 2 Chemokine mRNA expression in brains of AdCXCL10- and Adμgal-infected mice. (**A**, **C**) CXC and CC chemokine transcripts were detected within the brains of infected mice using a multitemplate probe set as described in Materials and Methods. Shown are representative mice at indicated times post infection. (**B**, **D**) Quantitative gene expression analysis. Densitometric analysis of chemokine mRNA transcripts obtained from the scanned autoradiograph using NIH 1.61 image software. Bands were normalized to the L32 probe, which is included in the probe set to verify consistency in RNA loading and assay performance. AdCXCL10-infected mice exhibited an increase in transcript levels for CXCL10, CXCL9, and CCL2 at all time points analyzed ($*P \leq .05$). Sham-infected mice displayed no detectable chemokines at any time points analyzed. Data represent three separate experiments with a minimum of three mice per experiment.

Table 1 Increased T-cell infiltration into the CNS of AdCXCL10-infected mice[a]

Mouse	*Days p.i.*	*CD4*	*CD8*	*F4/80*
Adμgal	3	$6.9 \leq 2.2$	$5.3 \leq 1.1$	$7.8 \leq 2.5$
	7	$6.6 \leq 1.3$	$5.4 \leq 1.7$	$9.2 \leq 3.7$
	14	$3.2 \leq 0.4$	$3.1 \leq 1.9$	$9.7 \leq 2.1$
	21	<1	<1	$7.3 \leq 3.4$
AdCXCL10[b]	3	$18.0 \leq 4.7$	$24.9 \leq 3.9$	$10.8 \leq 8.2$
	7	$22.1 \leq 1.8$	$23.2 \leq 1.6$	$11.2 \leq 2.5$
	14	$11.5 \leq 1.2$	$26.1 \leq 3.9$	$11.6 \leq 3.8$
	21	$11.9 \leq 8.2$	$17.4 \leq 2.1$	$10.2 \leq 1.2$
Sham		<1	<1	7.1

[a]Data represent three separate experiments with a minimum of 3 mice/group. Data presented as mean $\leq$ SEM.
[b]$CD4^+$ and $CD8^+$ T-cell infiltration into the CNS of AdCXCL10-infected mice is significantly increased ($P \leq .05$) as compared to Adμgal-infected animals at all time points analyzed. Data presented represent the percentage of positive cells within the gated population.

in the frequency of T-cell infiltration within the brains of AdCXCL10-infected mice at all time points analyzed (Table 1). Mice infected with AdCXCL10 displayed a dramatic increase in T cell infiltration at 3 days p.i. that peaked at 7 days p.i. By day 14 p.i., numbers of $CD4^+$ T cells had dropped by approximately 50% as compared to levels present at day 7 p.i. However, there was no further decrease in $CD4^+$ T-cell infiltration into the CNS at day 21 p.i. In contrast, there was sustained $CD8^+$ T-cell infiltration into the CNS of AdCXCL10-infected mice at day 14 p.i., and this dropped only slightly by day 21 p.i., suggesting that $CD8^+$ T cells were more responsive to CXCL10-induced chemotaxis as compared to $CD4^+$ T cells at later times p.i. Analysis of Adμgal-infected mice indicated increased infiltration of both $CD4^+$ and $CD8^+$ T cells as compared to sham-infected animals at days 3, 7, and 14 p.i., although this was significantly reduced as compared to AdCXCL10-infected mice. Although T-cell infiltration was enhanced in mice infected with AdCXCL10, there was no increase in macrophage infiltration (as assessed by F4/80 antigen expression) as compared to Adμgal-infected mice.

Instillation of AdCXCL10 into the CNS results in limited neuropathology

Brains from infected mice were examined for pathological alterations by analyzing tissue sections stained with hematoxylin/eosin combined with Luxol-fast blue (H&E-LFB) at defined times p.i. to assess inflammation and myelin loss. Such analysis revealed that although there was increased cellular infiltration into the CNS of AdCXCL10-infected mice as early as day 3 p.i., neuropathology was detectable only at later time points. Compared to Adμgal-infected mice, AdCXCL10-infected animals displayed increased cellular accumulation within the brain characterized by perivascular and meningeal inflammatory lesions at day 21 p.i. (Figure 3**A**). These cells appear to be lymphocytes based on morphology (inset to Figure 3**A**), which is consistent with the fluorescence-activated cell sorting (FACS) data presented in Table 1. Increased numbers of cells were detectable within the olfactory bulb as well as the thalamus, indicating that cells were able to invade the brain parenchyma (Figure 3**C** and **E**, respectively). Cellular infiltrates were located primarily within the internal granular layer of the olfactory bulb, although cells could be detected in the external plexiform layer separating the mitral and glomerular layer neurons (Figure 3**C**). At this time, the cell type is unknown; however, the cellular morphology is suggestive of either plasma cells or neutrophils (inset to Figure 3**C**). Infiltration into the thalamus was accompanied by mild grey and white matter destruction (Figure 3**E**). In marked contrast, histologic analysis of the brains of Adμgal-infected mice at day 21 p.i. indicated there were no detectable inflammatory cells lining endothelial vessel walls (Figure 3**B**) nor present in olfactory bulb or thalamus (Figure 3**D** and **F**). These data are not surprising based on flow analysis of brains from these mice indicating that T-cell infiltration is not detectable at this time point.

Discussion

Recent investigations indicate that chemokines such as CXCL10 contribute to neurodegenerative disease, in part, by contributing to leukocyte infiltration into the CNS (Fife *et al*, 2001; Sorensen *et al*, 1999; Simpson *et al*, 2000; Xia *et al*, 2000). For example, Liu *et al* (2001b) have demonstrated that chronic expression of CXCL10 by astrocytes within the CNS of mice persistently infected with MHV results in T-cell accumulation accompanied by robust demyelination, suggesting a role for CXCL10 in disease pathogenesis. Indeed, antibody-mediated neutralization of CXCL10 resulted in a pronounced reduction in neurologic disease severity and demyelination, accompanied by reduced T-cell accumulation within the CNS. Similar results were observed following MHV infection of $CXCL10^{\leq/\leq}$ mice (Dufour *et al*, 2002). Furthermore, evidence from human neurologic diseases including MS, Alzheimer's disease, and viral meningitis have all implicated a role for CXCL10 in attracting T cells into the CNS (Xia *et al*, 2000; Liu *et al*, 2000; Lahrtz *et al*, 1997; Sorensen *et al*, 1999; Simpson *et al*, 2000; Balashov *et al*, 1999). Therefore, the cumulative evidence points to a significant role for CXCL10 in regulating leukocyte trafficking into the CNS and disease progression in a variety of neuroinflammatory diseases.

The present study was undertaken to better understand the contributions of CXCL10 in the development of neuropathology. To this end, we have constructed a replication-deficient adenovirus vector that constitutively expresses murine CXCL10. Instillation of AdCXCL10 into the CNS of C57BL/6 mice

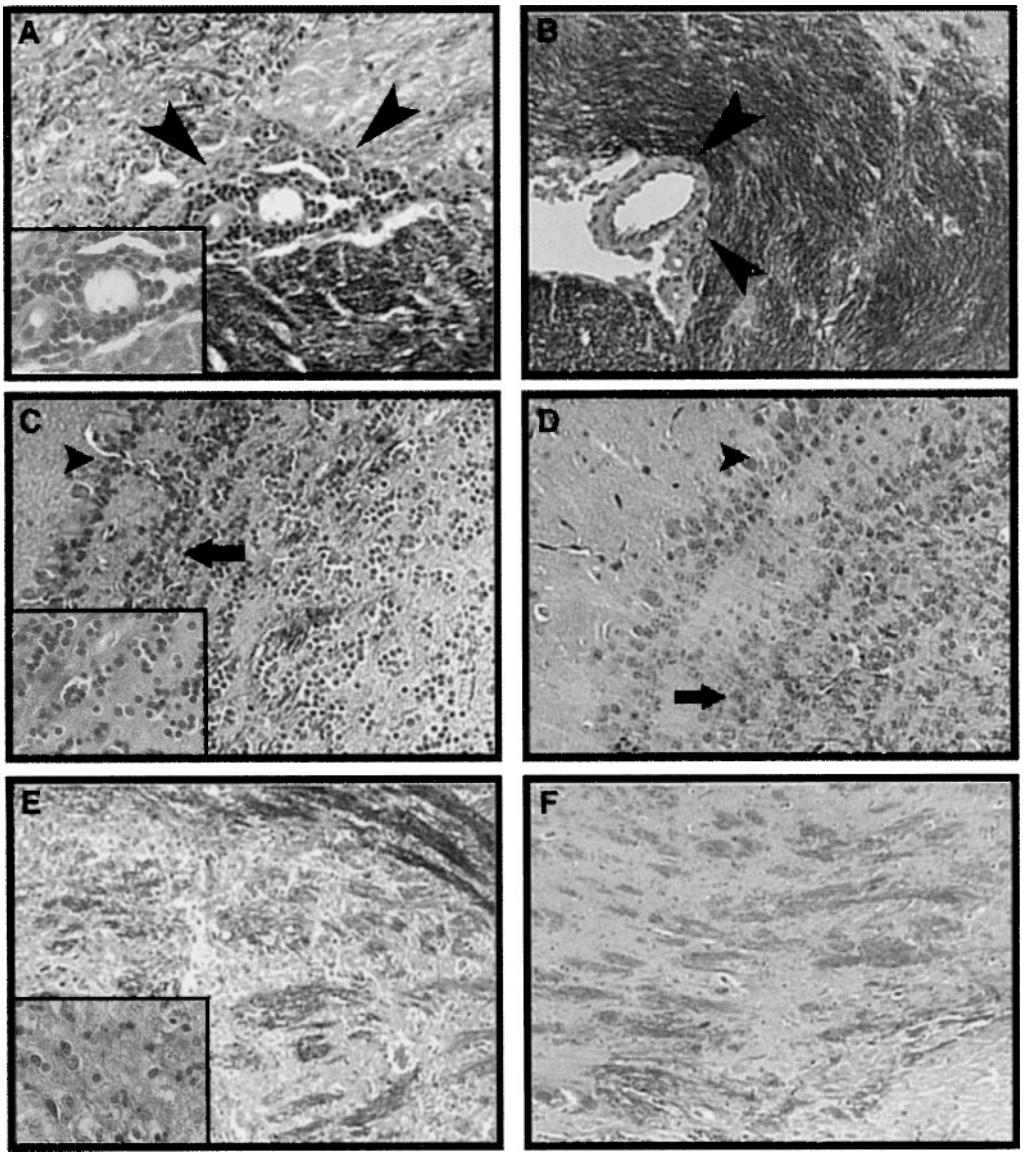

Figure 3 Leukocyte infiltration into the brains of AdCXCL10- and Adμgal-infected mice. Representative H&E-LFB stains of brains of infected mice at day 21 p.i. Shown is a perivascular inflammatory lesion (**A**) as well as leukocyte accumulation within the olfactory bulb (**C**) and cellular infiltration accompanied by grey and white matter damage in the thalamus (**E**) of a AdCXCL10-infected mouse. In contrast, there is no detectable inflammation present in vessels (**B**) nor the olfactory bulb (**D**) or thalamus (**F**) of Adμgal-infected mice. (**A**, **B**) Arrowheads point out vessel walls. (**C**, **D**) Arrowhead indicates glomerular layer neurons and arrow indicates mitral layer neurons. Original magnification: $\leq$200 (**A**, **B**); $\leq$100 (C–F).

resulted in expression of CXCL10 mRNA transcripts up to 21 days p.i. In addition, elevated levels of transcripts for the chemokines CXCL9, CCL2, and CCL5 were detected in the brains of AdCXCL10-infected mice at all time points analyzed. Mice infected with Adμgal exhibited a slight increase in transcript levels for CXCL10; however, CXCL9 transcripts were not detected at any time point. Although previous reports have indicated an important role for IFN-μ in triggering CXCL9 expression in cells, examination of IFN-μ within the brains of both AdCXCL10- and Adμgal-infected mice revealed very low level expression of IFN-μ mRNA and no detectable differences between infected groups at any time point examined. These data imply that CXCL9 expression within the brain is not solely dependent on IFN-μ and other cytokine may be involved in inducing expression of this chemokine. In support of this are recent studies indicating that both IFN-μ as well as tumor necrosis factor (TNF)-μ can induce CXCL9 expression in the absence of IFN-μ costimulation (Stylianou *et al*, 2002; Janatpour *et al*, 2001). AdCXCL10-infected mice consistently expressed increased transcript levels for CCL2 at all time points examined compared to Adμgal-infected mice. Although Adμgal-infected mice also expressed CCL2 and CCL5, transcript levels for these chemokines were consistently reduced at the majority of time points analyzed. These results

were not completely surprising as these chemokines have been shown to be expressed following adenovirus infection of the CNS of C57BL/6 mice (Charles *et al*, 1999). Collectively, these data suggested that the overall increased expression of chemokine genes within the CNS of AdCXCL10-infected mice may reflect an enhanced level of neuroinflammation.

Correlating with CXCL10 expression within the CNS of AdCXCL10-infected mice was increased T-cell infiltration at all time points analyzed. Recruitment of $CD4^+$ and $CD8^+$ T cells peaked at 7 days p.i. and although infiltration of both T-cell subsets continued up to 21 days p.i., $CD8^+$ T cells exhibited an enhanced chemotactic response to CXCL10 as compared to $CD4^+$ T cells at the later time points. Both $CD4^+$ and $CD8^+$ T cells have previously been shown to express CXCR3 following viral infection of the CNS, therefore increased infiltration of these cells into the CNS of AdCXCL10-infected mice is not surprising (Liu *et al*, 2000). The prolonged infiltration of $CD8^+$ T cells as compared to $CD4^+$ T cells suggests differential expression profiles for CXCR3 exists on these two populations of lymphocytes. However, the exact reasons for this observation are not understood at this time. The data presented are consistent with a recent report by Boztug *et al* (2002) that showed increased T-cell accumulation within the CNS of transgenic mice in which CXCL10 is expressed behind the astrocyte-specific promoter glial fibrillary acidic protein (GFAP). Interestingly, T cells were unable to penetrate into the parenchyma of the CXCL10-transgenic mice, which is in contrast to the data presented in this report in which inflammatory cells were readily detected within the parencyhma of AdCXCL10-infected mice. Whether differences exist in the extracellular matrix that allow for increased leukocyte infiltration of adenovirus-infected mice as compared to CXCL10-transgenic mice remains to be determined. Alternatively, it is possible that mechanical disruption of the blood-brain barrier (BBB) following intracerebral (i.c.) injection of virus contributed to increased T-cell infiltration. However, although compromising the BBB could account for limited T-cell infiltration directly following i.c. infection as is observed in Adμgal-infected mice, it is unlikely to account for the persistent T-cell recruitment observed up to 21 days p.i., as shown in AdCXCL10-infected mice. The lack of macrophage infiltration into the CNS of AdCXCL10 or Adμgal-infected mice was somewhat surprising given the overall increased expression of the macrophage chemoattractants CCL2 and CCL5 in both populations of mice. Previous studies from our laboratory have indicated that both of these chemokines exert a potent macrophage chemotactic response following viral infection of the CNS (Lane *et al*, 2000; Chen *et al*, 2001). These results imply that additional factors may be necessary for macrophage trafficking into the CNS following adenovirus infection.

Other studies have utilized either transgenic technology or adenovirus-directed delivery to examine the effects of chemokine/cytokine expression within the CNS. These reports have revealed that chemokine/cytokine expression results in distinct pathologies depending upon the system studied. For example, CNS-targeted expression of TNF-μ or N51/KC resulted in pronounced inflammatory lesions and extensive demyelination and paralysis (Tani *et al*, 1996; Stalder *et al*, 1998). In contrast, other studies have shown that expression of either CXCL10, MIP-2, or transforming growth factor (TGF)-μ did not result in any overt pathology within the CNS (Boztug *et al*, 2002; Bell *et al*, 1996; Pang *et al*, 2000). The dramatic difference in results appears to be dependent upon the activation state of the infiltrating cell type. Indeed, instillation of N51/KC and TNF-μ results in recruitment and activation of neutrophils and macrophages, respectively (Tani *et al*, 1996; Stalder *et al*, 1998). In contrast, expression of MIP-2, a chemokine that is responsible for neutrophil chemotaxis but not activation, resulted in robust neutrophil accumulation within the CNS but no observable neuropathology (Bell *et al*, 1996). In addition, recent investigations have indicated that intraparenchymal instillation of chemokines can induce a directional migration of leukocytes into the CNS. For example, microinjection of C10 results in the recruitment of macrophages and T cells, suggesting that injection of a single chemokine can influence cellular trafficking into the target organ (Asensio *et al*, 1999).

Our results suggest that overexpression of CXCL10 within the CNS is able to attract both $CD4^+$ and $CD8^+$ T cells. However, T-cell infiltration only marginally enhances additional chemokine production and does not result in macrophage recruitment. Therefore, the ability of T cells within the CNS to trigger robust neuropathology appears to be dependent on their activation status, which is related to their ability to secrete additional chemokines/cytokines that lead to the development of neurologic disease. Based on a historical perspective, additional factors that trigger lymphocyte activation (as defined by proliferation and cytokine production) would include a virus capable of replication, e.g., MHV, or a previously defined endogenous antigens that are capable of eliciting an inflammatory response within the CNS, such as myelin basic protein (MBP) or proteolipid protein (PLP). In the absence of such potent stimulation, infiltrating T cells remain somewhat inert and are not able to contribute to tissue destruction.

Materials and methods

Construction of replication-deficient adenovirus expressing CXCL10 (AdCXCL10)

The cDNA for the coding sequence of murine CXCL10 (bases 33 to 399) was kindly provided by I. Campbell

(Scripps Research Institute, La Jolla, CA). The cDNA encoding CXCL10 was restricted with *Bam*HI and *Bgl*II, then ligated into the shuttle vector pCMVAddel under the control of the cytomegalovirus promoter. Verification of the sequence arrangement of recombinant plasmid constructs was achieved by restriction enzyme digests and DNA sequence analyses. Recombinant pCMVAddel containing CXCL10 sequence was cotransfected into HEK293 cells with helper plasmid containing Ad5 sequence with the E1 region deleted. Virus was isolated from plaques, grown on 293 cells, and purified by cesium chloride density equilibrium banding with ethidium bromide. A replication-deficient adenovirus containing μ-galactosidase (AdμGal) was kindly provided by L. Villarreal (Viral Vector Facility, University of California, Irvine).

Western blot

A murine astrocyte cell line (ASTL-1, kindly provided by M. Buchmeier, Scripps Research Institute) was infected with either AdCXCL10 or Adμgal (moi = 10). Supernatants were removed at 24 h p.i. and concentrated by centrifugation using Centricon Plus-20 columns (Fisher Scientific, Pittsburgh, PA). Recombinant murine CXCL10 was used as a positive control. Proteins were separated by 8% to 12% Bis-Tris sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Novex, San Diego, CA) and transferred to a nitrocellulose membrane and blocked for 1 h. Membranes were washed, incubated for 30 min with diluted (1:5000) polyclonal rabbit anti-CXCL10 antisera (Liu *et al*, 2000, 2001b), followed by incubation with secondary goat anti-rabbit antibody. Signal was detected by chemiluminescence (Novex).

Chemotaxis

Activated mononuclear cells were obtained by immunizing C57BL/6 mice with $5 \leq 10^5$ PFU of MHV. Previous studies have demonstrated that MHV infection results in accumulation of CXCR3-positive T cells being recruited to the site of infection, therefore this population of cells was used as target cells for CXCL10-induced chemotaxis (Liu *et al*, 2000). The capacity of supernatants obtained from ASTL-1 cells infected with either AdCXCL10 or Adμgal to chemoattract leukocytes from MHV-immunized mice was determined by a chemotaxis assay using Transwell plates (CoStar, Cambridge, MA) containing polycarbonate inserts with 5-μm pores. The chemotaxis assay was performed using a previously described protocol (Biddison *et al*, 1998). In attempt to determine if adenovirus-derived CXCL10 was responsible for chemotaxis, supernatants were incubated either in the presence or absence of neutralizing polyclonal anti-CXCL10 (10 μg/ml) antisera for 1 h prior to use in chemotaxis assays (Liu *et al*, 2000, 2001b). Cells that migrated to the lower chamber were counted and cell viability was assessed using trypan blue. Only live cells were used to determine total cell migration. All conditions were performed in triplicate.

Animals

Five- to six-week-old male C57BL/6 (H-2^b background) were used for all studies described. Following anesthetization by inhalation of methoxyflurane (Pitman-Moore, Washington Crossing, NJ), mice were injected intracerebrally into the right hemisphere of the brain with $1 \leq 10^7$ PFU of either AdCXCL10 or Adμgal suspended in 50 μl sterile phosphate-buffered saline (PBS). This type of injection routinely results in deposition of virus into the hippocampus as determined in studies with MHV (Lane *et al*, 1998). Control mice were injected with 50 μl sterile PBS alone. Brains were isolated at scheduled time points and one half was used for flow cytometric analysis. The remaining halves were used for RNA isolation or fixed in normal buffer formalin, followed by embedding in paraffin for histological analysis.

Ribonuclease protection assay (RPA)

Chemokine gene expression was determined using the multitemplate probe set mCK-5 (Pharmingen, San Diego, CA) using a previously described protocol (Lane *et al*, 2000). CXCL9 and CXCL10 transcripts were detected using previously described antisense riboprobes (Liu *et al*, 2001a). Target samples represented total RNA (15 μg) obtained from the brains of mice infected with either AdCXCL10 or Adμgal at defined times post-instillation of either virus. For quantification of signal intensity, autoradiographs were scanned and individual chemokine transcript signals were normalized as the ratio of band intensity to the internal L32 control present within each probe set. Analysis was performed using NIH Image 1.61 software (Liu *et al*, 2000; Lane *et al*, 2000).

Mononuclear cell isolation and flow cytometry

Mice were sacrificed at scheduled time points and a single cell suspension was obtained from brains of mice as previously described (Lane *et al*, 2000). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 and CD8 were used to detect infiltrating $CD4^+$ and $CD8^+$ T cells. FITC-conjugated rat anti-mouse F4/80 was used to detect activated macrophage/microglial cells. An isotype-matched FITC antibody was used as a control. Cells were incubated with antibodies for 30 min at 4≤C, washed, fixed in 1% paraformaldehyde, and analyzed on a FACStar (Becton Dickinson, Mountain View, CA) (Lane *et al*, 2000). Data are presented as the percentage of positive of cells within the gated population.

Statistical analysis

All data were analyzed by performing the Student's *t* test using Sigma-Stat 2.0 software. Values of $P \leq .05$ were considered significant.

References

Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reaman GH, Tosato G (1995). Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J Exp Med* **182:** 155–162.

Asensio VC, Lassmann S, Pagenstecher A, Steffensen SC, Henriksen SJ, Campbell IL (1999). C10 is a novel chemokine expressed in experimental inflammatory demyelinating disorders that promotes recruitment of macrophages to the central nervous system. *Am J Pathol* **154:** 1181–1189.

Balashov KE, Rottman JB, Weiner HL, Hancock WW (1999). CCR5+ and CXCR3+ T cells are increased in multiple sclersosis and their ligands MIP-1μ and IP-10 are expressed in demyelinating brain lesions. *Proc Natl Acad Sci U S A* **96:** 6873–6878.

Bell MD, Taub DD, Kunkel SJ, Strieter RM, Foley R, Gauldie J, Perry VH (1996). Recombinant human adenovirus with Rat MIP-2 gene insertion causes prolonged PMN recruitment to the murine brain. *Eur J Neurosci* **8:** 1803–1811.

Biddison WE, Cruikshank WW, Center DM, Pelfrey CM, Taub DD, Turner RV (1998). CD8+ myelin peptide-specific T cells can chemoattract CD4+ myelin peptide-specific T cells: importance of IFN-inducible protein 10. *J immunol* **160:** 444–448.

Bonecchi, RG, Bianchi, G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray A, Mantovani A, Sinigaglia F (1998). Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* **187:** 129–134.

Boztug K, Carson MJ, Pham-Mitchell N, Asensio VC, DeMartino J, Campbell IL (2002). Leukocyte infiltration but not neurodegeneration in the CNS of transgenic mice with astrocyte production of the CXC chemokine ligand 10. *J Immunol* **169:** 1505–1515.

Charles PC, Chen X, Horwitz MS, Brosnan CF (1999). Differential chemokine induction by the mouse adenovirus type-1 in the central nervous system of susceptible and resistant strains of mice. *J NeuroVirol* **5:** 55–64.

Chen BP, Kuziel WA, Lane TE (2001). Lack of CCR2 results in increased mortality and impaired leukocyte activation and trafficking following infection of the central system with a neurotropic coronavirus. *J Immunol* **167:** 4585–4592.

Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM (2001). IFN-inducible ELR$^{\leq}$ CXC chemokines display defensin-like antimicrobial activity. *J Immunol* **167:** 623–627.

Dufour JH, Dziejman M, Liu MT, Leung JH, Lane, TE, Luster AD (2002). IFN-μ-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol* **168:** 3195–3204.

Farber JM (1997). MIG and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* **61:** 246–257.

Fife BT, Kennedy KJ, Paniagua MC, Lukacs NW, Kunkel SL, Luster AD, Karpus WJ (2001). CXCL10 (IFN-μ-inducible protein-10) control of encephalitogenic CD4$^+$ T cell accumulation in the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* **166:** 7617–7624.

Flier J, Boorsma DM, van Beek PJ, Nieboer C, Stoof TJ, Willemze R, Tensen CP (2001). Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. *J Pathol* **194:** 398–405.

Gattass CR, King LB, Luster AD, Ashwell JD (1994). Constitutive expression of interferon gamma-inducible protein 10 in lymphoid organs and inducible expression in T cells and thymocytes. *J Exp Med* **179:** 1373–1378.

Gottlieb AB, Luster AD, Posnett DN, Carter DM (1988). Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. *J Exp Med* **168:** 941–948.

Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD (2001). Donor-derived IP-10 initiates development of acute allograft rejection. *J Exp Med* **193:** 975–980.

Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C (2000). Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med* **192:** 1515–1519.

Janatpour MJ, Hudak S, Sathe M, Sedgwick JD, McEvoy LM (2001). Tumor necrosis factor-dependent segmental control of MIG expression by high endothelial venules in inflamed lymph nodes regulates monocytes recruitment. *J Exp Med* **193:** 1375–1384.

Lahrtz F, Piali L, Nadal D, Pfister HW, Spanaus KS, Bagiolini M (1997). Chemotactic activity on mononuclear cells in the cerebrospinal fluid of patients with viral meningitis is mediated by interferon-gamma inducible protein-10 and monocyte chemotactic protein-1. *Eur J Immunol* **27:** 2484–2489.

Lane TE, Asensio VC, Yu N, Paoletti AD, Campbell IL, Buchmeier MJ. (1998). Dynamic regulation of μ- and μ-chemokine expression in the central nervous system during mouse hepatitis virus-induced demyelinating disease. *J Immunol* **160:** 970–978.

Lane TE, Liu MT, Chen BP, Asensio VC, Samawi RM, Paoletti AD, Campbell IL, Kunkel SL, Fox HS, Buchmeier MJ (2000). A central role for CD4$^+$ T cells and RANTES in virus-induced central nervous system inflammation and demyelination. *J Virol* **74:** 1415–1424.

Liu MT, Chen BP, Oertel P, Buchmeier MJ, Armstrong D, Hamilton TA, Lane TE (2000). Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurologic function in a viral model of multiple sclerosis. *J Immunol* **165:** 2327–2330.

Liu MT, Armstrong D, Hamilton TA, Lane TE (2001a). Expression of Mig (monokine induced by interferon-μ) is important in T lymphocyte recruitment and host defense following viral infection of the central nervous system. *J Immunol* **166:** 1790–1795.

Liu MT, Kierstead HS, Lane TE (2001b) Neutralization of the chemokine CXCL10 reduced inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. *J Immunol* **167:** 4091–4097.

Luster AD, Ravetch JV (1987). Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* **166:** 1084–1097.

Pang L, Ye W, Che X, Rossler BJ, Betz AL, Yang G (2000). Reduction of inflammatory response in the mouse brain

with adenoviral-mediated transforming growth factor-μ1 expression. *Stroke* **32:** 544–552.

Patel DD, Zachariah JP, Whichard LP (2001). CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. *Clin Immunol* **98:** 39–45.

Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR (1998). The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* **101:** 746–754.

Romagnani P, Beltrame C, Annunziato F, Lasagni L, Luconi M, Galli G, Cosmi L, Maggi E, Salvadori M, Pupilli C, Serio M (1999). Role for interactions between IP-10/Mig and CXCR3 in proliferative glomerulonephritis. *J Am Soc Nephrol* **10:** 2518–2526.

Shimada A, Morimoto J, Kodama K, Suzuki R, Oikawa Y, Funae O, Kasuga A, Saruta T, Narumi S (2001). Elevated serum IP-10 levels observed in type 1 diabetes. *Diabetes Care* **24:** 510–515.

Simpson JE, Newcombe J, Cuzner ML, Woodroofe MN (2000). Expression of the interferon-gamma-inducible chemokines IP-10 and Mig and their receptor, CXCR3, in multiple sclerosis lesions. *Neuropathol Appl Neurobiol* **26:** 133–142.

Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin S, Sellebjerg F, Strieter RM, Frederiksen JL, Ransohoff RM (1999). Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* **103:** 807–815.

Stalder AK, Carson MJ, Pagenstscher A, Asensio VC, Kincaid C, Benedict M, Powell HC, Masliah E, Campbell IL (1998). Late-onset chronic inflammatory encephalopathy in immune-competent and severe combined immune-deficient (SCID) mice with astrocyte-targeted expression of tumor necrosis factor. *Am J Pathol* **153:** 767–783.

Strieter RM, Kunkel SL, Arenberg DA, Burdick MD, Polverini PJ (1995). Interferon μ-inducible protein 10 (IP-10), a member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochem Biophys Res Commun* **210:** 51–57.

Stylianou E, Yndestad A, Sikkeland LI, Bjerkeli V, Damas JK, Haug T, Eiken HG, Aukrust P, FrOland SS (2002). Effects on interferon-alpha on gene expression of chemokines and members of the tumour necrosis factor superfamily in HIV-infected patients. *Clin Exp Immunol* **130:** 279–285.

Tani M, Fuentes ME, Peterson JW, Trapp BD, Durham SK, Loy JK, Bravo R, Ransohoff RM, Lira SA (1996). Neutrophil infiltration, glial reaction, and neurological disease in transgenic mice expressing the chemokine N51-KC in oligodendrocytes. *J Clin Invest* **98:** 529–539.

Vanguri P, Farber JM (1990). Identification of CRG-2. An interferon-inducible mRNA predicted to encode a murine monokine. *J Biol Chem* **265:** 15049–15057.

Wang X, Li X, Schmidt DB, Foley JJ, Barone FC, Ames RS, Sarau HM (2000). Identification and molecular characterization of rat CXCR3: receptor expression and interferon-inducible protein-10 binding are increased in focal stroke. *Mol Pharmacol* **57:** 1190–1198.

Xia MQ, Bacskai BJ, Knowles RB, Qin SX, Hyman BT (2000). Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. *J Neuroimmunol* **108:** 227–235.