CXC Chemokine Ligand 13 Plays a Role in Experimental Autoimmune Encephalomyelitis

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Experimental autoimmune encephalomyelitis (EAE) is a T cell–mediated autoimmune disease of the CNS that is widely used as an animal model of multiple sclerosis. In this study, we investigate the role of CXCL13, a chemokine involved in the development and organization of secondary lymphoid tissues, in the pathogenesis of EAE. We detected CXCL13 mRNA and protein in spinal cords of mice with EAE. CXCL13-deficient mice exhibited a mild, self-limited form of disease. CXCL13 appeared to be important for the establishment of chronic white matter lesions. Furthermore, adoptive transfer experiments with CXCL13-deficient hosts indicate that the chemokine plays a distinct role during the effector phase. Our findings raise the possibility that reagents that antagonize or inhibit CXCL13 might be useful for the treatment of neuroinflammatory diseases such as multiple sclerosis. The Journal of Immunology, 2006, 176: 7676–7685.

Lymphoid chemokines, including CCL19 (ELC), CCL21 (SLC), and CXCL13 (BLC), are constitutively expressed in secondary lymphoid tissues and are responsible for the distinctive architecture of those organs (1, 2). They direct leukocyte trafficking through the specialized compartments of lymph nodes (LNs) and spleen (3, 4). CCL19 and CCL21 attract CCR7+ naive and central memory T cells as well as activated dendritic cells (DC) (3) to T cell zones, whereas CXCL13 attracts CXCR5+ B cells and a subset of Th cells (termed follicular Th cells) to B cell–rich areas (5–8). Over the past several years, it has become evident that lymphoid chemokines are also expressed in nonlymphoid tissues during chronic inflammation, such as gastric mucosa in the setting of refractory Helicobacter pylori infection (9) and the liver in response to Propionibacterium acnes infection (10). Furthermore, transgenic expression of lymphoid chemokines in the skin or pancreas drives the development of LN-like structures in those tissues (11).

Although lymphoid chemokines might be expressed in nonlymphoid organs as part of an adaptive response against infection, they also have the potential to support autoimmune inflammation. Hence, CCL19 and CCL21 are up-regulated in the pancreas of NOD mice with diabetes (12), and CXCL13 is expressed in the thymus and kidneys of mice developing experimental lupus nephritis (13). With regard to autoimmune diseases in humans, CXCL13 was detected in salivary glands from patients with Sjögren’s syndrome and synovial tissues from patients with rheumatoid arthritis (14, 15). Nevertheless, the physiological role of endogenous lymphoid chemokines in the pathogenesis of autoimmune diseases has yet to be directly demonstrated.

In the current study, we investigate the role of CXCL13 in the development of experimental autoimmune encephalomyelitis (EAE). EAE is an inflammatory demyelinating disease of CNS white matter that frequently follows a relapsing or progressive clinical course. It is widely used as an animal model of multiple sclerosis (MS). Adoptive transfer studies have demonstrated that myelin-reactive CD4+ T cells initiate EAE (16). Nevertheless, macrophages and microglia and, in some models, B cells are major components of established CNS infiltrates and contribute to the end organ damage (demyelination and axonal injury) that results in neurological deficits (17, 18). Many laboratories have investigated the role of proinflammatory chemokines, such as CCL2, CCL3, CCL5, and CXCL10, in attracting circulating leukocytes to acute demyelinating lesions (19, 20). However, little is known about the contribution of lymphoid chemokines to the development of CNS inflammation during EAE or MS.

We found that CXCL13 is up-regulated in the CNS during EAE following the adoptive transfer of myelin-specific CD4+ T cells, as well as following active immunization with myelin peptides. CXCL13 is produced in the CNS at an early time point, and its levels rise progressively over the course of relapsing EAE. CXCL13 deficiency results in a milder course, characterized by more complete recovery and attenuation of white matter inflammation and gliosis during acute and chronic stages of disease. Furthermore, adoptive transfer experiments with CXCL13-deficient hosts or wild-type (WT) hosts treated with anti-CXCL13 mAbs demonstrate that the chemokine plays a distinct role during the effector phase.

Materials and Methods

Mice

SJL, B10.PL, and C57BL/6 mice were obtained from The Jackson Laboratory. CXCL13-deficient mice on the C57BL/6 background were a gift from Dr. J. Cyster (University of California, San Francisco, CA) (1, 21). Mice that express transgenic TCR Vβ8.2 and Vα4 chains specific for myelin basic protein (MBP) Ac1–11 in the context of I-Aκ (MBP-TCR) were originally provided by Dr. C. A. Janeway (Yale University School of Medicine, New Haven, CT) and bred in our facility. Animals were housed under specific pathogen-free, barrier facility conditions. All experiments

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were performed under University Committee on Animal Resources-approved protocols.

**Peptides**

Myelin peptides were synthesized by Macromolecular Resources and purified by HPLC. The sequences were as follows: proteolipid protein (PLP)_{39–151}, HSLKGLWHPDKF; MBP_{Ac1–11}, Ac-ASQKRPSoRHG; and myelin oligodendrocyte glycoprotein (MOG)_{ss,ss}, MEGVWYRSPFSRVVHYRNGK.

**Induction of EAE by active immunization**

Mice were immunized with 100 μg of the relevant myelin peptide emulsified in CFA (with 4 mg/ml heat-killed Mycobacterium tuberculosis H37Rv; v/v) by s.c. injection at four sites over the flanks. Bordetella pertussis toxin (List Laboratories) was injected i.p. (300 ng/mouse) on days 0 and 2 postchallenge. Animals were examined daily for signs of EAE and rated for severity of neurological impairment on a standard five-point scale (22) as follows: 0, no discernable deficits; 1, limp tail; 2, mild hindlimb weakness with an inability to flip over from a supine position; 3, obvious hindlimb and/or forelimb weakness; 4, paralysis; 5, moribund. A relapse is defined as an increase in clinical score by one or more points for 2 consecutive days or longer.

**Induction of EAE by adoptive transfer**

SJL mice were immunized with 100 μg of PLP_{39–151} emulsified in IFA (1:1) and C57BL/6 mice were immunized with MOG_{ss,ss} in CFA (1:1) by the s.c. route as described above, but without injection of pertussis toxin. Twelve to 17 days later, spleens or draining LNs (inguinal and axillary) were removed and processed as previously described (23). Cells were cultured in standard medium with myelin peptide (50 μg/ml PLP or 25 μg/ml MOG) with or without murine rIL-12 (5 ng/ml; R&D Systems). At 96 h, cells were harvested, washed, counted by trypan blue exclusion, and injected into naive syngeneic recipients (2–5 × 10^6 cells). For spleen cultures, live cells were enriched by Ficoll-Paque gradient before transfer. Recipient mice were examined daily and rated by observers blinded to treatment group or phenotype.

In some experiments, CD4+ T cells were purified from cultured LN cells or splenocytes using mouse-specific CD4 cell enrichment columns (R&D Systems) before injection into naive syngeneic recipients. Purity of isolated CD4+CD3+ cells was >97% as assessed by FACs.

**Anti-CXCL13 treatment in vivo**

Pooled spleen and LN cells from MBP-TCR-transgenic mice were stimulated in vitro with MBP_{Ac1–11} (50 μg/ml) and mIL-12 (5 μg/ml) for 4 days and then injected into naive B10.PL recipient mice (35–50 × 10^6 cells, i.p.). Recipients were injected with a neutralizing anti-mouse CXCL13 Ab (rat IgG2a, clone 143614; R&D Systems) or control rat IgG2a (clone 54447; Sigma-Aldrich) for 40 min at 37°C. Mice were then injected s.c. with 250 μl of recombinant murine CXCL13 (R&D Systems) or vehicle (PBS). Recipient mice were examined daily and rated by observers blinded to treatment group or phenotype.

**RNA analysis**

Total RNA was extracted from whole spinal cords or isolated mononuclear cells (MNCs) using TRIzol reagent (Invitrogen Life Technologies). Multiprobe RNase protection assays (RPA) were performed with the Riboquant Tiprobe RNase protection assays (RPA) were performed with the Riboquant kit (Pharmacia Biotech), washed with HBSS containing 3% FBS, and then injected into naive B10.PL recipient mice (35–50 × 10^6 cells, i.p.). Recipients were injected with a neutralizing anti-mouse CXCL13 Ab (rat IgG2a, clone 143614; R&D Systems) or control rat IgG2a (clone 54447; R&D Systems) at 0.3 μg/dose, i.p., on days 3, 6, and 10 after adoptive transfer. Clinical signs of EAE were evaluated by an observer who was blinded to the nature of treatment.

The differences in daily mean clinical scores between groups of SJL mice were immunized with 100 μg of PLP_{39–151} emulsified in IFA (1:1) and C57BL/6 mice were immunized with MOG_{ss,ss} in CFA (1:1) by the s.c. route as described above, but without injection of pertussis toxin. Twelve to 17 days later, spleens or draining LNs (inguinal and axillary) were removed and processed as previously described (23). Cells were cultured in standard medium with myelin peptide (50 μg/ml PLP or 25 μg/ml MOG) with or without murine rIL-12 (5 ng/ml; R&D Systems). At 96 h, cells were harvested, washed, counted by trypan blue exclusion, and injected into naive syngeneic recipients (2–5 × 10^6 cells). For spleen cultures, live cells were enriched by Ficoll-Paque gradient before transfer. Recipient mice were examined daily and rated by observers blinded to treatment group or phenotype.

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**Histopathological studies**

Spinal cords were dissected from mice following intracardiac perfusion with 4% paraformaldehyde. They were then decalcified in Immunocal and then injected into naive B10.PL recipient mice (35–50 × 10^6 cells, i.p.). Recipients were injected with a neutralizing anti-mouse CXCL13 Ab (rat IgG2a, clone 143614; R&D Systems) or control rat IgG2a (clone 54447; R&D Systems) at 0.3 μg/dose, i.p., on days 3, 6, and 10 after adoptive transfer. Clinical signs of EAE were evaluated by an observer who was blinded to the nature of treatment.

**CXCL13 mRNA was measured in spinal cord MNCs by semi-quantitative RT-PCR and normalized to HPRT. Southern blot hybridization was performed using radiolabeled internal oligo probes with the following sequences: CXCL13, GGTCCGACAGCAACTTCG; HPRT, AATAGCAGGTTGCTTCACTCCGT.

**Western blot analysis**

The assay was performed following published protocols (24). Detection was with goat anti-mouse CCL19, CCL21, CXCL13 Abs (R&D Systems), followed by anti-goat HRP (Jackson Immunoresearch Laboratories). Bands were visualized using chemiluminescence (Pierce Super Signal West Femto substrate). Positive controls included protein extract from spleen and recombinant chemokine proteins (R&D Systems).

**Isolation of spinal cord MNCs and enrichment of CD11c+ cells**

Mice were anesthetized with Avertin and perfused with PBS by the intracardiac route using a peristaltic pump. Intact vertebral columns were removed by gross dissection and cords were ejected under the pressure of an HBSS-filled syringe. Spinal cords were pooled from several mice, minced into small fragments, and digested with collagenase (2 mg/ml; CLS-4; Worthington Biochemical) and Dnase (1 mg/ml; DN25; Sigma-Aldrich) for 40 min at 37°C. MNCs were then isolated over a 30/70% Percoll gradient (Pharmacia Biotech), washed with HBSS containing 3% FBS, and used for FACs analysis or RT-PCR. CD11c+ cells were enriched from spinal cord MNCs using anti-FITC-coated magnetic beads (Miltenyi Biotech) following staining with FITC-conjugated anti-CD11c mAb (BD Pharmingen).

**Flow cytometric analysis**

Spinal cord MNCs were incubated with FCBlock Ab (BD Pharmingen) and stained with various combinations of fluorochrome-labeled Abs to mouse CD3 (clone 145-2C11), CD4 (RM4-5), CD45 (30-F11), CD44 (IM7), ICOS (C938.4A), CD127 (IL-7Rα, A7/34) (all from ebioscience); CD11c (HL-3), CD11b (M1/70), CD62L (MEL-14), and CXCR3 (2G8) (all from BD Pharmingen), or with isotype control Abs. Cells were washed twice and fixed with 1% paraformaldehyde in PBS before analysis on a BD Biosciences FACSCalibur instrument with CellQuest software.

**Lymphoproliferation**

LN cells (4 × 10^5 in 0.2 ml) or splenocytes (2 × 10^5 in 0.2 ml) were cultured with or without myelin peptide in quadruplicate for 4 days in 96-well flat-bottom plates. Wells were pulsed for the final 16 h of culture with 1 μCi of [3H]Tdr (NEN), and incorporated radioactivity was measured with a Betaplate scintillation counter (Wallac).

**Cytokine assays**

Splenocytes were cultured in 24-well plates (5 × 10^5 in 1 ml) or 10^5 in 2 ml) for 72 h. Supernatants were collected and analyzed using the OptEIA Mouse IFN-γ set (BD Pharmingen). ELISPOT assays were performed as previously described (25).

**Statistics**

The differences in daily mean clinical scores between groups of CXCL13–/– and WT mice were evaluated using the Mann-Whitney Wilcoxon test. The differences in disease incidence and prevalence of chronic relapsing EAE were assessed by Fisher’s exact test. Differences in proliferation, cytokine production, and mean cumulative clinical scores were assessed by Student’s t test.
Results

CXCL13 is expressed in the CNS during acute EAE, and its levels rise progressively during the course of relapsing disease

To determine whether CXCL13 is up-regulated in the CNS during EAE, we removed spinal cords from B10.PL mice following immunization with MBP peptide in CFA and performed RT-PCR. We detected CXCL13 mRNA in every cord from animals with clinical EAE, but not in cords from naive controls or from one MBP-primed mouse that remained asymptomatic (Fig. 1A). CXC5 mRNA was present in the CNS of some symptomatic mice. In synchrony with CXCL13, we detected markers indicative of T cell (CD3δ), DC (CIITA form I), and myeloid cell (lysozyme M) accumulation. Expression of another lymphoid chemokine, CCL19, and its receptor, CCR7, were also up-regulated. We did not detect CXCL13 in the spinal cords of control mice injected with PBS in CFA (data not shown), indicating that induction of CXCL13 in the CNS was not secondary to a nonspecific effect of microbial products contained in the adjuvant.

Next, lymphoid chemokine mRNA expression was measured during the course of relapsing-remitting disease in PLP-sensitized SJL mice by RPA. CXCL13 appeared in the CNS at the first exacerbation, and its levels rose steadily through the remission and relapse stages (Fig. 1B). CXCL13 mRNA was not detectable in cords from naive mice, as confirmed by RT-PCR and Southern blot hybridization (data not shown). CCL19 and CCL21 followed similar kinetics to CXCL13. By contrast, CNS expression of the “inflammatory” chemokines, MIP-1α, MIP-2, and RANTES, were highest during the initial exacerbation, fell during remission, and increased modestly during relapse (data not shown). These results suggest that inflammatory chemokines are dominant during acute EAE, whereas CXCL13 becomes prominent during chronic/relapsing stages of disease.

We questioned whether myelin-specific T cells alone are capable of inducing CXCL13 expression in the CNS. We have previously shown that LN cells from SJL mice immunized with an emulsion of PLP139–151 peptide in IFA (without mycobacteria) transfer EAE following in vitro stimulation with a combination of Ag and rIL-12. The same LN cells are not encephalitogenic if IL-12 is omitted from the culture (23). CXCL13 and CXC5 mRNA were consistently expressed in spinal cords from mice with acute EAE following the injection of PLP/IL-12 reactivated cells (Fig. 1C). By contrast, spinal cords harvested from asymptomatic control mice, injected with cells that had been reactivated with PLP only, failed to express either transcript. The appearance of CXCL13 in the CNS was associated with up-regulation of T cell and DC markers (CD3δ and CIITA form I, respectively).

Western blots were performed on pooled spinal cord tissues from mice with adoptively transferred EAE to determine whether CXCL13 was expressed at the protein level. We readily detected CXCL13 protein in inflamed spinal cords but not in spinal cords from naive controls (Fig. 1D). Similar results were obtained with regard to CCL19. In contrast, CCL21 protein was constitutively expressed in the CNS in the absence of inflammation, possibly reflecting a role of that chemokine in homeostatic lymphocyte trafficking through the CNS. It should be noted that our Western blot assay is not quantitative, because chemokines were initially concentrated from tissue lysate using heparin-Sepharose beads to increase sensitivity (24). Hence, we cannot exclude the possibility that CCL21 protein is up-regulated from baseline levels during EAE. Indeed, this appears to be the case, based on RNA studies described above.

CXCL13 mRNA is detected in CD11c+ spinal cord MNCs
CXCL13 is produced by stromal cells in spleen and LNs (3, 4). It is also secreted by hemopoietic cells, such as monocytes, macrophages, and myeloid DCs during inflammatory responses (13, 26, 27). Furthermore, murine peritoneal macrophages constitutively express CXCL13 (21, 28).
To investigate whether hemopoietic cells are the source of CXCL13 in the CNS during EAE, we isolated spinal cord MNCs and subjected them to RT-PCR analysis. We reproducibly found CXCL13 as well as CCL19 transcripts in spinal cord MNCs from MOG-sensitized C57BL/6 mice (Fig. 2A) and PLP-sensitized SJL mice (data not shown) sacrificed at the peak of the initial exacerbation. At this point, CNS infiltrates are composed of lymphoid and myeloid cells, including a significant subset that expresses the DC marker, CD11c (Fig. 2A). CXCL13 transcripts were elevated in MNC fractions enriched for CD11c+ cells (Fig. 2B), suggesting that DCs might be a significant source of CXCL13 in the CNS during initiation of EAE. By contrast, CXCL13 and CCL19 were not detectable in MNCs isolated from the spinal cords of naive mice (Fig. 2A), which are dominated by CD11b+ cells (resident microglia) and lack a CD11c+ cell population.

**CXCR5+ cells accumulate in EAE infiltrates**

CXCL13 is the ligand for CXCR5, a receptor expressed by B cells, recently activated T cells, and follicular helper CD4+ T cells (6–8). The presence of CXCR5+ cells in EAE infiltrates was confirmed by FACS analyses of spinal cord MNCs. In SJL mice with EAE, the vast majority of CNS infiltrating CXCR5+ cells were CD4+CD3+ T cells expressing an effector memory phenotype (CD62Llow, CD44high, ICOShigh) (Fig. 3A). We also found CXCR5+CD4+ T cells among spinal cord MNCs from MOG-sensitized C57BL/6 mice (see Fig. 6) (data not shown). The relative percentage of CXCR5+ cells within the CD4+ T cell compartment tended to increase during successive stages of relapsing disease in SJL mice (data not shown). Although B cells, which universally express CXCR5, could be found among the CNS-infiltrating cells, they were present in sparse numbers (~2% of cells in the lymphoid gate; data not shown).

Interestingly, we also consistently detected a small population of CD4+CD3+ cells. The majority of these cells expressed IL-7Rα, and a percentage expressed CXCR5 (Fig. 3B). The cell surface phenotype CXCR5+IL-7Rα+CD4+CD3− is suggestive of lymphoid tissue inducer cells, a unique cell subset implicated in the

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** CXCL13 and CCL19 transcripts are expressed in spinal cord MNCs from mice with EAE. A, Spinal cord MNCs were isolated from C57BL/6 mice with MOG35–55-induced EAE (first episode) or naive controls, and used for FACS analysis and RT-PCR. B, Spinal cord MNCs were isolated from C57BL/6 mice with EAE and separated based on the expression of CD11c with magnetic beads. Relative levels of CXCL13 mRNA in unseparated MNCs, CD11c-depleted, and CD11c-enriched fractions were determined by semiquantitative RT-PCR, Southern blot hybridization, and densitometry. Results are representative of two or more experiments.
FIGURE 3. CXCR5+/CD4+ T cells with an effector memory phenotype, and a subset of CD4+/CD3− cells, accumulate in the spinal cords of mice with EAE. Spinal cord MNCs were isolated from PLP139−151/CFA-sensitized SJL mice with EAE (day 19) and analyzed by FACS for the expression of CXCR5 and activation markers on CD4+ T cells (A), and expression of IL-7Ra and CXCR5 on CD4+CD3− cells (B). Lymphoid cells were gated based on light scatter characteristics. These experiments were performed three times with similar results.

EAE is attenuated in CXCL13-deficient mice

To assess the physiological significance of CXCL13 in EAE, we immunized C57BL/6 CXCL13−/− and WT mice with MOG peptide in CFA and monitored their clinical courses. CXCL13−/− mice exhibited a mild, self-limited form of EAE compared with WT mice (Fig. 4A). Clinical scores were generally lower in CXCL13−/− mice during the first episode, but the average day of onset and disease incidence did not differ significantly from WT mice. Differences between the groups were pronounced during later stages of EAE. When subjected to our immunization protocol, C57BL/6 WT mice typically exhibited neurological disability throughout the observation period of 30 days or longer. In pooled experiments, 39 of 52 WT mice showed deficits weeks following clinical onset. By contrast, CXCL13−/− mice experienced an accelerated and often complete recovery, the vast majority exhibiting a monophasic course. In pooled experiments, only 7 of 43 CXCL13−/− mice failed to regain full ambulatory capacities following acute EAE (Fig. 4, A and B).

Histopathological studies corroborated our clinical findings. Spinal cords from WT mice sacrificed during the presenting exacerbation were characterized by demyelination that involved multiple white matter tracts and extended deep into the parenchyma. By contrast, pathologic changes tended to be mild in CXCL13−/− mice and were restricted, in large part, to the subpial regions (Fig. 5A). Inflammatory infiltrates in CXCL13−/− mice contained relatively few macrophages and activated microglia, but there was no apparent difference in CD3+ T cell infiltration in comparison with WT mice (data not shown).

Differences between the groups were even more apparent during later stages of disease (at day 32 postimmunization). Spinal cords from WT mice revealed extensive gliosis and subpial and meningeal fibrosis (Fig. 5B). Collagen deposits (stained blue with Trichrome) were found adjacent to large demyelinated plaques that were heavily infiltrated by macrophages. By contrast, cords from CXCL13−/− mice showed minimal gliosis/fibrosis, and, as in the acute phase, pathological changes were restricted to subpial regions.

Inflammatory CNS infiltrates are diminished in CXCL13−/− mice

To investigate whether CXCL13 deficiency had influenced the recruitment and retention of leukocyte subsets in the CNS, we isolated MNCs from WT and CXCL13−/− spinal cords during early and late stages of EAE and analyzed them by flow cytometry. CXCL13−/− mice consistently yielded fewer MNCs per spinal cord than WT mice (Fig. 6A). During the presenting episode, the cell yield from pooled CXCL13−/− cords was 30–50% lower than from WT cords. The number of CNS MNCs fell dramatically between peak disease and subsequent remission in both groups. However, the decline was more pronounced in CXCL13−/− mice, which yielded 4- to 5-fold fewer spinal cord MNCs than their WT counterparts during remissions.

During acute EAE, there was no major difference in the subset composition of infiltrating cells. In particular, the percentages of CD4+ T cells were comparable in CXCL13−/− and WT infiltrates (Fig. 6B), and these cells uniformly expressed CD44 and ICOS, indicative of an activated state (data not shown). Percentages of myeloid cells (including CD45+CD11b+ macrophages and CD11c+ DCs) and CD4−CD3+ cells (primarily CD8+ T cells) were moderately reduced in CXCL13−/− CNS infiltrates (Figs. 6B and 7A; data not shown).

The percentage of CD4+ T cells among infiltrating leukocytes remained comparable between the groups at remission. Hence, the absolute number of infiltrating CD4+ T cells was 4- to 5-fold lower in CXCL13−/− cords, in direct proportion to the relative reduction in total MNCs (Fig. 6A). In contrast, monocytes/macrophages were disproportionately depleted in spinal cords of CXCL13−/− mice during late stages of disease, such that the absolute number of CD45+CD11b+ macrophages was, on average, 18-fold lower in CXCL13−/− than in WT cords (Fig. 7B). A similar trend was observed with respect to CD11c+ cells (data not shown).

Interestingly, CXCR5 was expressed on similar percentages of CNS-infiltrating cells in CXCL13−/− and WT mice (Fig. 6B). At peak disease, 10–20% of both CD4+ and CD4− T cells were CXCR5+ irrespective of host genotype (data not shown). Furthermore, the cellular composition of CXCR5+ cells did not differ. In both groups, ~50% of CXCR5+ cells were CD3+ T cells, and the other 50% included B cells, NK cells, and a subset of myeloid cells (our unpublished data).

Following active immunization, CXCL13-deficient mice generate myelin-reactive T cells that are capable of initiating CNS inflammation

CXCL13-deficient mice exhibit impaired lymphoid organogenesis, with a paucity of Peyer’s patches, LNs, and disorganized splenic architecture. Although these mice are not grossly immunodeficient, it was important to determine whether they could mount a myelin-specific T cell response sufficient to initiate CNS inflammation. Splenocytes harvested from CXCL13−/− mice immunized with MOG135−155 in CFA mounted significant proliferative, IL-2 and IFN-γ responses upon challenge with peptide in vitro (Fig. 8A). LN cells, although scarce in CXCL13−/− mice, also proliferated vigorously and secreted IFN-γ in an Ag-specific manner (data not shown). Spleens from immunized CXCL13−/− mice generally contained a lower frequency of IL-2- and IFN-γ-producing cells.
(on average 2- to 3-fold) than WT spleens both during acute EAE and chronic disease (Fig. 8A, lower panels). Nevertheless, individual responses varied, with some CXCL13−/− mice showing comparable cytokine production to WT mice.

We next assessed the ability of CXCL13−/− mice to generate encephalitogenic T cells in adoptive transfer experiments. Splenocytes from MOG/CFA-primed CXCL13−/− or WT mice were stimulated in vitro with MOG and IL-12 for 4 days, and then equal numbers of cells were injected into naive WT recipients. Donor cells from both groups induced a severe and persistent form of EAE (Fig. 8B).

MOG-specific CD4+ T cells harvested from the spleens of CXCL13−/− mice during EAE remissions induce a relatively mild form of EAE

Although we readily detected encephalitogenic T cells in spleens of CXCL13−/− mice during the initial episode of EAE, we questioned the extent to which these cells persist during the later stages when clinical disease is quiescent. Splenocytes were harvested from CXCL13−/− or WT mice during EAE remissions and reactivated with Ag and IL-12 ex vivo. Equal numbers of purified CD4+ T cells were then injected into naive syngeneic recipients. Cells from WT mice induced severe EAE, characterized by hindlimb paralysis at peak disease. In contrast, cells harvested from CXCL13−/− mice on the same day postimmunization induced a milder form of EAE wherein all of the recipients were free of deficits by day 17 (Fig. 8C).
FIGURE 6. CNS-infiltrating T cells are diminished in CXCL13-deficient mice during late stages of EAE. C57BL/6 CXCL13<sup>−/−</sup> and WT mice were immunized with MOG<sub>35–55</sub>/CFA. FACS analysis of pooled CNS MNCs was performed during the first episode of EAE (days 15–21), or during remission (day 42). A. The absolute number of infiltrating CD4<sup>+</sup> T cells/cord was calculated by multiplying their percentage within the inflammatory infiltrate (determined using FACS) by the total number of MNCs per cord. Results shown represent the mean of two representative experiments. B. Dot plots were generated using the lymphoid scatter gate. All experiments were performed three or more times with similar results.
CXCL13 derived from the CNS infiltrate were calculated as described in the legend to Fig. 6 and are all viable cells. AMNCs were isolated during the first episode or remission of EAE for FACS organ-specific autoimmune disease. We found that the lymphoid CXCL13 plays a pathogenic role in EAE, a CD4 T cell-mediated model of CNS infiltration. In this paper, we demonstrate that the lymphoid chemokine CXCL13 is up-regulated in the inflamed CNS of mice with EAE, reaching their height during the relapsing or progressive stages (Fig. 1). These results are corroborated by recent reports of lymphoid chemokine expression in the CNS during EAE from several independent laboratories (30–32). CXCL13 has also been detected in CNS tissues from individuals in the secondary progressive stage of MS (33, 34). As shown in Fig. 4, CXCL13−/− mice experienced a milder initial episode of EAE than their WT counterparts. Furthermore, the clinical courses of the two groups diverged dramatically during subsequent stages in pathogenesis. Spinal cords harvested from CXCL13−/− mice revealed diminished inflammation and demyelination compared with cords from chemokine-sufficient mice, and a reduction in the extent of gliosis and fibrosis (Fig. 5). Collectively, these data suggest an association between CNS CXCL13 and neuroinflammation.

A critical question raised by our study concerns the mechanism and site of action whereby CXCL13 exerts its effects. CXCL13−/− mice have rudimentary LNs and disorganized splenic microarchitecture (1). Hence, CXCL13 deficiency could potentially impair the priming and expansion of naive autoreactive T cells in peripheral lymphoid tissues. Nevertheless, we found that these mice are capable of mounting significant Ag-specific responses to myelin peptides (Fig. 8A). The magnitude of their responses was generally lower than those of WT mice. This could be responsible for the reduced severity of the initial EAE episode in CXCL13−/− animals as well as the slight decrease in encephalitogenicity exhibited by MOG-specific splenocytes primed in CXCL13−/− donors (Figs. 4A and 8B). However, suboptimal T cell priming during the preclinical phase of EAE is unlikely to fully account for the milder disease course in CXCL13−/− mice. First, analysis of the clinical courses of individual WT mice reveals no direct correlation between the severity of the first exacerbation and degree of disability during later stages (our unpublished observations). Moreover, those outlier CXCL13−/− mice that underwent relatively severe first episodes nevertheless typically experienced a full recovery. In contrast, MOG-stimulated T cells that were purified from CXCL13−/− spleens during EAE remissions, as opposed to earlier stages, were less potent than their WT counterparts in inducing EAE (Fig. 8C). This observation raises the possibility that CXCL13 is important for the generation and/or long-term survival of MOG-specific memory T cells. In ongoing experiments, we are investigating the role of CXCL13 in the induction of autoreactive memory cells during EAE.

Our data also indicate that CXCL13 plays a distinct role during the effector phase of EAE. In adoptive transfer models, CXCL13−/− hosts experience a milder form of EAE than WT hosts (Fig. 4C). They generally recover complete or near-complete ambulatory capacity within a short time period, whereas WT recipients exhibit persistent deficits. Thus, CXCL13 deficiency attenuates acute EAE at a point past the activation and differentiation of encephalitogenic T cells. Similarly, adoptively transferred EAE was suppressed in immunocompetent B10.PL mice by the administration of neutralizing Abs against CXCL13 (Fig. 4D). Collectively, these results suggest that the ameliorative effect of CXCL13 deficiency on EAE involves inhibition of effector cell migration and/or function, and is not solely attributable to a paucity or disorganization of peripheral lymphoid tissues.

CXCL13 could potentially mediate the recruitment or retention of CXCR5+ leukocytes in the CNS. Although the absolute number of infiltrating MNCs per spinal cord was lower in CXCL13−/− mice, the percentage of CXCR5+ cells and their subset composition did not differ substantially from WT mice (Fig. 6). Therefore, our findings do not suggest that the primary role of CXCL13 in

### Adoptively transferred EAE is attenuated in CXCL13−/− mice

Based on our observations that CXCL13 is up-regulated in the CNS during EAE, we speculated that it might play a distinct role during the effector stage of pathogenesis. Consequently, we compared clinical EAE in CXCL13-deficient and WT mice following the adoptive transfer of MOG-specific CD4 T cells from WT donors (Fig. 4C). WT recipients injected with 5 × 10^6 CD4+ T cells quickly progressed to severe EAE: 60% of mice died, and the surviving mice suffered conspicuous chronic deficits. By contrast, CXCL13−/− recipients underwent a significantly milder course of EAE with delayed onset, reduced mortality (28%), and accelerated recovery. The majority of mice enjoyed complete or near-complete recovery. Similar results were obtained in two additional experiments using LN cells from primed WT donors (data not shown).

In parallel experiments, we assessed the effects of CXCL13 neutralization on the course of adoptively transferred EAE in B10.PL mice. Recipient mice were treated with either an anti-mouse CXCL13 mAb or isotype-matched control mAb on days 3, 6, and 10 following the transfer of MBP-TCR transgenic cells. Anti-CXCL13 treatment significantly ameliorated clinical disease (Fig. 4D). A similar result was obtained with a neutralizing polyclonal goat anti-CXCL13 Ab following adoptive transfer of PLP/IL-12-stimulated cells in SJL mice (data not shown).

### Discussion

In this paper, we demonstrate that the lymphoid chemokine CXCL13 plays a pathogenic role in EAE, a CD4+ T cell-mediated organ-specific autoimmune disease. We found that the lymphoid chemokines, CXCL13, CCL21, and CCL19, are up-regulated in the inflamed CNS of mice with EAE, reaching their height during the relapsing or progressive stages (Fig. 1). These results are corroborated by recent reports of lymphoid chemokine expression in the CNS during EAE from several independent laboratories (30–32). CXCL13 has also been detected in CNS tissues from individuals in the secondary progressive stage of MS (33, 34). As shown in Fig. 4, CXCL13−/− mice experienced a milder initial episode of EAE than their WT counterparts. Furthermore, the clinical courses of the two groups diverged dramatically during subsequent stages in pathogenesis. Spinal cords harvested from CXCL13−/− mice revealed diminished inflammation and demyelination compared with cords from chemokine-sufficient mice, and a reduction in the extent of gliosis and fibrosis (Fig. 5). Collectively, these data suggest an association between CNS CXCL13 and neuroinflammation.

A critical question raised by our study concerns the mechanism and site of action whereby CXCL13 exerts its effects. CXCL13−/− mice have rudimentary LNs and disorganized splenic microarchitecture (1). Hence, CXCL13 deficiency could potentially impair the priming and expansion of naive autoreactive T cells in peripheral lymphoid tissues. Nevertheless, we found that these mice are capable of mounting significant Ag-specific responses to myelin peptides (Fig. 8A). The magnitude of their responses was generally lower than those of WT mice. This could be responsible for the reduced severity of the initial EAE episode in CXCL13−/− animals as well as the slight decrease in encephalitogenicity exhibited by MOG-specific splenocytes primed in CXCL13−/− donors (Figs. 4A and 8B). However, suboptimal T cell priming during the preclinical phase of EAE is unlikely to fully account for the milder disease course in CXCL13−/− mice. First, analysis of the clinical courses of individual WT mice reveals no direct correlation between the severity of the first exacerbation and degree of disability during later stages (our unpublished observations). Moreover, those outlier CXCL13−/− mice that underwent relatively severe first episodes nevertheless typically experienced a full recovery. In contrast, MOG-stimulated T cells that were purified from CXCL13−/− spleens during EAE remissions, as opposed to earlier stages, were less potent than their WT counterparts in inducing EAE (Fig. 8C). This observation raises the possibility that CXCL13 is important for the generation and/or long-term survival of MOG-specific memory T cells. In ongoing experiments, we are investigating the role of CXCL13 in the induction of autoreactive memory cells during EAE.

Our data also indicate that CXCL13 plays a distinct role during the effector phase of EAE. In adoptive transfer models, CXCL13−/− hosts experience a milder form of EAE than WT hosts (Fig. 4C). They generally recover complete or near-complete ambulatory capacity within a short time period, whereas WT recipients exhibit persistent deficits. Thus, CXCL13 deficiency attenuates acute EAE at a point past the activation and differentiation of encephalitogenic T cells. Similarly, adoptively transferred EAE was suppressed in immunocompetent B10.PL mice by the administration of neutralizing Abs against CXCL13 (Fig. 4D). Collectively, these results suggest that the ameliorative effect of CXCL13 deficiency on EAE involves inhibition of effector cell migration and/or function, and is not solely attributable to a paucity or disorganization of peripheral lymphoid tissues.
EAE is to attract CXCR5+ cells from the periphery to the CNS. Alternatively, CXCL13 might coordinate the colocalization of encephalitogenic CXCR5+/CD4+ T cells and APCs within the CNS itself. In lymphoid organs, CXCL13 plays a critical role in the positioning of lymphocyte subsets and DCs within B and T cell compartments. We propose that it might play an analogous role during neuroinflammation. Our data implicate CD11c+ myeloid cells as at least one cellular source of CXCL13 in inflamed spinal cords during initiation of EAE (Fig. 2). There is growing data that CD11c+ cells act as highly efficient APCs in the CNS during EAE (Ref. 35 and our unpublished observations). The majority of CXCR5+ cells in CNS infiltrates are CD3+ T cells (Fig. 3). By secreting CXCL13, CNS CD11c+ cells could drive autoreactive T cells to migrate into their vicinity, thereby greatly increasing the likelihood of cognate interaction. A recent study underscored the importance of T cell reactivation within the CNS for the recruitment of macrophages and clinical manifestation of EAE (25). It is possible that, in CXCL13−/− mice, CNS APCs fail to reactivate myelin-specific T cells at a sufficient rate to sustain the accumulation and activation of myeloid cells within EAE lesions, resulting in a milder, self-limited disease (Fig. 7).

We considered the possibility that CXCL13 could affect non-T cell CXCR5+ subsets within EAE infiltrates. In particular, we found IL-7Rα+CD4+CD3+ cells among CNS-infiltrating CXCR5+ cells. This phenotype is suggestive of lymphoid inducer cells that play an important role in the development of secondary lymphoid organs during embryogenesis (2, 29). These cells might also be involved in lymphoid neogenesis during chronic inflammation. CXCL13 could also affect B cells in the CNS infiltrates. However, it should be noted that, in our EAE models, B cells constituted a minor subset of the inflammatory infiltrate. B cells comprised 2% or less of spinal cord MNCs, with no significant differences between CXCL13−/− and WT mice. We found no difference in the percent of B cells among splenocytes of MOG-immunized CXCL13−/− and WT mice (data not shown). Furthermore, we detected activation-induced cytidine deaminase and γ3 circle transcripts at a similar frequency in spinal cords of CXCL13−/− and WT mice with acute EAE (data not shown). Activation-induced cytidine deaminase is an enzyme that is necessary for Ig somatic hypermutation and isotype switch recombination; circle transcripts are a by-product of isotype switch recombination. Hence, our findings reflect an ongoing Ab response in myelin-primed CXCL13−/−, as well as WT, mice. Other investigators have identified lymphoid follicle-like structures containing B cells within the meninges of murine models of chronic EAE (32) and in CNS specimens from patients with secondary progressive MS (33). Cognate T-B cell interactions are known to occur in the CNS of patients with MS, leading to Ab production in situ (34, 36).
Although we did not find any evidence that T-B cell interactions were inhibited in the CNS of CXCL13−/− mice, we cannot exclude the possibility that CXCL13 plays a role in the development of Ab responses against myelin Ags, with pathogenic consequences, in some forms or stages of autoimmune demyelinating disease.

In conclusion, the current study is the first to directly demonstrate a pathogenic role for CXCL13 in EAE. CXCL13 appears to participate in the effector stage of disease, being important for the establishment and maintenance of inflammatory infiltrates and glial/fibrotic lesions in the CNS. Our data suggest that agents that neutralize CXCL13 or block its receptor might be useful in the treatment of human autoimmune conditions, such as MS.

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Disclosures

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References