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IFN-Inducible Protein 10/CXC Chemokine Ligand 10-Independent Induction of Experimental Autoimmune Encephalomyelitis[1]

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In multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), autoaggressive T cells traffic into the CNS and induce disease. Infiltration of these pathogenic T cells into the CNS has been correlated with the expression of the chemokine IFN-inducible protein (IP)10/CXC chemokine ligand (CXCL)10, a chemoattractant for activated T cells, and its receptor CXCR3, in the CNS of both MS patients and mice with EAE. In the present study, we report that targeted deletion of IP-10 did not diminish the expression, severity, or histopathology of EAE induced by active immunization with 100 μg of myelin oligodendrocyte glycoprotein peptide (MOG)p35–55. However, we found that IP-10-deficient mice had a lower threshold for expression of disease compared with wild-type littermates. EAE induced by immunization with 5 μg of MOGp35–55 resulted in more severe disease characterized by a greater number of CNS lesions and infiltrating mononuclear cells in IP-10-deficient mice compared with wild-type controls. IP-10-deficient mice immunized with MOGp35–55 demonstrated increased levels of IFN-inducible T cell α-chemokine/CXCL11 mRNA in the CNS and decreased levels of monokine induced by IFN-γ/CXCL9 mRNA in draining lymph nodes, suggesting differential compensation for loss of IP-10 in lymphoid vs parenchymal tissue compartments. EAE in IP-10-deficient mice induced by low-dose immunization was associated with enhanced Ag-specific Th1 responses in the draining lymph node, which corresponded with diminished lymph node TGF-β1 expression. Our data demonstrated that IP-10 was not required for the trafficking of pathogenic T cells into the CNS in EAE but played an unexpected role in determining the threshold of disease susceptibility in the periphery. ***The Journal of Immunology*****, 2004, 172: 550–559.**

Multiple sclerosis (MS)[4] is a chronic, demyelinating disorder of the CNS that is thought to result from an autoimmune attack of the myelin sheath. Autoreactive T cells that enter the CNS are believed to be important initiators of the disease, controlling subsequent recruitment and activation of effector cells, such as macrophages. The molecular mechanisms controlling the trafficking of autoreactive T cells into the CNS is not known and of considerable interest. Experimental autoimmune encephalomyelitis (EAE), a demyelinating inflammatory disease of the murine CNS, shares both neuropathological and clinical features with MS (1, 2). EAE is characterized by demyelinating lesions containing myelin-specific and nonspecific $CD4^+$ and $CD8^+$ T cells and macrophages (3–5). EAE expression requires effective T cell activation and extravasation into the CNS. Thus, inhibition of molecules that affect these processes, such as very late Ag 4 and CD44, result in loss of the ability to induce EAE (6–10). Importantly, a recent placebo-controlled trial revealed that inhibition of very late Ag 4 led to fewer new inflammatory brain lesions and fewer relapses over a 6-mo period in patients with relapsing MS (6).

Chemokines, a superfamily of small, chemotactic cytokines that mediate leukocyte recruitment into tissues in homeostasis and inflammation (11), have become a focus of investigation into the mechanism of CNS autoimmunity. These chemoattractants are cell-type selective and expressed in unique compartments within tissues resulting in the fine control of leukocyte subset homing. Although studies of EAE in mice with targeted gene deletions for monocyte chemoattractant protein (MCP)-1/CC chemokine ligand (CCL)2 and its receptor CCR2 have suggested a prominent role for this chemokine-chemokine receptor system in the recruitment of monocytes during disease initiation (12–14), the question of signals responsible for lymphocyte recruitment remains to be established.

Although little is known regarding chemokine-mediated T cell recruitment in CNS autoimmune diseases, IFN-inducible protein (IP)-10, monokine induced by IFN-γ (Mig)/CXC chemokine ligand (CXCL)9, and IFN-inducible T cell α-chemoattractant (I-TAC)/CXCL11 are attractive candidates to mediate this process. IP-10, Mig, and I-TAC are expressed within CNS lesions in both EAE and MS, and the receptor that binds these ligands, CXCR3, is expressed on T cells infiltrating EAE and MS lesions as well as on T cells in the cerebrospinal fluid and periphery of MS patients during exacerbations (15–25). Furthermore, IP-10 is expressed at high levels in the CNS of patients with viral meningitis and levels

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[4] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; IP-10, IFN-inducible protein 10; CXCL, CXC chemokine ligand; Mig, monokine induced by IFN-γ; CCL, CC chemokine ligand; I-TAC, IFN-inducible T cell α-chemoattractant; LNC, lymph node cell; MCP, monocyte chemoattractant protein; MBP, myelin basic protein; MMDS, mean maximal disease severity; MOG, myelin oligodendrocyte glycoprotein; PTX, pertussis toxin.

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correlate with the numbers of CXCR3-positive T cells found in the CNS (26), again suggestive of a functional relationship.

Recent studies using inhibitory Abs and gene-targeted mice have suggested a role for IP-10 and CXCR3 in the trafficking of activated T cells into tissue, including the CNS, in certain animal models (27–32). However, the overall effect of loss of IP-10 on CNS inflammation has been contradictory. In a model of viral-induced encephalomyelitis, mice treated with Abs to IP-10, as well as IP-10-deficient mice, had a delay in CNS viral clearance that was associated with decreased numbers of infiltrating $CD4^+$ and $CD8^+$ T cells (28, 32). These mice were also spared the demyelination that normally follows acute infection of wild-type or control Ab-treated mice (32, 33). Furthermore, treatment of mice with Abs against IP-10 or vaccination of mice with naked IP-10 DNA, resulting in anti-IP-10 Abs, have both been reported to inhibit the induction of EAE (29, 34). In contrast to these studies, treatment of rats with anti-IP-10 mAbs was found to exacerbate the induction of EAE in an active immunization model via decreased retention of encephalitogenic lymphocytes in draining lymph nodes (35). Given these conflicting results, we sought to determine whether targeted deletion of IP-10 would affect T cell recruitment and influence the induction or development of EAE.

In this report, we describe the expression of EAE in IP-10-deficient mice. These mice not only showed unaltered expression of EAE after immunization with a standard dose of myelin oligodendrocyte glycoprotein peptide (MOG)p35–55 (100 μg), but also demonstrated an enhanced susceptibility to EAE, developing marked disease after immunization with doses of MOGp35–55 (5 μg) that produced limited disease in wild-type littermates. Induction of EAE in IP-10-deficient mice was associated with up-regulation of I-TAC in the CNS and decreased expression of Mig in draining lymph nodes, suggesting that loss of IP-10 results in dysregulation of CXCR3 ligand expression. Th1-type responses of lymph node cells (LNCs) from IP-$10^{-/-}$ mice were enhanced at the lower immunizing dose, compared with responses of IP-$10^{+/+}$ mice. This was associated with a dose-dependent decrease in lymph node TGF-β1 levels, suggesting that IP-10 may play a role in the delivery or expression of T cell regulatory activity in the lymph node.

Materials and Methods

Animals and Abs

All mice were bred and maintained in identical specific pathogen-free conditions (Massachusetts General Hospital Center for Comparative Research, Charlestown, MA). IP-$10^{-/-}$ and littermate IP-$10^{+/+}$ control mice (129sv; C57BL/6) were generated as previously described (32). The 129sv;C57BL/6 IP-$10^{-/-}$ mice were backcrossed for seven generations into C57BL/6 IP-$10^{-/-}$ mice to place the IP-$10^{-/-}$ allele into the C57BL/6 background. These C57BL/6 IP-$10^{-/-}$ mice were used in experiments examining the effect of low-dose MOGp35–55 immunization on the induction of EAE. C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA) were used as controls compared with IP-$10^{+/+}$ animals only in Ab experiments. For Ab experiments, monoclonal anti-IP-10 Abs were generated as previously described (27) and isotype control Syrian Hamster gamma-globulin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescent-conjugated Abs against CD3, CD4, CD8, CD11b, and CD45 (FITC, PE, or allophycocyanin) were purchased from BD PharMingen (San Diego, CA).

EAE induction

Active EAE was induced in 6- to 8-wk-old female IP-$10^{+/+}$, IP-$10^{-/-}$, C57BL/6 or SJL/PL F_1 (The Jackson Laboratory, Bar Harbor, ME) mice by s.c. immunization with myelin peptides MOGp35–55 (100 μg, 20 μg, 5 μg, or 0.5 μc) or myelin basic protein (MBP) peptide Ac-1-11 (50 μg) in PBS emulsified 1:1 with CFA (Difco, Detroit, MI) for a total 100 μl immunizing dose, followed by i.v. injection of 150 ng of pertussis toxin (PTX; Sigma-Aldrich, St. Louis, MO) on days 0 and 2 postimmunization, as previously described (13). For Ab treatment experiments, anti-IP-10 mAbs or Ig isotype control Abs (100 μg/mouse) were injected i.p. on days 0, 2, 4, 6, 8, 10, and 12 postimmunization. Mice from all experiments were graded for clinical manifestations of EAE by the following criteria: 1) tail weakness; 2) difficulty righting; 3) hind limb paralysis; 4) forelimb weakness or paralysis; and 5) moribund or dead.

CNS histology

Brains and spinal cords were collected on days 9, 15, 21, and 28 postimmunization, fixed in 10% formaldehyde in PBS, embedded in paraffin, sectioned longitudinally 60 μm apart for two sections, and stained with H&E. Sections were evaluated and inflammatory lesions were quantitated by a blinded neuropathologist (R.A.S.).

Flow cytometry

CNS tissue from mice with EAE was dissected on day 14 postimmunization and dispersed into single cell suspension in RPMI 1640 with 10% FCS. Cells were washed in RPMI 1640 and viable cells were separated by FCS gradient then Percoll gradient (70/30%) centrifugation. Cells were placed in blocking solution containing 10% normal mouse serum and stained with fluorescently conjugated Abs for CD3, CD4, CD8, CD11b, and CD45 (10 μg/ml) in PBS/1% FCS. For the detection of dead cells, the intercalating DNA-dye 7-amino actinomycin D was used (BD Biosciences, San Jose, CA). Stained cells were fixed with 2% paraformaldehyde in PBS at 4°C. Data collection and analysis were conducted using a FACSCaliber flow cytometer with CellQuest software (BD Biosciences). For fluorescence analysis, a bivariate dot plot of forward vs side scatter was generated, and separate regions for lymphocytes and monocytes were delineated. Fluorescent analysis displays then were generated by gating through either lymphocytes or monocyte/macrophages.

RNase protection assays and Northern blot analysis

Brains from mice immunized with 5 and 100 μg doses of MOGp35–55 were collected on days 9, 15, 21, and 28 postimmunization and snap frozen in liquid nitrogen. Total RNA was prepared via standard guanidine isothiocyanate $CsCl_2$ gradient centrifugation. A total of 3×10^5 cpm/μl [α-^{32}P]UTP (3000 Ci/mmol, NEN, Boston, MA) labeled-cytokine, -chemokine, and -chemokine receptor and GAPDH RNA probes were hybridized to total RNA (5 μg) according to the manufacturer's instructions (BD PharMingen). Samples were digested with RNase A and RNase Ti (BD PharMingen) and electrophoresed through a 5% acrylamide urea-containing gel, which was dried and exposed to film (Eastman Kodak, Rochester, NY) for 16–170 h. For Northern blot analyses, total brain RNA (10 μg) prepared from similarly immunized animals at the same time points was fractionated by 1.2% agarose formaldehyde gel electrophoresis and transferred to a GeneScreen membrane (NEN). Membranes were prehybridized and then hybridized with [α-^{32}P]dCTP-labeled cDNA probes generated using the DECAprime II kit (Ambion, Austin, TX), with 2×10^7 cpm/μl [α-^{32}P]dCTP (NEN). The following probes were used: a 480-bp fragment of RANTES cDNA (provided by G. Dranoff, Harvard Medical School, Boston, MA), a 1-kb fragment of CXCR3 cDNA, a 1.4-kb full-length fragment of the murine Mig cDNA (provided by J. Farber, National Institutes of Health, Bethesda, MD), and a 829-bp fragment of the murine I-TAC cDNA (provided by G. Werner-Felmayer, University of Innsbruck, Innsbruck, Austria). GAPDH (provided by M. Prystowsky, Albert Einstein College of Medicine, Bronx, NY) was used as a control for RNA loading. Signal quantitation was determined using IPLab image analysis software.

LNC numbers, cytokine production, and proliferation

Draining lymph nodes were collected from IP-$10^{-/-}$ and IP-$10^{+/-}$ and IP-$10^{+/+}$ mice on days 9 and 14 postimmunization with MOGp35–55. Single cell suspensions of LNCs were prepared and quantitated on a standard hemocytometer. Day 9 LNCs were cultured at 4×10^6 cells/ml in serum-free X-vivo 20 medium (BioWhittaker, Walkersville, MD) with 100 μg of MOGp35–55. Supernatants were collected at 24 h for IL-2 and IL-4 levels, and 48 h for IFN-γ and IL-10. Cytokine levels were measured by quantitative ELISA (Promega, Madison, WI) as previously described (13). MOGp35–55-specific proliferation of LNCs was determined after 96 h as previously described (13).

Quantitative RT-PCR

CNS tissues and popliteal lymph nodes were collected from 129/Bl6 IP-$10^{-/-}$ and IP-$10^{+/+}$ mice on day 14 postimmunization with 100 and 5 μg MOGp35–55, snap frozen in liquid nitrogen and used to prepare total RNA via Qiagen RNA extraction kit (Valencia, CA). Total RNA was treated with DNase I (Amp-Grade; Life Technologies, Grand Island, NY) at room temperature for 10 min followed by heat-inactivation in the presence of EDTA and quantitated via RiboGreen (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. For quantitative RT-PCR, 2

μg of total RNA from each sample was used as template for the reverse transcription reaction. A total of 100 μl of cDNA was synthesized using oligo(dT)$_{15}$, random hexamers, and multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). All samples were reverse transcribed under the same conditions and from the same reverse transcription master mix to minimize differences in reverse transcription efficiency. All oligonucleotide primers for quantitative RT-PCR were synthesized by Invitrogen (San Diego, CA). The following primers (5′-3′) were used: 1) GAPDH, GGCAAATTCAACGGCACAGT (f) and AGATGGTGATG GGCTTCCC (r); 2) I-TAC, GCCCTGGCTGCGATCAT (f) and CAG CGCCCCTGTTTGAAC (r); 3) Mig, AATGCACGATGCTCCTGCA (f) and AGGTCTTTGCGGGATTTGTAGTGG (r); 4) CXCR3, TGCTGTGC TACTGAGTCAGCG (f) and CTACAGCCAGGTGGAGCAGG (r); 5) TGF-β1, AAACGGAAGCGCATCGAA (f) and GGGACTGGCGAGC CTTAGTT (r); 6) IL-10, TTTGAATTCCCTGGGTGAGAA (f) and GGAG AAATCGATGACAGCGC (r); 7) KC, GTGTTGCCCTCAGGGCC (f) and GCCTCGCGACCATTCTTG (r); and 8) ENA-78 (LIX), CTCGC CATTCATGCGGAT (f) and CTTCAGCTAGATGCTGCGGC (r). Oligonucleotide primers were designed using Primer Express software 1.0 (Perkin-Elmer Applied Biosystems, Foster City, CA). The PCR was performed in 30 μl with 4 μl of cDNA, 15 μl of 2× SYBR Green master mix (Applied Biosystems), and 60 pmol of each of the appropriate primers. The quantitative RT-PCR was performed in optical 96-well strips with optical caps (Stratagene, La Jolla, CA) using the MX4000 Multiplex quantitative RT-PCR system (Stratagene). The same thermal profile conditions were used for all primer sets. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min then 40 cycles of 95°C for 15 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured three times during the annealing/extension phase, and amplification plots were analyzed using the MX4000 software version 2.0 (Stratagene). A series of standards was prepared by performing 10-fold serial dilutions of full-length cDNAs in the range of 20 million copies to two copies per PCR. Subsequent analysis was performed on the data output from the MX4000 software using Microsoft Excel. Quantity values generated for gene expression were generated by comparison of the fluorescence generated by each sample with standard curves of known quantities and were divided by the quantity of total RNA present in each quantitative RT-PCR.

Results

Loss of IP-10 did not affect induction of EAE via active immunization

To examine the requirement for IP-10 in the initiation and progression of EAE, groups of IP-10$^{-/-}$ and IP-10$^{+/+}$ littermate mice were immunized with a standard dose (100 μg) of the encephalitogenic peptide MOGp35–55 and scored for signs of disease. As these mice were generated via crossing 129sv;C57BL/6 parents heterozygous at the IP-10 locus, they express the H-2^{b} MHC haplotype and are thus susceptible to MOGp35–55-induced EAE. Wild-type offspring and their IP-10-deficient littermates developed clinical EAE with mode incidences of 80% and 100%, respectively. The mean maximal disease severity (MMDS) was consistently higher in the IP-10-deficient animals, although this difference was not statistically significant. Of a total of 61 animals immunized, 33/33 IP-10$^{+/+}$ and 29/31 IP-10$^{-/-}$ developed EAE, with IP-10-deficient animals developing slightly more severe symptoms (MMDS, 2.9 for IP-10$^{-/-}$ vs 2.5 for IP-10$^{+/+}$). A representative experiment is shown in Fig. 1. Thus, IP-10-deficient mice were able to develop EAE in an active disease model.

Administration of anti-IP-10 mAbs did not affect induction of EAE

Other investigators have found that anti-IP-10 Ab treatment before adoptive transfer of encephalitogenic T cells significantly decreased the severity of clinical disease compared with control normal rabbit serum control Abs (29). We tested the effect of anti-IP-10 neutralizing mAb (clone 1F11), which was effectively used in other models of lymphocyte mediated inflammatory disease (27, 29–31), on the development and progression of EAE in 129sv; C57BL/6 IP-10$^{+/+}$ and C57BL/6 mice immunized with MOGp35–55 as well as in SJL/PL F$_1$ mice immunized with MBP-Ac1-11 (Fig. 2). In all experiments, animals received 100 μg of

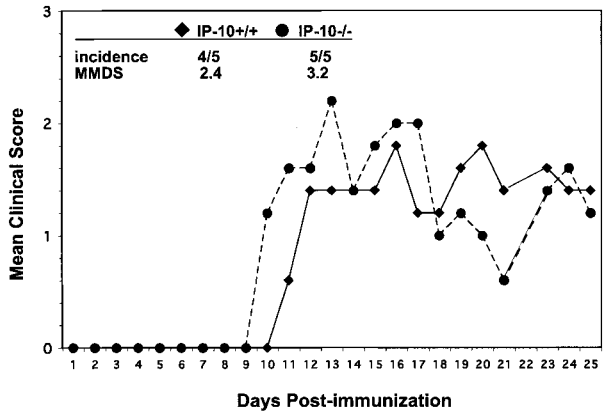

FIGURE 1. Incidence and severity of disease in IP-10$^{-/-}$ (●) and IP-10$^{+/+}$ (◆) 129S;C57BL/6 littermates immunized with 100 μg of MOGp35–55. Groups of 5–10 mice were immunized with MOGp35–55 in CFA and received two i.v. injections of PTX (150 ng/injection). Results are expressed as mean disease score. This experiment is representative of $n = 4$, with five to six animals per group per experiment.

anti-IP-10 mAb on days 0, 2, 4, 6, 8, 10, and 12 postimmunization with MOGp35–55. All mice immunized with MOGp35–55 developed EAE regardless of pretreatments with either anti-IP-10 mAbs or control IgG (Fig. 2). Differences in MMDS between anti-IP-10 and control IgG-treated groups were not significant. SJL/PL F$_1$ mice treated with anti-IP-10 mAbs or control IgG and immunized with MBP-Ac1-11 developed EAE with MMDS's of 4.0 and 2.8 for each group, respectively. This difference was also not significant. These results are consistent with those obtained using IP-10-deficient mice and demonstrated that neutralization of IP-10 did not affect the development of EAE induced by standard immunizations in active disease models that use mice of various strains.

IP-10-deficient mice were more susceptible to EAE

We had noted that in many of our experiments comparing the development and progression of EAE in IP-10$^{-/-}$ and IP-10$^{+/+}$ mice, incidences and MMDS's were consistently slightly higher in the IP-10-deficient group. We hypothesized that loss of IP-10 might actually increase the susceptibility of mice to EAE and tested this by immunizing groups of mice with lower doses of MOGp35–55. We found that immunization of animals with 20 and 5 μg of MOGp35–55 induced enhanced disease in IP-10$^{-/-}$ mice compared with similarly immunized IP-10$^{+/+}$ mice (Fig. 3, *A* and *B*). Indeed, the incidences and MMDS's of IP-10$^{-/-}$ mice immunized with 5 μg of MOGp35–55 were comparable to both IP-10$^{+/+}$ and IP-10$^{-/-}$ mice immunized with standard 100 μg dose of MOGp35–55 (Figs. 1 and 3, *A* and *B*). Differences in MMDS were statistically significant for IP-10$^{+/+}$ and IP-10$^{-/-}$ mice immunized with 5 μg MOGp35–55 ($p < 0.05$). Immunization of mice with 0.5 μg of MOGp35–55 did not induce signs of EAE in IP-10$^{+/+}$ mice, whereas it produced tail weakness in three of five IP-10$^{-/-}$ mice (data not shown). Low-dose immunization of IP-10$^{-/-}$ mice backcrossed seven generations into the C57BL/6 strain also induced higher levels of disease incidence and MMDS (Fig. 3*C*). These data suggest that loss of IP-10 may affect the susceptibility to induction of EAE via active immunization.

Histopathology of IP-10-independent EAE

To examine the particular basis of our in vivo findings, we analyzed CNS tissues from IP-10$^{-/-}$ and IP-10$^{+/+}$ mice immunized with both standard (100 μg) and low (5 μg) doses of MOGp35–55

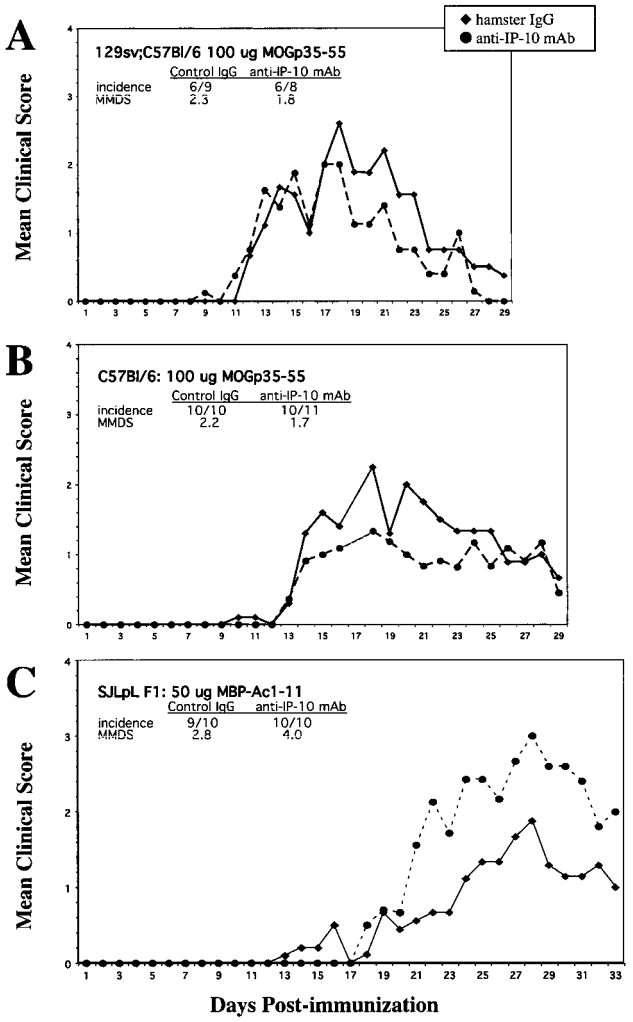

FIGURE 2. Anti-IP-10 mAbs do not affect the incidence and severity of EAE induced with 100 μg of MOGp35–55. *A*, 129S;C57BL/6 (IP-$10^{+/+}$ littermates), C57BL/6 (*B*), and SJLpl F_1 (*C*) mice were monitored for the development of clinical disease following EAE induction with MOGp35–55. Mice were treated i.p. with 100 μg of anti-IP-10 mAb (●) or normal hamster IgG (◆) on days 0, 2, 4, 6, 8, 10, 12, and 14 after immunization with 100 μg of MOGp35–55 in CFA plus PTX (150 ng/injection). Results are expressed as mean disease score; *p* values derived from Student's *t* test. Data are representative of one to two experiments per strain, with 5–10 animals per group per experiment.

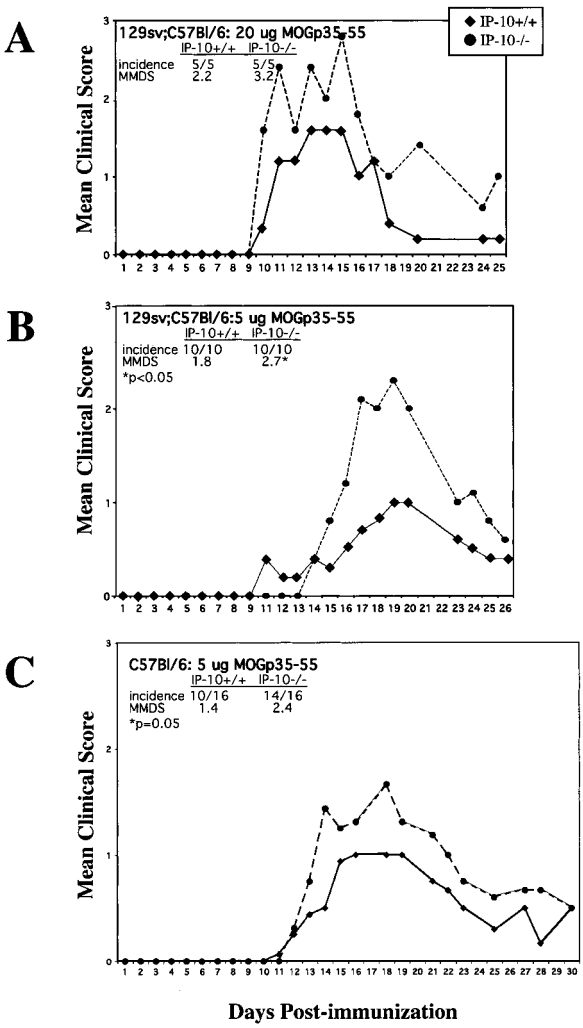

FIGURE 3. IP-10-deficient mice are more susceptible to MOGp35–55-induced EAE. IP-$10^{-/-}$ (●) and IP-$10^{+/+}$ (◆) littermates in the 1295; C57BL/6 (*A* and *B*) and C57BL/6 (*C*) backgrounds were immunized with 20 μg (*A*) or 5 μg (*B* and *C*) of MOGp35–55 in CFA plus PTX (150 ng/injection). Results are expressed as mean disease score. These experiments are representative of one to two experiments per strain for 5–10 animals per group per experiment.

(Fig. 4). Histological examination of CNS tissues from mice immunized with standard dose MOGp35–55 ($n = 4$ mice per group) demonstrated numerous meningeal and CNS parenchymal inflammatory/demyelinating lesions in all mice with clinical EAE examined (Fig. 4, *A*, *B*, and *G*). These lesions consisted mainly of lymphocytic and monocytic infiltrates with few granulocytes (Fig. 4, *A* and *B*). The numbers of lesions were similar in both groups on day 15 after immunization with 100 μg of MOGp35–55 (Fig. 4*G*), which represents the time of peak disease severity. Histological examination of CNS tissue from mice immunized with 5 μg dose MOGp35–55 ($n = 6$ mice per group) revealed that CNS tissues of IP-$10^{-/-}$ mice in this low-dose group had numerous lesions the numbers of which peaked on day 15 postimmunization, correlating with the time of peak severity of disease in this group (Fig. 4, *C*, *E*, and *H*). At most time points examined, these lesions also consisted mostly of mononuclear cells. However, IP-10-deficient animals examined on day 9 postimmunization with the 5 μg dose MOGp35–55 had only meningeal lesions that contained numerous neutrophils in addition to mononuclear cells (Fig. 4*E*, inset). The lack of parenchymal/demyelinating lesions in these animals was consistent with the lack of clinical findings at the day 9 time point (Fig. 3*B*). The appearance of neutrophilic meningitis at day 9 postimmunization is not characteristic of EAE generated via immunization with MOGp35–55 at any dose, both for the early appearance of lesions in the course of the disease and for the presence of granulocytes. Analysis of the mRNA expression of murine neutrophil chemoattractants KC/CXCL1, MIP-2/CXCL2, and ENA 78 (LIX)/CXCL5 via quantitative RT-PCR of CNS tissues obtained at days 7 or 9 postimmunization did not reveal significant differences between IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with low-dose MOGp35–55 (data not shown). In addition, IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with 5 μg of MOGp35–55 demonstrated similar up-regulation of CNS MIP-1α/CCL3 mRNA that peaked on day 15 postimmunization (data not shown), as reported (13).

Consistent with the clinical findings, CNS tissues of IP-$10^{+/+}$ mice immunized with 5 μg of MOGp35–55 had very few lesions, correlating with the attenuated disease observed in this group (Fig.

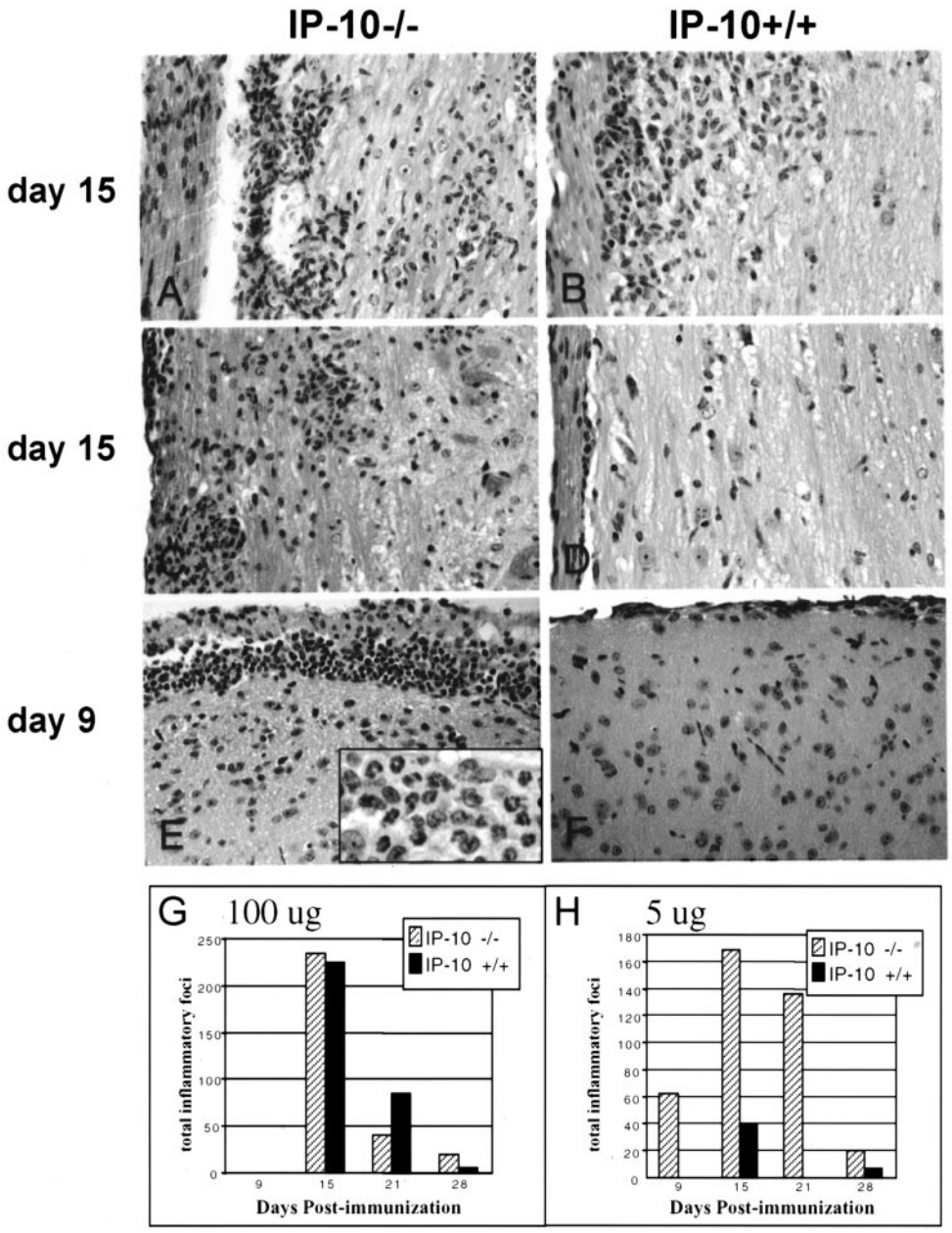

FIGURE 4. Histological analysis of CNS tissue from IP-$10^{-/-}$ and IP-$10^{+/+}$ littermates immunized with MOGp35–55. *A* and *B*, Meningeal and parenchymal mononuclear cell infiltrates typical of acute EAE in the spinal cord of IP-$10^{-/-}$ (*A*) and IP-$10^{+/+}$ (*B*) mice immunized with 100 μg of MOG peptide and sacrificed on day 15 postimmunization. Each animal showed a clinical grade of 3. Spinal nerve roots (peripheral nervous system, *left side* of each field) do not shown inflammation. *C* and *D*, Typical meningeal and parenchymal mononuclear cell infiltrates in the spinal cord of an IP-$10^{-/-}$ mouse (*C*) immunized with 5 μg of MOG peptide and sacrificed on day 15 postimmunization (clinical grade = 3). Only minimal meningitis was present in an IP-$10^{+/+}$ mouse (*D*) similarly immunized and sacrificed on day 15 postimmunization (clinical grade = 1). *E* and *F*, CNS samples of IP-$10^{-/-}$ (*E*) and IP-$10^{+/+}$ (*F*) mice immunized with 5 μg of MOG peptide and sacrificed on day 9 postimmunization. *E*, In IP-$10^{-/-}$ mice (clinical grade = 0), the infiltrate is restricted to leptomeninges and consists of a mixture of mononuclear cells and neutrophils (inset). There is no inflammation in CNS sample from an IP-$10^{+/+}$ mouse (clinical score = 0) immunized similarly and sacrificed at the same time point (*F*). All original magnification is ×350, except inset which is ×524. *G* and *H*, Quantification of CNS lesion numbers in IP-$10^{-/-}$ and IP-$10^{+/+}$ mice immunized with 100 μg of MOGp35–55 (*G*) and 5 μg of MOG (*H*). Histological sections from CNS tissues of IP-$10^{-/-}$ (▨) and IP-$10^{+/+}$ (■) mice immunized with 100 μg (*G*) and 5 μg (*H*) of MOG peptide were examined blinded by a neuropathologist for total numbers of inflammatory lesions (n = 4 mice per group).

4, *D*, *F*, and *H*). The few lesions detected consisted mostly of mononuclear cells and were found only in CNS from day 15 postimmunization time point (Fig. 4, *D* and *G*). Thus, loss of IP-10 appeared to increase the susceptibility of mice to EAE, allowing them to develop significant disease after immunization with MOGp35–55 at doses that do not produce EAE in wild-type littermate control mice.

IP-10-deficient mice with EAE had characteristic CNS lymphocyte profiles

Our histological results demonstrated that IP-10-deficient mice developed severe EAE with numerous characteristic lesions containing mostly mononuclear cells. To examine whether a deficiency of IP-10 influenced the recruitment of leukocyte subsets into the CNS, we

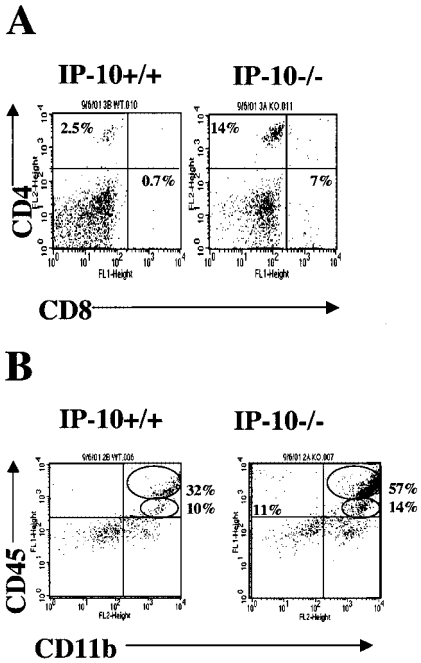

FIGURE 5. Patterns of CNS infiltrating leukocytes in IP-$10^{-/-}$ and IP-$10^{+/+}$ mice immunized with 5 μg of MOGp35–55. IP-$10^{-/-}$ and IP-$10^{+/+}$ control littermates were immunized with 5 μg of MOGp35–55 in CFA with PTX (150 ng/injection) and sacrificed ~2 wk postimmunization, at peak clinical EAE (clinical score = 3). Cells were isolated from the CNS and stained with CD8-FITC and CD4-PE mAbs (*A*) or anti-CD45 and anti-CD11b mAbs (*B*). There was a 7–10-fold increase in CNS-infiltrating $CD4^+$ and $CD8^+$ cells (A) and a 4-fold increase in CNS-infiltrating $CD45^+CD11b^+$ macrophages (*B*) in IP-$10^{-/-}$ vs IP-$10^{+/+}$ mice immunized with 5 μg of MOG peptide. Fluorescent analyses were generated by exclusion gating of dead cells and then gating either lymphocytes or monocyte/macrophages as observed using a bivariate dot plot of forward vs side scatter. Data are representative of two experiments with similar results, n = 3 mice per group.

analyzed CNS leukocyte infiltrates in IP-$10^{-/-}$ and IP-$10^{+/+}$ mice immunized with 100 and 5 μg doses of MOGp35–55 via flow cytometry (Fig. 5). Mononuclear cells were isolated from pooled the CNS tissues (brain and spinal cord) of IP-$10^{+/+}$ and IP-$10^{-/-}$ mice (three to four mice per group) at the peak severity of disease (14 days) postimmunization and examined for expression of CD3, CD4, CD8, CD45, and CD11b. In experiments using immunization with 100 μg dose MOGp35–55, groups of mice with comparable disease severity were chosen for consistency. IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with 100 μg dose MOGp35–55 had similar percentages of CNS infiltrating $CD4^+$ (25 vs 22%; data not shown) and $CD8^+$ T (11 vs 11%; data not shown) cells and $CD45^{high}CD11b^+$ cells (infiltrating macrophages) (19 vs 21%; data not shown). Similar analyses performed on IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with 5 μg dose of MOGp35–55 revealed vastly different percentages of CNS $CD4^+$ (2.5 vs 14%; Fig. 5*A*) and $CD8^+$ T cells (0.7 vs 7%; Fig. 5*A*) and $CD45^{high}CD11b^+$ cells (32 vs 57%; Fig. 5*B*). Total numbers of infiltrating mononuclear cells are compiled in Table I. These data were consistent with the levels of clinical disease, histology and numbers of inflammatory lesions observed in all these groups of mice and demonstrate that there was similar recruitment of leukocytes into the CNS of IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with 100 μg MOGp35–55 but significantly fewer leukocytes recruited into the CNS of IP-$10^{+/+}$ mice immunized with 5 μg MOGp35–55 compared with similarly immunized IP-$10^{-/-}$ mice.

CNS I-TAC mRNA levels are increased in IP-$10^{-/-}$ mice with EAE

To address the possible mechanisms of lymphocyte recruitment in the absence of IP-10, we examined the CNS chemokine mRNA levels of IP-$10^{+/+}$ and IP-$10^{-/-}$ mice during EAE induced by immunization with both 100 and 5 μg of MOGp35–55 via RNase protection assay (data not shown) and Northern blot (Fig. 6) analyses. Following immunization with 100 μg of MOGp35–55, both IP-$10^{+/+}$ and IP-$10^{-/-}$ mice demonstrated similar up-regulation of CNS RANTES/CCL5, MCP-1, Mig, and CXCR3 mRNA levels 15 days after immunization, as we have previously reported (Fig. 6*A*). However, IP-$10^{-/-}$ mice immunized with 5 μg MOGp35–55 demonstrated substantially increased induction in CNS RANTES, MCP-1, and CXCR3 mRNA levels 15 days postimmunization, compared with IP-$10^{+/+}$ mice (Fig. 6*A*), reflecting the overall increase in inflammatory infiltrates observed in the IP-10-deficient mice immunized with this lower dose. Interestingly, levels of CNS I-TAC mRNA were significantly up-regulated in only IP-$10^{-/-}$ mice regardless of the dose of MOGp35–55 (Fig. 6, *A* and *B*). This result was confirmed via quantitative RT-PCR analysis of CNS tissues obtained from groups of IP-$10^{+/+}$ vs IP-$10^{-/-}$ mice immunized with both 5 and 100 μg of MOGp35–55 (Fig. 6*B*). These results suggest that I-TAC expression in the CNS may compensate for the loss of IP-10 during development of EAE in susceptible mice.

Mig mRNA levels are decreased in IP-$10^{-/-}$ mice. Given that loss of IP-10 did not affect the trafficking of lymphocytes into the CNS and was instead associated with the up-regulation of the alternative CXCR3 ligand, I-TAC, we examined the expression of IP-10, Mig, and I-TAC at the draining lymph nodes of IP-$10^{+/+}$ and IP-$10^{-/-}$ animals 14 days postimmunization with 5 and 100 μg MOGp35–55 via real-time quantitative RT-PCR (Fig. 7). Interestingly, in contrast to the CNS, loss of IP-10 in the lymph node was associated with significantly decreased expression of Mig in IP-$10^{-/-}$ mice compared with IP-$10^{+/+}$ mice regardless of immunizing dose and no compensatory up-regulation of I-TAC (Fig. 7). Loss of these alternative ligands for CXCR3 was associated with decreased levels of CXCR3 mRNA in these lymph nodes, suggesting loss of recruitment of CXCR3-expressing lymphocytes. Although others have observed in a rat model of EAE that neutralization of IP-10 using anti-IP-10 mAbs treatment decreased numbers of LNCs in the draining lymph nodes of immunized animals during peak clinical EAE (35), we did not observe significant differences in overall LNC numbers between IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with MOGp35–55 (data not shown).

Table I. *Total numbers of immunophenotyped mononuclear cells*[a]

Dose (μg)	Genotype	Cell Recovery/CNS	$CD4^+$	$CD8^+$	$CD45^{high}/CD11b^+$
100	IP-10+/+	1.5×10^6	2.6×10^5	1.2×10^5	2.0×10^5
	IP-10−/−	1×10^6	2.2×10^5	1.1×10^5	2.3×10^5
5	IP-10+/+	0.6×10^6	0.1×10^5	0.4×10^4	0.6×10^5
	IP-10−/−	1.5×10^6	2.3×10^5*	1.1×10^5*	7.5×10^5*

[a] Data are expressed as mean of six to eight mice/group from two separate experiments.
*, $P < 0.05$.

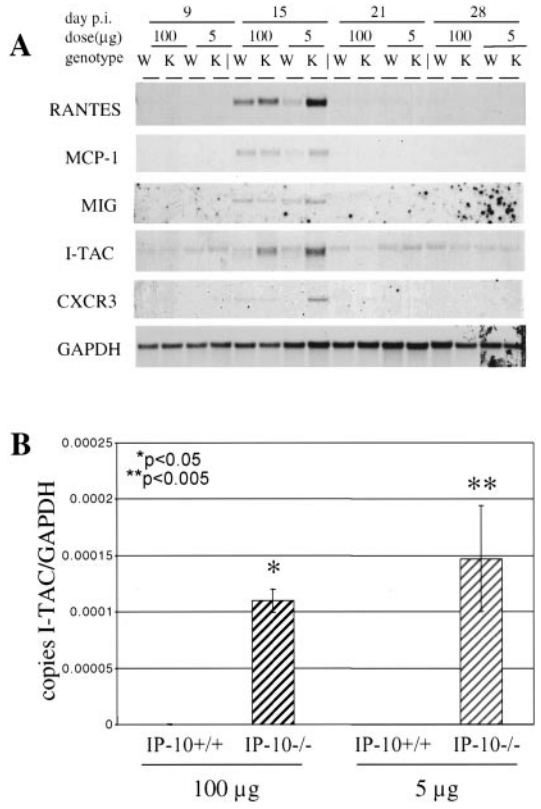

FIGURE 6. Chemokine expression in CNS of MOGp35–55 immunized mice. *A*, Northern blot analysis of total RNA isolated from CNS of IP-$10^{+/+}$ wild-type (W) and IP-$10^{-/-}$ knockout (K) mice at the indicated times postimmunization (day p.i.) with 100 or 5 μg MOG of peptide (dose in micrograms) for expression of RANTES, MCP-1, Mig, I-TAC, and CXCR3 mRNAs. Blots were reprobed for expression of GAPDH mRNA to control for RNA loading. Exposure times: RANTES and MCP-1 = 2 days; Mig and I-TAC = 4 days; CXCR3 = 14 days; GAPDH = 4 h. *B*, CNS I-TAC expression. A total of 5 μg of total RNA obtained from CNS tissues of IP-$10^{+/+}$ (■) and IP-$10^{-/-}$ (▨) 15 days postimmunization with 100 μg (black) or 5 μg (gray) of MOGp35–55 was analyzed by quantitative RT-PCR (n = 3 mice per group).

These data suggest that loss of IP-10 differentially affects the chemokine network at different tissue sites, which may contribute to alterations in the activation and trafficking of lymphocyte subpopulations.

Ag-specific T cell responses in the absence of IP-10

Given that loss of IP-10 affects the expression of alternate CXCR3 ligands, we next determined whether loss of IP-10 affected MOGp35–55-specific T cell responses. We analyzed immune responses of IP-$10^{+/+}$ and IP-$10^{-/-}$ mice following immunization with 100 or 5 μg of MOGp35–55 (Fig. 8). Draining LNCs were harvested from mice 9 days after immunization and restimulated with Ag for 72 h. Cells were then analyzed for Ag-induced production of Th1 (IFN-γ, IL-2) and Th2 (IL-10, IL-4) cytokines and Ag-specific proliferation with stimulation index. Ag-specific IFN-γ production and proliferation of LNCs from IP-$10^{-/-}$ mice immunized with 100 μg of Ag were decreased by ~1.9-fold and 3.5-fold, respectively, compared with LNCs from similarly immunized IP-$10^{+/+}$ animals, whereas IL-2 levels in these groups of mice were essentially identical (Fig. 8*A*). By contrast, identical analysis of LNCs obtained from animals immunized with the 5 μg dose of MOGp35–55 determined that Ag-specific cytokine responses of LNCs from IP-10-deficient animals were 3.7-fold

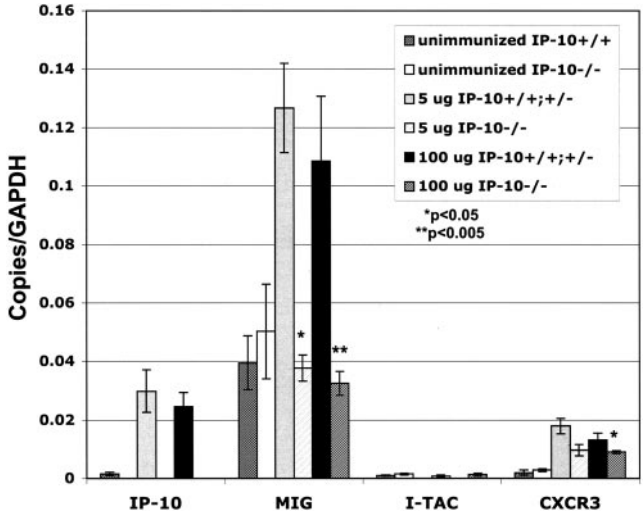

FIGURE 7. Expression of CXCR3 ligands in lymph node tissues after immunization with MOGp35–55. A total of 5 μg of total RNA obtained from draining lymph nodes of IP-$10^{+/+}$ and IP-$10^{+/-}$ (■) and IP-$10^{-/-}$ (▨) 9 days postimmunization with 100 μg (black) or 5 μg (gray) MOGp35–55 was analyzed by quantitative RT-PCR for expression of IP-10, Mig, I-TAC, and CXCR3. Data are expressed as mean (n = 3) in each group and is representative of combined data from n = 2 experiments.

(IFN-γ) and 250-fold (IL-2) higher, whereas proliferative responses were 2.5-fold (stimulation index) higher, than those of LNCs from wild-type littermates (Fig. 8*B*). Th2 cytokine responses were undetectable in all groups (data not shown). These data suggest that the optimal immunizing dose for effector T cell activation might actually decrease in the absence of IP-10.

Lymph node TGF-β1 mRNA levels are decreased in IP-$10^{-/-}$ mice. Given that chemoattractants have been observed to affect the production of cytokines by dendritic cells (36), the observed loss of expression of CXCR3 chemoattractants at the draining lymph nodes and the observed ability to induce EAE with lower doses of MOGp35–55 in IP-10-deficient mice suggested that loss of IP-10 may decrease levels of immunosuppressive cytokines during T cell priming. The immunoregulatory cytokine TGF-β1 has been shown to modulate a variety of Ag-specific T cell functions

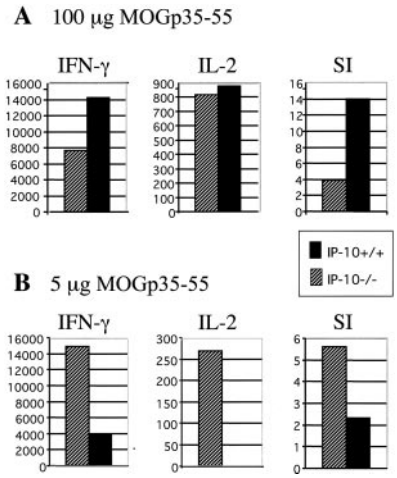

FIGURE 8. Ag-specific cytokine production and proliferative responses of lymph node cells from IP-$10^{+/+}$ and IP-$10^{-/-}$ mice. Cells were isolated on day 9 postimmunization with 100 μg (*A*) and 5 μg (*B*) of MOG peptide (pooled from three to six mice per group) and stimulated in vitro with 100 μg of MOGp35–55.

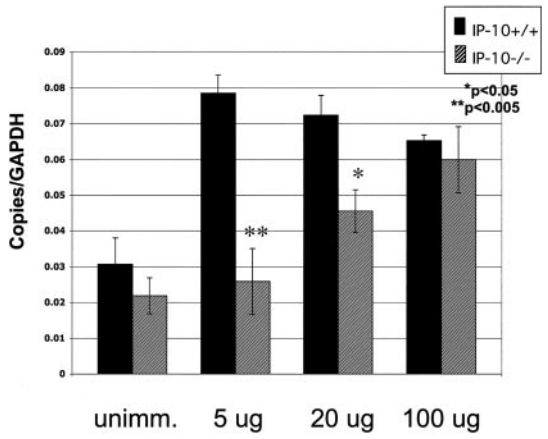

FIGURE 9. Expression of TGF-β1 mRNA in lymph node tissues after immunization with MOGp35–55. A total of 5 μg of total RNA obtained from lymph nodes of IP-$10^{+/+}$ (■) and IP-$10^{-/-}$ (▨) 9 days after immunization with 5 μg, 20 μg, and 100 μg of MOG peptide was analyzed for expression of TGF-β1 mRNA using quantitative RT-PCR. Data are expressed as mean (n = 2–4) in each group and are representative of combined data from n = 2 experiments.

and has been implicated in the suppression of various autoimmune diseases including EAE (37–42). Examination of draining lymph nodes in IP-$10^{+/+}$ and IP-$10^{-/-}$ mice immunized with 5 μg, 20 μg, and 100 μg of MOGp35–55 for TGF-β1 mRNA levels was performed via quantitative RT-PCR (Fig. 9). Although the lymph node TGF-β1 mRNA levels of IP-$10^{+/+}$ mice were similar regardless of the immunizing dose of MOGp35–55, there was a dose-dependent increase in TGF-β1 expression in IP-$10^{-/-}$ mice. Similar analysis for the expression of the immunoregulatory cytokine IL-10 did not reveal a similar dose-dependent effect (data not shown). These data suggest that the decreased threshold for induction of EAE in IP-10-deficient mice may be due to an inability to mount a counter regulatory response with lowered levels of immunizing Ag.

Discussion

IP-10 has been detected in the CSF and brain parenchyma of patients with a variety of neuroinflammatory diseases (15, 26, 43, 44) and is a potent chemoattractant for activated T lymphocytes and NK cells (11). In EAE, an animal model for MS, IP-10 levels in the CNS have been correlated with the development of clinical disease and the recruitment of CXCR3-expressing pathogenic T cells (19, 20, 45). Treatment of SJL mice with Abs to IP-10 before adoptive transfer of encephalitogenic T cells or immunizing mice with naked IP-10 DNA decreased the severity of EAE (29). Based on these observations, IP-10 is thought to be essential for the development of CNS mononuclear infiltrates, and its receptor CXCR3, is considered a putative therapeutic target for diseases involving the trafficking of inflammatory T cells. However, the absolute requirement for IP-10 in EAE has never been directly examined.

In this study, we sought to determine whether IP-10 is required for the development of EAE by analyzing disease in IP-10-deficient mice. Our data showed that IP-10 is not essential for the development of EAE. Neurological disease induced in IP-$10^{+/+}$ and IP-$10^{-/-}$ mice by a standard immunizing dose of MOGp35–55 (100 μg) was clinically and histologically equivalent. Administration of anti-IP-10 mAb did not affect clinical disease in experiments using three different strains of mice immunized with either MOGp35–55 (129sv;C57BL/6 and C57BL/6 strains) or MBP-Acp1–11 (SJL/PL F_1 strain). This correlated with our data in IP-10-deficient animals and suggests that IP-10-independent EAE may be a generalized feature of many murine genetic strains. These results differed from a study be Fife et al. (29) in which anti-IP-10 neutralizing Abs blocked the induction of EAE using an adoptive transfer model. This disparity may be due to effects of in vitro vs in vivo priming of T cells on Th1 chemokine receptor expression patterns or to the differences in time to disease between the two protocols, which may allow the development of compensatory mechanisms to occur in the immunization model.

The induction of EAE in IP-10-deficient mice suggests that trafficking of encephalitogenic $CD4^+$ T cells from the lymph node into the CNS remains functionally intact in the absence of IP-10 signaling. Indeed, phenotypic analysis of mononuclear cell accumulation in the CNS of IP-$10^{+/+}$ and IP-$10^{-/-}$ mice with EAE induced by immunization with 100 μg of MOGp35–55 demonstrated similar percentages and phenotypes of T cells. The induction of EAE was associated with increased CNS levels of RANTES and CXCR3 mRNAs, likely reflecting the infiltration of activated lymphocytes, as these have been shown to be the cellular sources of this chemokine and chemokine receptor during EAE (20, 46). Interestingly, we found that IP-$10^{-/-}$ mice up-regulated I-TAC mRNA at both the 5 and 100 μg MOGp35–55 doses, compared with similarly immunized IP-$10^{+/+}$ mice. The mechanism and ability to generalize this compensatory up-regulation of I-TAC in IP-$10^{-/-}$ mice is not known. However, the overall trafficking of mononuclear cells into the CNS during the induction of EAE was not impaired by the loss of IP-10 through targeted gene deletion or via administration of neutralizing Abs and suggests that other CXCR3 ligands, or other chemokine-chemokine receptor systems, are operational or can compensate for the loss of IP-10 to orchestrate T cell recruitment into the CNS in this model.

IP-10 is also expressed in secondary lymphoid tissue, suggesting it may also affect T cell development and activation. In support of this potential role, IP-10 has been shown to enhance IFN-γ release in Ag-stimulated PBMC cultures (47), to enhance Ag-specific T cell proliferative responses in secondary lymphoid tissue (32), and to stabilize Th1-dendritic cell clusters in the lymph node (48). Given our in vivo finding that loss of IP-10 did not affect the induction of an Ag-specific T cell inflammatory response, we analyzed the phenotype of T cells generated by immunization with MOGp35–55 in IP-$10^{-/-}$ and IP-$10^{+/+}$ mice. Consistent with previous observations using IP-10-deficient mice (32), we observed decreased Ag-specific proliferation and production of the Th1 cytokine IFN-γ by LNCs from IP-$10^{-/-}$ mice immunized with 100 μg of MOGp35–55, compared with similarly immunized IP-$10^{+/+}$ animals. Thus, IP-10-deficient mice immunized at a standard dose of MOGp35–55 appeared to generate T cells with decreased effector activities in vitro that were nevertheless sufficiently encephalitogenic in vivo to induce EAE.

Interestingly, similar experiments using LNCs from IP-$10^{-/-}$ and IP-$10^{+/+}$ mice immunized with 5 μg of MOGp35–55 demonstrated the reverse in vitro finding, with enhanced effector T cell functions observed with LNCs from IP-10-deficient animals. This finding likely explains our observation that IP-10-deficient mice developed severe EAE at an immunizing dose of MOGp35–55 (5 μg) that produced limited disease in their IP-$10^{+/+}$ littermates. However, it is also interesting to note that a similar increase in susceptibility to EAE was seen when the IFN-γ pathway was inhibited. Treatment of mice with anti-IFN-γ mAbs or gene deletion of the IFN-γ receptor exacerbated EAE or converted the normally resistant 129 strain to a susceptible strain to MOG-induced EAE (49–52). Examination of the pattern of chemokine expression in the CNS of these mice demonstrated the striking absence of IP-10. In addition, histologic examination of CNS tissues from IFN-γ

receptor gene deleted mice with EAE revealed extensive involvement of neutrophils (51), similar to our findings in IP-10-deficient mice immunized with low dose MOGp35–55. As has been suggested for the osteopontin gene deletion affects on EAE, the difference between EAE induced by low-dose immunization may be more significant in mice on the 129sv;C57BL/6 background than on the pure C57BL/6 background due to polymorphic genes closely linked to the IP-10 locus that in the 129sv strain have a greater contribution to disease expression (53). Thus, IFN-γ and one of its downstream targets, IP-10, seem to have a paradoxical affect on EAE that is more apparent in strains of mice with inherently more resistance to EAE induction.

Resistance to EAE induction has been associated with enhanced immunoregulatory responses, such as the production of immunoregulatory cytokines. TGF-β1 and IL-10 are two pleiotropic cytokines that have been correlated with the inhibition of autoimmune diseases, including EAE (40, 42, 54–58). In the EAE model, TGF-β1 has been shown to mediate resistance to the disease via the actions of regulatory $CD4^+$ T cells (38). The role of IL-10 in EAE is more controversial, with elevated levels associated with suppression of disease in some models and no effect on disease in others (59–62). We observed that TGF-β1, but not IL-10 (data not shown), expression exhibited a dose-dependent increase in response to immunization with MOGp35–55 compared with similarly immunized wild-type littermates. This correlated with an increased Ag-specific Th1 peripheral immune response and more severe clinical disease. Thus, lower levels of TGF-β1 in the lymph node were associated with a lower threshold to induce EAE in this model. The enhancement of immune responses to low-dose MOGp35–55 may be the result of loss of immunoregulatory mechanisms in IP-10-deficient animals that may reflect alterations in the trafficking of TGF-β1 expressing regulatory T cells. In this regard, the CXCR3 ligand, I-TAC, has been shown to be chemotactic for $CD4^+CD25^+$ regulatory T cells (63). Alternatively, loss of IP-10 may interfere with dendritic cell-Th1 cell interactions in the lymph node as has been seen in a murine model of granulomatous liver disease (48). This could alter lymph node physiology as well as the activation and cytokine profile of Th1 cells.

In summary, loss of IP-10 signaling did not abrogate the induction or expression of EAE at standard immunizing doses but paradoxically increased susceptibility to disease at lower immunizing doses, which may be more apparent in more resistant strains such as 129sv. This increased susceptibility was the result of a lower threshold to induce a MOGp35–55-specific peripheral immune response in IP-10-deficient mice. We correlated this lower threshold of T cell activation in IP-10-deficient mice with decreased lymph node levels of TGF-β1, suggesting that IP-10 plays a role in the delivery or expression of regulatory T cells in the lymphoid compartment in certain genetic backgrounds. Based on these observations, IP-10 should be approached with caution as a target for MS therapy.

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