Absence of Monocyte Chemoattractant Protein 1 in Mice Leads to Decreased Local Macrophage Recruitment and Antigen-specific T Helper Cell Type 1 Immune Response in Experimental Autoimmune Encephalomyelitis

By DeRen Huang,* Jintang Wang,* Pia Kivisakk,* Barrett J. Rollins,‡ and Richard M. Ransohoff*

From the *Department of Neurosciences, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195; and the ‡Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Abstract

Monocyte chemoattractant protein (MCP)-1 plays a critical role in innate immunity by directing the migration of monocytes into inflammatory sites. Recent data indicated a function for this chemokine in adaptive immunity as a regulator of T cell commitment to T helper cell type 2 (Th2) effector function. Studies in a Th1-dependent animal model, experimental autoimmune encephalomyelitis (EAE), showed that MCP-1 was highly expressed in the central nervous system (CNS) of affected rodents, and MCP-1 antibodies could block relapses of the disease. Mice deficient for the major MCP-1 receptor, CC chemokine receptor (CCR)2, did not develop EAE after active immunization but generated effector cells that could transfer the disease to naive wild-type recipients. We analyzed EAE in mice deficient for MCP-1 to define the relevant ligand for CCR2, which responds to murine MCP-1, MCP-2, MCP-3, and MCP-5. We found that C57BL/6 MCP-1–null mice were markedly resistant to EAE after active immunization, with drastically impaired recruitment of macrophages to the CNS, yet able to generate effector T cells that transferred severe disease to naive wild-type recipients. By contrast, adoptive transfer of primed T cells from wild-type mice into naïve MCP-1–null recipients did not mediate clinical EAE. On the SJL background, disruption of the MCP-1 gene produced a milder EAE phenotype with diminished relapses that mimicked previous findings using anti–MCP-1 antibodies. There was no compensatory upregulation of MCP-2, MCP-3, or MCP-5 in MCP-1–null mice with EAE. These results indicated that MCP-1 is the major CCR2 ligand in mice with EAE, and provided an opportunity to define the role of MCP-1 in EAE. Compared with wild-type littermates, MCP-1−/− mice exhibited reduced expression of interferon γ in draining lymph node and CNS and increased antigen-specific immunoglobulin G1 antibody production. Taken together, these data demonstrated that MCP-1 is crucial for Th1 immune responses in EAE induction and that macrophage recruitment to the inflamed CNS target organ is required for primed T cells to execute a Th1 effector program in EAE.

Key words: autoimmune disease • chemokine • chemokine receptor • macrophage • T helper cell type 1/T helper cell type 2

Introduction

Chemokines are small proteins (8–12 kD) divided into four subfamilies (CXC, CC, C, and CX3C) according to the organization of positionally conserved cysteine residues (1). Monocyte chemoattractant protein 1 (MCP-1) is a prototype CC chemokine, active towards monocytes, dendritic cells, and NK cells, thereby playing an important role in inflammatory responses.
nate immunity (2–7). However, MCP-1 is also a crucial factor for the development of adaptive Th2 responses. In this regard, MCP-1 directs the differentiation of Th0 cells to Th2 in vitro (8) by a mechanism dependent on IL-4. Administration of anti–MCP-1 Abs (9) or disruption of the MCP-1 gene (2) significantly reduced the size of schistosome egg antigen (SEA) secondary granulomata, a Th2-dominant disease model. Conversely, local overexpression of MCP-1 increased the size of SEA secondary granulomata (10). Immunization with trinitrophenol–derivated ovalbumin plus IFA elicited a reduced Th2 and unaltered Th1 response in MCP-1-deficient (MCP-1−/−) mice. MCP-1−/− mice of Balb/c strain were relatively resistant to *Leishmania major* infection, indicating that lack of MCP-1 led to reduced Th2 immunity (11).

Experimental autoimmune encephalomyelitis (EAE), a model for autoimmune demyelination of the central nervous system (CNS), has been widely employed to explore pathogenic mechanisms underlying the human disease multiple sclerosis (MS [12, 13]). The generation of myelin protein–reactive T cells is an immunological hallmark of both EAE and MS and is required for disease expression in EAE. These autoreactive T cells traffic to the CNS, and initiate inflammation and destruction of CNS myelin with consequent neurological impairment (14, 15).

Th1-type T cells, producing IFN-γ, IL-2, and TNF-β, are associated with cellular immune responses, delayed-type hypersensitivity, and macrophage activation, whereas Th2-type T cells, producing IL-4, IL-5, and IL-10, are important for humoral immune responses (16, 17). The dynamic interplay and reciprocal inhibition between Th1 and Th2 cytokines has been demonstrated in numerous research reports. IL-4 is a major factor that governs Th2 differentiation and inhibits the development of IFN-γ–secreting cells (18). The activation of macrophages and the production of Th1 cytokines such as IFN-γ can also be inhibited by IL-10 (19). Most encephalitogenic T cell clones examined are Th1 polarized (20–22), although exceptions have been reported (23). Th1 cytokines are markedly elevated in the CNS of animals during EAE attacks whereas Th2 cytokines are associated with disease recovery (24). IL-4–induced immune deviation is beneficial for recovery from EAE (25); EAE can be prevented and/or reversed by myelin antigen–specific T cells that are genetically transduced with either IL-4 or IL-10 genes (26, 27); anti–IL-4 treatment reverses the tolerance induced by an altered peptide ligand (28), and absence of IL-4 in gene–targeted mice increases the severity of EAE (29). In summary, Th1 immune responses are pathogenic and Th2 responses are protective in the initiation and evolution of EAE.

However, antigen-specific T cells constitute only a small proportion of infiltrating leukocytes in EAE or MS lesions (30). Secondarily recruited inflammatory cells account for the vast majority of infiltrating cells and play a pivotal role in CNS tissue damage (31). Although the detailed mechanisms by which inflammatory cells influx into the CNS compartment are not completely understood, increasing evidence suggests that chemokines, in concert with adhesion molecules, are essential for this process (32). In EAE, elevated expression of MCP-1 by CNS parenchymal cells, tightly linked to clinical disease, has been demonstrated repeatedly (33–35). Further, anti–MCP-1 Abs blocked relapses of adoptive transfer EAE in SJL mice (36). Additionally, mice that lacked CC chemokine receptor 2 (CCR2), the major receptor on monocytes for MCP-1, failed to develop EAE after active immunization and were resistant to induction of EAE by the adoptive transfer of primed T cells from syngeneic wild-type mice (37, 38). It was uncertain whether MCP-1 was the relevant ligand for CCR2 in these experiments, as this receptor also responds to MCP-2, MCP-3, and MCP-5. However, there is also support for the possibility that regulation of Th2 responses by MCP-1 could be important for the pathogenesis of EAE; in particular, MCP-1 was critical for the development of tolerance after oral administration of a proteolipid protein (PLP) peptide containing residues 139–151 (39).

Therefore, the phenotype of EAE in MCP-1–deficient mice could not readily be predicted. On one hand, defective MCP-1–dependent monocyte recruitment might lead to attenuated disease. Alternatively, if functional replacement of MCP-1 by another MCP mediated monocyte accumulation in the CNS of these mice, defective Th2 responses might lead to very severe, nonremitting disease. Finally, in view of redundancy in the immune/inflammatory system, it remained possible that MCP-1–null mice would manifest EAE identically to wild-type animals. Given these considerations, the role of MCP-1 in the pathogenesis of EAE merited further characterization.

These concerns are also likely pertinent for the human disorder MS. Patients with active disease, manifest by clinical attacks, showed significantly decreased MCP-1 in the cerebrospinal fluid, as compared with controls. Of eight chemokines measured, only MCP-1 was reduced in the cerebrospinal fluid of patients with active MS. However, abundant MCP-1 has been readily detected by immunohistochemistry in autopsy brain sections containing MS lesions (40–42). Therefore, the role of MCP-1 in the pathogenesis of MS remains to be clarified.

In this report, we describe the phenotype of EAE in MCP-1–null mice. These mice exhibited markedly reduced clinical and histological EAE after active immunization and did not develop clinical disease after receiving encephalitogenic T cells from wild-type animals. Expressions of MCP-2, MCP-3, and MCP-5 in the CNS of both wild-type and MCP-1–null mice with EAE were virtually identical. These findings indicated that MCP-1 was the major ligand for CCR2 in murine EAE. In this EAE model, we found that disruption of the MCP-1 gene led to an attenuated Th1 autoimmune response and complimentarily increased Th2 response. These results indicated a crucial role for MCP-1 in generating CNS inflammatory reactions that mediate the effector phase of myelin–specific Th1 autoimmune responses. Therefore, the data suggested that primed encephalitogenic Th1 cells cannot manifest effector functions in the CNS without recruiting hematogenous macrophages.
**Materials and Methods**

**Mice.** The disruption of the MCP-1 gene has been described previously (2). MCP-1−/− mice were backcrossed onto the C57BL/6 (B6) strain for eight generations. One F8 MCP-1−/− mouse was further backcrossed to a B6 mouse (obtained from The Jackson Laboratory). The heterozygous offspring were intercrossed to produce F9 wild-type (+/−), heterozygous (−/−), and MCP-1−/− mice. F10 mice were generated in a similar manner. MCP-1+/+, MCP-1−/−, and MCP-1−/− F9 and F10 mice on the B6 background were used in this study.

MCP-1−/− deficient mice on the B6/129 background were also backcrossed onto SJL for seven generations. MCP-1−/− and MCP-1−/− F7 mice on SJL background were used in this study.

Mice were genotyped using a PCR-based analysis of genomic DNA extracted from tail clips. Primers MCP-1F, 5′-GGA GCA TCC ACG TGT TGG C-3′ and MCP-1R, 5′-ACA GCT TCT-3′ amplified a DNA fragment within the MCP-1 gene. Primers NeoF, 5′-CGC TTC TTT CCT TTT GAG AC-3′ and NeoR, 5′-ATC CTC GCC GTC GGG CAT GC-3′ amplified a fragment in the neomycin resistance gene insert. PCR reactions were performed in a PerkinElmer 9700 cycler (annealing temperature, 50°C) and products were visualized by electrophoresis on ethidium bromide−stained NuSeive GTG® agarose gel.

**Rat Myelin Oligodendroglial Glycoprotein and Mouse Proteolipid Protein Peptides.** Rat myelin oligodendroglial glycoprotein (MOG)35−55 and mouse PLP139−151 peptides were obtained from (BIO-SYNTHESIS) and purified by HPLC with a purity of 98%. The sequence of MOG35−55 was MEVGWYRSPFS-K and that of PLP139−151 was HSLGKWL-Q. MCP-2, MCP-3, and MCP-5 cDNA were amplified from spinal cord RNA by reverse transcription (MPO35−55 and anti-CD3e Ab at 5% CO2, and 95% humidity. The supernatants were collected after 24, 48, and 72 h of in vitro restimulation and kept at −80°C until assay. Levels of IFN-γ, IL-4, and IL-10 in sera and cell culture supernatants were determined using ELISA kits commercially obtained from R&D Systems. The standard curves were made on the same occasion and the sensitivities for the methods were 2.0, 2.0, and 4.0 pg/ml for IFN-γ, IL-4, and IL-10, respectively. All samples were measured in duplicate and diluted if necessary.

**Analysis of Chemokine and Chemokine Receptor mRNA Levels by RNase Protection Assay.** Mice were anesthetized with sodium pentobarbital and intracardially perfused through the left ventricle with ice-cold PBS. Spinal cords were extruded by flushing the vertebral canal with PBS, rinsed in PBS, and kept at −80°C. Total cellular RNA was prepared from spinal cord tissue by TRIzol (Life Technologies). Quantification of CC chemokines and chemokine receptors in CNS tissue was done by RT-PCR using specific primer sets and SYBR® green I (Bio-Rad) in a 96-well format. GAPDH was used as a control for each sample. The expression levels of MCP-1, MCP-2, MCP-3, and MCP-5 in spinal cord RNA were determined by RT-PCR using a standard curve (10−6, 10−5, 10−4, 10−3, 10−2, and 10−1) for each primer. The PCR products were visualized on a 2% agarose gel.

**Active Induction of EAE with MOG and PLP Peptides and Clinical Evaluation.** Mice of 8–9 wk of age were subcutaneously injected with 300 µg MOG35−55 emulsified in CFA (Difco) containing 400 µg Mycobacterium tuberculosis. Mice were intravenously injected with pertussis toxin (Sigma-Aldrich) as indicated in the figure legends on day 0 and 2 postimmunization (dpi). The immunization in SJL mice was carried out as described previously (43). All mice were weighed, examined, and graded daily for neurological signs in a double blind manner by one of us (J. Wang) as follows: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; and 5, moribund state. Disease relapse was determined when an increase of one EAE score unit was observed. Signs of neurological impairment were typically accompanied by an abrupt, substantial weight loss (>7%). The average day of EAE onset was calculated by adding the first day of clinical signs for individual mice and divided by the number of mice in the group. Day of EAE onset in mice that showed no clinical EAE was deliberately regarded as 1 day after the experiment was terminated (44). The EAE index was calculated by adding all the daily EAE scores to obtain cumulative score and dividing by day of EAE onset. Active immunization with MOG35−55 induced monophasic EAE in B6 mice and was followed for 65 d. Chronic relapsing EAE induced by PLP139−151 was monitored for 90 d.

**T Cell Proliferation Assay.** Mice were killed and draining lymph nodes (popliteal and inguinal lymph nodes [PLNs]) were dissected on day 10 pi. Single cell suspensions (5 × 10⁶/ml) were prepared and cultured in triplicate in 96-well flat-bottomed plates (Falcon; Becton Dickinson) in 200 µl/well in the presence or absence of MOG35−55 and anti-CD3e antibody (clone GHPDK). The disruption of the MCP-1 gene has been described previously (2). MCP-2, MCP-3, and MCP-5 cDNA was amplified by specific primer pairs, mcp-2f, 5′-GGA GCA TCC ACG TGT TGG C-3′ and mcp-5R, 5′-ACA GCT TCT-3′ amplified a fragment in the neomycin resistance gene insert. PCR reactions were performed in a PerkinElmer 9700 cycler (annealing temperature, 50°C) and products were visualized by electrophoresis on ethidium bromide−stained NuSeive GTG® agarose gel.

**Reverse Transcription PCR Detection for Levels of MCPs, Chemokines, and Chemokine Receptor mRNA Levels by RNase Protection Assay.** Mice were anesthetized with sodium pentobarbital and intracardially perfused through the left ventricle with ice-cold PBS. Spinal cords were extruded by flushing the vertebral canal with PBS, rinsed in PBS, and kept at −80°C. Total cellular RNA was prepared from spinal cord tissue by TRIzol (Life Technologies). Quantification of CC chemokines and chemokine receptors in CNS tissue was done by RT-PCR using specific primer sets and SYBR® green I (Bio-Rad) in a 96-well format. GAPDH was used as a control for each sample. The expression levels of MCP-1, MCP-2, MCP-3, and MCP-5 in spinal cord RNA were determined by RT-PCR using a standard curve (10−6, 10−5, 10−4, 10−3, 10−2, and 10−1) for each primer. The PCR products were visualized on a 2% agarose gel.

**Glyceraldehyde−3−phosphate dehydrogenase (GAPDH) expression was used as an unregulated control and amplified using primers: GAPDHf, 5′-GTA GGT GGT CGG AGT CAA CG-3′ and GAPDHr, 5′-CAA GTG TGT CAT GGA TCA CG-3′, MCP-2, MCP-3, and MCP-5 cDNA were amplified by specific primer pairs, mcp-2f, 5′-ATC CTA CCT GCT TGG TGT GGA AAA C-3′ and mcp-2r, 5′-ACT AAA GCT GAA GAT CCC CCT TCG-3′; mcp-3f, 5′-CAC ATT CCT ACA GAC AGC TC-3′ and mcp-3r, 5′-AGC TAC AGA GAG ATG ACC ACC AG-3′, and mcp-5f, 5′-CTC ATT CAC TGA CAT TGT GGT CC-3′ and mcp-5r, 5′-TCT CCC TCC ACC TCC ACC ATG CAG AG-3′. IFN−γ, IL−4, and IL−10 were amplified with primer pairs: IFNgf, 5′-AGC GGG TCA ATG GAT GTA AGC CTA GAT TG-3′ and IFNgr, 5′-GCA ACA GTT TTC AGC TGT CAT ATA GGG-3′; il4f, 5′-TCC GCA TTT TTA AGC ACC AGC TT-3′ and il4r, 5′-GGA AAC CCC GAA AGA GTG TC-3′; and il10f, 5′-CAT CAT GTA TGC TAT TAT GC-3′ and il10r, 5′-TAC CTG GTA GAA GTG ATC CGC-3′. CD3e was detected with primer pair: CD3cf, 5′-ATG GAC GAG AGG AAG GGT CTG-3′ and CD3er, 5′-TCA CTT CCT CTT CAG TTG GGT-3′. Levels of CD8 cDNA were detected using primer pair: CD8f, 5′-TCT GTC GTC CCA GTC CCT C-3′ and CD8r, 5′-TCT CTC TGT CTG ACT AGC GG-3′, while that of Mb−1 (the gene encoding IgGα, expressed by B cells; reference 45); Mb−1f, 5′-GCC AGG GGT TCT AGA AGC-3′ and Mb−1r, 715

Huang et al. on July 13, 2017 from jem.rupress.org Downloaded from
Results

C57BL/6 MCP-1–deficient Mice Are Relatively Resistant to Active EAE Induction with MOG35–55. MCP-1+/+, MCP-1−/−, and MCP-1−/+ F9 mice (n = 9 in each group) were immunized with 300 μg MOG35–55 plus 500 ng pertussis toxin intravenous injection on day 0 and 2 pi. One MCP-1−/+ mouse died of immunization on day 4 pi. The remainder of the mice, regardless of genotype, developed clinical EAE. MCP-1−/+ mice showed EAE signs around day 10 pi, consistent with previously reported results in C57BL/6 mice (44, 48). MCP-1−/− manifested significantly delayed EAE with an average onset on day 21 pi (Fig. 1). Three out of nine wild-type mice died of EAE, and another three had to be killed because of moribund state. None of the MCP-1−/− mice died of EAE or had to be killed throughout the experiment and they recovered from the disease significantly faster and more completely than wild-type littermate controls. Heterozygote MCP-1−/+ mice developed EAE with an intermediate kinetics and severity. None of the MCP-1−/+ mice died of EAE, whereas three were killed in a moribund state due to severe EAE. Analyses of CNS tissue histology revealed massive inflammatory infiltrates in wild-type control mice (+ + + to + + + + in regions of lumbar and sacral spinal cord, n = 4; Fig. 2 A) but markedly reduced inflammatory reaction in MCP-1−/−null mice (+ to + + in regions of corresponding levels of affected spinal cord, n = 4; Fig. 2 B; reference 49). Examination of demyelination using Luxol Fast Blue stain-
ing revealed significant reduction in MCP-1−/− mice compared with their littermates controls 65 d after immunization (data not shown).

F10 MCP-1−/− mice and wild-type littermate controls were analyzed in a subsequent experiment. As pertussis toxin has been shown to increase the permeability of the blood-brain barrier (BBB [50, 51]), enhance delayed type hypersensitivity (DTH) responses and the production of IFN-γ (52–54), augment expression of CD80, CD86 on antigen-presenting cells and CD28 on T cells (55), and T cell immune responses (55, 56), we reduced the amount of pertussis toxin in the immunization to 200 ng per injection in an attempt to reduce the high death rate observed in wild-type mice in the F9 experiments. In this experiment, all MCP-1+/+ and MCP-1−/− mice showed signs of clinical EAE; none died of EAE or required killing. In contrast to the MCP-1+/+ mice that developed full-blown EAE, the disease was largely suppressed with significantly delayed onset and milder neurological impairment (Fig. 3) and significantly less weight loss (data not shown) in MCP-1−/− mice. These data demonstrated that MCP-1-null mice and CCR2-deficient mice exhibited strikingly similar EAE phenotypes (37, 38) and suggested that MCP-1 may be the relevant ligand for CCR2 in this model.

Disruption of MCP-1 Gene Attenuates the Severity of PLP-induced EAE and Reduces the Number of Relapses in SJL Mice. 11 MCP-1+/+ and 10 MCP-1−/− mice on SJL background were immunized with PLP139–151 peptide emulsified in CFA. One MCP-1+/+ and two MCP-1−/− mice died of immunization within the first week after immunization. The reminder of MCP-1+/+ mice (10/10) and 7 out of 8 MCP-1−/− mice showed clinical EAE. Compared with the MCP-1−/− group, a significantly higher percentage of MCP-1+/+ mice died of EAE during the first attack and relapses thereafter. Among the mice that survived the first attack, six MCP-1+/+ mice had eight relapses (four mice had one attack each and two had two attacks each), whereas three out of seven MCP-1−/− mice had single attacks. At day 90 pi, the average EAE index was significantly higher in MCP-1+/+ group (n = 4) than that in MCP-1−/− group (n = 7) (Fig. 4). These results implicated a role for MCP-1 in eliciting relapses of EAE in this model. These findings were consistent with previous reports showing reduction of relapses using anti–MCP-1 Abs in a passive EAE model (36) and in mice receiving vaccine containing naked DNA encoding for MCP-1 (57).

Figure 3. Attenuated MOG35–55-induced EAE in MCP-1-null mice. F10 MCP-1−/− mice (n = 6) and their littermate wild-type controls (n = 6) were immunized with MOG35–55 in CFA and intravenously injected with pertussis toxin (PT; 200 ng/injection). Shown are EAE score (mean ± SD) of individuals in each group.
There Is No Compensatory Upregulation of MCP-2, MCP-3, or MCP-5 in the CNS of MCP-1−/− Mice with EAE. Because CCR2 is shared in common among all MCPs (58–60), we analyzed MCP-2, MCP-3, and MCP-5 mRNA expression in the CNS of C57BL/6 mice with MOG-induced EAE, using quantitative real-time reverse transcription (RT)-PCR. Levels of MCP-2 and MCP-3 but not MCP-5 were elevated in CNS tissue from EAE mice. There was no significant difference in MCP-2 expression in CNS tissue between MCP-1+/+ and MCP-1−/− group (23.8 ± 0.4 vs. 23.7 ± 0.5, mean ± SD; n = 5 in each group). No significant difference of MCP-3 expression in CNS tissue from MCP-1+/+ (31.0 ± 0.6, n = 5) and MCP-1−/− (33.1 ± 2.6, n = 5, P = 0.4) mice was found, whereas CNS MCP-5 expression in mice with EAE was essentially undetectable (data not shown). The unaltered levels of MCP-2 and MCP-5 and a trend towards decreased expression of MCP-3 in MCP-1−/− mice (higher PCR cycle number) uncovered no compensatory expression of other MCPs in this system, consistent with a previous report in autoimmune kidney disease model in MCP-1−/− MRL-Fas<sup>−/−</sup> mice (61). These findings supported the hypothesis that the similarity of EAE phenotype in MCP-1−/− and CCR2−/− deficient mice is caused by absence of the ligand, i.e., MCP-1 signaling pathway through its major receptor CCR2.

T Cell Proliferation to MOG35–55 Peptide. To examine the afferent limb of the immune response to MOG peptide in wild-type and MCP-1−/− mice, MNC suspensions were prepared from PILNs primed with 200 μg MOG35–55 peptide in CFA and rechallenged with MOG35–55 in vitro. MCP-1−/− mice showed a nonsignificantly higher recall T cell response than wild-type controls. No T cell recall response was induced by stimulation with PLP139–151 in vitro, a specificity control. No difference in anti-CD3ε–induced T cell proliferation was found between wild-type and MCP-1−/− deficient mice (data not shown). These data indicated that the CD3 pathway was intact in MCP-1−/− mice, and that MOG35–55–specific T cells can be generated in MCP-1−/− mice.

MCP-1−/− Mice Do Not Develop Clinical EAE in Passive Transfer Model. An adoptive transfer EAE model was used to further address whether MCP-1−/− mice could develop pathogenic autoimmune responses to MOG35–55 peptide. MCP-1+/+ and MCP-1−/− B6 mice were immunized with MOG35–55 peptide in CFA, and MNCs isolated from draining lymph nodes were cultured in the presence of MOG35–55 peptide and IL-12 before transfer into MCP-1−/− mice or littermate controls. As expected, MCP-1+/+ mice receiving MCP-1+/+ T cells developed clinical EAE (Fig. 5). MCP-1−/− T cells showed approximately the same encephalitogenic capacity in this adoptive transfer model, resulting in a comparable incidence, severity, and clinical course of EAE in MCP-1−/− recipients. In contrast, MCP-1−/− mice that received MCP-1+/+ T cells failed to develop clinical EAE (Fig. 5). This result indicated that the attenuated EAE in MCP-1−/− mice was not caused by impaired generation of encephalitogenic T cells. The data also demonstrated that absence of MCP-1 expression in the recipient rendered the mice unable to respond to encephalitogenic signals produced by wild-type T cells.

To dissect the mechanisms underlying the relatively resistance to EAE induction in MCP-1−/− deficient mice, the MOG35–55–induced EAE model was used in the following mechanistic studies. To obtain samples from MCP-1−/− mice with full-blown EAE, F10 mice were immunized with MOG35–55 in CFA plus 500 ng pertussis toxin per injection.

Reduced CD11b<sup>+</sup>CD4<sup>+</sup>/CD4<sup>+</sup> Ratio but Unchanged Levels of CD3ε and CD8 Transcripts in CNS Tissue from MCP-1−/− Mice with EAE. Results described above suggested that MCP-1−/− mice were deficient in recruiting mono-
cytes to the CNS during EAE. To address this issue, leukocytes were isolated from CNS tissue of wild-type and MCP-1–null mice with comparable severity of EAE, and analyzed with flow cytometry. Cell numbers in preparations isolated from MCP-1−/− mice during EAE attacks (score 4) were ∼1/3 of those from MCP-1+/+ littermate controls with comparable EAE severity. Compared with MCP-1+/+ littermate controls, MCP-1−/− mice showed a sharply reduced percentage of CD11b+CD4− cells in the CNS during EAE attacks (71.8 ± 4.6% vs. 44.0 ± 4.2%, mean ± SD, n = 5 and 4, respectively; P < 0.0001). In contrast, percentages of CD4+ cells were relatively increased in MCP-1−/− mice (20.2 ± 5.9% vs. 39.8 ± 2.7%, mean ± SD, n = 6 and 4, respectively; P < 0.001; Fig. 6).

The CD4+ infiltrating T cells expressed high levels of CD11b (α5β2 integrin) both in MCP-1+/+ (79.0 ± 5.6, n = 5) and MCP-1−/− (78.1 ± 6.8, n = 5) mice, indicating that most infiltrating CD4+ T cells are activated.

To normalize percentage of CD4+ T cells in the CNS infiltrates of wild-type and MCP-1–null mice with EAE, total CNS T cells were analyzed by determining levels of CD3e in the CNS. We found no significant difference in CNS CD3e mRNA levels between wild-type (0.789, n = 5; CD3e/GAPDH) and MCP-1−/− (0.791, n = 5; CD3e/GAPDH) mice with clinical EAE scores of 3.5–4.0, suggesting that total T cell numbers in the CNS of wild-type and MCP-1–null mice with EAE were equivalent. Therefore, the increased proportion of CD4+ cells in the CNS leukocyte infiltrates of MCP-1–null mice was caused by a marked reduction in the number of CD11b+CD4− cells in the CNS of MCP-1–deficient mice with EAE.

CD8+ T cells play an important downregulatory role in the pathogenesis of EAE (62). The possibility that the milder clinical EAE phenotype observed in MCP-1−/− mice might be due to an increased number of CD8+ T cells in the CNS infiltrates was unlikely based on the fact that levels of CD8-specific mRNA were virtually identical in MCP-1−/− (24.08 ± 0.3, n = 5) and MCP-1+/+ (24.21 ± 0.2, n = 5) CNS tissue during EAE attack. The passive transfer EAE model was used to examine further if CD8+ T cells can be preferentially recruited into CNS in the absence of MCP-1. MOG35–55–reactive MCP-1+/+ T cells were incubated in the presence of MOG35–55 and IL-12, and were injected intravenously into MCP-1+/+ and MCP-1−/− mice. To reduce the influence of secondarily recruited macrophages in the CNS, recipient mice were killed at day 3 and 4 after T cell transfer, before the onset of clinical EAE. CNS-infiltrating T cells were recovered and analyzed using flow cytometry. No difference was found between MCP-1+/+ and MCP-1−/− mice (data not shown). These results demonstrated that disruption of MCP-1 gene exerts no significant impact on the recruitment of adoptively transferred T cells into inflammatory CNS tissue. No significant difference in Mb-1 mRNA levels was found in EAE-affected CNS tissue from MCP-1+/+ and MCP-1−/− mice (data not shown).

We also examined the leukocyte infiltrates in MCP-1−/− mice at day 14 pi when the MCP-1+/+ controls were undergoing EAE attacks while the MCP-1−/− mice were still free of EAE signs. Shown in Fig. 6 C are numerous CD45+CD11b+ (mainly containing macrophages/activated microglia and activated T cells) isolated from MCP-1−/− mice during EAE attack on day 14 pi. In contrast, the majority of cells isolated from MCP-1−/− mice were CD45+CD11b− microglia, and the components of the infiltrates (Fig. 6 D) were virtually the same as those from healthy unimmunized mice (data not shown).

**Diminished MOG35–55–specific Th1 Cytokine Responses in MCP-1−/− Mice.** Significant changes in cytokine production have been described in the Th1 immune response that typifies MOG35–55 peptide–induced EAE in B6 mice (35). Serum concentrations of IFN-γ, IL-4, and IL-10 were determined by ELISA from mice immunized with MOG35–55 on days 8 and 10 pi and at the peak of EAE (day 14 pi in wild-type controls and 25 pi in MCP-1–deficient mice). At day 8 pi, concentrations of IFN-γ were slightly but significantly higher in wild-type than in MCP-1–deficient mice. This difference between wild-type and MCP-1–deficient mice became strikingly evident on day 10 pi near the onset of EAE in wild-type controls. The onset of EAE was also associated with increased serum levels.
of IFN-γ in MCP-1−/− deficient mice, but the magnitude of increase was significantly less than in MCP-1+/+ mice (Fig. 7 A). Circulating IL-10 was detected at low levels in both MCP-1+/+ and MCP-1−/− mice before and after the onset of EAE. Before EAE onset at day 8 pi (Fig. 7 B), serum IL-10 was slightly but significantly higher in MCP-1−/− mice than in wild-type controls. Serum IL-4 remained below the limits of detection in both MCP-1+/+ and MCP-1−/− mice at all examined occasions during the cell culture, PILN cells from mice immunized with MOG35–55 in CFA. Upon rechallenge with MOG35–55 peptide in vitro, at all examined occasions during the cell culture, PILN cells from MCP-1−/− mice secreted ~50% less IFN-γ than cells from MCP-1+/+ mice (Fig. 8, left). IL-4 and IL-10, signature Th2 cytokines in EAE (26, 29, 63, 64), were also measured. Although low levels of IL-10 were found in the culture supernatants of restimulated PILN cells from both MCP-1+/+ and MCP-1−/− mice, significantly higher levels of IL-10 were detected in cultures of cells from MCP-1−/− mice (Fig. 8, right). IL-4 was undetectable in all cell culture supernatants.

CNS Cytokine mRNA Accumulation in MCP-1−/− and Wild-type Mice with EAE. Local expression of IFN-γ in the CNS was analyzed by real-time RT-PCR. Significantly higher geometric mean levels of IFN-γ were found in spinal cord tissue from MCP-1+/+ mice at the peak of EAE attacks (MCP-1+/+: 27.7 ± 0.3, mean ± SD, n = 4; MCP-1−/−: 29.9 ± 0.8, n = 4, P < 0.05). This result indicated approximately a fourfold difference in the CNS expression of IFN-γ between mice with intact and disrupted MCP-1 genes, despite equal numbers of CNS-infiltrating T cells (see above). There was no difference in IL-10 expression in CNS tissue between MCP-1+/+ and MCP-1−/− mice with full-blown EAE. IL-4 gene expression was undetectable both in MCP-1+/+ and MCP-1−/− mice (data not shown).

Decreased Expression of IFN-γ–inducible 10-kD Protein, Macrophage Inflammatory Protein 1α, and RANTES in CNS Tissue from MCP-1−/− Mice with EAE. CNS chemokine expression was quantified using RPA, in tissues from MCP-1+/+ and MCP-1−/− mice equally affected by EAE (score 3.5–4.0). MCP-1−/− mice had significantly lower levels of IFN-γ–inducible 10-kD protein (IP-10), macrophage inflammatory protein (MIP)-1α, and regulated upon activation, normal T cell expressed and secreted (RANTES) transcripts compared with wild-type littermate controls (Fig. 9). Expression of MCP-3 was low in MCP-1+/+ and MCP-1−/− mice at the peak of EAE attack without significant differences between MCP-1−/− mice and littermate controls, supporting the results obtained using real-time RT-PCR. T cell activation gene (TCA)-3 expression in the CNS of MCP-1+/+ and MCP-1−/− mice with EAE was near the lower limits of detection. No significant difference was found in CCR gene expression (data not shown). However, there was a nonsignificant trend towards decreased levels of CCR2 expression in MCP-1−/− EAE CNS tissue compared with wild-type controls. In the ab-
sence of MCP-1, the presence of CCR2 in the affected tissue might indicate the action of other CCR2 ligand(s). Alternatively, the migration of CCR2-bearing cells into CNS might be a bystander phenomenon.

Compelling evidence has shown that IP-10, MIP-1α, and RANTES are potent factors that attract Th1 T cells into sites of inflammation (65–70). Further, Th1/Th2 T cells have been recently reported to differentially secrete RANTES, lymphotactin, and TCA-3, respectively (71). Enhanced expression of IP-10, MIP-1α, and RANTES in MCP-1<sup>+/+</sup> EAE CNS tissue and undetectable TCA-3 expression in either MCP-1<sup>−/−</sup> or MCP-1<sup>+/−</sup> support the notion that immune reactions within the CNS during EAE attacks are Th1 biased and such responses are more pronounced in mice with an intact MCP-1 gene.

**Anti-MOG Ig Isotypes in MCP-1<sup>−/−</sup> and Wild-type Mice.** Sera from MCP-1<sup>−/−</sup> and MCP-1<sup>+/−</sup> mice with EAE were analyzed for total IgG, IgG1, and IgG2a Abs against the immunizing MOG35–55 peptide. Wild-type and MCP-1−deficient mice produced similar amounts of total MOG-specific IgG (Fig. 10). Despite the disparity in clinical severity between wild-type and MCP-1-null mice, this finding was not unexpected, given the results of experiments using B cell–deficient mice, which showed that Ig does not play an important pathogenic role in MOG35–55 peptide-induced EAE in B6 mice (48).

However, Ig isotype analyses differentiated the wild-type and MCP-1-deficient mice. Levels of anti-MOG35–55 IgG1 Abs in wild-type controls remained low from day 14 pi through day 60 pi (Fig. 10) and were not elevated at intermediate time points (data not shown). In contrast, significantly higher levels of anti-MOG35–55 IgG1 Abs were evident in MCP-1<sup>−/−</sup> mice (Fig. 10). Levels of anti-MOG35–55 IgG2a Abs showed a trend towards elevated levels in wild-type controls on day 14 pi, whereas no difference between wild-type and MCP-1−null mice was found on day 60 pi (Fig. 10).

Taken together, these results suggest that a polarized MOG35–55–induced Th1 immune response in wild-type mice leads to a suppressed Th2 response, characterized by undetectable IL-4, lower levels of IL-10, lower levels of anti-MOG35–55 IgG1, and higher levels of IgG2a. In this model, the absence of MCP-1 results in a shift towards a Th2-biased response, with reduced production of IFN-γ, enhanced secretion of IL-10, and higher levels of IgG1.

**Discussion**

We and others have previously shown that MCP-1 was markedly elevated in the CNS of SJL and B6 mice with EAE (33, 35) and levels of MCP-1 expression correlated with the severity of relapsing EAE (72). Anti-MCP-1 Abs blocked relapses of EAE (36). CNS MCP-1 is largely produced by parenchymal astrocytes (34). MCP-1 expression by astrocytes in MS brain lesions has also been convincingly documented (40–42).

However, these studies did not establish a primary role for MCP-1 in disease pathogenesis. In recent definitive studies of EAE using CCR2-deficient mice (37, 38), the relevant ligand for the deleted receptor was not defined. Moreover, MCP-1 exhibits attributes that argue for a role in restraining autoimmune demyelination. In particular, MCP-1 exerts a direct or indirect (via IL-4) impact on Th2 T cell development (11). Further, the presence of MCP-1 in vitro cell culture systems decreased the encephalitogenic potential of T cells directed to PLP139–151 (36). NK cells that inhibited the encephalitogenic potential of autoaggressive T cells in DA rats produced high levels of MCP-1 in vitro (73). The role(s) of MCP-1 in the pathogenesis and development of EAE (MS) has therefore been uncertain. Using gene-targeted mice, we demonstrate that lack of MCP-1 delays the onset of EAE and ameliorates its severity, by reducing the accumulation of inflammatory leukocytes within CNS. This phenotype was associated with impaired MOG35–55–specific Th1 immune responses.

Impaired macrophage recruitment into the CNS, as indicated by reduced total number of cells and percentage of CD11b<sup>+</sup>CD45<sup>+</sup>CD4<sup>+</sup> cells recovered from CNS in MCP-1−deficient mice in our study, is consistent with the reduction of macrophages in MCP-1<sup>−/−</sup> mice in contact hypersensitivity responses (2), in kidney and lung lesions of MCP-1−/− MRL-<sup>Faslpr</sup> mice (74), in aortic walls of MCP-1−/− and low density lipoprotein receptor double-deficient mice (75), and in atherosclerosis plaques from MCP-1<sup>−/−</sup> mice that overexpress apolipoprotein B (76).

Compelling evidence suggests that macrophages and their products can be detrimental in EAE and human MS. Expression of MHC class II is markedly elevated in the CNS tissue and undetectable TCA-3 expression in either MCP-1<sup>−/−</sup> or MCP-1<sup>+/−</sup> mice with EAE (33, 35) and levels of MCP-1 expression correlated with the severity of relapsing EAE (72). Anti–MCP-1 Abs blocked relapses of EAE (36). CNS MCP-1 is largely produced by parenchymal astrocytes (34). MCP-1 expression by astrocytes in MS brain lesions has also been convincingly documented (40–42).

However, these studies did not establish a primary role for MCP-1 in disease pathogenesis. In recent definitive studies of EAE using CCR2-deficient mice (37, 38), the relevant ligand for the deleted receptor was not defined. Moreover, MCP-1 exhibits attributes that argue for a role in restraining autoimmune demyelination. In particular, MCP-1 exerts a direct or indirect (via IL-4) impact on Th2 T cell development (11). Further, the presence of MCP-1 in in vitro cell culture systems decreased the encephalitogenic potential of T cells directed to PLP139–151 (36). NK cells that inhibited the encephalitogenic potential of autoaggressive T cells in DA rats produced high levels of MCP-1 in vitro (73). The role(s) of MCP-1 in the pathogenesis and development of EAE (MS) has therefore been uncertain. Using gene-targeted mice, we demonstrate that lack of MCP-1 delays the onset of EAE and ameliorates its severity, by reducing the accumulation of inflammatory leukocytes within CNS. This phenotype was associated with impaired MOG35–55–specific Th1 immune responses.

Impaired macrophage recruitment into the CNS, as indicated by reduced total number of cells and percentage of CD11b<sup>+</sup>CD45<sup>+</sup>CD4<sup>+</sup> cells recovered from CNS in MCP-1−deficient mice in our study, is consistent with the reduction of macrophages in MCP-1<sup>−/−</sup> mice in contact hypersensitivity responses (2), in kidney and lung lesions of MCP-1−/− MRL-<sup>Faslpr</sup> mice (74), in aortic walls of MCP-1−/− and low density lipoprotein receptor double-deficient mice (75), and in atherosclerosis plaques from MCP-1<sup>−/−</sup> mice that overexpress apolipoprotein B (76).

Compelling evidence suggests that macrophages and their products can be detrimental in EAE and human MS. Expression of MHC class II is markedly elevated in the CNS tissue and undetectable TCA-3 expression in either MCP-1<sup>−/−</sup> or MCP-1<sup>+/−</sup> mice with EAE (33, 35) and levels of MCP-1 expression correlated with the severity of relapsing EAE (72). Anti–MCP-1 Abs blocked relapses of EAE (36). CNS MCP-1 is largely produced by parenchymal astrocytes (34). MCP-1 expression by astrocytes in MS brain lesions has also been convincingly documented (40–42).

However, these studies did not establish a primary role for MCP-1 in disease pathogenesis. In recent definitive studies of EAE using CCR2-deficient mice (37, 38), the relevant ligand for the deleted receptor was not defined. Moreover, MCP-1 exhibits attributes that argue for a role in restraining autoimmune demyelination. In particular, MCP-1 exerts a direct or indirect (via IL-4) impact on Th2 T cell development (11). Further, the presence of MCP-1 in in vitro cell culture systems decreased the encephalitogenic potential of T cells directed to PLP139–151 (36). NK cells that inhibited the encephalitogenic potential of autoaggressive T cells in DA rats produced high levels of MCP-1 in vitro (73). The role(s) of MCP-1 in the pathogenesis and development of EAE (MS) has therefore been uncertain. Using gene-targeted mice, we demonstrate that lack of MCP-1 delays the onset of EAE and ameliorates its severity, by reducing the accumulation of inflammatory leukocytes within CNS. This phenotype was associated with impaired MOG35–55–specific Th1 immune responses.

Impaired macrophage recruitment into the CNS, as indicated by reduced total number of cells and percentage of CD11b<sup>+</sup>CD45<sup>+</sup>CD4<sup>+</sup> cells recovered from CNS in MCP-1−deficient mice in our study, is consistent with the reduction of macrophages in MCP-1<sup>−/−</sup> mice in contact hypersensitivity responses (2), in kidney and lung lesions of MCP-1−/− MRL-<sup>Faslpr</sup> mice (74), in aortic walls of MCP-1−/− and low density lipoprotein receptor double-deficient mice (75), and in atherosclerosis plaques from MCP-1<sup>−/−</sup> mice that overexpress apolipoprotein B (76).
clinical relapses of EAE (79). B7–1/B7–2−/− mice are resistent to EAE induction (47). Similarly, blocking of interactions between CD40 on macrophages and CD40L on T cells has been shown to effectively prevent EAE (80). Products of macrophages like TNF-α, IFN-γ, and nitric oxide have also been demonstrated to be critical in the effector phase of EAE (81, 82). Macrophage depletion inhibits the induction of EAE (83). The absence of clinical EAE in MCP−1−/− recipients of wild-type encephalitogenic T cells further indicates the importance of CNS MCP-1 expression in recruiting macrophages to the CNS. We propose that is the failure to recruit significant number of macrophages into CNS that constitutes the principal mechanism for resistance to EAE induction in MCP−1−/− deficient mice. Based on these studies, we cannot exclude the possibility that MCP−1 may directly alter trafficking pattern of dendritic cells in periphery or CNS, expression of costimulators, inflammatory cytokines, and adhesion molecules. Further studies are underway to address these issues.

As several studies have shown that MCP−1 is a critical factor for T cell commitment to the Th2 phenotype, we did not anticipate that MCP−1 gene disruption would result in reduced MOG35−55−specific Th1 immune response in these EAE experiments. Our results show that MOG35−55−specific MCP−1−/− T cells secreted a large amount of IFN-γ, although less than MCP−1+/+ T cells, but undetectable levels of IL-4. In view of the reciprocal regulation between IFN-γ and IL-10, the enhanced in vitro secretion of IL-10 by MCP−1−/− T cells might be secondary to reduced levels of IFN-γ. However, increased expression of IL-10 was not observed in vivo as demonstrated by the equal amount of IL-10 transcripts in MCP−1+/+ and MCP−1−/− EAE CNS tissue. The fact that MOG35−55−reactive MCP−1−/− T cells mediated severe EAE in wild-type recipient mice in the passive transfer EAE model further suggests that they were Th1 polarized. An explanation for the equal encephalitogenic capacity of MCP−1−/− T cells compared with MCP−1+/+ T cells could be that the defective IFN-γ production by MCP−1−/− T cells might be corrected by the presence of IL-12 in the cell culture system (84). Alternatively, IFN-γ concentrations beyond a threshold may be dispensable for encephalitogenic potential. T cells from MCP−1–null mice were shown to produce lower levels of IFN-γ in vitro upon MOG35–55 restimulation in our studies, and MCP−1−/− splenocytes secreted ~50% less IFN-γ when restimulated with Schistosoma mansoni eggs in vitro (2), implying a role for MCP-1 in maximal expression of this cytokine under some circumstances. On the premise that MCP-1 is the major ligand for CCR2 in this model, our results are consistent with what has been recently reported in CCR2-deficient mice (38). Thus, a dual function is suggested for MCP-1 in regulating T cell immune responses: promoting Th2 immune responses in certain circumstance while facilitating Th1 responses in others. Such difference is not uncommon when molecules were tested in different animal strains, disease models, and using different immunogens.

We propose that the role of MCP−1 in EAE became manifest because of the extreme Th1 polarization implicated in this model. The impaired ability to mount Th2 responses was not relevant in these experiments because the disease was severely attenuated by the reduction of macrophage recruitment to the CNS. Such reduction of macrophage reaction might subsequently result in reduced Th1 immune responses. Remarkably, in the absence of recruited macrophages, highly polarized Th1 cells became unable to express the Th1 effector program, most clearly demonstrated by decreased circulating and CNS IFN-γ and failure to elicit EAE in MCP−1-deficient mice by MOG−primed MCP−1+/+ encephalitogenic T cells.

Taken in the context of recent reports (37, 38), our results indicate that the MCP-1/CCR2 ligand/receptor pair is critical for the expression of EAE in mice. In turn, these findings motivate a continuing effort to characterize the function of this multipotential chemokine in human disease.

We thank Dr. W.J. Karpus (Northwestern University) for helpful discussion and sharing data before publication.

This work was supported by the National Institutes of Health (2RO1 NS32151 and 1PO1 NS38667 to R.M. Ransohoff; 2RO1 CA53091 to B.J. Rollins), and The National Multiple Sclerosis Society with a Pilot Project award to B.J. Rollins. We gratefully acknowledge the Williams Family Foundation for MS Research. D. Huang is a scholar of the Morgenthaler Family Foundation.

Submitted: 2 October 2000
Revised: 8 February 2001
Accepted: 13 February 2001

References

70. Sallusto, F., C.R. Mackay, and A. Lanzavecchia. 1997. Selec-


