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Chemokine-Like Receptor-1 Expression by Central Nervous System-Infiltrating Leukocytes and Involvement in a Model of Autoimmune Demyelinating Disease[1]

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Abstract

We examined the involvement of chemokine-like receptor-1 (CMKLR1) in experimental autoimmune encephalomyelitis (EAE), a model of human multiple sclerosis. Upon EAE induction by active immunization with myelin oligodendrocyte glycoprotein amino acids 35–55 ($MOG_{35–55}$), microglial cells and CNS-infiltrating myeloid dendritic cells expressed CMKLR1, as determined by flow cytometric analysis. In addition, chemerin, a natural ligand for CMKLR1, was up-regulated in the CNS of mice with EAE. We found that CMKLR1-deficient (CMKLR1 knockout (KO)) mice develop less severe clinical and histologic disease than their wild-type (WT) counterparts. CMKLR1 KO lymphocytes proliferate and produce proinflammatory cytokines in vitro, yet $MOG_{35–55}$-reactive CMKLR1 KO lymphocytes are deficient in their ability to induce EAE by adoptive transfer to WT or CMKLR1 KO recipients. Moreover, CMKLR1 KO recipients fail to fully support EAE induction by transferred MOG-reactive WT lymphocytes. The results imply involvement of CMKLR1 in both the induction and effector phases of disease. We conclude that CMKLR1 participates in the inflammatory mechanisms of EAE and represents a potential therapeutic target in multiple sclerosis.

Experimental autoimmune encephalomyelitis (EAE)[3] is a widely studied animal model of multiple sclerosis (MS), an inflammatory demyelinating disease of the CNS of unknown etiology. Early tissue injury in EAE and MS is mediated by myelin Ag-specific $CD4^+$ T lymphocytes (1) that require expression of αA integrin for recruitment from the blood and entry into the CNS parenchyma (2). Blockade of αA integrin suppresses MS and EAE (3,4). However, the development of EAE and MS is marked by tightly controlled regulation of other adhesion molecules, as well as numerous chemoattractant receptors and their respective ligands (5) that are thought to help control immune cell recruitment, microenvironmental positioning,

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Disclosures

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[3]Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; CMKLR1, chemokine-like receptor-1; DC, dendritic cell; KO, knockout; LN, lymph node; mDC, myeloid DC; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; pDC, plasmacytoid DC; p.i., postimmunization; PLC, peritoneal lavage cell; WT, wild type.

and function within the inflamed CNS. In addition to $CD4^+$ T cells, MS and EAE lesions contain several other cell types, including recruited B lymphocytes, $CD8^+$ T lymphocytes, macrophages, and CNS-resident cells (e.g., astrocytes and microglia) (reviewed in Ref. 6). Trafficking and chemoattractant receptors differentially expressed by these populations thus offer a rich potential for regulating pathogenic CNS inflammation.

Chemokine-like receptor-1 (CMKLR1; also known as ChemR23 or Dez) is a chemoattractant receptor that is expressed by unique subsets of dendritic cells (DC), as well as tissue-resident macrophages in mice and humans (7–9). CMKLR1-expressing cells migrate to chemerin, a proteolytically regulated chemoattractant (9–11). TLR ligands and cytokines regulate CMKLR1 expression on ex vivo mouse macrophages (8). Macrophages and DC are prominent in EAE and MS inflammatory lesions and have critical roles in mediating tissue injury. These cells can contribute to the disease process through multiple mechanisms, including production of proinflammatory cytokines, Ag processing and presentation to autoreactive T lymphocytes, and production of reactive oxygen species that directly induce damage to myelin (1,12,13). We hypothesized that CMKLR1 plays a role in inflammatory processes within the CNS. In this report, we evaluated the role of CMKLR1 in the EAE model of MS.

Materials and Methods

Mice

CMKLR1 knockout (KO) mice were obtained from Deltagen and fully backcrossed (nine generations) onto the C57BL/6 background. C57BL/6 control mice were purchased from The Jackson Laboratory. Female mice (8–12 wk old) were used in all experiments. All animal experiments were conducted in accordance with approved Stanford and National Institutes of Health Institutional Animal Care and Use Committee guidelines.

EAE induction by active immunization

Myelin oligodendrocyte glycoprotein (MOG) peptide amino acids 35–35 (MEVGWYRSPFSRVVHLYRNGK; $MOG_{35–55}$) was synthesized by the Stanford Protein and Nucleic Acid Facility (Stanford, CA). For active EAE, $MOG_{35–55}$ was dissolved in PBS at 2 mg/ml. The $MOG_{35–55}$ solution was then emulsified in an equal volume of CFA, consisting of incomplete Freund's adjuvant (Difco) plus 4 mg/ml heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco). The emulsion (100 μl) was injected s.c.; mice were given an i.v. boost of 400 ng pertussis toxin (List Biological Laboratories) at the time of immunization and again 2 days later. Clinical EAE was assessed daily as previously described (14).

EAE induction by adoptive transfer

Mice were immunized s.c. with 100 μg $MOG_{35–55}$ in CFA. Draining lymph nodes (LN) and spleen cells were harvested 10 days later and resuspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, and 2-ME. $MOG_{35–55}$ was added at 10 μg/ml and recombinant murine IL-12 (R&D Systems) at 10 ng/ml. After 4 days in culture (37°C, 8% CO_2), lymphocytes were isolated using Lympholyte-Mammal (Cedarlane Laboratories), pooled, and resuspended in HBSS for transfer. Mice received $1–2 \times 10^7$ viable cells i.v. Pertussis toxin (400 ng) was given i.v. immediately after cell transfer and again 2 days later. Transferred lymphocytes were >95% $CD3^+$, as determined by flow cytometric analysis (data not shown).

Generating the anti-CMKLR1 mAb BZ186

CMKLR1 KO mice were immunized via s.c. injection of $\sim 4 \times 10^7$ wild-type (WT) peritoneal exudate cells. For the first immunization, cells were suspended in saline and emulsified in CFA;

incomplete Freund's adjuvant was used for two subsequent injections. Hybridomas producing anti-mouse CMKLR1 mAb were subcloned. Specificity was confirmed by reactivity with mouse CMKLR1 transfectants and lack of reactivity with human CMKLR1 transfectants. ELISA (BD Pharmingen) was used to determine the isotype (IgG1κ) of the resulting mouse anti-mouse CMKLR1 mAb, designated BZ186.

ELISAs

Mice were immunized s.c. with 100 μg $MOG_{35–55}$ in CFA. After 10 days, draining LN and spleen cells were harvested and resuspended in RPMI 1640 with supplements. Cells were plated at 2×10^5 cells/well in flat-bottom 96-well plates, $MOG_{35–55}$ peptide was added at various concentrations, and cells were incubated at 37°C, 8% CO_2. Culture supernatants were harvested at 72 h and levels of IFN-γ, TNF (both from BD Pharmingen), and IL-17 (eBioscience) in triplicate wells were determined by sandwich ELISA according to the manufacturer's instructions.

Proliferation assays

LN and spleen cells were cultured as described above for 72 h in triplicate wells with a range of $MOG_{35–55}$ concentrations. [^{3}H]Thymidine was added for the last 18–24 h of culture and thymidine incorporation was assessed using a β-plate scintillation counter.

CNS mononuclear cell preparation

Mice were perfused through the heart with 30 ml cold PBS. Spinal cords were extracted, minced, and incubated with HBSS containing 0.2 U of Liberase R1 (Roche), 50 μg/ml DNase I (Roche), and 25 mM HEPES for 30 min at 37°C. Digested tissue was forced through stainless steel mesh and mononuclear cells were collected from 30:70% discontinuous Percoll gradients.

Flow cytometry

mAbs directed against mouse CD3 (145–2C11), CD11c (HL3), CD11b (M1/70), CD19 (1D3), CD45 (30-F11), and CD45R/B220 (RA3–6B2) were from eBioscience or BD Pharmingen. For flow cytometric analysis of CMKLR1 expression, PE-conjugated rat anti-mouse IgG1 (BD Pharmingen) was used to detect BZ186 or its isotype control, mAb DREG200 (mIgG1 with specificity for human L-selectin (CD62L)). Staining buffer consisted of PBS containing 2% BSA plus 0.1% sodium azide. Data were acquired using an LSRII flow cytometer and analyzed with FlowJo Software.

Histology

Brains and spinal cords were extracted and fixed in 10% buffered formalin. Formalin-fixed tissue was embedded in paraffin and sections were stained with Luxol fast blue-H&E stain. CNS inflammatory foci (>10 mononuclear cells/focus) in leptomeninges and parenchyma were counted in each mouse sample in a blinded fashion by one of the authors (R.A.S.).

For immunohistochemical analysis, sections of paraffin-embedded brain and spinal cord tissue were subjected to sodium citrate Ag retrieval, followed by immunostaining with anti-F4/80 mAb (Serotec). Biotinylated, species-specific secondary was added (Vector Laboratories), followed by incubation with ABC reagent (Vector Laboratories). The reactions were developed using diaminobenzidine (Sigma-Aldrich) and visualized by light microscopy.

The incidence of demyelinating lesions in the CNS tissues was assessed using a semiquantitative scoring system. The presence (score = 1) or absence (score = 0) of a demyelinated lesion as indicated by loss of blue staining in Luxol fast blue-stained sections was determined in four white matter regions (posterior columns, anterior columns, and two

lateral columns), in two cross-sections of the spinal cord, the brain stem, and cerebellar white matter in each mouse. The maximum total score for each mouse was 10.

Statistics

Nonparametric clinical EAE data were analyzed using the Mann-Whitney *U* test. All parametric data were analyzed using the Student's *t* test. Fisher's exact test was used to compare disease incidence. Values of p less than 0.05 were considered statistically significant.

Results

Attenuation of clinical EAE in CMKLR1 KO mice

CMKLR1 KO mice develop normally; they are healthy, fertile, and have no overt immune system abnormalities or defects. We evaluated the cellular composition of blood, thymus, bone marrow, spleen, and LN derived from WT and CMKLR1 KO mice. There were no differences between the two groups with respect to total numbers or percentages of numerous leukocyte subsets, including T cells, B cells, NK cells, monocytes/macrophages, granulocytes, or DC in any of the tissues analyzed (data not shown).

To evaluate the role of CMKLR1 in a model of autoimmune demyelinating disease, we induced EAE in CMKLR1 KO mice by active immunization. There was no significant difference between CMKLR1 KO and WT mice with respect to day of disease onset (Fig. 1 and Table I). Disease incidence was also similar between the two groups: 33 of 33 (100%) WT mice developed clinical EAE vs 30 of 33 (91%) of CMKLR1 KO mice (Table I). CMKLR1 KO mice did not, however, develop the same severity of acute EAE as their WT counterparts. The average maximal disease score for WT mice was 3.4, compared with 2.6 for CMKLR1 KO mice ($p < 0.005$, as determined by Mann-Whitney *U* test; Table I). In addition, clinical EAE in CMKLR1 KO mice was significantly reduced throughout the chronic phase of disease (Fig. 1). Together, these data indicate that CMKLR1 KO mice are susceptible to EAE induction by active immunization, but that CMKLR1 is required for maximal acute EAE. Also, CMKLR1 may promote chronic/progressive EAE.

Reduced histological EAE in CMKLR1 KO mice

EAE and MS are characterized by perivascular inflammatory cell infiltrates located predominantly in the CNS white matter. We evaluated CNS lesions in WT and CMKLR1 KO mice, analyzing brain and spinal cord tissue harvested either during the acute or chronic phase of EAE (13 or 46 days post-immunization (p.i.), respectively). As shown in Table II, WT and CMKLR1 KO mice had similar numbers of parenchymal inflammatory foci at day 13 p.i. In contrast, CMKLR1 KO mice had significantly fewer meningeal inflammatory foci at this time point (Fig. 2, *A* and *B*). CMKLR1 KO mice with chronic EAE (day 46 p.i.) also had significantly fewer meningeal and parenchymal inflammatory lesions than their WT counterparts (Table II and Fig. 2, *C* and *D*). Consistent with reduced inflammation, there was a trend toward reduced demyelination in CMKLR1 KO mice with chronic EAE (day 46 p.i.). Although the difference did not achieve statistical significance ($p = 0.06$), mice with the most extensive demyelination may have died before the end of the experiment (mortality rate was 9 of 33 (27%) in WT mice vs 2 of 33 (6.1%) in CMKLR1 KO animals). Thus, our data do not exclude a role for CMKLR1 in the vulnerability of the myelin to demyelination or the capacity of oligodendrocytes to remyelinate lesions. Demyelination at the early (day 13 p.i.) time point was generally limited to perivascular areas associated with inflammatory cuffs. At the later time point (day 46 p.i.), demyelinated foci were generally larger and less inflammatory in both WT and CMKLR1 KO mice (Table II).

CNS inflammatory lesions of CMKLR1 KO mice contain fewer F4/80$^+$ cells

Because CMKLR1 is expressed by F4/80$^+$CD11b$^+$ mouse macrophages (8), we evaluated CNS tissue from mice with EAE for F4/80$^+$ cells by immunohistochemistry. Analysis of CNS tissue harvested at day 13 p.i. revealed no qualitative or quantitative differences in F4/80 staining between WT and CMKLR1 KO mice (data not shown). In contrast, WT mice with chronic EAE had more F4/80$^+$ macrophages in the leptomeninges than CMKLR1 KO animals (day 46 p.i.). Compared with CMKLR1 KO mice, WT CNS tissue also contained more foamy macrophages and microglia in parenchymal lesions (Fig. 2, *E* and *F*). Together, these results are consistent with a contribution of CMKLR1 to recruitment of F4/80$^+$ cells to the CNS.

We used flow cytometry to quantify leukocyte subsets isolated from the spinal cords of WT and CMKLR1 KO mice during the preclinical and acute phases of EAE. There were no differences between WT and CMKLR1 KO mice with EAE with respect to absolute numbers or percentages of CD4$^+$ or CD8$^+$ T cells, B cells, CD11b$^+$ microglia, or CD11c$^+$ DC in the spinal cord (data not shown). Thus, although there is more inflammation in WT mice, CNS inflammatory lesions in KO and WT mice appear to recruit similar leukocyte subsets.

Lymphocyte proliferation and cytokine production

To determine whether lymphocyte activation defects contributed to differences in inflammatory cell infiltration of the CNS, we assessed recall proliferation and cytokine responses of WT and CMKLR1 KO lymphocytes to $MOG_{35–55}$ peptide. When they were restimulated with $MOG_{35–55}$ in vitro, CMKLR1 KO-draining LN cells and splenocytes proliferated at levels comparable to their WT counterparts (Fig. 3*A*). CMKLR1 KO-draining LN cells generally produced lower levels of IFN-γ, IL-17, and TNF than WT LN cells, but these differences did not reach statistical significance in pair-wise comparisons for most of the $MOG_{35–55}$ peptide concentrations tested (Fig. 3*B*, *left panels*). In contrast, CMKLR1 KO splenocytes produced IFN-γ, IL-17, and TNF at levels comparable or superior to their WT counterparts (Fig. 3*B*, *right panels*).

To further evaluate the immune activation and Ag-presenting capabilities of WT and CMKLR1 KO cells, we isolated CD3$^+$ T cells from the draining LN or spleens of MOG-immunized mice, followed by stimulation with $MOG_{35–55}$ in presence of mitomycin C-treated splenocytes. In an effort to distinguish between potential CMKLR1-related T cell and APC effects, we used all possible combinations of WT and CMKLR1 KO T lymphocytes and APC in these coculture studies. We found that CMKLR1 KO T cells were fully capable of proliferative and cytokine responses when stimulated with WT or CMKLR1 KO APC. In addition, CMKLR1 KO splenocytes induced robust proliferation and cytokine production by WT and CMKLR1 KO T cells (supplemental Fig. 1).[4] Thus, the reduced severity of EAE in CMKLR1 KO mice is not due to intrinsic defects in lymphocyte or APC functional capacity.

Induction of EAE by adoptive transfer

To determine the relative role of CMKLR1 expression on effector leukocytes vs recipient cells and tissues, we performed adoptive transfer experiments. $MOG_{35–55}$-reactive WT or CMKLR1 KO lymphocytes were generated and transferred into WT recipient mice (WT:WT or KO:WT transfers, respectively). CMKLR1 KO lymphocytes were much less effective than their WT counterparts at transferring EAE to WT recipients (Fig. 4). In addition, WT:KO and KO: KO transfers did not produce EAE with the same frequency as WT:WT transfers. Moreover, histologic disease was less severe when KO mice were either the donors or recipients of MOG-specific lymphocytes (Table III). These studies indicate that CMKLR1 expression on donor

[4]The online version of this article contains supplemental material.

mononuclear cells and on recipient cells or tissues is required for maximal EAE induction in an adoptive transfer setting. The failure of CMKLR1 KO recipients to develop EAE upon transfer with WT MOG-specific lymphocytes, cells that effectively induce EAE in WT recipients, indicates that CMKLR1 is involved in CNS inflammation during the effector phase of disease. Furthermore, the inability of MOG-reactive CMKLR1 KO lymphocytes to induce EAE upon adoptive transfer to WT hosts suggests an unexpected role for CMKLR1 in the induction phase of disease.

Peritoneal macrophage responses

We next evaluated WT and CMKLR1 KO peritoneal lavage cells (PLCs) for their responses to proinflammatory stimuli. To enrich for macrophages, nonadherent PLCs were removed by washing. Adherent cells were stimulated in vitro with LPS plus IFN-γ (LPS/IFN-γ). Resident PLCs derived from CMKLR1 KO mice produced IL-6 and TNF at levels similar to WT mice upon LPS/IFN-γ stimulation (supplemental Fig. 2*A*). To elicit inflammatory macrophages, we gave WT and CMKLR1 KO mice an i.p. injection of thioglycollate. Thioglycollate-elicited peritoneal macrophages from CMKLR1 KO mice were also competent in their ability to produce IL-6 and TNF in response to stimulation with LPS/IFN-γ (supplemental Fig. 2*B*). Thus, CMKLR1 KO resident and inflammatory macrophages are fully capable of mounting effector cytokine responses.

Analysis of CNS mononuclear cells by flow cytometry

CMKLR1 expression has been reported on APCs in mice and humans (7–10), as well as on human NK cells (15). Using a novel anti-CMKLR1 mAb, we analyzed CMKLR1 expression on mouse spinal cord mononuclear cells by flow cytometry. An earlier anti-CMKLR1 mAb, designated BZ194, was generated by immunizing rats with an amino-terminal CMKLR1 peptide. As reported previously (8), this mAb specifically stains mouse macrophages but not NK cells, even though NK cells express the receptor. The mAb used in the present study (BZ186) was generated by immunizing CMKLR1 KO mice with WT peritoneal exudate cells; it stains both mouse macrophages and NK cells (supplemental Fig. 4).

CMKLR1 was expressed at relatively low levels on microglia, defined as $CD3^{-}CD19^{-}CD11b^{+}CD45^{low}$ (16), in naive mice (data not shown), as well as in mice with acute EAE (Fig. 5*A*). Additional characterization of CNS mononuclear cells demonstrated CMKLR1 expression by a small subset of CNS-infiltrating macrophages ($CD45^{high}CD3^{-}CD19^{-}CD11b^{+}CD11c^{-}$; Fig. 5*C*). CNS-infiltrating $CD45^{high}CD3^{-}CD19^{-}CD11b^{-}CD11c^{int}B220^{+}$ plasmacytoid DC (pDC) are $CMKLR1^{-}$ (Fig. 5*D*). Conversely, $CD45^{high}CD3^{-}CD19^{-}CD11b^{+}CD11c^{high}B220^{-}$ myeloid DC (mDC) isolated from the spinal cords of EAE mice are $CMKLR1^{+}$ (Fig. 5*E*). $CMKLR1^{+}$ macrophages were also detectable in the draining LN and spleens of mice with preclinical and acute EAE, though at much lower frequency than in the CNS (data not shown). CMKLR1 was not detectable on macrophages or DC derived from the LN or spleen of naive mice (data not shown).

Chemerin, a natural ligand for CMKLR1, is up-regulated in the CNS of mice with EAE

We also asked whether chemerin, a natural ligand for CMKLR1, is expressed in the CNS. Chemerin mRNA is up-regulated in the spinal cords of mice with EAE (supplemental Fig. 3). Levels of CMKLR1 transcripts were also higher in spinal cords from mice with acute EAE, although the increase did not achieve statistical significance (data not shown). Collectively, the data implicate the CNS as a source of active chemerin for CMKLR1-expressing cells.

Discussion

In this report, we show that mice deficient in CMKLR1 develop less severe clinical EAE than their WT counterparts. Onset of symptoms is not delayed, but clinical score is significantly reduced in CMKLR1 KO mice, correlating with a significant reduction in histologically assessed CNS inflammation. CMKLR1 is expressed on a subset of CNS-infiltrating macrophages in the inflamed CNS, as well as on microglia and on mDC in mice with EAE. Consistent with reduced inflammation, there was a trend toward reduced demyelination in CMKLR1 KO mice with chronic EAE (day 46 p.i.). Together, our findings demonstrate an important contribution of CMKLR1 to EAE pathogenesis, although the underlying role(s) of receptor expression by microglia, and by CNS-infiltrating macrophages and mDC during EAE, remain to be clarified.

CMKLR1 directs chemotaxis of macrophages and DC subsets in response to its proteolytically activated ligand, chemerin (7–9). Thus it is attractive to postulate a direct role for the receptor in recruitment of macrophages and $CMKLR1^+$ DC subsets into the inflamed CNS, as well as participation of CMKLR1 in cell positioning and cell-to-cell interactions in inflammatory lesions. Indeed, compared with WT mice, CMKLR1 KO mice with chronic EAE had fewer $F4/80^+$ macrophages in the CNS, implying reduced $F4/80^+$ cell recruitment or survival. Because macrophages can amplify the inflammatory cascade, reduced macrophage numbers could be a primary determinant of the overall reduced CNS inflammation seen in CMKLR1 KO mice in the current studies. However, because the relative frequency of different immune cell subsets was generally similar in WT vs KO mice, one cannot exclude the possibility that the reduced macrophage numbers are secondary to other CMKLR1-dependent phenomena.

Data presented in this report, as well as experiments using radiation bone marrow chimeric mice (K.L.G. and E.C.B., unpublished observations), show that $CMKLR1^+$ mDC and macrophages are recruited to the CNS during EAE. CMKLR1 has been implicated in DC recruitment from the blood into lymphoid tissues and inflamed skin in humans (10,17,18), and it is likely to contribute to DC recruitment and/or tissue interactions in EAE as well. In this context, it is interesting that we observed high levels of expression of CMKLR1 by mDC in acute EAE in the present studies, but no expression by pDC. This is the first clear demonstration of CMKLR1 expression by endogenous mouse DC in our hands as, in contrast to clear expression by pDC in humans, neither we nor others have detected robust CMKLR1 expression by significant populations of peripheral lymphoid or extralymphoid tissue DC in adult mice (8). Several groups have probed the contributions of DC to the pathogenesis of EAE. Greter and colleagues demonstrated that $CD11c^+$ DC present Ag to autoaggressive T cells in vivo, which facilitates the development of EAE (13). Bailey and colleagues reported that CNS mDC colocalize with myelin Ag-specific $CD4^+$ T cells within perivascular spaces of the inflamed CNS. Moreover, mDC appear to be highly efficient at presenting endogenous myelin Ags and driving Th17 differentiation within the CNS during relapsing EAE (19). The same group reported that pDC negatively regulate a relapsing-remitting model of EAE through direct suppression of mDC activity (20). Furthermore, pDC are major sources of type I IFNs (21), and recent studies support a protective role for type I IFNs in EAE (22,23). Collectively, the data suggest that the recruitment of mDC, pDC, and their microenvironmental localization within the CNS may contribute significantly to the pathogenesis of EAE.

We were surprised to find that $MOG_{35–55}$ reactive CMKLR1 KO lymphocytes are not fully capable of transferring EAE to WT recipients (Fig. 4 and Table III). Importantly, CMKLR1 deficiency had no significant defects on lymphocyte cytokine or proliferative responses assessed in vitro (Fig. 3 and supplemental Fig. 1). This suggests that lymphocytes generated in a CMKLR1 KO environment may have other functional properties that are not addressed by these in vitro analyses. We hypothesize that CMKLR1 expression by CNS resident cells is

a key determinant of EAE pathophysiology, because there is no difference between WT and CMKLR1 KO mice with respect to peripheral immune cell activation. In light of this possibility, we were intrigued to find that microglia express CMKLR1 protein on the cell surface, extending early reports of CMKLR1 transcript expression by a mouse microglial cell line (24). The significance of CMKLR1 expression by microglial cells is currently unclear, but microglial cell activation is critical to EAE pathogenesis (1,6,25). CMKLR1 could influence microglial cell localization or functional activity during CNS inflammation.

Chemerin, a natural ligand for CMKLR1, also binds GPR1 (26), previously an orphan G-protein-coupled receptor that is phylogenetically related to CMKLR1 (27). The relevance of chemerin: GPR1 interactions in vivo remains to be determined, although the expression of GPR1 mRNA by human fetal and simian adult astrocytes (28) points to possible CNS-related functions. We have also recently identified chemerin as a ligand for the orphan serpentine receptor CCRL2. Chemerin:CCRL2 binding does not trigger cell migration or intracellular calcium mobilization. Rather, CCRL2 appears to concentrate and present bioactive chemerin to CMKLR1-expressing cells (29). We are currently evaluating the role of CCRL2 in EAE.

The regulation of chemerin activity in vivo is incompletely understood. We and others have shown that the chemoattractant ability of chemerin is activated via cleavage of inhibitory C-terminal amino acids by proteases that have roles in the coagulation, fibrinolytic, and inflammatory cascades (7,9,11,30), pathways that are known or likely to be engaged during CNS inflammation in EAE and MS. Proteases involved in these cascades may promote EAE pathogenesis in part via cleavage of chemerin, with subsequent recruitment of or functional impact on CMKLR1-expressing cells. For example, tissue plasminogen activator activates chemerin and is expressed at high levels by astrocytes within the CNS of mice with EAE (31). Transcripts for mast cell tryptase, for which chemerin is also a substrate, are increased in MS lesions (32). Chemerin can be cleaved and activated by neutrophil-derived serine proteases (11,30). Thus, the activation of chemerin in vivo may be mediated by proteases derived from both recruited and CNS-resident cells in an inflammatory setting. It is possible that chemerin production or activation is up-regulated in a localized manner (e.g., within unique niches of the CNS parenchyma) during an inflammatory response, thus helping recruit $CMKLR1^+$ cells to specialized compartments. Indeed, Lande et al. recently reported chemerin colocalization with intralesional endothelial cells in the brains of MS patients (33).

In a recent report, Cash and colleagues reported that chemerin-derived C-terminal peptides possess anti-inflammatory properties in a murine model of zymosan-induced peritonitis (34). These properties were dependent on CMKLR1, as indicated by analysis of CMKLR1 KO mice. It will be of interest to determine whether similar C-terminal chemerin peptides are beneficial in other models of inflammatory disease. Our data suggest that chemerin promotes inflammatory responses during EAE, raising the possibility that chemerin has disparate roles in peritoneal vs CNS inflammation or in different inflammatory settings. It is likely that chemerin, like other cytokines and chemokines, interacts functionally with other mediators in a complex manner to regulate inflammatory responses in vivo. It is also possible that CMKLR1 functions independently of chemerin binding in the EAE model.

As mentioned previously, human blood pDC express CMKLR1 (9,10) and recent studies show that a subset of pDC in the brains of human MS patients expresses the receptor as well (33). However, mouse pDC in lymphoid tissues and blood (8), and in normal and inflamed CNS as shown here, do not express detectable CMKLR1, highlighting the challenges associated with extrapolating observations made in mouse models to human systems. Although mouse and human pDC share key functions, they differ significantly in their expression of a number of surface Ags, in addition to CMKLR1 (21). CMKLR1 is only weakly expressed, if at all, by circulating human mDC (9,10) and has not been reported on endogenous mouse mDC, but is

transiently induced to high levels on both mouse bone marrow-derived and human monocyte-derived mDC generated in response to GM-CSF or Flt-3 ligand in vitro (8). The present finding of expression by mouse mDC in EAE supports the potential for CMKLR1 to participate in mDC recruitment and functions in select inflammatory settings. In contrast to its distinctive patterns of expression by human vs mouse DC subsets, CMKLR1 in both species is highly expressed by tissue macrophages, including macrophages in the CNS of mice with EAE.

In summary, our results demonstrate an important contribution of CMKLR1 in the pathogenesis of EAE, with potential involvement in the regulation of DC dynamics and macrophage accumulation in CNS inflammation. They point to CMKLR1:chemerin interactions as a potential target for therapy of chronic and/or progressive MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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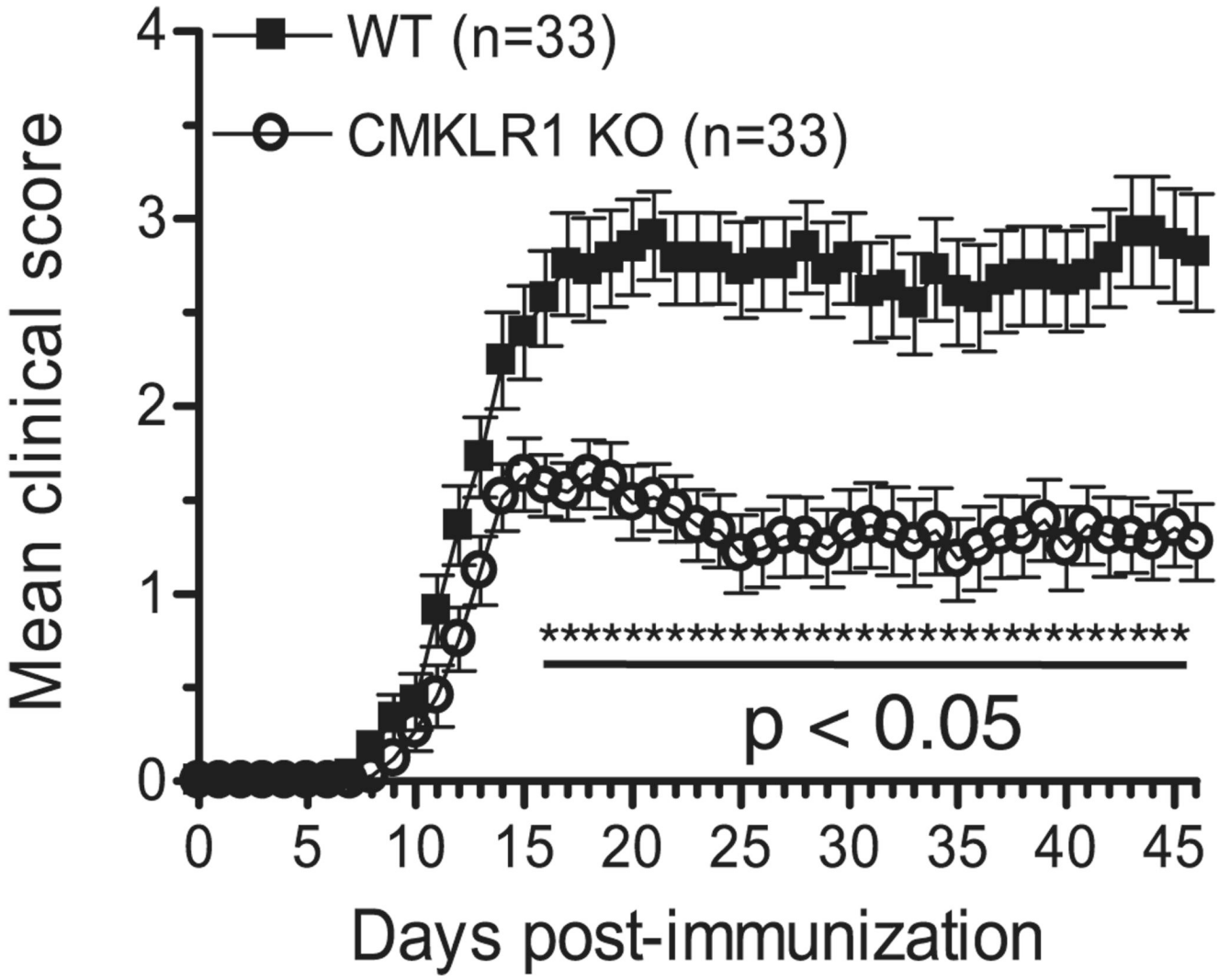

FIGURE 1.

Reduced clinical EAE in CMKLR1 KO mice. EAE was induced by active immunization and mice were monitored daily for clinical disease as described in *Materials and Methods*. Data are pooled from five independent experiments, each consisting of 4–10 mice per group, and are presented as mean clinical score ± SEM vs time. *, $p < 0.05$, as determined by Mann-Whitney *U* test.

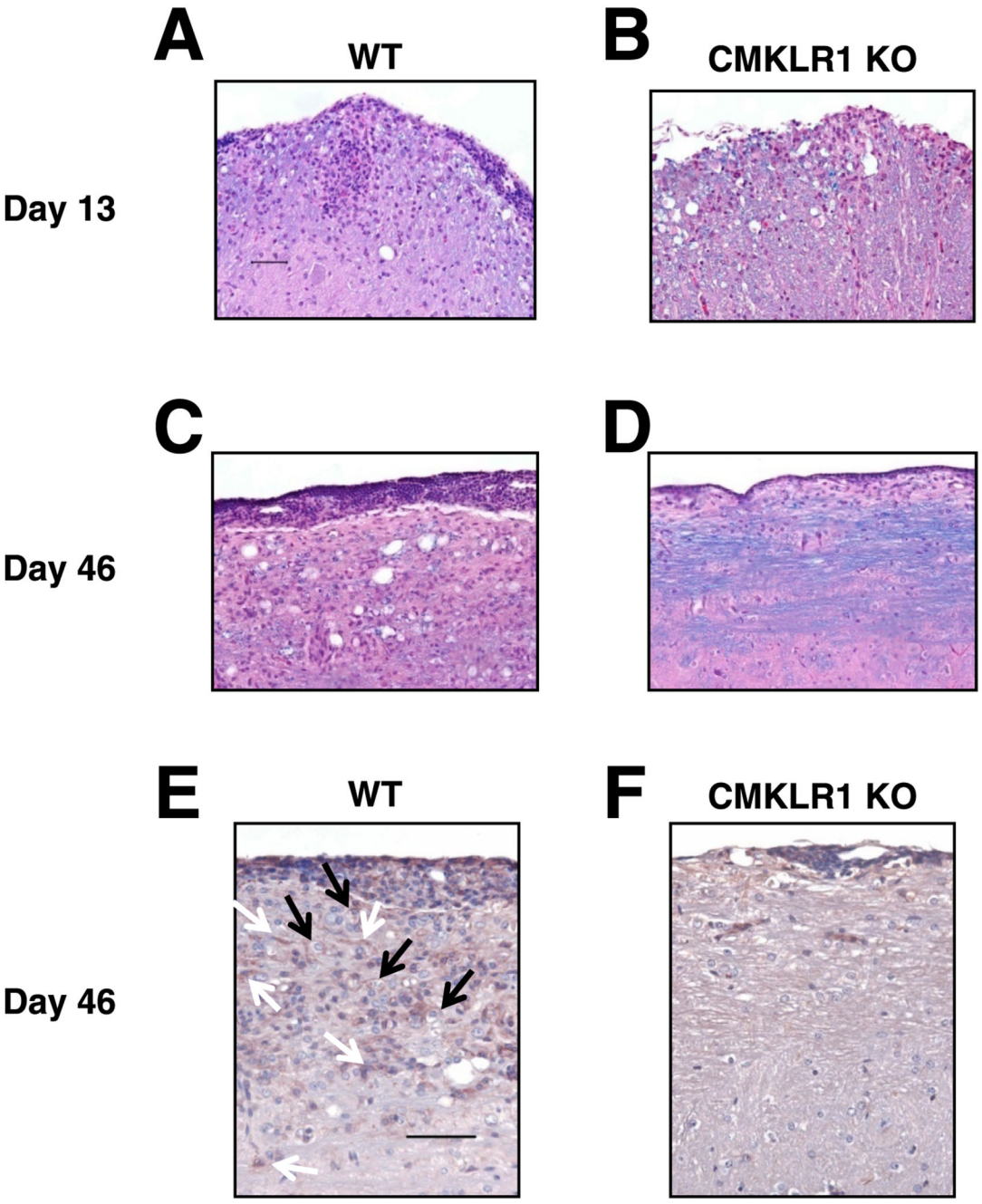

FIGURE 2.

Reduced histological EAE in CMKLR1 KO mice. Representative spinal cord sections are shown from actively immunized WT (*left panels*) or CMKLR1 KO mice (*right panels*) that were killed at 13 (*A* and *B*) or 46 (*C* and *D*) days p.i. *A*, Meningeal and parenchymal mononuclear cell infiltrates typical of acute EAE in the spinal cord of a WT mouse sacrificed on day 13 p.i. *B*, Less meningeal infiltration in the spinal cord of CMKLR1 KO mouse with EAE at day 13 p.i. *C*, Typical meningeal and parenchymal mononuclear cell infiltrates in the spinal cord of a WT mouse with chronic EAE. *D*, Meningitis and mild parenchymal inflammation are present in the spinal cord of a CMKLR1 KO mouse sacrificed at day 46 p.i. Sections from paraffin-embedded WT (*E*) and CMKLR1 KO (*F*) brain and spinal cord tissue

harvested at day 46 p.i. were subjected to immunostaining with anti-F4/80 mAb. Reactions were developed with diaminobenzidine chromogen and counter-stained with hematoxylin. White arrows highlight microglia, black arrows indicate foamy macrophages. Magnification = 160×. Bar = 50 μm.

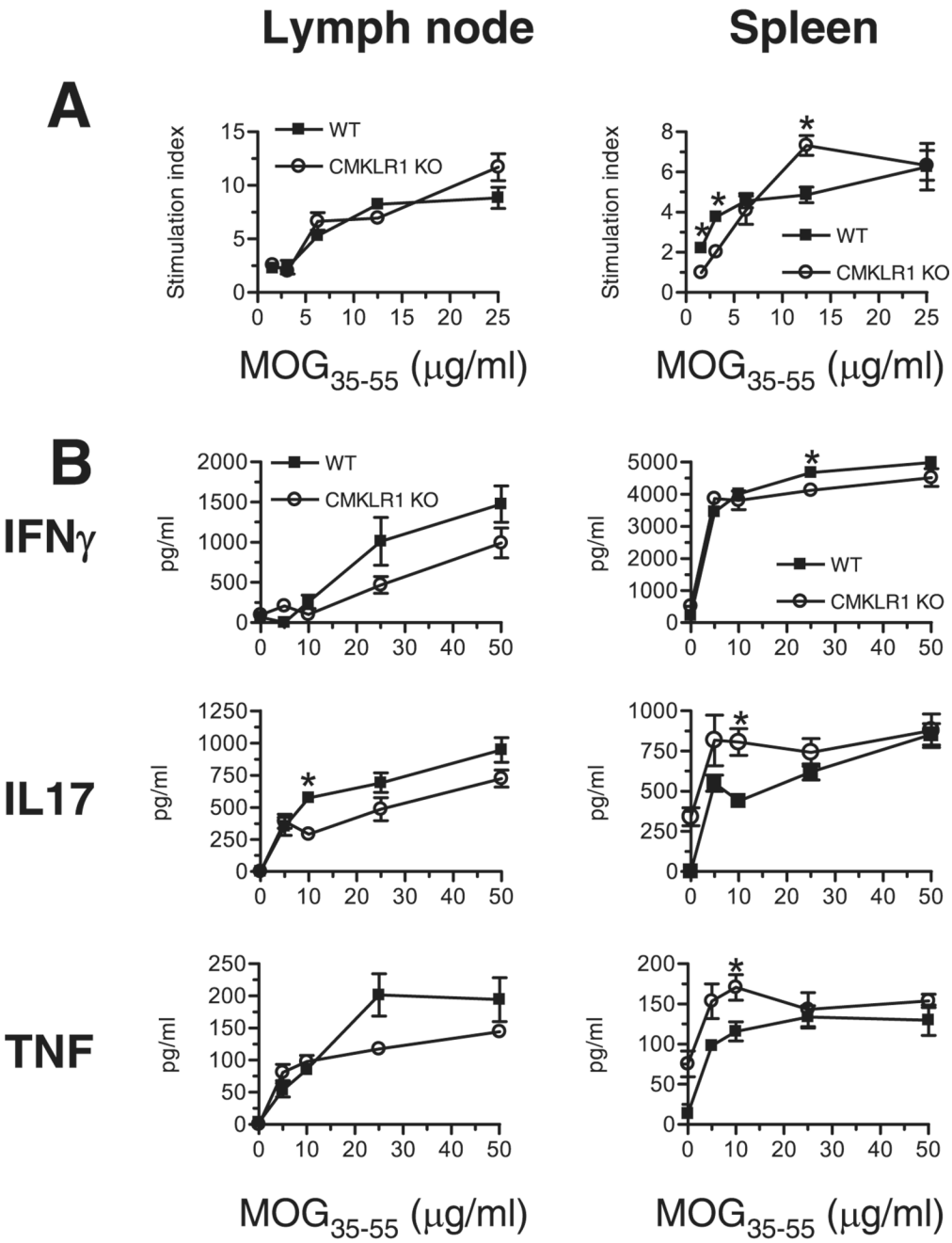

FIGURE 3.

Recall proliferation and cytokine responses of lymphocytes from CMKLR1 KO mice. Mice were immunized with $MOG_{35–55}$ emulsified in CFA. After 10 days, spleen cells and draining LN cells were restimulated in vitro with the indicated concentrations of $MOG_{35–55}$. *A*, After 72 h of stimulation, proliferation of LN (*left panel*) or spleen cells (*right panel*) was assessed by [^{3}H]thymidine incorporation assay. Data are presented as a stimulation index (mean cpm with Ag ÷ mean cpm without Ag); bars represent SEM. *B*, Culture supernatants were collected after 72 h and levels of the indicated cytokines produced by LN (*left panels*) or spleen cells (*right panels*) were measured by ELISA. *, $p < 0.05$, as determined by Student's *t* test. Data shown are representative of two independent experiments with similar results.

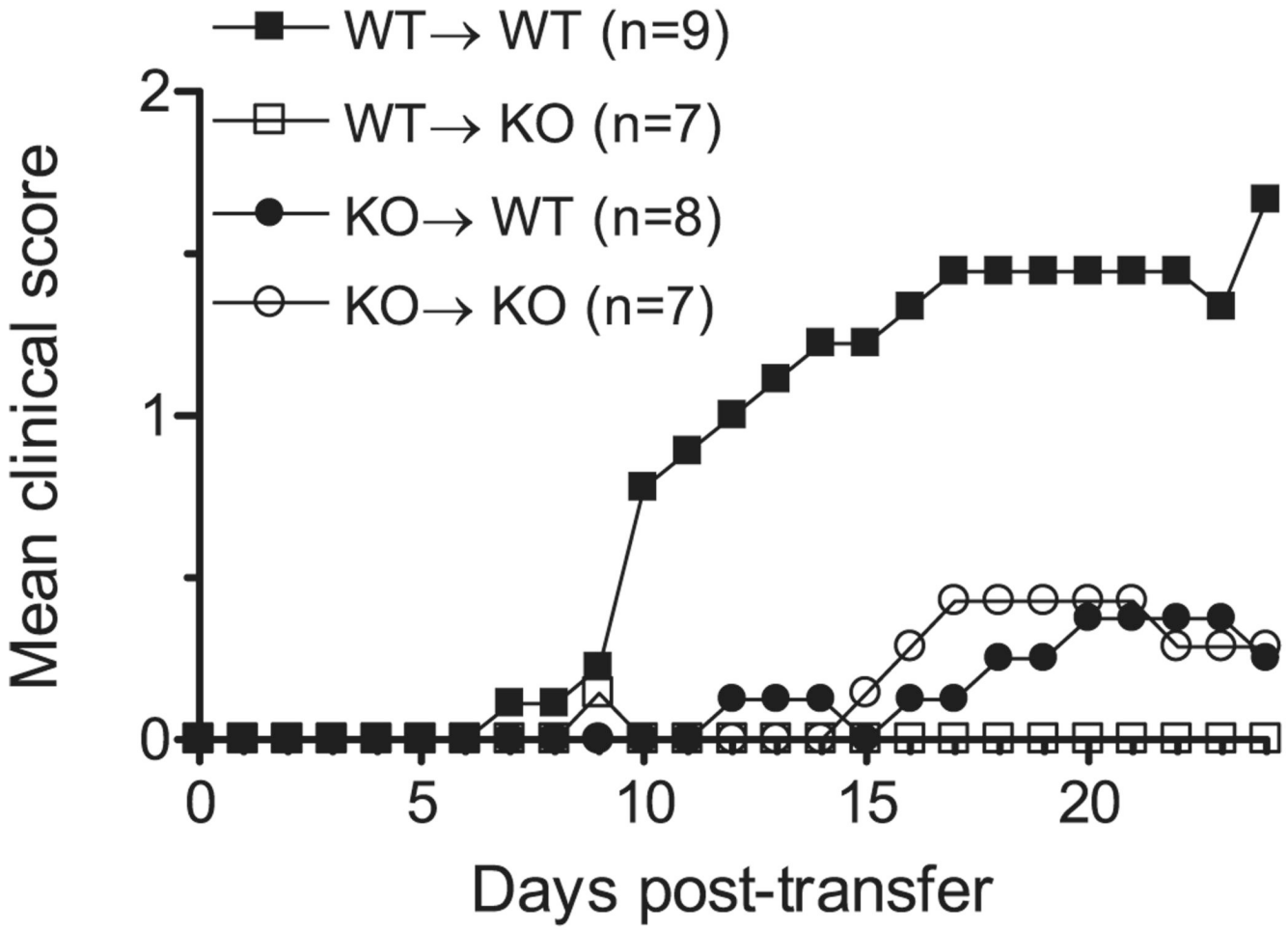

FIGURE 4.

Induction of EAE by adoptive transfer of MOG-reactive lymphocytes. EAE was induced in WT or CMKLR1 KO mice by passive transfer of WT or CMKLR1 KO $MOG_{35–55}$-reactive lymphocytes as described in *Materials and Methods*. Data shown are pooled from adoptive transfers that were performed at various times (eight independent experiments, n = 1–4 recipient mice per group). Values are presented as mean clinical score vs time.

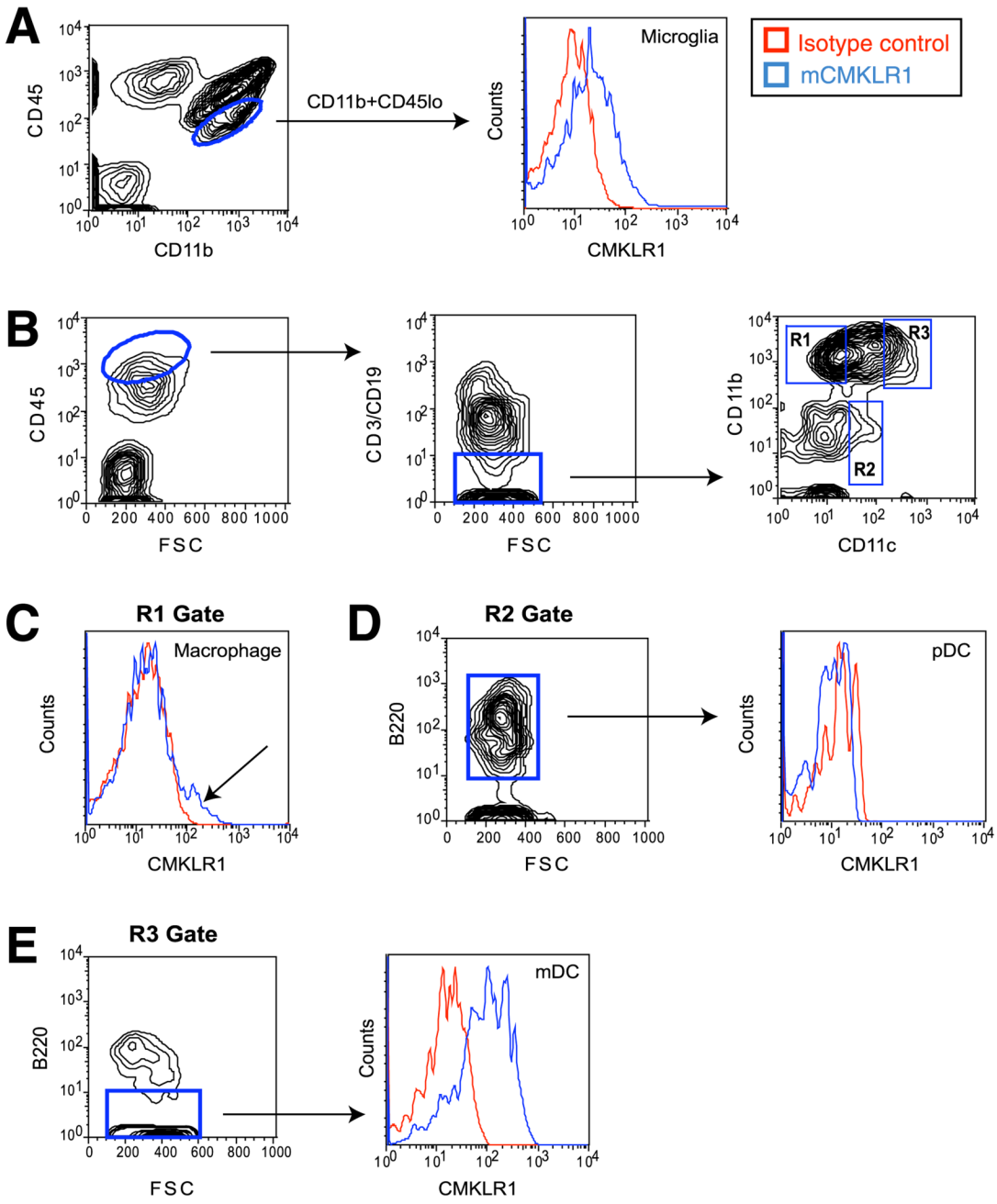

FIGURE 5.

Detection of CMKLR1^{+} cells in CNS of mice with EAE. Mononuclear cells were isolated from the spinal cords of mice with acute EAE as described in *Materials and Methods*. Cells were stained with anti-mouse CMKLR1 mAb BZ186 or DREG200 isotype control mAb, followed by incubation with PE-conjugated anti-mouse IgGl. Lastly, mAbs directly conjugated to cell surface Ags were added and cells were analyzed by flow cytometry. For all histograms, the red line represents isotype control mAb staining. *A*, CD3^{-}CD19^{-}CD11b^{+}CD45^{low} microglia were analyzed for expression of CMKLR1. *B*, Cells were gated as CD45^{high} and cells in the CD3^{-}CD19^{-} gate were analyzed for expression of CD11b and CD11c. Leukocytes were gated on macrophages (R1), pDC (R2), or mDC (R3), which were then analyzed for expression of

CMKLR1. *C*, A subset of $CD11c^{-}CD11b^{+}$ macrophages (R1 gate) expresses CMKLR1 (indicated by the arrow). *D*, $CD11c^{int}CD11b^{-}B220^{+}$ pDC (R2 gate) are CMKLR1-negative. *E*, $CD11c^{high}CD11b^{+}B220^{-}$ mDC (R3 gate) express CMKLR1. CNS cells were pooled from three mice for analysis; data shown are representative of three independent experiments with similar results. FSC, forward scatter.

Table I

Clinical EAE in actively immunized WT and CMKLR1 KO mice[a]

	Incidence of Clinical EAE	Mean Day of Onset (SEM)	Mean Maximal Score (SEM)
WT	33/33 (100%)	12.2 (0.7)	3.45 (0.2)
CMKLR1 KO	30/33 (91%)	13.1 (0.8)	2.60 (0.2)*

[a]Data are pooled from five independent experiments with each experiment consisting of 4–10 mice per group.

*$p < 0.05$, as determined by Mann-Whitney *U* test.

Table II

Histological EAE in actively immunized WT and CMKLR1 KO mice[a]

	Number of Mice	Parenchymal Foci (SEM)	Meningeal Foci (SEM)	Demyelination Score (SEM)
Day 13 p.i.				
WT	5	98.0 (12.4)	116 (13.3)	7.8 (0.9)
CMKLR1 KO	7	85.7 (7.4)	74.7 (5.1)*	8.23 (0.5)
Day 46 p.i.				
WT	7	47.4 (9.9)	35.9 (5.4)	5.86 (0.6)
CMKLR1 KO	14	21.4 (4.8)*	20.8 (3.8)*	4.23 (0.7)†

[a] Brain and spinal cord tissue was harvested 13 or 46 days after induction of EAE by active immunization. Histological changes were evaluated as described in *Materials and Methods*.

* $p < 0.05$, as determined by Student's *t* test.

† The difference in the demyelination score between WT and CMKLR1 KO mice at day 46 p.i. did not reach statistical significance ($p = 0.06$).

Table III

Clinical and histological EAE in mice induced to develop disease by passive transfer[a]

Donor:Recipient	Incidence (Percentage)	Parenchymal Foci (SEM)	Meningeal Foci (SEM)
WT:WT	9/9 (100%)	31.9 (10.0)	46.4 (10.3)
WT:KO	1/7 (14%)*	0.0 (0.0)	0.29 (0.29)†
KO:WT	2/8 (25%)*	12.1 (12.1)	12.9 (12.4)
KO:KO	2/7 (28%)*	14.4 (7.8)	13.4 (6.5)†

[a] Brain and spinal cord tissue was harvested 24 days after induction of EAE by passive transfer. Data are pooled from eight independent experiments, with each experiment containing one to four recipient mice per group.

* $p < 0.05$ (compared to WT:WT group), as determined by Fisher's exact test.

† $p < 0.05$ (compared to WT:WT group), as determined by Student's *t* test.