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IL-9 Promotes Th17 Cell Migration into the Central Nervous System via CC Chemokine Ligand-20 Produced by Astrocytes

Yan Zhou,*1 Yoshifumi Sonobe,*1 Tomohiko Akahori,* Shijie Jin,* Jun Kawanokuchi,* Mariko Noda,* Yoichiro Iwakura,† Tetsuya Mizuno,* and Akio Suzumura*

Newly discovered IL-9–producing helper T cells (Th9) reportedly exert both aggravating and suppressive roles on experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. However, it is still unclear whether Th9 is a distinct Th cell subset and how IL-9 functions in the CNS. In this study, we show that IL-9 is produced by naive CD4+ T cells that were stimulated with anti-CD3 and anti-CD28 Abs under the conditions of Th2-, inducible regulatory T cell-, Th17-, and Th9-polarizing conditions and that IL-9 production is significantly suppressed in the absence of IL-4, suggesting that IL-4 is critical for the induction of IL-9 by each producing cell. The IL-9 receptor complex, IL-9R and IL-2Rγ, is constitutively expressed on astrocytes. IL-9 induces astrocytes to produce CCL-20 but not other chemokines, including CCL-2, CCL-3, and CXCL-2 by astrocytes. The conditioned medium of IL-9–stimulated astrocytes induces Th17 cell migration in vitro, which is cancelled by adding anti–CCL-20 neutralizing Abs. Treating with anti–IL-9 neutralizing Abs attenuates experimental autoimmune encephalomyelitis, decreases the number of infiltrating Th17 cells, and reduces CCL-20 expression in astrocytes. These results suggest that IL-9 is produced by several Th cell subsets in the presence of IL-4 and induces CCL-20 production by astrocytes to induce the migration of Th17 cells into the CNS. The Journal of Immunology, 2011, 186: 4415–4421.

Interleukin-9 was originally classified as a Th2 cytokine and it induces STAT1, STAT3, and STAT5 phosphorylation in cells through the receptor complex that consists of the IL-9R and IL-2Rγ (1, 2). Previous studies have suggested that IL-9 induces allergic responses, including airway hyperresponsiveness with eosinophilia and IgE hyperproduction in the serum (3–6).

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis that is characteristic of demyelination in the white matter and the focal infiltration of inflammatory cells, including CD4+ helper T cells and macrophages. Th17 cells play an important role in EAE development by disrupting the blood–brain barrier (7). IL-17 mRNA expression is significantly upregulated in CD4+ T cells in the spinal cords of mice before the onset of EAE (8). Constitutive CCL-20 expression in endothelial cells of the choroid plexus is associated with the initial entry of CCR6+ Th17 cells into the CNS in EAE (9). Th1 cells have also been associated with the induction of EAE. Adoptively transferred Th1-polarized myelin-specific T cell lines can cause EAE (10, 11). IFN-γ mRNA expression is increased in the later phase of EAE (8). Th1 cells are also considered to be the primary mediators in classic EAE, which reflects targeting to the spinal cord (11). Thus, although Th17 cells appear to be critical for the development of EAE, Th1 cells may also play an important role in the disease progression. Therefore, it is important to elucidate the role of each Th subset in EAE.

IL-9–producing helper T cells (Th9) were recently discovered and have been reported to be induced by the stimulating naive CD4+ T cells with TGF-β alone (12–17). However, other reports have suggested that both Th2 and Th17 cells produce IL-9 (14, 15). Thus, it remains unclear whether Th9 is a distinct subset and how IL-9 is produced by each T cell subset.

Myelin oligodendrocyte glycoprotein (MOG)-specific Th9 cells are able to induce EAE upon adoptive transfer (18). It has also been reported that genetically ablating IL-9R or blocking IL-9 with neutralizing Abs attenuated EAE due to fewer Th17 cells in the CNS (15, 19), whereas others have suggested that an IL-9R deficiency exacerbated EAE (14). Thus, the precise function of IL-9 and its role in the CNS also remain unclear.

In this study, we show that IL-9 can be optimally induced in the presence of IL-4 in various subsets of Th cells. IL-9 is produced under the conditions of Th2-, inducible regulatory T cell (iTreg)-, Th17-, and Th9-polarizing conditions but is significantly suppressed in the complete absence of IL-4 in vitro and in vivo. Furthermore, the IL-9 receptor complex is constitutively expressed on astrocytes in the CNS. IL-9 induces astrocytes to produce CCL-20 but not other chemokines, including CCL-2, CCL-3, and CXCL-2 by astrocytes. IL-9–stimulated conditioned medium from astrocytes induces Th17 cell migration in vitro but this effect is

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; iTreg, inducible regulatory T cell; MOG, myelin oligodendrocyte glycoprotein.

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cancelled by the addition of anti–CCL-20 neutralizing Abs. Additionally, anti–IL-9 neutralizing Abs attenuate EAE, decrease the number of Th17 cells, and reduce CCL-20 expression in astrocytes. These results indicate that IL-9 is induced in several subsets of CD4+ T cells in the presence of IL-4, and it induces CCL-20 production by astrocytes to enhance the infiltration of Th17 cells into the CNS.

Materials and Methods

Reagents

MOG peptide 35–55 (MOG35–55 MEVGWYRSPFSRVHLYRNGK) was synthesized and purified by Operon Biotechnologies. IFA was obtained from Sigma-Aldrich. Heat-killed Mycobacterium tuberculosis H37Ra was obtained from Difco, and pertussis toxin was obtained from List Biological Laboratories. The following Abs were used in this study: PE-Cy5-anti-CD4 (GK1.5; BD Biosciences), PE- or Alexa 488-anti-mouse IL-17A (TC11-18H10; BD Biosciences), FITC-anti-mouse IFN-γ (XM16-2; BD Biosciences), PE-anti-mouse IL-9 (RM9A4; BioLegend), PE-anti-mouse CCR6 (140706; R&D Systems), anti-mouse IL-9R (17J-1; Santa Cruz Biotechnology), anti-mouse IL-2R (Z07N; Santa Cruz Biotechnology), and anti-mouse glial fibrillary acidic protein (GFAP) (Dako).

Animals

IL-4-deficient mice (C57BL/6) were generated as described previously (20) and provided by Dr. Y. Iwakura (University of Tokyo). Mice were bred in the animal facility of the Research Institute of Environmental Medicine at Nagoya University. The protocols for animal experiments were approved by the Animal Experiment Committee of Nagoya University.

Helper T cell differentiation

CD4+CD62L+ T cells were isolated using MACS beads according to the manufacturer’s protocol (Miltenyi Biotech). CD4+ T cells were stimulated with plate-bound anti-CD3 Abs (5 μg/ml) (145-2C11; BD Biosciences) and soluble anti-CD28 Abs (2 μg/ml) (37.51; BD Biosciences) for 4 d in RPMI 1640 medium supplemented with 2 mM sodium pyruvate, l-glutamine, 10% FBS, and IL-2 (20 ng/ml) in the presence of recombinant cytokines. T cells were polarized with recombinant mouse IL-12 (10 ng/ml; R&D Systems), mouse IFN-γ (5 ng/ml; R&D Systems) plus anti–IL-4 (11B.11; 10 μg/ml; BD Biosciences) for Th1, mouse IL-4 (2 ng/ml; R&D Systems) plus anti–IFN-γ (XMG1.2; 10 μg/ml; BD Biosciences) for Th2, human TGF-β1 (5 ng/ml), IL-6 (30 ng/ml) plus anti–IFN-γ for Th17, human TGF-β1 (5 ng/ml) for iTreg, and IL-4 (2 ng/ml) plus human TGF-β1 (5 ng/ml) for Th9.

RT-PCR

Total RNA was extracted from the cells using the RNasy Mini kit (Qiagen). After 0.2 μg total RNA was denatured for 5 min at 65°C, the reverse transcription reaction was performed. Then, real-time RT-PCR was performed as previously described (21) using the following specific primer sets: IL-9 forward, 5’-ATGCAATCGTACGGTCTG-3’, IL-9 reverse, 5’-ATGCAGGCTCCCTGTCGTAGT-3’, CCL-2 reverse, 5’-GTTCACCTGAAGGGTCTACGTA-3’, CCL-3 forward, 5’-CTCAACCATGTAAGGTCT-3’, CCL-3 reverse, 5’-GGGATCCTGACCCGAGGTC-3’, CCL-2 forward, 5’-CCGGCTCTCAGTGCTG-3’, CCL-2 reverse, 5’-GGGATCACGTGGTTGCTTCTT-3’, GAPDH forward, 5’-ACTCAAGGCAAATTCAACGG-3’, GAPDH reverse, 5’-CCCTGTGACTGACGGCTA-3’.

ELISA

The levels of IL-4 (BD Biosciences), IL-9 (BD Biosciences), and CCL-20 (R&D Systems) in the culture supernatants were measured by ELISA according to the manufacturers’ instructions.

Flow cytometry

Cells were stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin. The cells were then fixed and permeabilized with Cytotox/Cytoperm reagent (BD Biosciences) for cytokine staining and then stained with the specific Abs. The cells were subsequently analyzed by flow cytometry using a Cytomics FC500 (Beckman Coulter).

Isolation of CNS mononuclear cells

CNS cells were prepared as previously described (22). Briefly, mice were intracardially perfused with PBS through the left ventricle. Then, the brain and spinal cord were removed and passed through a nylon mesh (NB 60; NBC, Tokyo). The mononuclear cells were enriched using a 30% Percoll gradient. Then, the cells were examined for the intracellular cytokine staining. The number of cytokine-producing cells was determined by multiplying the frequency of each population by the total number of cells isolated from each treatment group.

Experimental autoimmune encephalomyelitis

MOG-induced EAE was induced as previously described (22). Briefly, mice were injected s.c. with 0.2 ml emulsion containing 200 μg MOG35–55 in PBS combined with an equal volume of CFA containing 300 μg killed Mycobacterium tuberculosis H37Ra. Mice were injected with pertussis toxin i.p. on the day of immunization and 2 d after immunization (400 ng/mouse). Anti–IL-9 Abs or the isotype controls were injected i.v. twice per week. The mice were scored daily as follows: 0, normal; 1, limp tail or mild hind limb weakness; 2, moderate hind limb weakness or mild ataxia; 3, moderate to severe hind limb weakness; 4, severe hind limb weakness; 5, paraplegia with moderate forelimb weakness; and 6, paraplegia with severe forelimb weakness, severe ataxia, or moribund.

In vitro priming of MOG-reactive CD4+ T cells

Mononuclear cells were harvested from the spleen and the CNS as previously described (22). CD4+ T cells were purified using MACS according to the manufacturer’s protocol (Miltenyi Biotech). The purity of the CD4+ T cell samples was >98% as determined by CD4-specific immunostaining. Purified CD4+ T cells (2 × 106 cells/ml) were cultured with mitomycin C-treated feeder cells (2 × 106 cells/ml) in the presence of 20 μg/ml MOG35–55 for 3 d. The supernatant was collected and the IL-9 levels were measured by ELISA.

Isolation of primary cells

Primary astrocytes were isolated as previously described (23). Briefly, microglial cells were removed from mixed glial cell cultures from newborn mice, and the remaining cultures were trypsinized and replated in Petri dishes. Cultures that had been passaged twice were used as astrocytes. The purity of the cultures was >95% as determined by immunostaining with anti–GFAP Abs.

FIGURE 1. IL-9 is critical for the optimal induction of IL-9 in Th cells. CD4+ CD62L+ naive T cells were stimulated with anti-CD3 and anti-CD28 Abs for 4 d in the presence of the corresponding cytokines. A, IL-9 mRNA expression relative to GAPDH was assessed by real time RT-PCR. B, IL-9 levels in the culture supernatants were measured by ELISA. Data are represented as the means ± SEM. *p < 0.05 (n = 5).
Primary microglia were isolated from mixed glial cell cultures prepared from newborn mice on day 14 using the “shaking off” method as previously described (24). The purity of the cultures was almost 100% as determined by immunostaining with anti-CD11b Abs.

Primary neurons were prepared from the neocortices of embryonic day 17 embryos as previously described (25). The purity of the cultures was 95% as determined by NeuN-specific immunostaining.

Immunocytochemistry

Astrocytes plated on 8-well chamber slides were fixed with 2% paraformaldehyde for 30 min and then incubated with anti–GFAP (Dako), anti–IL-9R (Santa Cruz Biotechnology), and anti–IL-2Rg (Santa Cruz Biotechnology) Abs. Then the cells were incubated with Alexa 488- or Alexa 568-conjugated secondary Abs (Invitrogen) for 30 min. Cells were examined with a deconvolution fluorescence microscope system (Bio Zero; Keyence, Osaka, Japan).

Migration assay

Astrocytes were stimulated with IL-9 for 48 h and then the supernatants were collected and used as IL-9-conditioned medium. Migration assays were performed as previously described with some modifications (21). Briefly, IL-9-conditioned medium with or without anti–CCL-20 Abs were added to the 12-well plates. Then, in vitro-induced Th17 cells were loaded onto the luminal side on the Transwell inserts (3 μm pore size; Corning) and incubated for 24 h at 37°C/5% CO2. Following migration, 50 μl 0.5 M EDTA was added to the abluminal side and the plates were placed on a shaker for 15 min to mobilize cells. Cells in the abluminal side were then harvested and counted by flow cytometry (Cytomics FC500; Beckman Coulter).

Immunohistochemistry

Frozen spinal cord sections were fixed with 4% paraformaldehyde and stained for CCL-20 and GFAP followed by Alexa 488-conjugated anti-rat IgG and Alexa 568-conjugated anti-rabbit IgG as previously described (21). The stained sections were examined with a deconvolution fluorescence microscope system (BZ-8000; Keyence).

Statistical analysis

Differences between the means of experimental groups were analyzed using the two-tailed Student t test with Welch’s correction.

Results

IL-9–producing CD4+ T cells are induced in an IL-4–dependent manner

First, we analyzed IL-9 production by examining mRNA expression and the IL-9 levels in the culture supernatants for each Th subset. IL-9 was induced in naïve T cells from wild-type mice under culture conditions containing IL-4 plus anti–IFN-γ (Th2), TGF-β–alone (iTreg), IL-6 plus TGF-β plus anti–IFN-γ (Th17), TGF-β plus IL-4 (Th9), TGF-β plus IL-25, or TGF-β plus IL-1β (Fig. 1). IL-9 production was significantly lower in the subset of helper T cells derived from IL-4–deficient mice under conditions that induce iTreg or Th17 or in the presence of TGF-β, plus IL-25 or TGF-β plus IL-1β (Fig. 1). This
significant reduction in IL-9 production was not observed in Th2 and Th9 cells from IL-4-deficient mice because IL-4 was used to induce these subsets. In adoptive transfer experiments, IL-23-induced Th17 cells in the absence of IL-4 were capable to induce EAE, but TGF-β plus IL-6-induced Th17 cells were not (Supplemental Fig. 1). IL-4-deficient mice developed more severe weakness in the later phase of EAE as compared with wild-type mice (Supplemental Fig. 2). However, when rIL-4 was administered in IL-4-deficient mice, the mice developed clinical signs of EAE earlier and the severity in the later phase was less than in untreated IL-4-deficient mice (Supplemental Fig. 2). The number of IL-9-producing CD4+ cells was significantly lower in IL-4-deficient mice compared with wild-type mice (Fig. 2A, 2B, Supplemental Fig. 3A, 3B). Inconsistent with this, the number of IL-17-producing CD4+ cells was also significantly lower in IL-4-deficient mice as compared with wild-type mice (Supplemental Figs. 3A, 3C, 4A, 4B). However, when IL-4 was injected into the IL-4-deficient mice, the number of IL-9-producing cells that was restored in CNS and splenic CD4+ T cells by administering IL-4 (Fig. 2C).

Taken together, these results suggest that IL-9 is induced in several CD4+ T cell subsets in the presence of IL-4.

**The IL-9 receptor complex is expressed on astrocytes in the CNS**

IL-9 was detected in the CNS during EAE but its function in the CNS is still unknown. Thus, we analyzed IL-9R and IL-2Rγ mRNA and protein expression in the CNS. Both IL-9R and IL-2Rγ mRNA were detected in the whole brain tissue (data not shown). Primary astrocytes expressed both IL-9R and IL-2Rγ mRNA, whereas neurons and microglia expressed only IL-2Rγ mRNA (Fig. 3A). Immunocytochemistry showed that astrocytes expressed the IL-9R and IL-2Rγ proteins on their surface (Fig. 3B). These results suggest that the IL-9 receptor complex is expressed on astrocytes and that astrocytes are the target of IL-9 in the CNS.

**IL-9 induces CCL-20 production by astrocytes**

It has been shown that IL-9 induces various chemotactic factors in mouse and human lung epithelial cells (4, 26). Thus, we explored the effects of IL-9 on the chemokine production by astrocytes. IL-9 dose-dependently induced CCL-20 mRNA expression in astrocytes but did not induce mRNA expression of other chemokines including CCL-2, CCL-3, and CXCL-2 mRNA (Fig. 4A). An ELISA confirmed that IL-9 induced CCL-20 production by astrocytes in a dose-dependent manner (Fig. 4B).

**IL-9 induces Th17 cell migration via CCL-20**

It has been reported that CCL-20 is important for the migration of Th17 cells to inflamed sites in autoimmune disease models including the SKG mouse model of rheumatoid arthritis and EAE (9, 27, 28). Thus, we investigated whether CCL-20 produced by astrocytes induced Th17 cell migration in vitro. Almost all of the IL-17-producing helper T cells expressed CCR6 (92.1%), a CCL-20 receptor (Fig. 5A). Furthermore, a transmigration assay revealed that conditioned medium from IL-9–stimulated astrocytes induced Th17 cell migration and that these effects were cancelled by adding anti–CCL-20 neutralizing Abs (Fig. 5B). These results suggest that CCL-20 produced by IL-9–stimulated astrocytes induces Th17 cell migration.

**Blocking of IL-9 suppresses CCL-20 expression in astrocytes and inhibits Th17 cell migration into the CNS in EAE**

To confirm that IL-9 induces CCL-20 expression in astrocytes and subsequent Th17 cell migration in vivo, we analyzed CCL-20 expression in astrocytes and the prevalence of Th17 cells in the CNS during EAE. The addition of anti–IL-9 neutralizing Abs...
attenuated the clinical signs of EAE (Fig. 6A) and was accompanied by decreased CCL-20 expression in astrocytes (Fig. 6B) and a reduced number of IL-17- and IFN-γ-producing CD4+ T cells in the spinal cord (Fig. 6C, 6D). However, the number of IFN-γ-producing CD4+ T cells was comparable between mice that were administered anti–IL-9 Abs and the isotype control (Fig. 6C, 6D). Thus, these results further support the notion that IL-9 induces CCL-20 expression in astrocytes to induce the migration of Th17 cells into the CNS in EAE.

Discussion

Th9 cells are a newly discovered subset of Th cells that have been proposed to be distinct from other Th subsets, including Th1, Th2, and Th17 cells (17). However, other studies have contradicted these findings by showing that Th2- and Th17-polarizing conditions as well as TGF-β alone induce IL-9 production by anti-CD3 and anti-CD28 Ab-stimulated CD4+ T cells (14, 15). Our study showed that IL-9 is produced under culture conditions that induce Th2 cells, iTreg, and Th17 cells as well as Th9 cells, and it therefore supports the latter hypothesis. It is reported that human Th17 cells also produce IL-9 (29), although the authors did not mentioned the role of IL-4. We showed that significantly lower levels of IL-9 were produced under Th17- and iTreg-polarizing conditions by cells derived from IL-4–deficient mice compared with wild-type mice. These results suggest that IL-4 is a key regulator that induces IL-9 in CD4+ T cells. In fact, STAT6, IFN regulatory factor-4, and PU.1, which were previously shown to be critical transcription factors that induce IL-9, are also critical for IL-4–induced Th2 differentiation (17, 30, 31). These findings suggest that IL-4 signaling is required for the optimal induction of IL-9 in CD4+ T cells. We also found that TGF-β plus IL-25 and TGF-β plus IL-1β induce IL-9 production by CD4+ T cells and that this IL-9 production is suppressed when IL-4 is genetically ablated (Fig. 1) or when anti–IL-4 Abs are added (data not shown), indicating that IL-9 production by naive CD4+ T cells stimulated with TGF-β plus IL-25 or IL-1β is also IL-4–dependent. However, others have claimed that IL-9 production under these conditions is IL-4–independent (12, 16). Although the reason for this discrepancy requires further studies, IL-25 was originally associated with Th2 differentiation via IL-4 production and STAT6-mediated signal transduction (32, 33). Furthermore, it was also shown that IL-1 interferes with tolerance induction of IL-4–producing T cells (34) and increases Con A-induced IL-4 mRNA expression (34, 35). These findings suggest that both IL-25 and
IL-1β are associated with the induction of IL-4 in CD4+ T cells. Our present study suggests that IL-4 is required for the optimal induction of IL-9 in CD4+ T cells. However, we do not exclude the possibility that factors other than IL-4 are also closely linked to IL-9 induction because IL-9 production is not completely suppressed in the absence of IL-4.

Nowak et al. (15) showed that either blocking of IL-9 or an IL-9R-deficiency attenuated EAE and decreased the number of Th17 cells in the CNS. Jäger et al. (18) also suggested that Th9 cells have an effector function against EAE. In contrast, other studies have demonstrated that the absence of IL-9R weakened the suppressive activity of naturally occurring regulatory T cells and exacerbated EAE (14). Although additional studies are needed to explain these conflicting results, our data indicated that IL-9 promoted Th17 cell migration into the CNS via CCL-20produced by astrocytes. Because Th17 cells play a critical role in EAE development (36, 37), IL-9 produced by a variety of Th cells has an effector function on EAE, at least on Th17-mediated inflammatory responses.

In this study we showed that when IL-4 was injected into IL-4–deficient mice, the mice developed EAE earlier but the severity in the later phase was less as compared with IL-4–deficient mice. IL-4 reportedly plays suppressive roles on microglia. We and others demonstrated previously that IL-4 suppressed MHC class II expression and production of inflammatory mediators such as TNF-α and NO in microglia (38–40). Bettelli et al. (41) also showed that IL-4–deficient mice exhibited delayed onset but exacerbated EAE in the later phase. Thus, although IL-4 induced IL-9 production and Th17 cell migration into the CNS via CCL-20 in an early phase of EAE, IL-4 may also act on microglia to suppress the inflammatory responses in the later phase of the disease.

We showed in this study that astrocytes expressed IL-9R but not neurons and microglia. A recent report demonstrated IL-9R on neurons and in developing brains (42). We used primary neuronal culture, which had been prepared from embryonic day 17 and extracted total RNA from days in vitro 14 to 21. The neurons in our cultures have synapse formation and glutamate receptor expression. Thus, the neurons used in this study are mature. In contrast, Fontaine et al. (42) used neuron cultures prepared from embryonic day 14.5 embryonic mice and IL-9R mRNA expression was analyzed at day in vitro 7. The difference may suggests that developing immature neurons express IL-9R. Actually, they also showed that IL-9R mRNA expression in developing brain decreased after birth. Additionally, since inflammatory factors including TNF-α and IFN-γ are upregulated in the CNS during EAE, these factors may affect IL-9R expression in neurons or glial cells including astrocytes. Therefore, it is possible that IL-9 may play a role on other neural cells.

Previous studies have shown that the main source of CCL-20 in the CNS is TNF-α or IL-1β–activated astrocytes (43). Moreover, CCL-20 mRNA expression is reportedly upregulated in the CNS during EAE, which is closely related to the disease severity (44). Our data provide additional evidence that CCL-20 is also induced in astrocytes by IL-9. Furthermore, CCL-20 is reportedly expressed in the epithelial cells in the brain as well as in the intestine (9, 45). We also found that CCL-20 was constitutively expressed in the spinal cord (Fig. 6B). Based on this morphology and the previous reports, it is possible that epithelial cells also express CCL-20. Interestingly, uniform CCL-20 expression in epithelial cells is thought to be important to allow the initial entry of CCR6+ Th17 cells into the CNS (9). Thus, since the CCR6–CCL-20 interaction is critical for the migration of Th17 cells into inflamed sites to develop EAE, our data suggest that IL-9–induced CCL-20 expression in astrocytes causes a second wave to further promote Th17 cell migration into the inflamed sites of EAE and to exacerbate the disease. The fact that anti-IL-9 neutralizing Abs suppressed disease in the later phase of EAE further supports this hypothesis. Although it is not clear which types of the cells produce IL-9 in the first place to start the cascade, Jäger et al. (18) showed that MOG-reactive Th9 cells infiltrated into the CNS upon adoptive transfer, suggesting that at least Th9 cells are the cells locally producing IL-9 to start the cascade.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1 IL-23–induced Th17 cells in the absence of IL-4 are still encephalitogenic. Splenocytes were harvested from IL-4–deficient C57BL/6 mice 10 days after immunization with MOG_{35-55} and cultured in the presence of MOG_{35-55} for 4 days. Then CD4^+ T cells were sorted using microbeads. Cells were then injected intravenously into naive C57BL/6 mice (5 x 10^6 cells/mouse). Mice were scored daily as described in Materials and Methods. Data shown are the average of three mice per group.
Supplementary Figure 2 Clinical scores of EAE in wild-type, IL-4–deficient mice, and IL-4–deficient mice that received rIL-4. EAE was induced by immunizing wild-type, IL-4–deficient mice, with MOG35-55 and i.p. injections of PTX on days 0 and 2 after immunization. PBS or rIL-4 (200 ng/mouse) was administered from day 0 to day 28 every other day. Mice were scored daily as described in Materials and Methods. Data shown are the average of six mice per group. *, p < 0.05 (IL-4KO + rIL-4 VS IL-4KO), †, p < 0.05 (IL-4KO + rIL-4 VS WT).
Supplementary Figure 3  Total cell numbers and the ratios of IL-9– and IL-17–producing CD4+ T cells in the CNS harvested at 13-15 days after immunization with MOG_{35-55}. EAE was induced by immunizing wild-type and IL-4–deficient mice with MOG_{35-55} and i.p. injections of PTX on days 0 and 2 after immunization. PBS or rIL-4 (200 ng/mouse) was administered from day 0 every other day. CNS cells were harvested at 13-15 days after immunization with MOG_{35-55} and total cell number was determined by counting and the ratios of IL-9– and IL-17–producing CD4+ T cells were assessed by flow cytometry. *, p < 0.05.
Supplementary Figure 4  The number of IL-17–producing CD4+ T cells decreases in the CNS of IL-4–deficient mice at the onset of EAE. (A, B) EAE was induced by immunizing wild-type, IL-4–deficient mice, with MOG$_{35-55}$ and i.p. injections of PTX on days 0 and 2 after immunization. PBS or rIL-4 (200 ng/ mouse) was administered from day 0 every other day. CNS cells were harvested at 13-15 days after immunization with MOG$_{35-55}$ and the number of IL-17–producing CD4+ T cells were determined by multiplying the frequency of each population by the total number of cells isolated per treatment group (n = 5). *, p < 0.05.