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Neutralization of IL-9 Ameliorates Experimental Autoimmune Encephalomyelitis by Decreasing the Effector T Cell Population

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Multiple sclerosis is a CD4+ T cell-mediated autoimmune disease affecting the CNS. Multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), have been thought to be Th1-mediated diseases. However, recent studies provide strong evidence that the major pathogenic T cell subsets in EAE are Th17 cells. IL-9, a hematopoietic growth factor, is considered to be a mediator of Th17 cells, but the precise mechanisms of its action are largely unknown. The present study was designed to investigate the role of IL-9 in autoimmune demyelination. IL-9 blockade with anti–IL-9 mAb inhibited the development of EAE, reduced the serum levels of IL-17, the CNS mRNA expression of IL-17, IL-6, IFN-γ, and TNF-α, and the myelin oligodendrocyte glycoprotein (MOG)-induced IL-17, IFN-γ secretion of lymphocytes. Furthermore, anti–IL-9 mAb in culture suppressed IL-17 production of MOG-reactive T cells and their potency in adoptive transfer EAE. These findings indicate that the protective effect of IL-9 blockade in EAE was likely mediated via inhibition of the development of MOG peptide-specific T cells, which in turn led to reduced infiltration of T cells into the CNS. Thus, anti–IL-9 mAb treatment may provide an effective therapeutic strategy against autoimmune diseases. The Journal of Immunology, 2010, 185: 4095–4100.
Materials and Methods

Mice

C57BL/6j mice were obtained from The Jackson Laboratory (Bar Harbor, ME). For all experiments, 8- to 10-wk-old female mice were used. All animals were housed under specific pathogen-free conditions and animal protocols were approved by the Thomas Jefferson University Animal Care and Use Committee. Paralyzed mice were afforded easy access to food and water.

Induction of EAE

Mice were immunized s.c. with 200 μg myelin oligodendrocyte glycoprotein (MOG35–55) peptide emulsified in CFA and injected twice with pertussis toxin (27). The severity of EAE was monitored and graded on a scale of 0–5: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and forelimb paralysis; and 5, moribund and death.

Anti–IL-9 mAb treatment

Anti–IL-9 mAb (clone D9302C12; BD Pharmingen, San Diego, CA) was confirmed in vitro to have inhibitory effects upon IL-9 bioactivity (28). In a pilot study, we sought to ascertain the optimal dose of anti–IL-9 mAb in vivo. In brief, anti–IL-9 mAb at doses of 0.3 mg, 1.2 mg, 4.8 mg (n = 3 in each group) or isotype-matched hamster IgG at a dose of 4.8 mg (n = 3) was injected i.p. once starting with day -1 of EAE induction. Blood from the orbital vein was obtained via a capillary tube on day 19. Anti–IL-9 mAb was measured by ELISA (eBioscience). We found that a dose of 0.3 mg is optimal (data not shown).

For IL-9 blockade, mice were i.p. treated with 0.3 mg hamster isotype control IgG (Hamster IgG, BD Pharmingen) or anti–IL-9 mAb (clone D9302C12, Hamster IgG; BD Pharmingen) every other day starting on day –1 postimmunization (p.i.). Other groups of mice received 10 ng rIL-9 (Akron Biotech, Philadelphia, PA) dissolved in 0.3 ml PBS daily beginning on the day of EAE induction.

CNS histology

Spinal cords were carefully dissected and immersion fixed in 4% formaldehyde in PBS (27). In brief, fixed tissues were embedded in paraffin wax and sections were cut from the mid-lumbar spinal cord (L3). Sections were stained with H&E or Luxol fast blue (myelin stain) for analyses of inflammation and demyelination, respectively. Slides were assessed in a blind fashion for inflammation and demyelination as described previously (29, 30). Briefly, inflammation was scored as follows: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. Demyelination was scored as follows: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination.

Isolation of CNS-infiltrating cells

Mice were perfused via the left atrium with glucose containing PBS, and spinal cords were removed. Mononuclear cells were isolated by digestion of the spinal cord homogenate with collagenase and DNase, followed by Percoll gradient centrifugation. CNS homogenates were pooled for each treatment group prior to analysis. For flow cytometry analyses, these cells were incubated with Abs to murine CD11b, CD11c, CD4, CD45, CD8α, and B220 (all from BD Pharmingen). The absolute numbers for each population were calculated by multiplying the frequency of each population by the total number of cells isolated per treatment group.

Gene expression analysis by real-time PCR

Mice were sacrificed at the peak of actively induced EAE disease; IgG-treated group (n = 8; mean score, 3.25 ± 0.14; range, 3–3.5). Anti–IL-9 mAb-treated group (n = 4). Additionally, six healthy mice were sacrificed to serve as controls. Animals were perfused transcardially with PBS, and spinal cords were removed and stored in RNAlater (Ambion, Austin, TX). Tissues were homogenized with a TissueLyser (Qiagen, Valencia, CA) and RNA was extracted using RNeasy Lipid Tissue Midi kits (Qiagen). cDNA was synthesized using SuperScript II first-strand synthesis kits (Invitrogen, Carlsbad, CA) and gene expression was measured by TaqMan real-time PCR (Applied Biosystems, Foster City, CA). β-actin was used as an endogenous control in all samples, and levels of gene expression were compared with healthy controls. Relative expression was calculated following the previously described protocol (27).

Adoptive EAE

Female 8- to 10-wk-old wild-type mice were immunized s.c. with 100 μg MOG35–55 containing 200 μg Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI) on day 0 and day 7. The immunized mice were treated with anti–IL-9 mAb (300 μg) once on days −1, 0, 2, 4, 6, 9, and 10 or with 20 ng IL-9 every day beginning on the day after injection. Control mice were treated with PBS. Lymph node cells were harvested on day 12 p.i. and cultured for 3 d in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, l-glutamine, HEPES, sodium pyruvate, and 2-ME. All cells were cultured in the presence of MOG35–55 (50 μg/ml) and IL-23 (10 ng/ml). After 72 h, cells were harvested, washed in PBS, and transferred to naïve recipient mice (2 × 107 cells/mouse) via the tail vein. The mice were given two doses (250 ng/mouse) of pertussis toxin i.p. on days 0 and 2 after cell transfer. EAE disease induction was assessed as described above.

Intracellular cytokine staining

Draining lymph node cells were stimulated with 15 μg/ml MOG35–55 peptide for 72 h and restimulated with 50 ng/ml PMA and 750 ng/ml ionomycin for the last 4 h in the presence of 10 μg/ml brefeldin A. To analyze lymphocytes infiltrated into the CNS, spinal cords were removed as described above, followed by digestion with collagenase D (5.0 mg/ml; Roche Diagnostics, Indianapolis, IN). Cells were isolated by Percoll centrifugation as previously described (27) and surface-stained with Abs against CD4 (RM4-5; BD Biosciences, San Jose, CA). Intracellular cytokine staining and FoxP3 staining were performed as described previously (27, 31). Cells were analyzed by FACS Aria flow cytometer (BD Biosciences), and data obtained were analyzed by FlowJo software (Tree Star, Ashland, OR).

Recall response and T cell cytokine production

Spleens were taken from mice sacrificed 8 d after MOG35–55 immunization. Cells were gently dispersed through nylon mesh into a single-cell suspension, washed, and cultured at 106 cells/ml in complete RPMI (RPMI 1640 containing 10% heat-inactivated FCS [CSL., Parkville, Victoria, Australia], 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were either stimulated with MOG35–55 (15 μg/ml) or anti-CD3 (1 μg/ml) plus CD28 (1 μg/ml) or left without any exogenous stimuli. For proliferation, [3H]thymidine (1 μCi/well) was added at 60 h, and incorporation of thymidine in DNA was measured after 12 h by a Wallac 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer Life Sciences, Foster City, CA). Supernatants were collected at 72 h, and quantitative ELISA was performed for cytokines using paired mAbs as recommended by the manufacturer (R&D Systems, Minneapolis, MN). For serum cytokine ELISA, blood was taken during the course of EAE via the tail vein, and serum samples were prepared using Z-Gel microtubes (Sarstedt, Newton, NC). Mouse IL-17 was detected with a Quantikine immunoassay kit (R&D Systems).

In vitro Th17 cell differentiation

Spleens were removed from naive mice and passed through a 100-μm cell strainer. Erythrocytes were lysed. CD4+ T cells, purified using magnetic bead separation kits (Miltenyi Biotec, Auburn, CA), were cultured with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) Abs (BD Pharmingen), TGF-β (3 ng/ml), IL-6 (30 ng/ml), and anti–IL-4 (10 μg/ml) (all from Invitrogen) in the presence or absence of recombinant mouse IL-9 (10 ng/ml) (Akron Biotech), or anti–IL-9 mAb (100 ng/ml).

Statistical analysis

The two-tailed Student t test and the χ2 test were used to analyze the significance of results. Additionally, the Mann-Whitney U test was performed for nonparametric analyses. Differences were considered significant at p ≤ 0.05.

Results

Anti–IL-9 mAb treatment suppressed acute EAE

To determine the preventive efficacy of IL-9 blockade in autoimmune inflammatory disease, we tested the effect of anti–IL-9 mAb on EAE. Anti–IL-9 mAb treatment delayed the onset of clinical disease, alleviated the severity of EAE (Fig. 1A), and reduced its incidence from 100 to 25% (Fig. 1B). This result is consistent with studies by Nowak et al. (17) demonstrating that targeted genetic disruption of IL-9R results in resistance to EAE.
Anti–IL-9 mAb-treated mice are devoid of CNS-infiltrated cells

To investigate the population of mononuclear cells that infiltrated into the CNS, we recovered mononuclear cells from spinal cords and stained them with CD4, CD8, B220, and CD11b Abs. At the peak of EAE, CD4+ T cells, CD8+ T cells, B cells, and macrophages were detected in control IgG-treated mice (Fig. 3A). In anti–IL-9 mAb-treated mice, the numbers of these cells were significantly reduced (Fig. 3A). Compared to control IgG-treated mice, the CNS of anti–IL-9 mAb-treated mice expressed significantly lower levels of IL-17, IL-6, IFN-γ, and TNF-α, but higher levels of FoxP3 mRNA (all \( p < 0.05-0.001 \); Fig. 3B).

Induction of MOG-specific T cells was suppressed in anti–IL-9 mAb-treated mice

To investigate whether the deficiency of CNS-infiltrating CD4+ T cells in anti–IL-9 mAb-treated mice was due to a T cell priming defect, we analyzed MOG35–55 peptide-specific CD4+ T cells from draining lymph nodes. In the lymphocytes prepared from inguinal lymph nodes at priming stage (8 d after Ag immunization), the development of Th17 cells as well as Th1 cells was significantly suppressed in anti–IL-9 mAb-treated mice compared with control mice (Fig. 4C). These results suggest that suppression of the differentiation of MOG35–55–reactive Th17 and Th1 cells by IL-9 blockade contributes to the protective effect against EAE.

Effect of anti–IL-9 mAb on Th17 differentiation in vitro

Given the role of IL-9 as a mediator of Th17-driven inflammatory disease (17), we next examined whether anti–IL-9 mAb had a direct effect on Th17 cell differentiation. Indeed, CD4+ T cells cultured with anti–IL-9 mAb showed significantly lower levels (3-fold decrease) of IL-17 production compared with wild-type CD4+ T cells when cultured with IL-6 plus TGF-β, suggesting that neutralizing IL-9 with anti–IL-9 mAb decreased the development of Th17 cells (Fig. 5A). IL-17 production was enhanced in the presence of recombinant IL-9 (Fig. 5A), suggesting an amplification loop in which IL-9 produced by Th17 cells participates in enhancing further differentiation of Th17 cells.
Anti–IL-9 mAb inhibits the generation of MOG-specific T cells in vivo

Because in vitro treatment of T cells with IL-9 mAb inhibited the differentiation of Th17 cells, we next investigated whether IL-9 mAb treatment is capable of inhibiting the generation of encephalitogenic T cells in vivo. Donor mice were treated with anti–IL-9 mAb or rIL-9; T cells from these donor mice were stimulated with MOG and IL-23 for 3 d in vitro and then transferred to recipient mice. In the control group, EAE was induced by adoptive transfer of enriched Th17 cells from donor mice that received only PBS. Mice that received MOG-specific T cells from IL-9 mAb-treated donor mice exhibited significantly less severe clinical signs and disease severity compared with the control EAE group (Fig. 5B). In the control group, EAE was induced by adoptive transfer of enriched Th17 cells from donor mice that received only PBS. Mice that received MOG-specific T cells from IL-9 mAb-treated donor mice exhibited significantly less severe clinical signs and disease severity compared with the control EAE group (Fig. 5B). In the control group, EAE was induced by adoptive transfer of enriched Th17 cells from donor mice that received only PBS. Mice that received MOG-specific T cells from IL-9 mAb-treated donor mice exhibited significantly less severe clinical signs and disease severity compared with the control EAE group (Fig. 5B). In the control group, EAE was induced by adoptive transfer of enriched Th17 cells from donor mice that received only PBS. Mice that received MOG-specific T cells from IL-9 mAb-treated donor mice exhibited significantly less severe clinical signs and disease severity compared with the control EAE group (Fig. 5B). In the control group, EAE was induced by adoptive transfer of enriched Th17 cells from donor mice that received only PBS. 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Mice that received MOG-specific T cells from IL-9 mAb-treated donor mice exhibited significantly less severe clinical signs and disease severity compared with the control EAE group (Fig. 5B).
cells transferred significantly less severe EAE to naive animals. Importantly, anti–IL-9 mAb negated this effect, as demonstrated by a low level of IL-17 production in anti–IL-9 mAb-treated cultures. Consistent with these observations, rIL-9 in culture enhanced IL-17 production and the potency of MOG-reactive T cells to transfer EAE. These findings provide further evidence that suppression of adoptive transfer EAE by anti–IL-9 mAb is mediated by Th17 suppression.

In this study, we observed that anti–IL-9 mAb treatment decreased the induction of Th1 cells during the priming stage of lymph nodes, suggesting that IL-9 may regulate the differentiation of Th1 cells. In agreement with this, it has been reported that IL-9 can induce IFN-γ production and suppress Th2 immune response in *Mycobacterium* infections (35). The effects of IL-9 were not limited to antagonizing suppressive factors in *Mycobacterium* infections; it also amplified the stimulatory activities of IL-2 (35, 46) and IL-6 (35). This general stimulation of cytolytic activities was accompanied by and dependent on IFN-γ, as the IFN-γ message was up-regulated by IL-9 (35). Moreover, Ag- and IL-18-stimulated Th1 cells can strongly produce IFN-γ and IL-9 (47). Culturing of sorted IFN-γ+ Th1 cells with OVA, IL-2, and IL-18 in the presence of APCs led to an increase in IL-9, IL-13, IFN-γ, and GM-CSF, but not IL-4 production (48). These results indicate a positive correlation between IL-9 and Th1 cells. Thus, decreased Th1 response after anti–IL-9 treatment, together with suppressed Th17 cells, represents an important mechanism underlying anti–IL-9–induced EAE suppression.

When comparing the developmental suppression of Th17 and Th1 in peripheral lymphoid tissue of anti–IL-9 mAb-treated mice, the frequency of Foxp3+ cells was also slightly diminished at the priming stage. At the same time, the effect of IL-9 blockade against the proliferation of MOG35–55 peptide-specific periphery T cells suggests that the deficiency of CNS-infiltrating CD4+ T cells during the priming stage of EAE is mediated not only via suppression of IL-9–induced inflammatory reactions, but also via inhibition of the induction of MOG35–55 peptide–specific Th17 and Th1 cells.

![FIGURE 4](http://www.jimmunol.org/). IL-9 blockade suppressed the induction of MOG35–55 peptide–specific T cells in peripheral lymphoid tissue. Intracellular staining of lymphocytes stimulated with MOG35–55 peptide is shown. Inguinal lymph node cells were recovered at day 8 p.i. All plots were gated on CD4+ T cells (A, B). C, IL-9 blockade suppressed the Ag-specific cytokine production of IL-17 and IFN-γ from lymphocytes. Splenocytes were recovered at the peak of disease after immunization and restimulated with 50 μg/ml MOG35–55 peptide for 72 h. IL-17 and IFN-γ concentrations in the supernatants were determined. D, IL-9 blockade suppressed the serum level of IL-17. Mice were treated with anti–IL-9 mAb or control IgG, and serum samples were prepared at the peak of EAE after Ag immunization. IL-17 cytokine concentrations in the serum were analyzed. 

![FIGURE 5](http://www.jimmunol.org/). Effect of anti–IL-9 mAb on the encephalitogenicity of MOG-specific Th17 cells. A. IL-17 production by naive CD4+ T cells cultures under optimal Th17 conditions and in the presence of either IL-9 (10 ng/ml) or anti–IL-9 mAb (100 ng/ml). B. Donor mice were immunized with MOG, IFA, and *M. tuberculosis*. Immunized mice were treated with either rIL-9 (20 ng/mouse), anti–IL-9 mAb (300 μg/mouse), or PBS. On day 12 p.i., mice were sacrificed and total lymph node cells were further primed with MOG35–55 (50 μg/ml) and IL-23 (10 ng/ml) for 3 d. A total of 2 × 10^7 viable MOG-specific Th17 cells were adoptively transferred to naive recipient mice (n = 5/group). Mice were examined for clinical signs every day until 20 d postransfer. *, Comparison between rIL-9–treated mice and wild-type mice. **p < 0.05; **p < 0.001; ***p < 0.0001, comparison between rIL-9–treated mice and wild-type mice.
Th1 cells, which in turn leads to reduced infiltration of T cells into the CNS. These findings not only provide evidence for an important pathogenic role of IL-9 in EAE, but also indicate that anti-IL-9 mAb treatment might represent a promising therapy for human MS and other Th17-mediated chronic autoimmune diseases.

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Disclosures
The authors have no financial conflicts of interest.

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