Amelioration of Experimental Autoimmune Encephalomyelitis in IL-4R α ^{-/-} Mice Implicates Compensatory Up-Regulation of Th2-Type Cytokines

Stefanie Gaupp,* Barbara Cannella,*
and Cedric S. Raine*†‡

From the Departments of Pathology,* Neurology,† and
Neuroscience,‡ Albert Einstein College of Medicine, Bronx,
New York

The cytokine receptor interleukin (IL)-4R, expressed by lymphocytes, is well known for its role in immunomodulatory signaling and has also been documented on oligodendrocytes, suggesting involvement in glial cell interactions. In the present study, we investigated the clinical course and pathology of experimental autoimmune encephalomyelitis in mice demonstrating deletion of IL-4R and found a correlation with cytokine expression during acute and chronic disease. Wild-type (WT) littermates served as controls. Although IL-4R^{-/-} mice displayed a milder course throughout, they showed comparable pathology to WT in the acute phase. However, during the chronic phase, IL-4R^{-/-} mice exhibited extensive remyelination and an apparent increase in oligodendrocytes. Cytokine patterns were examined by immunocytochemistry, fluorescence-activated cell sorting, and enzyme-linked immunosorbent assay and were strongly proinflammatory within the central nervous system during the acute phase in WT mice whereas IL-4R^{-/-} animals expressed higher levels of IL-6 and IL-10 that became more pronounced with time. The milder experimental autoimmune encephalomyelitis and enhanced remyelination in IL-4R^{-/-} mice appeared to be related to a shift toward a Th-2 pattern involving mainly IL-6 and IL-10. These data suggest that IL-4R exerts a negative regulatory role on oligodendrocytes that when deleted results in enhanced myelin repair. (*Am J Pathol* 2008, 173:119–129; DOI: 10.2353/ajpath.2008.071156)

Multiple sclerosis (MS) is a debilitating condition of the central nervous system (CNS) characterized clinically by relapses and remissions and pathologically, by myelin

loss, inflammation, and axonal damage.¹ Experimental autoimmune encephalomyelitis (EAE), a major animal model, shares with MS many clinical and histopathological features. In the human disease and EAE, considerable evidence exists to suggest a central role for T-cell-mediated immune responses in disease pathogenesis.² Autoantigen-specific T cells in MS and EAE are believed to cause tissue damage through the production of Th1-type (proinflammatory) cytokines, direct lysis of cells expressing self antigens (cytotoxicity), or through the recruitment of inflammatory cells and antibody production. Less commonly, autoimmune demyelination can be mediated by Th2-type cells.^{3,4} Numerous studies have shown changes in the Th1/Th2 balance to be important during both pathogenesis and/or regulation of EAE.⁵ Immunoregulatory cytokines, such as interleukin (IL)-4 and IL-10, favor Th2-type reactions and either block development of Th1-type responses or directly inhibit proinflammatory cytokines secreted by Th1-type cells.^{6–8}

Previous studies on IL-4 in EAE in different mouse strains focused on its immunomodulatory function. For example, intraperitoneal administration of SJL mice with IL-4 led to amelioration⁹; C57B/6 and BALB/c mice deficient in IL-4 were found to be differently susceptible to EAE¹⁰; gene therapy of Biozzi mice with IL-4 inhibited progression of disease¹¹; and non-EAE-susceptible BALB/c mice treated with neutralizing antibody to IL-4 developed disease.¹² Interestingly, IL-4 and IL-10 and their receptors are not only expressed by cells of the immune system but are also constitutively expressed by glial cells in the human CNS, with some up-regulation in MS,^{13,14} and contribute to the innate immune repertoire of the CNS.¹⁵ In this regard, the presence of the immuno-

Supported in part by the National Multiple Sclerosis Society (grants NMSS RG-1001-K11 and NMSS CA-1022-A-1), the National Institutes of Health (NS 08952 and NS 11920), and the Wollwicz Foundation.

Accepted for publication April 10, 2008.

Present address of S.G.: Ruhr-Universität Bochum, Zentrum Klinische Forschung, Neuroimmunologisches Labor, Bochum, Germany.

Address reprint requests to C.S. Raine, Dept. of Pathology, F140, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. E-mail: raine@aecom.yu.edu.

regulatory cytokine receptor, IL-4R, on oligodendrocytes in the human CNS,¹³ may be relevant to oligodendrocyte biology. In this study, we have investigated the effect of IL-4R deletion on the development of EAE to ask whether this has any impact on myelin pathology. Our results have shown that animals lacking IL-4R α experienced a less severe clinical course and within the CNS, displayed milder pathology. This translated during the chronic phase into enhanced remyelination. The underlying mechanisms appeared to correlate with reduced proinflammatory cytokine levels and an increased immunoregulatory (IL-6/IL-10) response within the CNS of IL-4R $\alpha^{-/-}$ mice.

Materials and Methods

Animals

Six- to eight-week-old IL-4R $\alpha^{-/-}$ BALB/c mice (BALB/c-IL4ra^{tm1sz}) from The Jackson Laboratory (Bar Harbor, ME) were used. This strain has been backcrossed 10 times onto the BALB/c background. Age- and gender-matched normal BALB/c mice, also from The Jackson Laboratory, served as controls.

Antigen and Antibodies

Myelin was prepared from guinea pig spinal cord according to established procedures.¹⁶ Monoclonal antibodies were used for immunohistochemistry, enzyme-linked immunosorbent assay, and fluorescence-activated cell sorting (FACS): CD4 T cells (L3T4), CD8 T cells (Lyt-2), macrophages (F4/80), IL-10, tumor necrosis factor (TNF)- α (AbD Serotec, Raleigh, NC), interferon (IFN)- γ (BD Pharmingen, San Diego, CA), IL-4R (R&D Systems, Minneapolis, MN), IL-6R (Santa Cruz Biotechnology, Santa Cruz, CA), and IL-10R (Genetex, San Antonio, TX). For phenotypic controls, mAbs against CNPase, MAG, and PLP (Chemicon, Temecula, CA), were used.

Sensitization for Active EAE and Assessment of Clinical Signs

On day 0, BALB/c mice were sensitized for active EAE by subcutaneous injection (two sites, dorsal flanks) with 700 μ g of myelin emulsified in incomplete Freund's adjuvant, containing 35 μ g of *Mycobacterium tuberculosis* (Difco, Detroit, MI). As a booster, on days 0, 2, and 7, 100 ng of pertussis toxin was given intraperitoneally. Animals were assessed daily for clinical signs and evaluated according to the following scale: grade 0, no abnormalities; grade 1, weak tail; grade 2, hind-limb weakness; grade 3, hind-limb paraparesis; grade 4, tetraparesis; grade 5, moribund/death. A total of 75 BALB/c mice (both sexes), were sensitized and monitored daily for up to 68 days after immunization, for clinical signs.

Histopathology

Light microscopy was performed on glutaraldehyde/osmium-fixed tissue from optic nerve, cerebrum, cerebellum, and spinal cord (six levels). The tissue was dehydrated and embedded in epoxy resin. One- μ m sections were cut and stained with toluidine blue. Inflammation, demyelination, Wallerian degeneration (WD), and remyelination were scored on a scale of 0 to 5, as described previously.¹⁷ For electron microscopy, thin sections were placed on copper grids, contrasted with lead and uranium salts, carbon-coated, and scanned in a HS600 electron microscope (Hitachi, Tokyo, Japan).

Immunohistochemistry

Blocks were made of phosphate-buffered saline-perfused cervical, thoracic, and lumbar spinal cord (three blocks each level), which were snap-frozen in liquid nitrogen. Cryostat sections (10 μ m) were fixed with cold acetone and methanol for 5 minutes each. After quenching with 0.03% hydrogen peroxide and blocking with 10% bovine serum albumin, sections were incubated with primary antibodies overnight at 4°C. Secondary biotinylated antibodies were applied for 1 hour at room temperature, followed by the avidin-biotin-horseradish peroxidase complex (ABC) reagent (Vectastain Elite kit; Vector Laboratories, Burlingame, CA). 3'-3'-Diaminobenzidine (KPL, Gaithersburg, MD) served as the substrate for horseradish peroxidase. Double-label immunofluorescence confirmed the identity of positive cells, using anti-mouse IgG1-Texas Red (Southern Biotechnology, Birmingham, AL), anti-rat IgG-Alexa 588, or biotinylated anti-mouse IgG followed by streptavidin-Alexa 488 (Molecular Probes, Eugene, OR). Controls comprised use of isotype-matched nonspecific antibodies and omission of primary antibody. Immunohistochemistry was also performed on 1- μ m epoxy sections etched for 1 hour with saturated sodium ethoxide diluted 1:1. Sections were then incubated with anti-MAG antibody for 48 hours, washed, and developed with the ABC kit with 3'-3'-diaminobenzidine as the chromogen.

FACS Analysis

Popliteal lymph node cells, sampled from three to seven pooled BALB/c mice at 9 days after immunization, were cultured with 2.5 μ g/ml of concanavalin A for 12 hours in the presence of Golgi Stop (BD Pharmingen). Cells were fixed with FixPerm (BD Pharmingen) and stained with anti-CD4-FITC, anti-CD8, anti-IL-6, anti-IL-4, anti-IL-13, anti-TNF, anti-IFN- γ , anti-IL-10, or anti-IL-10R antibodies for 1 hour at 4°C. Subsequently, the fluorochrome-labeled antibody rat anti-mouse IgG-Fab-Alexa 568 (BD Pharmingen) was applied for 30 minutes at 4°C. Cell fluorescence was measured using a FACScan flow cytometer (BD Pharmingen), and data were analyzed using WinMDI2.8 software (J. Trotter, Scripps Research Institute, La Jolla, CA). Isotype-specific antibodies were used as controls.

Enzyme-Linked Immunosorbent Assay

Popliteal lymph node cells from BALB/c mice at 9 days after immunization, were cultured for 9 days with 30 μ g/ml of irradiated (3000 rad) myelin for 54 hours. To determine the cytokine content of TNF- α , IFN- γ , IL-10, and IL-4 in the supernatant, the enzyme-linked immunosorbent assay Duo-Set kits from R&D Systems were used according to the manufacturer's protocol. For this investigation lymph node and spleen cells from animals from each group were pooled. A total of 9 WT and 11 IL-4R $\alpha^{-/-}$ animals from three different experiments was investigated.

Statistical Analysis

For statistical evaluation of the clinical course during the indicated observation period analysis of variance for two variables was performed (GraphPad Prism 3.0; GraphPad Software, San Diego, CA). Student's *t*-test was used for comparison of means of FACS data from two to three different experiments. *P* values were considered significant at *P* < 0.05 and highly significant at *P* < 0.01 or *P* < 0.0001.

Results

Lack of IL-4R α Decreases Clinical Severity of EAE

We investigated the effect of the lack of IL-4 receptor on the course of EAE in IL-4R $\alpha^{-/-}$ mice (*n* = 25), for up to 60 days after immunization. For comparison, immunized wild-type (WT) BALB/c mice (*n* = 29), served as controls. Although both groups showed similar clinical signs of EAE, IL-4R $\alpha^{-/-}$ mice consistently showed reduced accumulative disease scores compared to WT animals (Figure 1A). By peak of disease (day 17 after immunization), all animals were ataxic but WT mice also displayed hind-limb paralysis. Both groups recovered in a comparable manner after the first episode. Relapsing activity was not evident. No signs of arthritis attributable to Freund's adjuvant was noted.

Minor Histopathological Differences Seen between Groups during Acute EAE

In contrast to the modest differences in clinical course between WT and IL-4R $\alpha^{-/-}$ mice, at the tissue level, we found negligible differences in pathology during the acute phase (Figure 1B). Cerebral white matter displayed perivascular inflammation but little demyelination in both groups. Spinal cord displayed infiltration by small lymphocytes, neutrophils, and monocytes, restricted mainly to meningeal spaces and subpial zones with WT animals displaying a greater preponderance of neutrophils (Figure 2, A and B). With regard to myelin pathology, scattered demyelinated axons occurred in both groups throughout the zone of infiltration, adjacent to areas con-

Figure 1. **A:** The clinical courses of WT and IL-4R $\alpha^{-/-}$ mice with EAE are presented from one of four experiments throughout a 51-day period. Note that WT mice display a slightly more severe course that was statistically significant (*P* < 0.0001, analysis of variance). **B:** Histograms of histopathology during acute EAE, two animals each group (day 17), reveal minimal differences between WT and IL-4R $\alpha^{-/-}$ groups. Differences were not statistically significant. **C:** During chronic EAE, day 52 after immunization, two animals each group, only inflammation (*P* < 0.01) was statistically significant between the two groups. Student's *t*-test.

taining normally myelinated axons. WD was seen at low levels in both groups.

Remyelination Is a Major Feature in IL-4R $\alpha^{-/-}$ Mice during Chronic EAE

During the chronic phase of EAE, there were minor differences only in the degree of infiltration and demyelination. Some infiltration was present in both groups and demyelinated axons existed in considerable numbers. Some WD was apparent in both groups (Figure 1C). Low-power examination of the lumbar spinal cord revealed broad zones of demyelination in subpial regions. WT mice (Figure 2, C and E) revealed most axons to be chronically demyelinated except for a few thinly myelinated (remyelinated) fibers in deeper layers adjacent to normally myelinated white matter where astrocytes predominated and oligodendrocytes were rare (Figure 2E). On the other hand, many IL-4R $\alpha^{-/-}$

mice displayed a broad band of uniformly thinly remyelinated axons along the inner margins of which an apparent increase in oligodendrocytes was seen (Figure 2, D and F). Immunocytochemistry of 1- μ m epoxy sections reacted with anti-MAG antibody confirmed the apparent increase in oligodendrocytes in IL-4R $\alpha^{-/-}$ mice (Figure 2, G and H). Electron microscopy of the acute phase confirmed the preponderance of neutrophils in the infiltrates in WT mice, the infrequency of WD, and the presence of primary demyelination (Figures 3, A and B). In the chronic phase, demyelination predominated in WT mice with EAE in which astrocytes were the major cellular component, whereas IL-4R $\alpha^{-/-}$ mice displayed an abundance of remyelination and oligodendrocytes (Figure 3, C and D).

Decreased Migration of Cells to CNS during Acute EAE in IL-4R $\alpha^{-/-}$ Mice

Immunocytochemistry of spinal cord sampled during acute EAE (day 15) showed greater numbers of CD4 $^{+}$ T cells within the CNS of WT than in IL-4R $\alpha^{-/-}$ animals (Figure 4, A and B). CD8 $^{+}$ cells were present in both groups in very low numbers only (Figure 4, C and D). Interestingly, examination of peripheral CD4 $^{+}$ and CD8 $^{+}$ cells in lymph node showed comparable levels of CD4 $^{+}$ cells in IL-4R $\alpha^{-/-}$ mice and WT (Figure 5A). Peripheral levels of CD8 $^{+}$ T cells were lower than CD4 $^{+}$ levels, but were slightly increased in IL-4R $\alpha^{-/-}$ mice (Figure 5A). However, the number of CD8 $^{+}$ T cells in the CNS was too low for evaluation (Figure 4, C and D). The number of macrophages in the infiltrates were found to be similar in the two groups but neutrophils were dramatically over-represented in the CNS of WT mice (Figure 2A; Figure 3A; Figure 4, E and F), a feature perhaps related to the differences in clinical course in the WT group (Figure 1).

Elevated Th1- and Th2-Type Cytokines in Lymph Node Cells in IL-4R $\alpha^{-/-}$ Mice during Acute EAE

Within the periphery during acute EAE, levels of proinflammatory and regulatory cytokines produced by lymph node cells showed differences in expression. For example, by FACS analysis, IFN- γ levels were slightly lower in IL-4R $\alpha^{-/-}$ mice (Figure 5B). At the same time, IL-10 and IL-10R expression were increased to statistically significant levels in the knockout (KO) group, perhaps indica-

tive of a simultaneous compensatory mechanism. Furthermore, there was a slight increase in IL-13 and a significant increase in IL-4. In comparison, within the CNS, immunocytochemistry showed that Th1-type cytokines (TNF- α and IFN- γ), were higher in WT mice with acute EAE (Figure 6, A and B), and were associated with macrophages and infiltrating cells. IL-6 and IL-10 were expressed at slightly higher levels in the CNS of IL-4R $\alpha^{-/-}$ mice (Figure 6, rows C and D; left), and were expressed by glial cells (mainly astrocytes). This was further supported by observations on the CNS of mice with chronic EAE, see below.

Elevated Th2-Type Cytokines in CNS of IL-4R $\alpha^{-/-}$ Mice during Chronic EAE

During chronic disease, comparable levels of TNF- α were seen, whereas IFN- γ was higher in WT mice (Figure 6, rows A and B; right). These cytokines were expressed by astrocytes and microglia. The pleiotropic cytokine, IL-6, and to a lesser extent, the anti-inflammatory cytokine, IL-10, were elevated during chronic EAE at higher levels in IL-4R $\alpha^{-/-}$ mice and were expressed mainly by astrocytes (Figure 6, rows C and D; right). It follows then that the enhanced CNS myelin repair occurring in IL-4R $\alpha^{-/-}$ mice (see Figures 2 and 3), may have been associated with an increased anti-inflammatory response.

Anti-Inflammatory Cytokine Receptors Occur on Mouse Oligodendrocytes

It is known that receptors for IL-4, IL-6, and IL-10 occur on human oligodendrocytes¹³ and that expression may increase during inflammatory pathology. In WT mice with EAE, IL-4R was readily demonstrable on CNPase $^{+}$ oligodendrocytes in the spinal cord (Figure 7A). During acute EAE, in both WT and IL-4R $\alpha^{-/-}$ mice, oligodendrocytes and astrocyte processes displayed immunoreactivity for IL-6R and IL-10R (Figure 7, rows B and C; left). During chronic disease, IL-4R $\alpha^{-/-}$ mice displayed somewhat higher level expression of both IL-6R and IL-10R on oligodendrocytes and some astrocyte processes, compared to WT levels (Figure 7, rows B and C; right). Double staining confirmed the identification of oligodendrocytes (Figure 7, rows D and E). Thus, it appeared that there was compensatory up-regulation of IL-6R and IL-10R on glial cells in the IL-4R α KO mice.

Figure 2. Histopathology of lumbar spinal cord in 1- μ m epoxy sections stained with toluidine blue. **A:** Acute EAE: WT, day 17 after immunization. Note prominent inflammation in meningeal space (**top**), which contains an abundance of densely staining neutrophils. The affected spinal cord parenchyma, below, displays extensive demyelination and gliosis. **B:** Acute EAE: IL-4R $\alpha^{-/-}$, day 17 after immunization. Meningeal infiltration is shown but contains few neutrophils. Demyelination and gliosis are also present within spinal cord white matter. **C:** Chronic EAE: WT, day 52 after immunization. A large demyelinated lesion in the subpial zone extends along the ventral fissure. Note the large number of naked axons. Inflammation is seen within the meninges (**left**). Some WD in the form of vacuolated or collapsed myelin sheaths, is also shown. **D:** Chronic EAE: IL-4R $\alpha^{-/-}$, day 52 after immunization. A lesion similar to **C** is shown except that nearly all affected fibers display thin myelin sheaths (remyelination). Some WD is also present. **E:** Detail from **C**. Note the well-preserved naked axons and the many fibrous astrocytes (a). A few fibers at the lesion margins (**bottom right**), display thin remyelination. **F:** Detail from the lesion shown in **D**. Note the widespread remyelination and the abundance of oligodendrocytes (**arrows**) and scattered astrocytes (a). **G:** Immunocytochemistry of an adjacent block of lumbar spinal cord from a WT mouse with chronic EAE 52 days after immunization. A demyelinated lesion contains a few lightly stained oligodendrocytes (**arrows**) and an abundance of astrocytes (a), identifiable by the perinuclear rim of dense chromatin and no cytoplasmic staining. One μ m epoxy section immunoreacted with anti-MAG antibody. **H:** Comparable section to **F** from an IL-4R $\alpha^{-/-}$ animal at 52 days after immunization, immunostained for MAG. Note the abundance of oligodendrocytes that display cytoplasmic staining for MAG. Astrocytes (a) are less numerous. Original magnifications: $\times 300$ (**A–D**); $\times 750$ (**E–G**).

Figure 3. Electron microscopy of acute and chronic EAE in WT and IL-4R^{-/-} mice. **A:** Acute EAE: WT, day 17 after immunization. Note the many polymorphonuclear neutrophils within the meningeal infiltrates and the demyelinated axons (a) within the spinal cord parenchyma below. **B:** Acute EAE: IL-4R^{-/-}, day 17 after immunization. The meningeal infiltrate contains fewer neutrophils and the spinal cord also displays demyelinated axons (a). **C:** Chronic EAE: WT, day 52 after immunization. A subpial lesion displays extensive demyelination. A few axons are thinly remyelinated. Fibrous astrocytes (A) are common in the parenchyma and two plasma cells can be seen in the leptomeningeal space, above. **D:** Chronic EAE: IL-4R^{-/-}, day 52 after immunization. Note the widespread remyelination and the abundance of oligodendrocytes (asterisk). Original magnifications: $\times 2000$ (A, C, D); 2500 (B).

Discussion

As has been the experience with a number of KO models, in this study on the effect of the deletion of a gene important in immunoregulation, IL-4R α , on the course and pathology of EAE, findings at variance with those anticipated were the

result. In this case, instead of a worsening effect, IL-4R α ^{-/-} mice displayed a somewhat ameliorated course in comparison to WT controls. These findings are in accord with those of Radu and colleagues,¹⁸ which showed in a diabetic model, that genetically altered IL-4R protected against disease. This strongly suggested the existence in our model of

Figure 4. Immunocytochemistry of spinal cord infiltrates during acute EAE, day 15 after immunization. **A:** Wt EAE; CD4 T cells. Intense infiltration by CD4⁺ cells is shown along penetrating blood vessels and throughout the parenchyma. **B:** IL-4R^{-/-} EAE; CD4 T cells. Note the reduced involvement of CD4⁺ cells in comparison to the WT. **C** and **D:** CD8 T cells. Comparable low-level expression of CD8 is seen in both groups. **E:** Wt EAE; neutrophils. Diffuse infiltration of neutrophils is seen. **F:** IL-4R^{-/-} EAE, neutrophils. Few neutrophils are present. Original magnifications: $\times 20$ (A–F); $\times 40$ (A–F, insets).

a compensatory effect of a different gene(s) with similar function, and indeed, protein levels showed an increase in IL-6 and IL-10 in the KO group that became more apparent in chronically affected animals. At the structural level, the most significant observation from this study was the appearance of enhanced remyelination in the IL-4R $\alpha^{-/-}$ group, a phenomenon associated with an apparent increase in oligodendrocytes over levels seen in WT mice. This increased myelin repair correlated with elevated IL-6 and IL-10 expression in chronic animals. Because IL-4R is known to be present on immune cells and glia and because an accumulation of CD4⁺ and CD8⁺ cells occurred within the pe-

riphery, it is possible that the lack of IL-4R may have served to protect the CNS against inflammation-related injury. However, precisely what role IL-4R plays on oligodendrocytes remains to be determined but the occurrence of an apparent increase of these cells in IL-4R $\alpha^{-/-}$ mice suggests that in the normal CNS, IL-4R may act as a negative regulator of oligodendrocyte development as well as being immunomodulatory.

The essential requirement of cytokines in Th cell differentiation is challenged by several studies *in vivo*,^{19,20} in which specific cytokines were absent from the environment. In support of a compensatory theory, such studies have shown that Th1 and Th2 CD4⁺ T cells can develop in the absence of IL-4.^{21,22} It has also been shown that IL-4R is not required for the development of Th2 cells.²³ However, a lack of IL-4R in the context of EAE has not been tested in detail, evidence for its role being indirect and derived from studies on IL-4, a cytokine known to be protective during the progression of EAE^{10,24} and in studies on Th17 function in EAE.²⁵ In the latter case, it was shown that IL-13 (which shares the IL-4R α chain with IL-4), is required for Th17 suppression in IL-25-mediated protection from EAE. Although IL-13 was not examined here in detail in IL-4R $\alpha^{-/-}$ mice, the question of whether the effects observed reflect an IL-13 response (rather than an IL-4 effect), cannot be excluded, particularly in view of the milder disease course observed in the IL-13^{-/-} group described in the above study.²⁵ Therefore, the present results showing milder EAE in IL-4R $\alpha^{-/-}$ mice may have been influenced by the known protective effect of IL-13 during autoimmune demyelination, perhaps through interactions with dendritic cells.²⁶ In contrast, other studies using mice deficient for IL-4,²⁷ or treated with IL-4 neutralizing antibodies,¹² reported striking effects on Th1/Th2 development. Some studies on active EAE in IL-4^{-/-} mice propose a less critical role for the cytokine in disease regulation.^{6,28–31} Although specific responses were significantly down-regulated in the absence of IL-4, none were completely abrogated. Furthermore, relapses were reported to be less frequent and less distinct in IL-4^{-/-} mice,²⁴ and in Lewis rats, IL-4 expression did not correlate with disease pathogenesis.³² These findings are of interest in view of the many and sometimes conflicting reports on the modulation of

Figure 5. Analysis of lymph node cell expression of T cells and cytokines during acute EAE day 11 after immunization. **A:** FACS analysis showing that CD4 T-cell levels were comparable between groups whereas CD8 T-cell levels were higher in the IL-4R^{-/-} group. Data represents pooled results of three experiments (three animals per group), using cells from 18 mice. **B:** FACS showing that immunomodulatory cytokines (IL-13, IL-4, IL-10, and IL-10R) were uniformly higher in the IL-4R^{-/-} group. Data represents pooled results from two experiments (14 mice). Differences in IL-4, IL-10, and IL-10R between the two groups were statistically significant: * $P \leq 0.05$, ** $P \leq 0.01$. **C:** Enzyme-linked immunosorbent assay of pooled lymph node and spleen cells showing cytokine profiles similar to FACS analysis in **B**. Results are drawn from three experiments (20 mice total).

Figure 6. Immunocytochemistry of cytokine levels in the spinal cords of WT and IL-4R^{-/-} mice sampled during acute and chronic EAE. In each animal, frozen sections were examined from cervical, thoracic, and lumbar spinal cord levels. **A:** TNF expression during the acute phase is higher in WT mice compared to IL-4R^{-/-} mice (**left**), but is comparable during the chronic phase (**right**). **B:** IFN- γ levels are higher in the WT group during both acute and chronic disease. **C:** IL-10 immunoreactivity, mainly astrocytic, is elevated in the IL-4R^{-/-} animals during acute and chronic EAE. **D:** IL-6 positivity on glial cells is increased in the IL-4R^{-/-} group during both acute and chronic phases. Original magnifications: $\times 200$.

encephalogenicity by IL-4,⁹ and the reported ability of Th2 T cells to protect against ongoing EAE.³³ It is known that IL-4 signaling might even promote Th1 responses and play a role in the development of dendritic cells,³⁴ suggesting that in IL-4R α ^{-/-} mice, dendritic cells may be less effective in polarizing CD4 responses toward a Th1 phenotype.

The presence of TNF- α in the CNS during the chronic stage of EAE may be in line with the observed increased remyelination and enhanced numbers of oligodendrocytes demonstrated previously in TNF- α KO animals that displayed delayed remyelination and reduced proliferation of oligodendrocytes.³⁵ In contrast to TNF- α , it has been reported that IFN- γ inhibits the development of oligodendrocytes.^{36,37} Therefore, the relative decrease of this proinflammatory cytokine during the chronic phase in the present study might promote an increase in oligodendrocytes and remyelination.

The increase in IL-6 and IL-10 in the present work during the chronic phase raises a number of intriguing

possibilities, the most attractive of which is a Th1-Th2-type switch. IL-6 displays dual pro- and anti-inflammatory roles, depending on its origin, amount, and presence of other cytokines.³⁸ In the context of EAE, results vary with those of Willenborg and colleagues³⁹ showing that application of exogenous IL-6 inhibited EAE, whereas Gijbels and colleagues⁴⁰ reported that administration of neutralizing antibodies to IL-6 reduced EAE. Although initially thought to be proinflammatory,⁴¹ many studies indicate that IL-6 has prominent anti-inflammatory and immunosuppressive effects and may negatively regulate the acute phase response.^{41,42} It has been shown that IL-6 acts on the CNS to elicit the release of ACTH, increasing glucocorticoid levels, which suppress the synthesis of proinflammatory cytokines such as TNF- α .^{43,44} In support of such suppression, we found decreased levels of TNF in the CNS during acute EAE. A major role in regard to remyelination might be played by IL-6, which is expressed mainly by astrocytes.^{45,46} A recent study by Zhang and colleagues⁴⁷ reported that increased differ-

Figure 7. Cytokine receptor expression in the spinal cords of WT and IL-4R $\alpha^{-/-}$ mice sampled during acute and chronic EAE. **A:** IL-4R immunoreactivity on oligodendrocytes in WT animals is confirmed by double labeling with CNPase. **B:** IL-6R reactivity during acute disease was seen on inflammatory cells, oligodendrocytes, and astrocytes in both groups (left two panels). Expression was increased in the IL-4R $\alpha^{-/-}$ mice during the chronic phase (right two panels). **C:** IL-10R was expressed by inflammatory cells, oligodendrocytes and astrocytes in both groups during acute disease (left two panels) and was somewhat elevated during chronic EAE in the IL-4R $\alpha^{-/-}$ animals (right two panels). **D:** IL-6R expression by oligodendrocytes is verified by co-localization with anti-MAG in this section from an IL-4R $\alpha^{-/-}$ mouse with acute EAE. **E:** IL-10R immunoreactivity on oligodendrocytes is confirmed by double staining for PLP. Original magnifications: $\times 600$ (A, E); $\times 200$ (B, C); $\times 700$ (D).

entiation of oligodendrocyte precursors into mature myelinating cells can be achieved by administration of IL-6/IL-6R fusion protein, observations of relevance to our findings of increased levels of IL-6 being associated with

remyelination. Enhancement of oligodendrocyte differentiation is known to be mediated by IL-6 receptor signaling.⁴⁸ Whether the higher levels of IL-6 reported here were attributable to lack of IL-4 signaling⁴⁹ and led to a

diminished Th1 response in the CNS, remains a possibility. Recently, IL-6 was shown to inhibit Th1 differentiation, while up-regulating SOCS1, a suppressor of cytokine signaling, in activated CD4⁺ T cells.⁵⁰

With regard to IL-10, the expression of which was also elevated in the CNS of IL-4R $\alpha^{-/-}$ animals, it is well known that this cytokine promotes the development of Th2 responses and also down-regulates Th1 cells. Thus, the decrease in CD4⁺ T cells in the periphery might have been the result of this phenomenon. The importance of IL-10 is underlined by studies showing that IL-10 KO mice develop severe chronic EAE, in contrast to IL-4 KO animals.^{6,51} Additionally, IL-10 has a critical role in regulating established EAE, in that treatment with IL-10 antibodies before disease onset can exacerbate the disease.⁵² On the other hand, regulatory CD4⁺ T cells have been shown to suppress EAE via secretion of IL-10.⁵³

Thus, as has been the experience with a number of gene deletion paradigms, elimination of one pathway has not always resulted in an anticipated outcome. Despite this, a number of interesting observations have emerged. In the present case, it was clear that IL-4R had an apparent inhibitory role in the oligodendrocyte response to disease and in its absence, enhanced myelin repair occurred. Secondly, that subsequent to development of Th1-type responses in IL-4R-null animals, a compensatory Th2-type cascade resulted, which at the level of the CNS, involved IL-6 and IL-10. Understanding of the varied outcomes of cytokine manipulations in genetically altered animals with EAE should have beneficial ramifications for MS.

References

1. Frohman EM, Racke MK, Raine CS: Multiple sclerosis—the plaque and its pathogenesis. *N Engl J Med* 2006, 354:942–955
2. Sospedra M, Martin R: Antigen-specific therapies in multiple sclerosis. *Int Rev Immunol* 2005, 24:393–413
3. Lafaille JJ, Keere FV, Hsu AL, Baron JL, Haas W, Raine CS, Tonegawa S: Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J Exp Med* 1997, 186:307–312
4. Genain CP, Abel K, Belmar N, Villinger F, Rosenberg DP, Lington C, Raine CS, Hauser SL: Late complications of immune deviation therapy in a nonhuman primate. *Science* 1996, 274:2054–2057
5. Olsson T: Critical influences of the cytokine orchestration on the outcome of myelin antigen-specific T-cell autoimmunity in experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Rev* 1995, 144:245–268
6. Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK: IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* 1998, 161:3299–3306
7. Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM: Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc Natl Acad Sci USA* 1992, 89:6065–6069
8. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A: IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991, 146:3444–3451
9. Racke MK, Bonomo A, Scott DE, Cannella B, Levine A, Raine CS, Shvach EM, Rocken M: Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J Exp Med* 1994, 180:1961–1966
10. Falcone M, Rajan AJ, Bloom BR, Brosnan CF: A critical role for IL-4 in regulating disease severity in experimental allergic encephalomyelitis as demonstrated in IL-4-deficient C57BL/6 mice and BALB/c mice. *J Immunol* 1998, 160:4822–4830
11. Furlan R, Poliani PL, Marconi PC, Bergami A, Ruffini F, Adorini L, Glorioso JC, Comi G, Martino G: Central nervous system gene therapy with interleukin-4 inhibits progression of ongoing relapsing-remitting autoimmune encephalomyelitis in B10.S mice. *Gene Ther* 2001, 8:13–19
12. Constantinescu CS, Hilliard B, Ventura E, Wysocka M, Showe L, Lavi E, Fujioaka T, Scott P, Trinchieri G, Rostami A: Modulation of susceptibility and resistance to an autoimmune model of multiple sclerosis in prototypically susceptible and resistant strains by neutralization of interleukin-12 and interleukin-4, respectively. *Clin Immunol* 2001, 98:23–30
13. Cannella B, Raine CS: Multiple sclerosis: cytokine receptors on oligodendrocytes predict innate regulation. *Ann Neurol* 2004, 55:46–57
14. Hulshof S, Montagne L, De Groot CJ, Van Der Valk P: Cellular localization and expression patterns of interleukin-10, interleukin-4, and their receptors in multiple sclerosis lesions. *Glia* 2002, 38:24–35
15. Carson MJ, Sutcliffe JG: Balancing function vs. self defense: the CNS as an active regulator of immune responses. *J Neurosci Res* 1999, 55:1–8
16. Moore GR, Traugott U, Raine CS: Survival of oligodendrocytes in chronic relapsing experimental autoimmune encephalomyelitis. *J Neurol Sci* 1984, 65:137–145
17. Moore GR, Traugott U, Farooq M, Norton WT, Raine CS: Experimental autoimmune encephalomyelitis. Augmentation of demyelination by different myelin lipids. *Lab Invest* 1984, 51:416–424
18. Radu DL, Noben-Trauth N, Hu-Li J, Paul WE, Bona CA: A targeted mutation in the IL-4R α gene protects mice against autoimmune diabetes. *Proc Natl Acad Sci USA* 2000, 97:12700–12704
19. Svetić A, Jian YC, Lu P, Finkelman FD, Gause WC: Brucella abortus induces a novel cytokine gene expression pattern characterized by elevated IL-10 and IFN-gamma in CD4⁺ T cells. *Int Immunol* 1993, 5:877–883
20. Noben-Trauth N, Kropf P, Muller I: Susceptibility to Leishmania major infection in interleukin-4-deficient mice. *Science* 1996, 271:987–990
21. Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K: Extent of T cell receptor ligation can determine the functional differentiation of naive CD4⁺ T cells. *J Exp Med* 1995, 182:1591–1596
22. Croft M, Swain SL: Recently activated naive CD4 T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. *J Immunol* 1995, 154:4269–4282
23. Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Paul WE, Sher A: Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4⁺ lymphocytes with a Th2 cytokine profile. *J Immunol* 2000, 164:3047–3055
24. Zhao ML, Fritz RB: Acute and relapsing experimental autoimmune encephalomyelitis in IL-4- and alpha/beta T cell-deficient C57BL/6 mice. *J Neuroimmunol* 1998, 87:171–178
25. Kleinschek MA, Owyang AM, Joyce-Shaikh B, Langrish CL, Chen Y, Gorman DM, Blumenschein WM, McClanahan T, Brombacher F, Hurst SD, Kastelein RA, Cua DJ: IL-25 regulates Th17 function in autoimmune inflammation. *J Exp Med* 2007, 204:161–170
26. Cash E, Minty A, Ferrara P, Caput D, Fradelizi D, Rott O: Macrophage-inactivating IL-13 suppresses experimental autoimmune encephalomyelitis in rats. *J Immunol* 1994, 153:4258–4267
27. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G: Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993, 362:245–248
28. Liblau R, Steinman L, Brocke S: Experimental autoimmune encephalomyelitis in IL-4-deficient mice. *Int Immunol* 1997, 9:799–803
29. Khoruts A, Miller SD, Jenkins MK: Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J Immunol* 1995, 155:5011–5017
30. Lafaille JJ: The role of helper T cell subsets in autoimmune diseases. *Cytokine Growth Factor Rev* 1998, 9:139–151
31. Nagelkerken L, Blauw B, Tieleman M: IL-4 abrogates the inhibitory effect of IL-10 on the development of experimental allergic encephalomyelitis in SJL mice. *Int Immunol* 1997, 9:1243–1251
32. Issazadeh S, Mustafa M, Ljungdahl A, Hojberg B, Dagerlind A, Elde R, Olsson T: Interferon gamma, interleukin 4 and transforming growth

- factor beta in experimental autoimmune encephalomyelitis in Lewis rats: dynamics of cellular mRNA expression in the central nervous system and lymphoid cells. *J Neurosci Res* 1995, 40:579–590
33. Khoury SJ, Hancock WW, Weiner HL: Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor beta, interleukin 4, and prostaglandin E expression in the brain. *J Exp Med* 1992, 176:1355–1364
34. Hochrein H, O'Keeffe M, Luft T, Vandenabeele S, Grumont RJ, Maraskovsky E, Shortman K: Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J Exp Med* 2000, 192:823–833
35. Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP: TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci* 2001, 4:1116–1122
36. Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, Martino G, Schwartz M: Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci* 2006, 31:149–160
37. Butovsky O, Landa G, Kunis G, Ziv Y, Avidan H, Greenberg N, Schwartz A, Smirnov I, Pollack A, Jung S, Schwartz M: Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J Clin Invest* 2006, 116:905–915
38. Lipsky PE: Interleukin-6 and rheumatic diseases. *Arthritis Res Ther* 2006, 8(Suppl 2):S4,1–5
39. Willenborg DO, Fordham SA, Cowden WB, Ramshaw IA: Cytokines and murine autoimmune encephalomyelitis: inhibition or enhancement of disease with antibodies to select cytokines, or by delivery of exogenous cytokines using a recombinant vaccinia virus system. *Scand J Immunol* 1995, 41:31–41
40. Gijbels K, Brocke S, Abrams JS, Steinman L: Administration of neutralizing antibodies to interleukin-6 (IL-6) reduces experimental autoimmune encephalomyelitis and is associated with elevated levels of IL-6 bioactivity in central nervous system and circulation. *Mol Med* 1995, 1:795–805
41. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK: IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998, 101:311–320
42. Tilg H, Dinarello CA, Mier JW: IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol Today* 1997, 18:428–432
43. Joyce DA, Steer JH, Abraham LJ: Glucocorticoid modulation of human monocyte/macrophage function: control of TNF-alpha secretion. *Inflamm Res* 1997, 46:447–451
44. Raber J, O'Shea RD, Bloom FE, Campbell IL: Modulation of hypothalamic-pituitary-adrenal function by transgenic expression of interleukin-6 in the CNS of mice. *J Neurosci* 1997, 17:9473–9480
45. Van Wagoner NJ, Oh JW, Repovic P, Benveniste EN: Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor. *J Neurosci* 1999, 19:5236–5244
46. Van Wagoner NJ, Benveniste EN: Interleukin-6 expression and regulation in astrocytes. *J Neuroimmunol* 1999, 100:124–139
47. Zhang PL, Izrael M, Ainbinder E, Ben-Simchon L, Chebath J, Revel M: Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein. *Mol Cell Neurosci* 2006, 31:387–398
48. Zhang P, Chebath J, Lonai P, Revel M: Enhancement of oligodendrocyte differentiation from murine embryonic stem cells by an activator of gp130 signaling. *Stem Cells* 2004, 22:344–354
49. te Velde AA, Huijbens RJ, Heije K, de Vries JE, Figdor CG: Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha, and IL-6 by human monocytes. *Blood* 1990, 76:1392–1397
50. Diehl S, Anguita J, Hoffmeyer A, Zaph T, Ihle JN, Fikrig E, Rincon M: Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity* 2000, 13:805–815
51. Samoilova EB, Horton JL, Chen Y: Acceleration of experimental autoimmune encephalomyelitis in interleukin-10-deficient mice: roles of interleukin-10 in disease progression and recovery. *Cell Immunol* 1998, 188:118–124
52. Cannella B, Gao YL, Brosnan C, Raine CS: IL-10 fails to abrogate experimental autoimmune encephalomyelitis. *J Neurosci Res* 1996, 45:735–746
53. Stohlman SA, Pei L, Cua DJ, Li Z, Hinton DR: Activation of regulatory cells suppresses experimental allergic encephalomyelitis via secretion of IL-10. *J Immunol* 1999, 163:6338–6344