IL-25 (IL-17E) is a recently identified member of the IL-17 family of cytokines (1). It has been implicated in the initiation of type 2 immunity by driving the expression of IL-4, IL-5, and IL-13 (1, 2). In vitro, IL-25 is found in activated Th2 cells, bone marrow–derived mast cells, and alveolar macrophages (1, 3, 4). Recently, we identified that IL-25 is produced by a population of CD4+ cells in mouse caecal patches (5). Expression of this cytokine is critical for immunity against helminth infections (5, 6). IL-25 protein administration or transgenic overexpression of IL-25 in mice resulted in Th2-like responses associated with inflammation characterized by elevated expression of IL-4, IL-5, and IL-13, inflammatory cell infiltration, and epithelial cell hyperplasia (1, 2, 7, 8).

These findings suggest that IL-25 may be an important mediator of type 2 immune pathologies.

Besides IL-25, there are five additional members that belong to the IL-17 family of cytokines. Although IL-25 promotes allergic responses, IL-17A had been characterized to promote chronic inflammatory responses (9, 10). We have recently reported that IL-17A is produced by a population of IL-23–dependent pathogenic T cells required for the induction of autoimmune inflammation (11–13). This finding has led to the proposal that these IL-17–producing cells (called Th17) represent a novel subset of CD4+ Th cells (14–17). Several recent reports have demonstrated that TGF-β and IL-6 are required for lineage commitment of IL-17–producing cells (18–20), and this novel differentiation pathway does not share transcription regulatory elements with Th1 and Th2 responses (21, 22). Indeed, we have identified an orphan nuclear receptor—retinoid-related orphan receptor γt (RORγt)—as the critical transcription regulator that controls TGF-β and IL-6–induced Th17 differentiation (23).
These remarkable findings provide definitive evidence that Th17 cells are indeed a bona fide T cell subset that is independently regulated from Th1 and Th2.

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune inflammatory disease of the central nervous system (CNS) that serves as a disease model for human multiple sclerosis. EAE can be induced by passive transfer of myelin antigen–specific Th17 or Th1, but not Th2 cells (13, 24, 25). In the inflamed CNS, high levels of proinflammatory cytokines, such as IL-17, IL-18, IFNγ, TNF, and IP10 are expressed with very low levels of Th2 cytokines such as IL-4 and -13 (26, 27). Studies have documented that redirecting the Th cell balance toward a type 2 response can suppress autoimmunity (28–30). It is well accepted that type 2 responses provide multiple signals that inhibit IFNγ-producing Th1 cells (31, 32). Recent in vitro studies have suggested that Th17 cells are also negatively regulated by IL-4 signaling pathways (21, 22). Nevertheless, very little is known about the in vivo regulation of Th17 cells. By generating IL-25–deficient mice, we investigated whether this potent Th2-promoting cytokine plays a physiological role in regulating the development and function of Th1 and/or Th17 encephalitogenic T cells.

RESULTS
IL-25 is locally expressed in the CNS and protects from EAE
IL-25 is a potent cytokine that drives the production of type 2 cytokines such as IL-4, IL-5, and IL-13 (1). During EAE priming, a broad range of cytokines is induced in C57BL/6 mice, including the proinflammatory cytokines IFNγ, IL-17, and IL-18, as well as the type 2 cytokines IL-4 and IL-13. To determine whether IL-25 plays a regulatory role during autoimmune inflammation, we analyzed the expression of this cytokine during the course of EAE. We observed that IL-25 mRNA is expressed in normal spinal cord as well as inflamed cytokine during the course of EAE. We observed that IL-25 is predominantly expressed by microglia in the normal CNS (Fig. 1 B). Remarkably, microglia expressed ninefold more IL-25 mRNA during active EAE. In contrast, inflammatory macrophages or infiltrating T cells do not express detectable levels of IL-25 (unpublished data).

We generated IL-25–deficient mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061738/DC1) to determine whether this cytokine has a role in regulating clinical EAE. These mice have no developmental defects, and they generate litters of normal size. Males and females are born in equivalent numbers. Analysis of IL-25–deficient spleens showed normal numbers and percentages of B cells, T cells, and granulocytes. IL-25–deficient thymi had the same number of cells and the same percentages of CD4 and CD8 double-negative, single-positive, and double-positive thymocytes, when compared with C57BL/6 mice.

After myelin oligodendrocyte glycoprotein (MOG) peptide/CFA immunization, IL-25–deficient (il25−/−) mice showed accelerated EAE onset and enhanced disease severity compared with WT C57BL/6 mice (Fig. 1 C). il25−/− mice consistently developed EAE 2–3 d earlier than WT mice, and all died (100% mortality at day 17) when immunized at doses optimized to give WT mice a nonlethal disease. In addition, incidence of disease reached 100% 6 d earlier in the il25−/− group (Fig. 1 C). Administration of IL-25 to MOG-immunized il25−/− completely protected the mice from EAE (Fig. S1 C), confirming that no other defects are responsible for the enhanced susceptibility of il25−/− mice to EAE. These results indicate that IL-25 may play an important role in suppressing the inflammatory responses that drive EAE pathogenesis.

IL-25 inhibits the Th17 response within the CNS
We have recently described an IL-23–dependent, IL-17–producing T cell population that is highly pathogenic in autoimmune inflammatory diseases (13). Here, we found the
same Th17 cells as well as IFNγ-producing T cells in the CNS of il25−/− mice at an earlier time point and in greater numbers than in the WT mice (Fig. 2). 2 d before expected disease onset, the CNS of il25−/− mice contained fivefold more IL-17+ cells compared with the WT (Fig. 2 A). In contrast, the total numbers of IFNγ-producing T cells were similar in the il25−/− and WT control mice. Also, the total number of inflammatory macrophages was comparable between il25−/− and WT mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061738/DC1). At the peak of disease, the il25−/− mice continue to have greater numbers of IL-17–producing T cells as well as IFNγ and TNF producers (Fig. 2 B).

To examine whether the disease exacerbation observed in the il25−/− mice was mediated by Th17 and/or Th1 cells, we injected anti–IL-17 or anti-IFNγ antagonist mAbs into EAE–primed mice. Blockade of IL-17 dramatically inhibited EAE, indicating that Th17 cells play a major role in EAE pathogenesis in il25−/− mice (Fig. 2 C). In contrast, anti-IFNγ treatment had no effect, consistent with studies demonstrating that Th1 cells only play a minor role in EAE (13). Thus, the absence of IL-25 leads to a more rapid and intense infiltration of pathogenic Th17 cells into the target organ, which drives an accelerated and exacerbated disease phenotype.

IL-25 is crucial for Th2 cytokine expression in the periphery

During EAE priming, myelin antigen–specific T cells are generated in lymph nodes draining the sites of immunization. In these lymphoid organs, T cell differentiation is dependent on the cytokines produced by antigen–presenting cells. In draining lymph node (DLN) cells from il25−/− mice, we found a twofold higher expression of IL-23p19 mRNA than in WT controls at day 7 after immunization (Fig. 3 A). Interestingly, the expression of IL-12p35 was not regulated by IL-25. To determine whether IL-25 affected T cell priming and T cell subset differentiation, we cultured DLN cells at day 7 after immunization in the presence of MOG peptide to assess myelin antigen–specific T cell responses. The Th2 cytokines IL-4, -5, and -13 were significantly reduced in DLN cultures from il25−/− mice. IL-17 and IFNγ levels were slightly higher in il25−/− DLN cultures; however, the difference did not reach statistical significance (Fig. 3 B). In addition to Th2 responses, regulatory T cells play a crucial role in limiting chronic inflammation. We found that il25−/−

![Figure 2. Increased numbers of CNS-infiltrating inflammatory Th17 cells in il25−/− mice.](image)

![Figure 3. Reduced Th2 cytokine production but normal regulatory T cell response in il25−/− DLN cells.](image)
mice generated Foxp3+ regulatory T cells in numbers similar to WT controls during the course of EAE (Fig. 3 C). These data suggest that IL-25 plays a role in driving IL-4, IL-5, and IL-13 production but does not affect regulatory T cell development.

**Exogenous IL-25 inhibits EAE development and progression**

We next determined whether exogenous IL-25 can suppress acute as well as relapse-remitting EAE. We used replication-deficient adenoviral vectors (rAdVs) to induce local CNS or systemic IL-25 expression (33). SJL mice were used for these studies because they exhibit a multiple relapse-remitting pattern of clinical disease. Using rAdV gene transfer vectors, we found that intracerebral delivery of IL-25–rAdV 1 d before immunization fully protected SJL mice from EAE (Fig. 4 A). Mice treated with GFP-control-rAdV were not protected. An intracerebral injection of $3 \times 10^9$ viral particles (Fig. 4 A) or i.v. injection of $10^{10}$ particles (not depicted) were equally effective. Injection of IL-25–rAdV 10 d after initial priming could also block EAE (Fig. 4 A). The histological analysis of spinal cords 18 d after immunization shows large numbers of parenchymal and perivascular infiltrates in the control group. IL-25–rAdV–treated mice, in contrast, show minimal to no infiltrate (Fig. 4 B and Table S1, available at http://www.jem.org/cgi/content/full/jem.20061738/DC1). We next examined whether IL-25 treatment can inhibit the effector phase of CNS inflammation using a passive transfer model of EAE. Recombinant adenoviral vector–mediated IL-25 expression prevented EAE development when the treatment was given at the time of cell transfer, or even 3 d after cell transfer (Fig. 4 C).

In multiple sclerosis, patients usually suffer from episodes of clinical disease relapse separated by periods of remission. Because IL-25–rAdV could protect from EAE when administered at either the priming stage (day 1 treatment) or during the effector stage (day 10 treatment), we tested whether IL-25 could be used therapeutically to modify clinical disease. Remarkably, we found that IL-25–rAdV completely blocked EAE relapse when the treatment was given after the first episode of paralysis (Fig. 4 D). In contrast, control-rAdV–treated mice suffered two rounds of disease relapse over a period of 51 d. Collectively, these results suggest that besides its ability to prevent disease, IL-25 may regulate effector T cells and reduce their pathogenic functions.

**IL-25 induced Th2 and suppressed Th17 responses**

IL-25–deficient mice exhibited increased Th17 responses that promoted severe EAE pathology (Fig. 2). This result led us to examine the in vivo mechanisms of action of IL-25 treatment during disease induction. Daily s.c. injection with mouse IL-25 (mIL)-25 protected the cytokine-treated mice from EAE (Fig. 5 A) as compared with saline-treated C57BL/6 mice as well as iIl25−/− mice. Given that the severe disease observed in iIl25−/− mice appeared to be driven by IL-17 rather than IFNγ (Fig. 2 C), we examined whether IL-25 could inhibit EAE in ifng−/− mice. Indeed, IL-25 treatment protected ifng−/− mice from EAE, suggesting that IL-25 regulation of the Th17 pathway is independent of IFNγ (Fig. 5 B).

To further characterize the immune responses regulated by IL-25, we profiled the ex vivo gene expression pattern of DLN cells 7 d after MOG/CFA priming. We observed a marked down-regulation of IL-17 in IL-25–treated C57BL/6
as well as ifng−/− mice (Fig. 5 C). Both genotypes showed an increased expression of IL-13 after IL-25 treatment. Interestingly, IL-25 did not regulate the expression of total IFNγ mRNA levels in response to priming with MOG peptide and CFA. It is notable that most of the IFNγ response in the DLNs is directed against the mycobacterial component of the adjuvant (only 1–2% MOG-specific T cell response and up to 10–30% anti-mycobacterial response). Thus, it is important to determine the myelin-specific T cell response. The in vitro MOG-specific cytokine recall analysis showed that DLN cells from IL-25–treated mice produced less IL-17 and IFNγ as compared with saline-treated controls (Fig. 5 D). Type 2 cytokines such as IL-4, IL-5, and IL-13 were markedly increased in IL-25–treated animals. Intracellular cytokine staining of DLN cell cultures revealed lower relative and absolute numbers of IL-17–producing CD4+ T cells in IL-25–treated ifng−/− as well as C57BL/6 mice (Fig. 5 E). These findings established that treatment with recombinant IL-25 can suppress autoimmune inflammation by inducing Th2 while suppressing Th1 and Th17 responses.

**IL-13 is required for IL-25–mediated protection from EAE and Th17 suppression**

Previous studies as well as data shown here have demonstrated that IL-25 strongly induced expression of IL-4 and -13, which are potent EAE inhibitors (27, 34). Therefore, we determined whether IL-25 suppression of EAE required the presence of these type 2 cytokines. Daily injection of mIL-25 protein protected Il4−/− mice from EAE (Fig. 6 A). However, IL-25 could not provide protection to Il13−/− mice (Fig. 6 B). The requirement for IL-13 signaling was confirmed by using il4ra−/− mice, which cannot respond to either IL-4 or IL-13. As shown in Fig. 6 C, IL-25 protein failed to protect the Il4ra−/− mice as well. These findings show that IL-13 plays an important role in IL-25–mediated suppression of EAE.

Following the observation that IL-25 regulates EAE through suppression of Th17 responses, we used an in vitro cell culture system to further investigate the underlying mechanism. Naïve DO11.10 CD4+ T cells were cocultured with LPS–activated CD11c+ DCs in the presence of neutralizing antibodies against IFNγ and IL-4. Addition of IL-23 to these cultures induced 10% of IL-17–producing T cells (Fig. 6 D), whereas untreated cultures only contained 0.3% of IL-17 producers (not depicted). The frequency of Th17 cells induced by IL-23 was reduced by half when the cells were grown in the presence of IL-25, showing that IL-25 blocked the in vitro Th17 response, independent of IL-4 (Fig. 6 D).

We also observed that IL-25 dramatically enhanced IL-13 production, even in the presence of IL-23, anti–IL-4, and anti–IFNγ (Fig. 6 E).

**Figure 5.** IL-25 induces Th2 and suppresses Th17 independent of IFNγ. (A) Clinical score of immunized WT and il25−/− mice compared with immunized WT mice injected daily s.c. with mIL-25 protein from day 1 to the end of the experiment. Results are representative for at least four experiments with five to six mice per group. (B) Clinical score of ifng−/− (GKO) mice (n = 5 per group) injected daily with PBS or mIL-25 (s.c.) from days 1–12. Results are representative of two experiments. (C) DLN cells from MOG-immunized WT or ifng−/− mice treated with IL-25 or saline were analyzed for cytokine gene expression at day 7 after immunization (five mice per group; DLN cells were processed in three different pools for mRNA analysis). Cytokine gene expression was normalized to ubiquitin. (D) In vitro cytokine recall response. DLN cells from MOG-immunized WT mice treated with PBS or mIL-25 daily s.c. were isolated at day 7 after immunization. Cells were stimulated with MOG peptide for 3 d and restimulated with anti-CD3 anti-CD28 for 2 additional days. Cytokine levels in supernatants were measured by CBA or ELISA. Cells from individual animals were cultured separately, and data are mean cytokine levels ± SD of three to five mice per treatment group. For IFNγ detection, nine mice per treatment group are shown. Data are representative of at least two experiments. *, P < 0.05. (E) DLN cells from MOG-immunized WT and ifng−/− (GKO) mice treated with IL-25 or saline (pool of four mice per group) were stimulated in vitro with MOG for 3 d followed by incubation with IL-2 for another 2 d. Frequencies (FACS plots) and absolute numbers (bar graph) of MOG-specific IL-17–producing CD4+ cells were assessed. Data shown are representative of two experiments.
IL-13 is required for Th17 suppression in IL-25-mediated protection from EAE. Clinical score of il4−/− (A), il13−/− (B), or il4rα−/− mice (C), with five mice per group injected daily with PBS or mIL-25 (s.c.) from days 1–12. Results are representative of two experiments. (D–F) Naive T cells and LPS-activated CD11c+ DCs were obtained from D011.10 × RAG−/− mice (pool of five mice) and incubated for 3 d with IL-23 in the presence of OVA peptide. IFNγ and IL-4 were neutralized in these cultures. (D) The effect of IL-25 addition on the number of IL-23–induced IL-17 producers was assessed by intracellular staining for IL-17. (E) IL-13 expression in these cultures was determined by LUMINEX. (F) The modulation of IL-17 protein production in IL-23–driven cultures by IL-25, IL-25 plus anti–IL-13, and IL-13 is shown in percentage of IL-23–induced IL-17 producers was assessed by intracellular staining for IL-17. (G) IL-13 expression in these cultures was determined by LUMINEX. (H) DCs were obtained from spleens and superficial lymph nodes and activated with LPS with or without addition of IL-13. After 24 h, mRNA expression relative to ubiquitin was measured for IL-23p19, IL-1β, and IL-6 by quantitative real-time PCR. Shown is an experiment using DCs from C57BL/6 mice representative of two C57BL/6 and two BALB/c experiments with a similar outcome (n = 3–5 mice pooled per experiment).

(Fig. 6 F). Moreover, blockade of IL-13 led to enhanced IL-17 production. Addition of recombinant IL-13 to IL-23–driven cultures reduced IL-17 production similar to the effects of adding recombinant IL-25 (Fig. 6 F). We confirmed that IL-13 is a suppressor of IL-17 production using cells from MOG33-55 TCR transgenic mice (Fig. 6 G). Because the IL-13 receptor is not expressed on T cells (35), we examined the effects of IL-13 on DC function. Remarkably, IL-13 suppressed LPS-mediated IL-23, IL-1, and IL-6 expression, which are required for Th17 differentiation and function (Fig. 6 H). Collectively, these data demonstrate that IL-25 mediates EAE protection as well as Th17 suppression via IL-13. In turn, IL-13 directly suppresses DC production of Th17-promoting factors.

DISCUSSION

In organ-specific autoimmunity, the balance of cytokines is a key determinant of resistance or susceptibility. In EAE, disease susceptibility is thought to correlate with the expression of proinflammatory cytokines such as IL-17, IFNγ, TNF, IL-6, and IL-1β. On the other hand, Th2 cytokines such as IL-4 and IL-13 have been shown to be important for preventing or ameliorating disease (26, 27, 36). In the current study, we show that IL-25, a Th2-promoting cytokine, can protect mice from EAE, and loss of IL-25 results in enhanced proinflammatory cytokine production and accelerated CNS pathology. This accelerated disease is also associated with an increased number of IL-17+, IFNγ+ and TNF-producing T cells that invade the CNS. Even though IFNγ+–producing cells can induce EAE and their presence within the CNS likely contributed to acute inflammation, neutralization of IFNγ in il25−/− mice did not lead to disease amelioration. In contrast, anti–IL-17 mAb treatment significantly inhibited EAE in il25−/− mice, suggesting that the IL-17 immune pathway plays an important role in the disease process. We also demonstrate that IL-25 treatment protects mice from EAE, and this protection clearly depends on the suppression of IL-17–producing T cells. Thus, the presence of IL-25 supports a cytokine environment that limits chronic inflammatory responses.

IL-25 is constitutively expressed in the CNS (37). Interestingly, EAE-resistant BALB/c mice constitutively expressed higher CNS levels of IL-25 mRNA compared with moderately susceptible C57BL/6 (unpublished data). In addition, we found that resident microglia is a primary cellular source of IL-25 in a normal CNS. Importantly, there is a ninefold induction of IL-25 expression in microglia during CNS inflammation. This IL-25 expression pattern supports the hypothesis that microglia have protective roles during chronic CNS inflammation. The observation of tissue-specific expression of IL-25 is consistent with our recent study in a helminth infection model, where we showed that locally expressed IL-25 is crucial for the ability of mice to clear the gut parasite Trichuris muris (5). Moreover, IL-25 is also constitutively expressed in the lungs (1). Again, highly specialized tissue–resident cells, in this case, alveolar macrophages, were shown to be the source of IL-25 (4). Thus, IL-25 is expressed in organ systems where regulation of inflammation is of critical importance. In healthy digestive and respiratory tracts, an anti-inflammatory environment has to be maintained because of the constant exposure to commensal microbes. In immune-privileged sites, such as the eyes or the CNS, inflammation must be constrained because of the detrimental consequences of tissue swelling responses in these organs.
During EAE priming, IL-25 induces IL-4, IL-5, and IL-13 in the periphery. Although IL-4 and IL-13 have been reported to be protective in EAE, there is little evidence that IL-5 plays a regulatory role in EAE (38). In contrast, the role of IL-4 in suppressing organ-specific autoimmunity is well documented (29, 33, 39–41). IL-4 is required for the differentiation of Th cells toward the Th2 pathway (42, 43). In EAE, deviation of the immune system toward a Th2 response correlates with disease resistance (44, 45), and delivery of IL-4 in vivo can modulate the immune response to ameliorate disease (29, 46). Surprisingly, IL-25 treatment still protected il4−/− mice from EAE, suggesting that the levels of IL-4 induced by IL-25 treatment are not sufficient for EAE suppression. In contrast to IL-4, we found that IL-13 is necessary for IL-25-mediated EAE suppression. Both IL-4Rα− (required for IL-13 signaling) and IL-13−deficient mice were not protected by IL-25 treatment, suggesting that IL-25 is acting via IL-13 to suppress disease. On its own, IL-13 protects rats from EAE, through its ability to deactivate inflammatory macrophages, as well as suppressing the production of nitric oxide, IL-1β, and TNF (27).

Here, we demonstrate a novel role for IL-13 in inhibiting Th17 differentiation and function. However, IL-13 can only partially inhibit IL-17 production. In contrast, IL-4 can completely block IL-17 response, consistent with two recent reports (21, 22). These results suggest that IL-13 and IL-4 may use different mechanisms to inhibit Th17 cells. Indeed, although T cells are shown to express the shared IL-4/IL-13 receptor chain IL-4Rα, the specific IL-13 receptor is not expressed on T cells (35). Therefore, IL-13 might act by inhibiting DC function (27). Indeed, we observed that IL-13 blocked Th17-promoting factors such as IL-1β, IL-6, and IL-23.

It is intriguing that in our Th17 cultures (DC-derived IL-6, IL-1β, TGF-β plus exogenous IL-23, anti–IL-4, and anti–IFNγ), IL-25 still induced IL-13 production. We have observed that IL-25 led to IL-13 production in CD4+ T cell populations in vitro (unpublished data). However, it is still unclear which T cell populations are responsive to IL-25 in vivo. It is also possible that IL-25 acts on NKT cells, which play an important role in the early IL-4 production during an immune response (47, 48). NKT cells secrete IL-4, IL-13, and IFNγ upon activation and have an important immunoregulatory role (49, 50). Interestingly, activation of NKT cells by the synthetic ligand α-galactosylceramide can induce IL-4 and IL-13, which protects mice from autoimmune inflammation (51–53). Another possible target of IL-25 may be antigen-presenting cells such as DCs. The IL-25 receptor, IL-17RB, has been found to be up-regulated in DCs under Th2-inducing conditions (54). Consistent with previous reports (55, 56), we observed that IL-25 could induce IL-13 mRNA expression in cultured DCs (unpublished data). Further studies are needed to understand how these IL-13-expressing cells regulate Th17 development.

We have shown that although IL-25 and IL-17 are members of the same cytokine family, they play opposing roles in the regulation of organ-specific autoimmunity. The type 2 responses promoted by IL-25 drive a novel regulatory mechanism for controlling Th17 responses. Unexpectedly, this regulation relies on IL-13 rather than IL-4, suggesting that IL-13 may be secreted at higher levels in the target organs during autoimmune inflammation. This protective mechanism is important because the lack of IL-25 causes devastating inflammatory responses during EAE. Thus, IL-25 expression by CNS-resident microglia may provide an important mechanism that limits brain and spinal cord inflammation. This unique property suggests that IL-25 may be a potential therapeutic agent for a range of organ-specific inflammatory disorders.

**MATERIALS AND METHODS**

**Animals.** il25−/− mice were generated in the C57BL/6 background (Fig. S1). A C57BL/6j genomic BAC library (Genome Systems) was screened with a full-length mLIL-25 cDNA probe, and a positive clone was identified and used to generate a shotgun plasmid library (SeqWright). Subclones were sequenced and assembled to generate 15,747 bp of sequence encompassing the il25 locus. Using a genomic subclone (F6), a BglII site was introduced in exon1 at the ATG via site-directed mutagenesis. This clone was digested with BglII, and a 5,442-bp 5′ region of homology was cloned into the unique BglII site of pgbl-basic (CLONTECH Laboratories, Inc.). A 1,049-bp 3′ region of homology was generated by PCR using a genomic subclone (C6) incorporating unique Spel and NotI restriction sites and cloned into pBS-lexoNeo-lox. This plasmid was digested with Sall, and the lexo-Neo-lox-3′ region of homology was ligated into the unique Sall site of the 5′ arm of pgbl-Base, yielding the final targeting vector. The Nhel-linearized targeting vector was electroporated into C57BL/6 ES cells, and Neo-resistant clones were analyzed for homologous recombination (HR) using a PCR-based screening strategy followed by Southern blot confirmation with 5′ and 3′ probes. A confirmed HR clone was electroporated with a Cre recombinase expression–containing plasmid, and several Neo-sensitive clones were screened using Southern blot to identify the Neo-flipped HR il25−/− ES line. This line was injected into C57BL/6 blastocysts, and il25−/− mice were obtained by intercrossing il25−/− animals. A PCR-based genotyping strategy was developed to track the il25 WT and knockin alleles. il25-targeted allele, sense, 5′-GCTGACTCTCAACATTCCTCCTCC-3′, and antisense, 5′-CCTGCTGTTCAAGTGGCCTCCT-3′; WT il25 allele, sense, 5′-CTACAGACAGGCTCCTACATGGAC-3′, and antisense, 5′-CCTCGTCTCAGGTAGGGGTCTT-3′.

C57BL/6j and il4−/− (C57BL/6-il4−/−Nino/j) mice were obtained from The Jackson Laboratory. il13−/−, il13−/−, ifng−/−, and DO11.10 × Rag−/− mice were from the DNAx colonies. Breeding pairs of il4−/− mice were obtained from F. Brombacher (University of Cape Town, Rondebosch, South Africa) and a colony maintained at DNAx. C57BL/6 MOG35-55 TCR transgenic mice were received from T. Backstrom (Malaghan Institute of Medical Research, Wellington, New Zealand). Animals were maintained in a specific pathogen-free environment and tested negative for pathogens in routine screening. All experiments were performed following the guidelines of the DNAx Institutional Animal Care and Use Committee.

**EAE induction.** For studies in gene-deficient mice, we used the C57BL/6-MOG35-55 model of EAE, as il25−/−, ifng−/−, il4−/−, il4−/−, and il13−/− were bred on a C57BL/6 background. C57BL/6 mice were immunized with 100 μg MOG35-55 peptide and CFA containing 1 mg H37Ra s.c. on day 0 and 100 ng pertussis toxin (PTX) i.v. on day 0 and again on day 2. For disease-prevention studies in relapse-remitting EAE, SJL mice were immunized with 50 μg PLP19–35 plus CFA containing 200 μg H37Ra s.c. on day 0. SJL mice were given 100 ng PTX on day 0 only. For passive transfer EAE, donor SJL mice were immunized with 50 μg PLP19–35 in CFA (no PTX adjuvant was used for the donor mice). 7–8 d after PLP priming, DLN cells were stimulated for 3–4 d with 20 μg/ml PLP19–35, and 2 × 10^6 viable cells

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were transferred into naive recipient SJL mice. Clinical scores were as follows: tail weakness, grade 1; hind limb weakness, grade 2; partial hind limb paralysis and weight loss, grade 3; paraplegia and incontinence, grade 4; quadriplegia and wasting, grade 5; and moribund, grade 6.

**IL-25, IL-25–rAdV, and antibody treatment.** Recombinant mouse IL-25 protein was administered daily s.c., beginning at 1 d before MOG immunization. For administration into the CNS, rAdV gene transfer vectors were suspended in 10 μl PBS and injected into the right-lateral cerebral ventricle using a 28-gauge needle, as described previously (33). For peripheral delivery, rAdVs suspended in 100 μl PBS were injected into the tail vein. Antibodies for neutralization of IFNγ (clone XMG1.2, rlgG1; 200 μg per mouse) and IL-17 (clone 1D10, rlgG1; 1 mg per mouse) and the corresponding isotype control (rat lgG1; 1 mg per mouse) were administered s.c. once a week starting 1 d before immunization.

**Isolation of CNS inflammatory cells.** C57BL/6 mice were immunized as described. At day 7 (before disease onset) and at peak disease (day 11 in the il25−/− and day 14 in WT), mice were killed, and CNS-infiltrating T cells, macrophages, and resident microglia were isolated by digesting brain and spinal cord homogenate with collagenase and DNase followed by Percoll gradient centrifugation as described previously (87). We determined the number of CD4+CD45Ro+ T cells and CD4+CD11b+CD45Ro+ inflammatory macrophages in the CNS by multiplying the percentage of lineage marker–positive cells by the total number of mononuclear cells isolated from the CNS. For further characterization, intracellular cytokine staining was performed (see the following section). For analysis of IL-25 mRNA expression in the CNS, inflammatory macrophages and resident microglia were isolated from irradiation bone marrow chimeric mice produced by the injection of CD45.1 B6 bone marrow cells into irradiated CD45.2 C57BL/6 mice. The CD11b+CD45.1+CD45R0+ inflammatory macrophages and CD11b+CD45.2+CD45R0+ resident microglia (radiation-resistant cells remaining with the host CD45 allotype) were purified by three-color flow cytometric sorting from >100 irradiation bone marrow chimeric mice (11). Purified and sorted cells from naive or diseased animals were pooled (microglia or inflammatory macrophages) and analyzed by quantitative real-time PCR for IL-25.

**Intracellular staining.** CNS-infiltrating cells were obtained from MOG/CFA-immunized mice as described in the previous paragraph. Cells were stained with anti–CD4–FITC, fixed, and permeabilized with Cytofix/Cytoperm (BD Biosciences). Intracellular staining with antibodies against IL-17, IFNγ, and TNF (BD Biosciences) was performed and analyzed by flow cytometry. T cells were identified by gating on CD4. The number of cytokine-producing cells was calculated from the percentage of cytokine–positive CD4+ T cells. We determined the number of CD4+ T cells in the CNS by multiplying the percentage of lineage marker–positive cells by the total number of mononuclear cells isolated from the CNS. For intracellular cytokine staining of DLN cells, DLNs were taken at day 7 after immunization. After 3 d of culture with MOG, cells were incubated with IL-2 for 2 d. Cells were then incubated with PMA/ionomycin in the presence of Golgi-Plug (BD Biosciences) for 5 h, and intracellular staining was performed as described. Absolute cell numbers were obtained by multiplying relative numbers with total number in culture plate well. For intracellular Foxp3-staining, DLNs were taken at day 7 after immunization and processed immediately for CD4, CD25, and Foxp3 staining with the Mouse regulatory T cell staining kit (eBioscience).

**Cytokine assays.** Mice were immunized as described. DLNs (axillary, brachial, and inguinal) were isolated at day 7, before disease onset. Single-cell suspensions were plated at 6 × 10⁶ cells/well in 24-well plates in the presence of 100 μg/ml MOG35-55 peptide in rCpG. Supernatants were collected after 3 d of culture. Cells were rested for 4 d in the presence of 1–2 ng/ml IL-2. After 7 d, cells were restimulated with 10 μg/ml αCD3 (plate bound) plus 5 μg/ml αCD28, and supernatants were collected at 24 and 72 h. Alternatively, supernatants were collected at 48 h after restimulation. Cytokine production was analyzed by sandwich ELISA (IL-13 and IL-17 kits; R&D Systems) or cytometric bead array (Mouse Th1/Th2 cytokine CBA and Inflammation CBA kits; BD Biosciences). For the CBA assay, 50 μl of supernatant was incubated with a mixture of beads coated with capture antibodies for IL-2, IL-4, IL-5, IFNγ, or TNF (for Th1/Th2 kit), or IL-6, IL-10, MCP-1, IFNγ, TNF, or IL-12p70 (for Inflammation kit). Addition of the PE-conjugated detection antibodies forms a sandwich complex. After 2 h of incubation and one wash, samples were analyzed by flow cytometry. Standard curves were generated from analysis of titrated cytokine standards (at least 10 serial dilutions) using the BD CBA analysis software, which runs on Excel (Microsoft). IL-13 and IL-17 in samples from in vitro Th17 assays were assessed using a multiplexing bead array (Lincos) according to the manufacturer’s recommendations. In brief, 25 μl of supernatants were incubated with assay buffer, and fluorescent beads were conjugated to capture antibodies for the cytokines of interest. After addition of the detection antibody mix and PE-conjugated streptavidin, plates were read on a LUMINEX 100 machine (Luminex). Data were analyzed using Masterplex QT (MiraLabs) and GraphPad Prism (GraphPad).

**Histological analysis.** Spinal cords were taken at day 18 after immunization. Fixed tissue was embedded in paraffin wax, and 5-μm-thick longitudinal sections were stained with hematoxylin and eosin. Lesions were counted, and each scored based was on the size of the inflammatory infiltrate (score 1, <10 cells; score 3, 10–50 cells; score 5, >50 cells). The total number of lesions was assessed per section, and mean and SD were calculated per treatment group. Cumulative score per section was divided by total number of lesion to obtain a mean lesion score per section. Mean and SD were calculated per treatment group.

**Quantitative real-time PCR.** DLNs were taken at day 7 after immunization from five mice per indicated group and processed in three different pools. Single-cell suspensions were prepared by mashing lymph nodes through a 70-μm cell strainer (BD Biosciences). CNS leukocytes were prepared and purified as described. DC cultures were prepared as stated in the following section. Total RNA was prepared from frozen cell pellets using RNeasy columns (QiAGEN) and 5 μg were subjected to treatment with DNase (Ambion) and reverse transcribed using Superscript II (Invitrogen) with oligo (dT)15 primers (Roche) and random hexamers (Promega). Cytokine-specific mRNA was measured by real-time quantitative PCR. (Applied Biosystems). Gene expression levels were normalized to ubiquitin.

**In vitro cultures.** Spleens and lymph nodes were taken from naive DO11.10 × RAG−/− BALB/c mice, which only produce OVA peptide–specific naive T cells. Cells were also isolated from naive C57BL/6 MOG35-55 TCR Tg (clone 2D2) mice or from WT C57BL/6 mice (for DC isolations). CD4+ as well as CD11c+ cells were purified by magnetic bead separation (MACS; Miltenyi Biotech) after collagenase digestion of tissue. For Th17 polarization, CD11c+ cells (5 × 10⁵ cells per well in a 24-well plate) were activated with 0.5 ng/ml LPS and incubated with OVA peptide as well as neutralizing antibodies against IL-4 (BVD4-1D11; 10 μg/ml) and IFNγ (XMGl1.2; 10 μg/ml) for 2 h before addition of CD4+ cells in a 1:1 ratio. 20 ng/ml IL-23 was added to expand Th17 cells. Depending on the experimental setup, 100 ng/ml IL-25, 100 ng/ml IL-3, or 10 μg/ml anti–IL-13 were added to the cultures. Cultures with no cytokine addition served as a control. After 72 h, cells were incubated with 5 ng/ml IL-2 for an additional 48 h. Supernatants were then sampled for cytokine analysis, and cells were stimulated with PMA/ionomycin for 5 h in the presence of Golgi-Plug (BD Biosciences). Intracellular staining for IL-17 was performed as described. For DC cultures, 10⁶ CD11c+ DCs were plated in a 48-well plate, rested overnight, and incubated with or without IL-13 1 h before addition of LPS. Cells were harvested after 24 h and prepared for gene expression analysis.

**Online supplemental material.** Fig. S1 contains a schematic representation of the WT il25−/− locus, the arms used for generating the targeted allele, and the
Neo-flipped-LacZ knockin allele. Fig. S2 shows similar numbers of CNS-infiltrating inflammatory macrophages and total T cells in WT versus il25−/− mice at several time points after MOG-EAE induction. Table S1 summarizes the results of the analysis of spinal cord sections from IL-25–treated versus untreated EAE mice (experiment shown in Fig. 4 B) and confirms the enhanced resistance of IL-25–treated mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061738/DC1.

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