

# IL-6 blockade inhibits the induction of myelin antigen-specific Th17 cells and Th1 cells in experimental autoimmune encephalomyelitis

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The development of Th17 cells is a key event in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis (MS). Previous studies have demonstrated that an IL-6-dependent pathway is involved in the differentiation of Th17 cells from naïve CD4-positive T cells *in vitro*. However, the role of IL-6 *in vivo* in the development of Th17 cells in EAE has remained unclear. In the present study, we found that IL-6 blockade by treatment with an anti-IL-6 receptor monoclonal antibody (anti-IL-6R mAb) inhibited the development of EAE and inhibited the induction of myelin oligodendrocyte glycoprotein (MOG) peptide-specific CD4-positive, CD8-positive, and Th17 T cells, in inguinal lymph nodes. Thus, the protective effect of IL-6 blockade in EAE is likely to be mediated via the inhibition of the development of MOG-peptide-specific Th17 cells and Th1 cells, which in turn leads to reduced infiltration of T cells into the CNS. These findings indicate that anti-IL-6R mAb treatment might represent a novel therapy for human MS.

autoimmunity | multiple sclerosis | T cells

**M**ultiple Sclerosis (MS) is an inflammatory demyelinating disease of the CNS. More than 2 million people worldwide are affected with this disease; however, an effective therapy for MS has not yet been established. Although the cause of MS is not fully understood, infiltration of CD4<sup>+</sup> T cell and CD8<sup>+</sup> T cell into the CNS is believed to be important in the pathogenesis of this disease (1).

Experimental autoimmune encephalomyelitis (EAE) is a murine model of human MS that shares many pathological and histological characteristics with human MS. Initially, EAE was considered to be a Th1-mediated disease; however, recent studies have revealed that the major pathogenic T cell subset in EAE are Th17 cells (2, 3), which are characterized by CD4-positive T cells producing IL-17A (IL-17) (4, 5). Th17 cells are believed to play an important role in host defense against extracellular pathogens, which are not effectively cleared by Th1 or Th2 cells. Because Th17 cells are highly proinflammatory, Th17 cells directed against self antigens cause severe autoimmune disease in mice, including EAE and collagen-induced arthritis (CIA) (3, 6).

Previous studies have suggested a pathogenic role for IL-17 in MS. Matuszevicius *et al.* (7) reported that IL-17-secreting lymphocytes were detected in the cerebrospinal fluid of MS. Lock *et al.* (8) revealed that increased levels of transcripts for IL-17 and IL-6 are detected in chronic lesions of patients with MS. Furthermore, Tzartos *et al.* (9) reported that IL-17-producing CD4<sup>+</sup> T cells are present within active areas of MS. Because IL-17 signaling is important for the production of various chemokines from fibroblasts and epithelial cells, which attract

antigen-presenting cells to the CNS, resulting in demyelination, the suppression of the development or the proliferation of Th17 cells may represent a promising therapy for MS.

Recently, three independent groups demonstrated that the cytokines IL-6 and TGF- $\beta$  synergistically induce the differentiation of Th17 cells in mice *in vitro* (10–12). More recently, IL-21 produced by Th17 cells themselves contributes to the amplification of differentiated Th17 cells (13–15). Moreover, IL-23 has been shown to contribute to the proliferation and stabilization of Th17 cells (12). Thus, because IL-6 is a regulator of Th17 differentiation *in vitro*, it represents a potential target for the inhibition of Th17 development *in vivo*. IL-6-deficient mice have been shown to be highly resistant to the induction of EAE (16, 17). However, data obtained from IL-6-deficient mice may not equate to data obtained by IL-6 blockade using neutralizing antibody, because the complete absence of IL-6 in knockout mice has been shown to display hematopoietic defects (18). Two independent groups have performed anti-IL-6 therapy against EAE, but the results were conflicting. Gijbels *et al.* (19) reported that treatment of anti-IL-6 antibody was protective against the development of EAE. By contrast, Willenborg *et al.* (20) reported that anti-IL-6 therapy has no significant protective effect in EAE. Therefore, the role of IL-6 in EAE remains unclear. Furthermore, because these studies were conducted before the discovery of the Th17 T cell subset, the effect of IL-6 blockade on T cell development in EAE, particularly highly proinflammatory Th17 cells, also remains unclear.

In the present study, we investigated the *in vivo* role of IL-6 in the development of T cells, particularly Th17 cells, in EAE, using an anti-IL-6R monoclonal antibody (anti-IL-6R mAb), which shows significant protective effect in CIA (21).

## Results

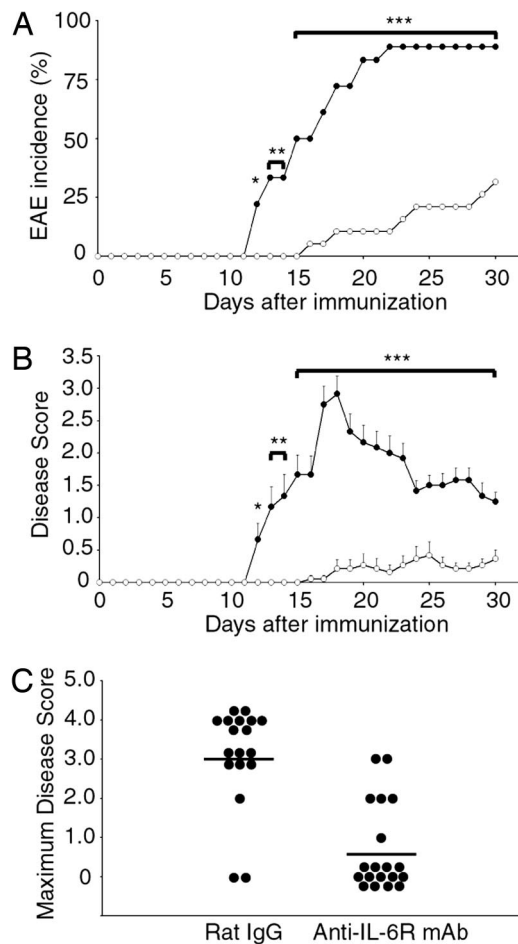
**Anti-IL-6R mAb Treatment Inhibited the Development of EAE.** To investigate a protective effect of anti-IL-6R mAb treatment against the development of EAE, we immunized C57BL/6J mice with MOG<sub>35–55</sub> peptide emulsified with CFA, followed by i.p. treatment of 8 mg of anti-IL-6R mAb or control rat IgG at the same day of immunization. Compared with a control rat-IgG-treated group, the incidence of EAE was reduced and the onset

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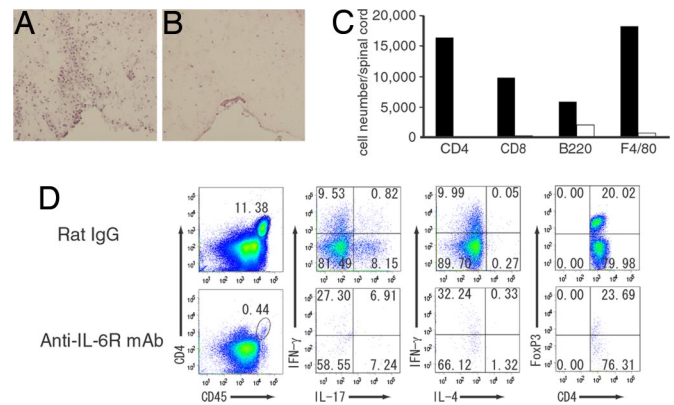
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of EAE was delayed in the anti-IL-6R-mAb-administered group (Fig. 1A). In anti-IL-6R-mAb-treated mice, the clinical scores were also significantly lower than those of control rat-IgG-treated mice (Fig. 1B and C).

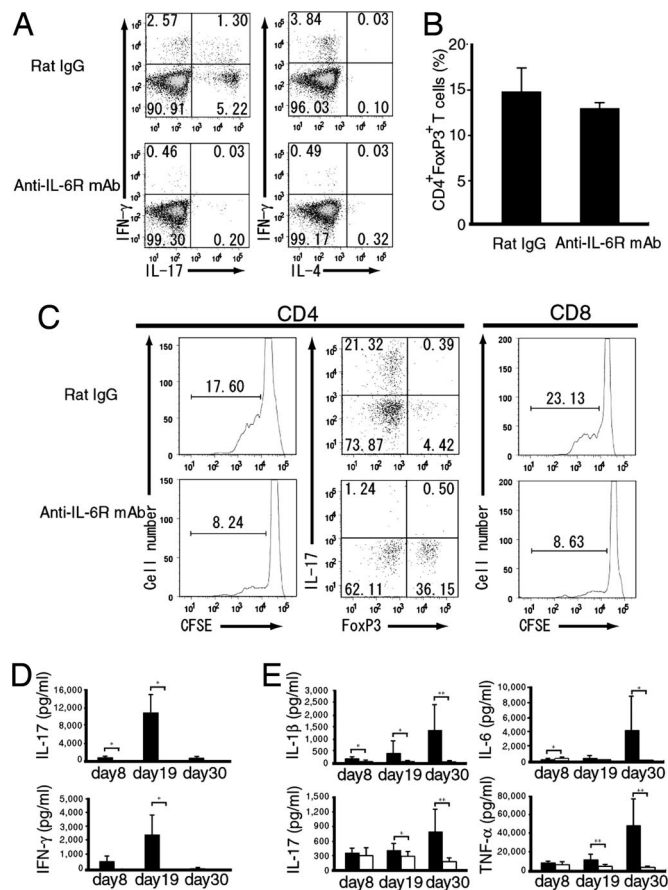
**Anti-IL-6R-mAb-Treated Mice Are Devoid of CNS-Infiltrated T Cells.** At the peak stage (19 days after antigen immunization) of EAE, histopathology of CNS in the control rat-IgG-treated mice showed intense infiltration of mononuclear cells into the white matter of spinal cords (Fig. 2A). In contrast, cellular infiltration was markedly reduced in anti-IL-6R-mAb-treated mice, which was consistent with their decreased clinical scores (Fig. 2B). Similarly, demyelination was also detected in control rat-IgG-received mice and not found in anti-IL-6R-mAb-treated mice (data not shown). To investigate the population of lymphocytes infiltrated into the CNS, we recovered mononuclear cells from the spinal cords and surface stained with CD4, CD8, B220, and F4/80 antibody. At peak stage of EAE,  $CD4^+$  T cells,  $CD8^+$  T cells, B cells, and macrophages were detected in control rat-IgG-treated mice (Fig. 2C). In anti-IL-6R-mAb-treated mice,  $CD4^+$  T cells,  $CD8^+$  T cells, and macrophages were hardly detected, whereas approximately one-third the number of B cells

Fig. 2. Anti-IL-6R-mAb-treated mice are devoid of CNS-infiltrated T cells. (A and B) H&E histology of spinal cords from antibody-treated mice taken at peak disease (19 days after antigen immunization). (Original magnifications:  $\times 20$ .) (C) Analysis of mononuclear cells infiltrating into the spinal cords. Mononuclear cells were recovered from spinal cord at 19 days after antigen immunization. Isolated cells were surface-stained with antibodies against CD45, CD4, CD8, B220, and F4/80. CD45 high populations were gated. (D) Intracellular cytokine staining and FoxP3 staining of spinal-cord-infiltrating cells. Mononuclear cells were recovered from spinal cord at 19 days after immunization, and cells were stimulated with PMA and ionomycin in the presence of Brefeldin A; all plots are gated on  $CD4^+ CD45^+$  T cells. Flow-cytometric analysis was done on pooled spinal cord from four mice per group, and results are representative of two independent experiments.



were found compared with control (Fig. 2C). Furthermore, intracellular cytokine staining of CNS-infiltrating lymphocytes demonstrated that intense infiltration of Th17, Th1, and FoxP3 positive regulatory T (RegT) cells were detected, whereas anti-IL-6R-mAb-treated mice had a paucity of cells in the CNS (Fig. 2D). Thus, anti-IL-6R mAb treatment on day 0 suppressed the presence of lymphocytes into the spinal cord.

**Anti-IL-6R mAb Treatment Suppressed the Induction of MOG<sub>35–55</sub>-Peptide-Specific T Cells in Peripheral Lymphoid Tissue.** To investigate whether the deficiency of CNS-infiltrating  $CD4^+$  T cells in anti-IL-6R-mAb-treated mice was due to a T cell priming defect or not, we analyzed MOG<sub>35–55</sub>-peptide-specific  $CD4^+$  T cells from draining lymph nodes. In the lymphocytes prepared from inguinal lymph nodes at priming stage (8 days after antigen immunization), the development of Th17 cells as well as Th1 cells was highly suppressed in anti-IL-6R-mAb-treated mice compared with rat-IgG-treated mice (Fig. 3A). In contrast, Th2 cells were not induced in mice treated with rat IgG or anti-IL-6R mAb in EAE (Fig. 3A). In addition, the population of FoxP3-positive RegT cells was also not changed significantly in anti-IL-6R-mAb-treated mice (Fig. 3B). To examine the effect of IL-6 blockade against the proliferation of MOG<sub>35–55</sub>-peptide-specific T cells, we performed CFSE dilution assay and found that anti-IL-6R mAb treatment at the same day of antigen immunization suppressed the proliferation of MOG<sub>35–55</sub>-peptide-specific  $CD4^+$  T cells *in vitro* (Fig. 3C Left). To further elucidate the subsets of MOG<sub>35–55</sub>-peptide-specific  $CD4^+$  T cells, CFSE<sup>low</sup>  $CD4^+$  T cells were gated and MOG<sub>35–55</sub>-peptide-specific Th17 and FoxP3<sup>+</sup> RegT cells were analyzed. An increased population of Th17 cells was detected in rat-IgG-treated mice. In contrast, although the absolute number of MOG<sub>35–55</sub>-peptide-responsive  $CD4^+$  T cells was lower than in the rat-IgG-treated group, a remarkably higher population of FoxP3<sup>+</sup> RegT cells and a lower population of Th17 cells were found in anti-IL-6R-mAb-treated group (Fig. 3C Center). In addition to the suppression of MOG<sub>35–55</sub>-peptide-specific  $CD4^+$  T cells, anti-IL-6R mAb also



**Fig. 3.** IL-6 blockade suppressed the induction of Th17 cells in the lymph nodes. (A) Intracellular cytokine staining of lymphocytes stimulated with MOG<sub>35-55</sub> peptide. Inguinal lymph node cells were recovered at 8 days after antigen immunization. All plots were gated on CD4<sup>+</sup> T cells. (B) FoxP3 staining of CD4<sup>+</sup> T cells recovered from inguinal lymph node at 8 days after immunization, and FoxP3-positive CD4<sup>+</sup> T cells analyzed by FACS. (C) Analysis of MOG<sub>35-55</sub>-peptide-specific T cells by CFSE dilution assay. All plots were gated on CD4<sup>+</sup> T or CD8<sup>+</sup> T and CFSE<sup>low</sup> populations, and a population of IL-17- or FoxP3-positive CD4<sup>+</sup> T cells was analyzed by FACS. Data are representative of three independent results. (D) IL-6 blockade suppressed the antigen-specific cytokine production of IL-17 and IFN-γ from lymphocytes. Inguinal lymph node cells were recovered at the indicated days after immunization, and lymph node cells were restimulated with 50 μg/ml MOG<sub>35-55</sub> peptide for 72 h. IL-17 and IFN-γ concentrations in the supernatant were determined by using BioPlex. (E) IL-6 blockade suppressed the serum proinflammatory cytokine levels. Mice were treated with anti-IL-6R mAb or rat IgG on day 0, and serum samples were prepared at the indicated days after antigen immunization. IL-1β, IL-6, IL-17, and TNF-α cytokine concentrations in the serum were analyzed by using BioPlex. All *p* values were determined by using the Student *t* test. \*, *P* < 0.05; \*\*, *P* < 0.005.

suppressed the proliferation of MOG<sub>35-55</sub>-peptide-specific CD8<sup>+</sup> T cells (Fig. 3C Right).

To investigate the effect of IL-6 blockade against MOG<sub>35-55</sub>-peptide-specific cytokine production, we quantified the concentrations of cytokine levels secreted into the culture supernatant of lymphocytes from inguinal lymph nodes, which were restimulated with MOG<sub>35-55</sub> peptide. We found that the production of IL-17 and IFN-γ was significantly suppressed during all stages in anti-IL-6R-mAb-treated mice, although these cytokine levels were higher at peak stage in control mice, which correlated with clinical scores (Fig. 3D). We could not detect IL-4, IL-6, or TNF-α in the culture supernatant. In contrast, the concentrations of serum cytokines including IL-1β, IL-6, IL-17, and TNF-α were significantly higher at the recovery stage than the peak

stage of rat-IgG-treated mice, and these serum cytokine levels were also suppressed in anti-IL-6R-mAb-treated mice during the peak and recovery stages (Fig. 3E). Serum cytokine levels of IFN-γ and IL-4 were below the limit of detection in both antibody-treated groups. At the recovery stage, increased serum cytokine levels, such as IL-17 and IL-6, in rat-IgG-treated mice might be mediated by activated lymphocytes that were migrated from the CNS to peripheral lymphoid tissues. These results suggest that suppression of the differentiation of MOG<sub>35-55</sub>-peptide-specific Th17 and Th1 cells by IL-6 blockade contributes to the protective effect against the development of EAE.

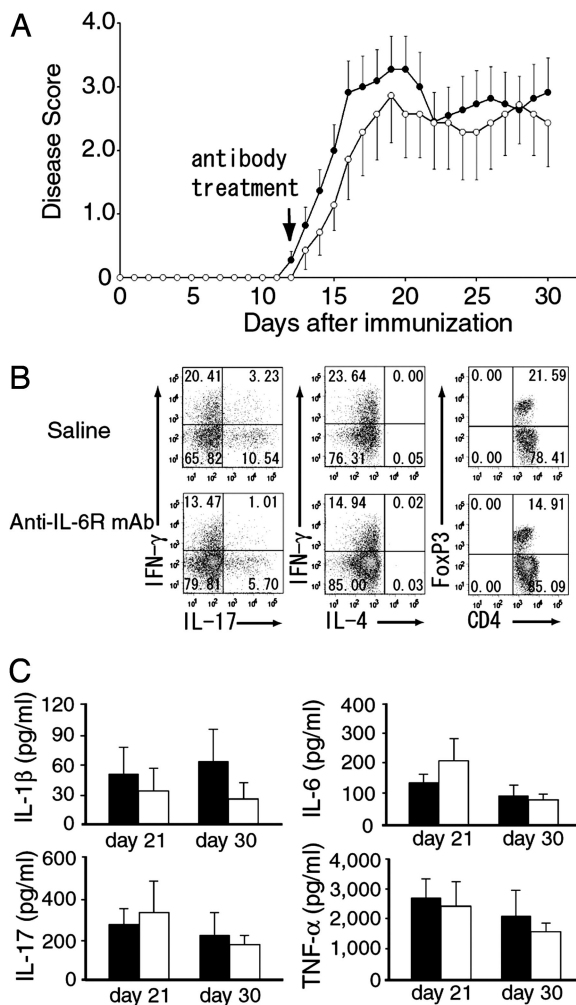
**Delayed Treatment of Anti-IL-6R mAb Failed to Suppress EAE.** To determine whether administration of anti-IL-6R mAb inhibits the proliferation of already committed Th17 cells *in vivo* or not, we treated antibody during onset stage (12 days after antigen immunization) and investigated the effect of anti-IL-6R mAb against EAE. There were no significant differences in the clinical scores between anti-IL-6R mAb and saline treated groups (Fig. 4A). Although the population of Th17, Th1 and FoxP3<sup>+</sup> RegT cells infiltrated into the spinal cord was lower in anti-IL-6R-mAb-treated mice than saline treated mice (Fig. 4B), it was not enough to reduce disease severity. In addition, there were no differences in serum cytokine levels (Fig. 4C). These results indicate that IL-6 blockade is not efficacious in inhibiting an established EAE.

**IL-6 Blockade Cannot Prevent the Infiltration of Activated Lymphocytes into the CNS.** Next, we investigated the effect of IL-6 blockade against passive induction of EAE to elucidate whether administration of anti-IL-6R mAb prevents the infiltration of activated lymphocytes into the spinal cord. IL-6 has been reported to induce cell-adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) in endothelial cells (22); therefore, IL-6 blockade might inhibit the infiltration of lymphocytes into the spinal cord. For the passive induction of EAE, lymphocytes from MOG<sub>35-55</sub> peptide/CFA-immunized C57BL/6J mice were stimulated with MOG<sub>35-55</sub> peptide for 4 days *in vitro*, and viable lymphocytes were transferred into naive wild-type mice. When mice were i.p. treated with 8 mg of anti-IL-6R mAb 1 day before transfer, there was no difference in the disease onset between anti-IL-6R-mAb- and rat-IgG-treated mice; however, clinical scores were partially inhibited in anti-IL-6R-mAb-treated mice (Fig. 5A). Histopathology of CNS in the anti-IL-6R-mAb-treated mice showed massive infiltration of mononuclear cells into the white matter of spinal cord, and it was comparable with those of control rat-IgG-treated mice (Fig. 5B). Moreover, there were also no differences in the absolute numbers of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, B cells, and macrophages infiltrating into the CNS (Fig. 5C). These results indicate that anti-IL-6R mAb treatment cannot prevent the infiltration of activated lymphocytes into the spinal cord.

### Discussion

The recent identification of the highly proinflammatory Th17 effector T cell subset has focused attention to the role of Th17 cells in the pathogenesis of autoimmune disease. In mice, autoantigen-specific Th17 cells have been shown to be the dominant pathogenic T cell subset in EAE (3). Moreover, IL-17-deficient mice have been reported to develop EAE with delayed onset and reduced severity (23). In humans, Th17 cells have been identified in the CNS of patients with MS (9). These studies highlight the importance of understanding the regulation of Th17 cell development in autoimmune disease.

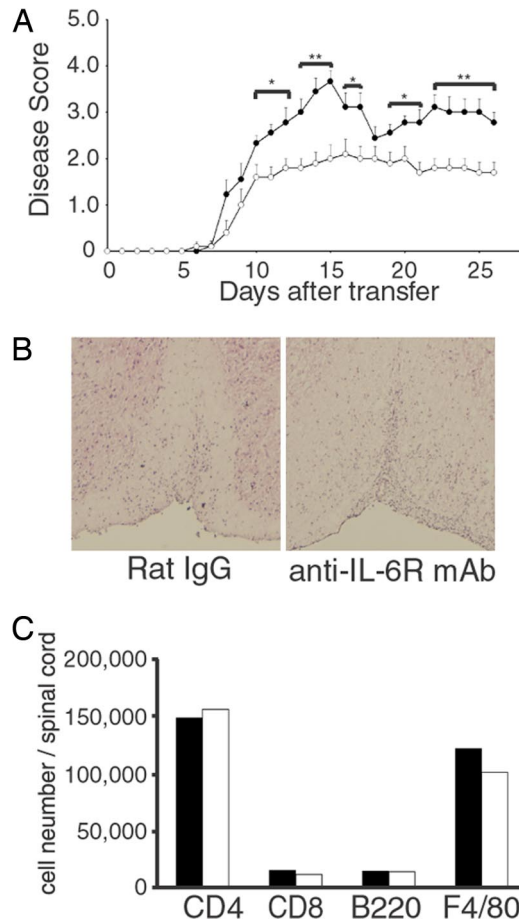
It has been demonstrated that IL-6 and TGF-β synergistically induce the differentiation of naïve CD4-positive T cells into Th17 cells in mice (10–12). However, our previous studies (24) and those of other groups (13–15) have highlighted both IL-6-



**Fig. 4.** Delayed treatment of anti-IL-6R mAb failed to suppress EAE. (A) Average clinical scores of mice treated with 8 mg of anti-IL-6R mAb ( $n = 7$ ) or saline ( $n = 11$ ) injected at 12 days after antigen immunization. Clinical scores (averages  $\pm$  SEM) combining two independent experiments are shown. (B) Intracellular cytokine staining and FoxP3 staining of spinal cord infiltrating cells recovered from peak stage (21 days after antigen immunization) of EAE. All plots were gated on CD4<sup>+</sup> CD45<sup>+</sup> T cells. Flow cytometric analysis was done on pooled spinal cord from 4 mice/group and results are representative of two independent experiments. (C) IL-6 blockade has no effect on the serum pro-inflammatory cytokine levels. Mice were treated with anti-IL-6R mAb or saline on day 12, and serum were prepared at indicated days after antigen immunization. IL-1 $\beta$ , IL-6, IL-17 and TNF- $\alpha$  cytokine concentrations in the serum were analyzed by BioPlex.

dependent and -independent pathways in the differentiation of Th17 cells *in vitro*. IL-6 knockout mice have been shown to be highly resistant to the development of EAE (16, 17). However, treatment with anti-IL-6 mAb has been reported to be protective in EAE in one study (19) and not protective in another study (20). Therefore, the role of IL-6 in the induction of T cells (particularly Th17 cells) in EAE remains unclear. In this study, we investigated the *in vivo* role of IL-6 in T cell development in EAE, using an anti-IL-6R mAb.

In this article, we showed that treatment with an anti-IL-6R mAb at the same day of antigen immunization effectively suppressed the disease incidence and severity of EAE (Fig. 1 A and B). Willenborg *et al.* (20), however, failed to suppress EAE disease using anti-IL-6 antibody; this may be due to the differences in the dose and/or timing of antibody treatment. Anti-IL-6R-mAb-treated mice were devoid of mononuclear cells in the



**Fig. 5.** IL-6 blockade cannot prevent the infiltration of activated lymphocytes into the CNS. (A) IL-6 blockade failed to suppress the disease onset but partially inhibits the disease severity of passive transfer EAE. Eight mg of anti-IL-6R mAb ( $n = 10$ ) or rat IgG ( $n = 9$ ) was treated at one day before transfer. EAE was passively induced and clinical scores (averages  $\pm$  SEM) are shown. Statistics were calculated with the Mann-Whitney *U* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ . (B) H&E histology of spinal cords from antibody-treated mice taken at peak disease. (Original magnification:  $\times 20$ .) Anti-IL-6R-mAb and rat-IgG-treated mice show inflammation in the white matter of the CNS. (C) Analysis of mononuclear cells infiltrating into the spinal cord. Mononuclear cells were recovered from pooled spinal cord from three mice per group at 14 days after transfer. Isolated cells were surface-stained with antibodies against CD45, CD4, CD8, B220, and F4/80, and analyzed by FACS. CD45 high populations were gated.

spinal cord (Fig. 2 B and C). These results are consistent with previous studies of EAE in IL-6-deficient mice (16, 17). Because a subset of CD8<sup>+</sup> T cells has been demonstrated to be suppressive against EAE (25), it is uncertain whether the decrease in the number of CD8<sup>+</sup> T cells infiltrated into the CNS contributes to the amelioration of the clinical score.

Importantly, anti-IL-6R mAb inhibited the induction of MOG<sub>35-55</sub>-peptide-specific Th17 cells (Fig. 3A) *in vivo*. As previously reported, the combination of IL-6 and TGF- $\beta$  is important for the differentiation of naive CD4-positive T cells into Th17 cells *in vitro* (10–12). Our data support the importance of IL-6 in the differentiation of Th17 cells *in vivo*.

We observed that anti-IL-6R mAb treatment suppressed the induction of Th1 cells during the priming stage of lymph nodes (Fig. 3A), whereas Th2 cells were not induced in EAE (Fig. 3A). It is not clear whether IL-6 directly regulates the differentiation of Th1 cells. Whereas IL-6 has been shown to suppress the induction of TGF- $\beta$ -inducible RegT cells from naive CD4<sup>+</sup> T

cells (10), the suppression of MOG<sub>35–55</sub>-peptide-specific Th1 cells might be mediated by MOG<sub>35–55</sub>-peptide-specific TGF- $\beta$ -inducible RegT cells, which were increased by IL-6 blockade in EAE (Fig. 3C). In agreement with this, it has been reported that IL-6 deficiency promotes the generation or expansion of MOG<sub>35–55</sub>-peptide-specific RegT cells and inhibits the induction of effector T cell responses, including Th17 and Th1 cells, although MOG<sub>35–55</sub>-peptide-specific CD8<sup>+</sup> T cells were not studied (13). Recently, Selvaraj *et al.* (26) reported that development of EAE was suppressed after passive transfer of inducible RegT cells and that the induction of both Th1 and Th17 cells was also suppressed, thus supporting a possible protective role for inducible RegT cells in anti-IL-6R-mAb-treated mice. When RegT cells were depleted from lymph node cells before CFSE dilution assay by using antibody against folate receptor 4 (a surface marker of RegT cells), we did not observe changes in the population of MOG<sub>35–55</sub>-peptide-specific Th1 cells (data not shown), suggesting that the differentiation of MOG<sub>35–55</sub>-peptide-specific Th1 cells is suppressed by RegT *in vivo* before the *in vitro* analysis, in anti-IL-6R-mAb-treated mice. In addition to the MOG<sub>35–55</sub>-peptide-specific CD4<sup>+</sup> T cells, anti-IL-6R-mAb-treated mice showed impaired proliferation of MOG<sub>35–55</sub>-peptide-specific CD8<sup>+</sup> T cells (Fig. 3C). It has been reported that the proliferation of antigen-specific CD8<sup>+</sup> T cells was suppressed by RegT cells (27). Therefore, the decreased population of MOG<sub>35–55</sub>-peptide-specific CD8<sup>+</sup> T cells might be mediated by the MOG<sub>35–55</sub>-peptide-specific TGF- $\beta$ -inducible RegT cells.

We also investigated the effect of anti-IL-6R mAb during disease onset stage, and no differences were found in the clinical score of EAE (Fig. 4A). Although the populations of Th17 and Th1 cells were partially decreased (Fig. 4B), this might be due to the prevention of newly differentiating Th17 cells from naive CD4<sup>+</sup> T cells by IL-6 blockade. However, a partial decrease in the populations of Th17 and Th1 cells was not sufficient to reduce disease severity significantly. These results indicate that IL-6 blockade is not effective in inhibiting the proliferation of already committed Th17 cells *in vivo*.

Infiltration of MOG<sub>35–55</sub>-peptide-specific CD4<sup>+</sup> T cells into the CNS is an important step in EAE disease onset (28), and IL-6 has been reported to induce ICAM-1 in endothelial cells (22), a protein possibly involved in the infiltration of lymphocytes through the blood–brain barrier (BBB) (28). Therefore, we investigated the effect of anti-IL-6R mAb treatment against passive induction of EAE to elucidate the effect of IL-6 blockade against infiltration of activated lymphocytes into the spinal cord. We did not observe any difference in the disease onset between anti-IL-6R mAb and rat-IgG-treated mice (Fig. 5A). Furthermore, there were no differences in the absolute number of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, B cells, and macrophages infiltrating into the CNS (Fig. 5C). These results indicate that IL-6 is dispensable for the infiltration of activated lymphocytes through the BBB and argues against a role for IL-6 in the induction of cell-adhesion molecules on the endothelial cells in EAE. Therefore, the absence of activated lymphocytes in the spinal cord of anti-IL-6R-mAb-treated mice (Fig. 2B) is likely to be mediated via the inhibition of the induction of MOG<sub>35–55</sub>-peptide-specific CD4<sup>+</sup> T cells, including Th17 cells, and CD8<sup>+</sup> T cells in the peripheral lymphoid tissue rather than via the inhibition of infiltration of activated lymphocytes into the spinal cord. Interestingly, the clinical score of passively induced EAE was partially inhibited in anti-IL-6R-mAb-treated mice compared with rat-IgG-treated mice (Fig. 5A). This indicates that IL-6 not only regulates Th17 differentiation but also affects T cells or other cells such as endothelial cells or CNS glial cells including astrocytes at effector phase in EAE. However, we could not find significant amelioration of the EAE clinical scores when anti-IL-6R mAb was administered after 12 days of antigen immunization (Fig. 4A). These differences in the protective efficacy of

IL-6 blockade at effector phase may be due to differences in serum cytokine levels between passively induced EAE and treatment of anti-IL-6R mAb at 12 days after antigen immunization against actively induced EAE. Thus, for example, because of prior immune activation, IL-17 (an inducer of IL-6 expression) levels are likely to be higher in the actively induced EAE model at day 12 (beginning of antibody administration) compared with IL-17 levels in the passively induced EAE model at the time of anti-IL-6R mAb administration.

Recent reports demonstrated differences in the regulation of Th17 cell development *in vitro* in humans compared with mice (29, 30). For the induction of human Th17 cells, TGF- $\beta$  is not required but inhibits their differentiation (29, 30). These groups concluded that IL-1 is an effective inducer of human Th17 differentiation. Furthermore, combination of IL-1 and IL-6 synergistically induces the differentiation of human Th17 cells *in vitro* (29), indicating that IL-6 is important for the induction of Th17 cells in both mice and humans *in vivo*. In mice, it has been reported that IL-1 augments Th17 differentiation induced by IL-6 and TGF- $\beta$  *in vitro* (12) and that IL-1 signaling is required for the induction of Th17 cells in EAE (31). Thus, our study indicates that the observed inhibition of Th17 induction in EAE after IL-6 blockade, may be mediated in part via the inhibition of the synergistic effect of IL-6 and IL-1 in the presence of TGF- $\beta$ .

In conclusion, we have demonstrated a key role for IL-6 in the differentiation of Th17 cells in EAE. Anti-IL-6R mAb treatment is most effective at the same day of antigen immunization, rather than after the commitment of Th17 cells in EAE. Furthermore, anti-IL-6R mAb therapy might be also effective in the ongoing or relapse of MS, because humanized anti-IL-6R mAb can be treated repeatedly without antigenicity. Moreover, not only effector cells but also naive T cells are thought to contribute to antigenic spread in a relapsing EAE model (32), and anti-IL-6R mAb might suppress the differentiation of Th17 cells from naive T cells at the relapsing phase.

Our studies suggest that the protective effect of anti-IL-6R mAb treatment in EAE is mediated not only via the suppression of IL-6-induced inflammatory reactions but also via the inhibition of the induction of MOG<sub>35–55</sub>-peptide-specific Th17 and Th1 cells, which in turn leads to the reduced infiltration of T cells into the CNS. These findings indicate that anti-IL-6R mAb treatment might represent a promising therapy for human MS and other Th17-mediated chronic autoimmune diseases.

## Materials and Methods

**Mice.** C57BL/6J mice were purchased from Charles River Laboratories. All experiments were conducted according to the institutional ethical guidelines for animal experimentation of the National Institute of Biomedical Innovation (Osaka).

**Active Induction of EAE.** Mice (9 weeks of age) were immunized s.c. with 300  $\mu$ g of MOG<sub>35–55</sub> peptide emulsified in CFA and injected with pertussis toxin twice. The severity of EAE was monitored and graded on a scale of 0–5: 0 = no disease; 1 = limb tail; 2 = hind limb weakness; 3 = hind limb paralysis; 4 = hind and fore limb paralysis; 5 = moribundity and death.

**Passive Induction of EAE.** Mice were immunized s.c. with MOG<sub>35–55</sub> peptide/CFA. Spleen cells and inguinal lymph node cells were harvested on day 15 after immunization and cultured for 4 days in the presence of 25  $\mu$ g/ml MOG<sub>35–55</sub> peptide, 10 ng/ml rMLL-23, and 5  $\mu$ g/ml anti-mIFN- $\gamma$  antibody. Viable lymphocytes ( $1.35 \times 10^7$ ) were transferred i.p. into naive C57BL/6J mice.

**Anti-IL-6R mAb Treatment.** For IL-6 blockade, mice were i.p. treated with 8 mg of anti-IL-6R mAb (clone MR16–1, rat IgG1) on days 0 or 12 postimmunization. Purified rat IgG (Cappel) or saline were administered as control. In the case of passive induction of EAE, 8 mg of anti-IL-6R mAb or rat IgG was administered i.p. into naive C57BL/6J mice one day before transfer.

**Histology.** Mice were perfused with PBS, and spinal cords were dissected and frozen in OCT compound. Sections 5  $\mu\text{m}$  in thickness from the spinal cord were stained with H&E.

**Intracellular Cytokine Staining.** Draining lymph node cells were stimulated with 50  $\mu\text{g}/\text{ml}$  MOG<sub>35–55</sub> peptide for 72 h and restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 750 ng/ml ionomycin for the last 4 h in the presence of 10  $\mu\text{g}/\text{ml}$  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). Cells were isolated by Percoll centrifugation as described in ref. 33 and surface-stained with antibodies against CD4 (Becton Dickinson; RM4-5), CD45 (Biolegend; 30-F11), CD8 (eBioscience, 53-6.7), B220 (eBioscience; RA3-6B2), and F4/80 (CALTAG Laboratories; Cl:A3-1). Intracellular cytokine staining and FoxP3 staining were performed as described in ref. 24. Cells were analyzed by using the FACSCanto flow cytometer (Becton Dickinson), and obtained data were analyzed by using FlowJo software (Tree Star).

**CFSE Assays.** Lymph node cells were incubated in 3  $\mu\text{M}$  CFSE (Molecular Probes). Stained cells ( $2 \times 10^5$ ) were cultured with 50  $\mu\text{g}/\text{ml}$  MOG<sub>35–55</sub>

peptide for 72 h. After stimulation, cells were stained with antibodies against CD4, CD8, IL-17, and FoxP3 and analyzed by using the FACSCanto flow cytometer.

**Cytokine Quantification.** For cytokine quantification, culture supernatants and serum were analyzed by using BioPlex (Bio-Rad) according to the manufacturer's instructions.

**Statistics.** The two-tailed Student *t* test,  $\chi^2$  test, or Mann–Whitney *U* test was used for the statistical analyses. Differences were considered significant when *p* values were  $<0.05$ .

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