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IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25$^+$CD4$^+$ regulatory T cells

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Abstract

CD25$^+$CD4$^+$ regulatory T cells inhibit the activation of autoreactive T cells *in vitro* and *in vivo*, and suppress organ-specific autoimmune diseases. The mechanism of CD25$^+$CD4$^+$ T cells in the regulation of experimental autoimmune encephalomyelitis (EAE) is poorly understood. To assess the role of CD25$^+$CD4$^+$ T cells in EAE, SJL mice were immunized with myelin proteolipid protein (PLP)$_{139–151}$ to develop EAE and were treated with anti-CD25 mAb. Treatment with anti-CD25 antibody following immunization resulted in a significant enhancement of EAE disease severity and mortality. There was increased inflammation in the central nervous system (CNS) of anti-CD25 mAb-treated mice. Anti-CD25 antibody treatment caused a decrease in the percentage of CD25$^+$CD4$^+$ T cells in blood, peripheral lymph node (LN) and spleen associated with increased production of IFN-γ and a decrease in IL-10 production by LN cells stimulated with PLP$_{130–151}$ *in vitro*. In addition, transfer of CD25$^+$CD4$^+$ regulatory T cells from naive SJL mice decreased the severity of active EAE. *In vitro*, anti-CD3-stimulated CD25$^+$CD4$^+$ T cells from naive SJL mice secreted IL-10 and IL-10 soluble receptor (sR) partially reversed the *in vitro* suppressive activity of CD25$^+$CD4$^+$ T cells. CD25$^+$CD4$^+$ T cells from IL-10-deficient mice were unable to suppress active EAE. These findings demonstrate that CD25$^+$CD4$^+$ T cells suppress pathogenic autoreactive T cells in actively induced EAE and suggest they may play an important natural regulatory function in controlling CNS autoimmune disease through a mechanism that involves IL-10.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory and demyelinating disease model that shares many clinical and histologic features with multiple sclerosis. In EAE-susceptible strains of mice, immunization with myelin protein antigens such as proteolipid protein (PLP), myelin basic protein and myelin oligodendrocyte glycoprotein (MOG) in complete Freund's adjuvant (CFA) induces a CD4$^+$ T_h1 cell-mediated inflammatory response in the central nervous system (CNS) (1). Even in genetically susceptible animals, spontaneous EAE occurs rarely and induced EAE is generally self-limited (2). Most self-reactive T cells are deleted in the thymus during T cell development, reducing the frequency, diversity and affinity of self-reactive T cells in the peripheral repertoire. Self-reactive cells that do not undergo negative selection are seeded to the periphery, and form the self-reactive repertoire for autoreactive T cells that can undergo expansion and lead to disease.

T cell anergy, deletion and immunological ignorance explain in part the maintenance of peripheral self-tolerance. Furthermore, accumulating evidence suggests that regulatory T cell-mediated control of self-reactive T cells also plays an important role in tolerance and in the prevention of autoimmune diseases (3,4). It has been shown that CD25$^+$CD4$^+$ regulatory T cells play a major role in regulating autoimmune responses in mice and these cells have also been identified in humans (5–14). Neonatal thymectomy of mice at day 3 leads to the development of a spectrum of organ-specific autoimmune diseases including gastritis, oophoritis and thyroiditis,

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which can be inhibited by transfer of $CD4^+$ T cells from normal mice (5–7). These thymectomized mice lacked $CD25^+CD4^+$ regulatory T cells in their peripheral lymphoid tissues. $CD25^+CD4^+$ T cells in normal mice are non-responsive to antigen-specific stimulation *in vitro* and, upon stimulation through the TCR, suppress the activation of other $CD4^+$ T cells in an antigen-non-specific manner (9,10). $CD25^+CD4^+$ regulatory T cells are naturally selected in the thymus, where $CD25^+CD4^+$ thymocytes require a TCR with high affinity for a self-peptide in order to be selected (15). However, the mechanism of action of $CD25^+CD4^+$ regulatory T cells is not completely understood, but involves cell–cell contact, and in some instances immunoregulatory cytokines such as IL-10 and transforming growth factor (TGF)-β [reviewed in (16,17)].

In addition to the neonatal thymectomy model, $CD25^+CD4^+$ T cells have been shown to play a role in other autoimmune disease models. Spontaneous diabetes is exacerbated in both CD80/CD86-deficient and CD28-deficient NOD mice which have a profound decrease in the number of immunoregulatory $CD25^+CD4^+$ T cells that normally control diabetes in pre-diabetic NOD mice. Transfer of a regulatory $CD25^+CD4^+$ cell population into CD28-deficent mice delayed and prevented diabetes (18). In an experimental model of inflammatory bowel disease, colitis in SCID mice induced by transfer of $CD45RB^{high}CD4^+$ T cells was inhibited by $CD45RB^{low}CD25^+CD4^+$ T cells (19). Furthermore, *in vivo* depletion of $CD25^+CD4^+$ T cells with anti-CD25 mAb provoked potent tumor-specific immune responses to eradicate syngeneic tumors (20,21), suggesting that immune suppression by $CD25^+CD4^+$ regulatory T cells is not restricted to experimentally autoimmune diseases. Recently, it was reported that transfer of $CD25^+CD4^+$ regulatory T cells from naive non-transgenic mice was poorly protective, and that antigen specificity was required for the selection and effector function of $CD25^+CD4^+$ regulatory T cells in the prevention of spontaneous EAE (22). Kohm *et al.* recently reported that adoptive transfer of $CD25^+CD4^+$ regulatory T cells significantly protected from EAE and decreased CNS infiltration (23). However, the mechanism of $CD25^+CD4^+$ T cell-mediated suppression in active EAE is not well understood. Here, we report that *in vivo* reduction of $CD25^+CD4^+$ T cells with anti-CD25 antibody increased the onset and severity of EAE, and was associated with a decreased IL-10 production. Furthermore, transfer of $CD25^+CD4^+$ T cells from naive SJL mice, but not IL-10-deficient mice, decreased the severity of active EAE, indicating that $CD25^+CD4^+$ T cells may play an important role in the down-regulation of the pathogenic T cell responses in EAE via a mechanism that involves IL-10.

Methods

Mice

Female SJL/J, C57BL/6J and IL-10-deficient mice on a C57BL/6J background (8–10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME), and housed in a pathogen-free animal facility at the Harvard Institutes of Medicine.

Antibodies and reagents

Anti-CD25 mAb (PC61) was prepared as previously described (20) and purified by Strategic Biosolution (Newark, DE). The endotoxin activity of purified antibody was measured with Limulus amebocyte lysate (0.3–0.5 endotoxin U/mg; Biowhittaker, Walkersville, MD). The following mAb were purchased from PharMingen (San Diego, CA): biotin-conjugated anti-CD25 (7D4), phycoerythrin (PE)-conjugated anti-CD25 (PC61), FITC-conjugated anti-CD25 (7D4) and PE- or FITC-conjugated or purified anti-CD4 (L3T4). Rat anti-mouse CD11b was from Serotec (Raleigh, NC; MCA74G). Mouse IL-10 soluble receptor (sR) and human IL-3 sR were purchased from R & D Systems (Minneapolis, MN).

Induction of EAE and in vivo depletion of $CD25^+CD4^+$ cells using anti-CD25 antibody

SJL and C57BL/6 mice were immunized s.c. in the flank with either 60 μg $PLP_{139–151}$ (SJL) (HSLGKWLGHPDKF; Biosource, Camarillo, CA) or 200 μg $MOG_{35–55}$ (C57BL/6J) (MEVGWYRSPFSRVVHLYRNGK; Biosource) in CFA containing 400 μg *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI) respectively. They also received two i.v. injections of 150 ng pertussis toxin (List Biological, Campbell, CA) on days 0 and 2 post-immunization. For depletion of $CD25^+CD4^+$ cells in SJL mice, mice were injected i.p. on day 1, 4 and 7 with 200 μl PBS, 400 μg rat IgG (ICN, Costa Mesa, CA) or 400 μg anti-CD25 antibody (PC61). Animals were monitored daily for symptoms of EAE and scored as follows: 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; 5, moribund or dead.

Flow cytometric analysis

Flow cytometry was performed as previously described (24). Briefly, $0.5–1.0 \times 10^6$ cells were incubated in PBS with 2% normal FCS and 0.1% sodium azide for 5 min. The cells were incubated with a mixture of PE-, FITC- or allophycocyanin-conjugated mAb on ice for 30 min. The cells were then washed twice and then fixed in 1% of formaldehyde. The analysis was performed on a FACScan flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA).

Cell purification and adoptive transfer

Spleen and lymph node (LN) (axillary, inguinal and mesenteric) cell suspensions were prepared. $CD4^+$ T cells were then enriched by negative selection using T cell subset enrichment columns (R & D Systems) according to the suggested protocol. To separate $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells, the enriched $CD4^+$ T cells were incubated with biotin-conjugated anti-CD25 (10 μg/10^8 cells) in PBS with 4% BSA on ice for 30 min and washed twice. The cells were then incubated with streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA) for 15 min at 4°C. Magnetic separation was performed with LS separation columns according to the manufacturer's instruction. For adoptive transfer experiments, purified $CD25^+$ or $CD25^-CD4^+$ T cells were immediately injected i.v. into mice.

Preparation of antigen-presenting cells (APC)

Splenocytes from naive mice were incubated with mouse CD90 MicroBeads (Miltenyi Biotec) at 4°C for 15 min. T cells were depleted by Magnetic LS separation columns according to the suggested protocol. T cell-depleted spleen cells were used as APC.

Proliferation assay

Purified $CD25^{-}CD4^{+}$ or $CD25^{+}CD4^{+}$ T cells (1.0×10^5/well) were cultured in 10% FCS DMEM in triplicate wells alone or with $CD25^{+}CD4^{+}$ T cells in the presence of APC (1.0×10^5 cells/well) and 0.5 μg/ml of soluble anti-CD3. Cultures were pulsed with 1 μCi [^{3}H]thymidine/well (NEN, Boston, MA), 48 h and harvested 12 h later. Popliteal LN (pLN) cells (5.0×10^5/well) were cultured in the presence of various concentrations of $PLP_{139–151}$ for 72 h and harvested 12 h later.

Cytokine ELISA

For cytokine assays, splenocytes or pLN cells were grown at 1.0×10^6 cells/well in 200 μl of serum-free medium X-VIVO 20 (Biowhittaker) with various antigen concentrations. Supernatants were collected after 24 h for IL-2, 48 h for IL-4, IL-10 and IFN-γ, and 72 h for TGF-β1. Purified $CD25^{+}CD4^{+}$ and $CD25^{-}CD4^{+}$ T cells were cultured in 48-well plates (0.5 ml/well) at 2×10^6/ml in serum-free medium X-VIVO 20 and plate-bound anti-CD3ε mAb (10 μg/ml). Supernatants were collected 24 48 and 72 h after culture. Quantitative ELISA for IL-2, IL-4, IL-10 and IFN-γ were performed using paired antibodies and recombinant cytokines from PharMingen (San Diego, CA) as per the manufacturer's recommendations. TGF-β1 was measured by the TGF-β Emax Immuno Assay System (Promega, Madison, WI) as previously described (24).

Preparation of tissues and histologic staining

Mice were anesthetized with pentobarbital and perfused by intracardiac puncture with 50 ml of cold PBS. Murine brains and spinal cords were removed, and frozen immediately and then stored at –80°C. Cryosections of tissues were fixed with cold methanol and paraformaldehyde, and stained with hematoxylin & eosin for visualization of inflammatory infiltrates. Fixed sections were also stained with anti-CD4 (PharMingen; 1:20) or anti-CD11b (PharMingen; 1:50) for 1 h at room temperature, followed by FITC-conjugated goat anti-rat IgG (Molecular Probes, Eugene, OR; 1:500) for 1 h.

Statistical analysis

Differences in days of onset of disease, mean maximum EAE scores and amounts of cytokines were analyzed for significance using the Student's *t*-test.

Results

In vivo reduction of $CD25^{+}CD4^{+}$ T cells exacerbates PLP-induced EAE

To assess the suppressive role of $CD25^{+}CD4^{+}$ regulatory T cells in EAE, SJL mice were injected with either 200 μl PBS, 0.4 mg normal rat IgG or 0.4 mg anti-CD25 antibody (PC61) i.p. on day 1, 4 and 7 post-immunization. EAE was induced with 60 μg $PLP_{139–151}$ in CFA in the flank plus two i.v. injections of 150 ng pertussis toxin on day 0 and 2. Mice were monitored for signs of EAE for 30 days post-immunization. As shown in Fig. 1, there was a significant decrease in the percentage of $CD25^{+}CD4^{+}$ T cells in blood (0.8 ± 0.2 versus 3.0 ± 0.4%), LN (2.3 ± 0.5 versus 4.8 ± 0.7%) and spleen (1.2 ± 0.3 versus 4.2 ± 0.5%) of antibody-treated mice on day10 post-immunization as compared with rat IgG-treated mice (average ± SD of three experiments). Rat IgG-treated mice developed EAE with 18.8% mortality and a mean maximum clinical score of 3.1 ± 1.2, similar to PBS-treated mice. In contrast, anti-CD25 antibody-treated mice showed hyperacute and atypical EAE with a mortality of 85% (17 of 20) (Table 1). The onset of disease in anti-CD25 antibody-treated mice occurred earlier than in the PBS- or rat IgG-treated mice. Anti-CD25 antibody-treated mice had malaise, ataxia, paralysis of forelimbs or/and hindlimbs, or complete hemi-paralysis, which were clinical features not observed in control animals with EAE. Paralysis of tail was not common. Most (88%) of the anti-CD25 antibody-treated mice (15 of 17) died within 3 days following the onset of disease. The surviving mice (three of 20) did not recover from the disease during the observation period (60 days). EAE did not develop in mice treated with anti-CD25 antibody or rat IgG in the absence of $PLP_{139–151}$ immunization, indicating that the depletion of $CD25^{+}CD4^{+}$ regulatory T cells by itself was not sufficient to induce disease.

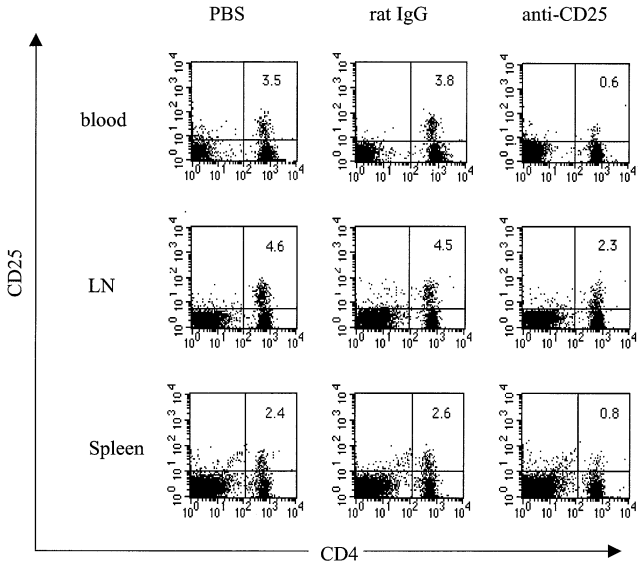

Fig. 1. Depletion of $CD25^{+}CD4^{+}$ T cells *in vivo* with anti-CD25 antibody. SJL mice were injected i.p. with 0.4 mg rat IgG or 0.4 mg anti-CD25 mAb (PC61) on day 1, 4 and 7 after immunization with 60 μg $PLP_{130–151}$. Mice were sacrificed on day10 post-immunization. Lymphoid cells of blood, LN and spleen were prepared, stained with FITC-conjugated anti-CD25 (7D4) and PE-conjugated anti-CD4, and then analyzed by flow cytometry. The data are from representative mice in three experiments.

Histologically, in EAE mice sacrificed on day 10 after immunization there were more inflammatory foci in the cerebral cortex in anti-CD25 antibody-treated mice compared with rat IgG-treated mice. The infiltrates were comprised of $CD4^{+}$ cells and $CD11b^{+}$ cells as detected by indirect immunofluorescence (Fig. 2). However, similar to the rat IgG-treated mice,

Table 1. Effect of reduction of CD25⁺CD4⁺ T cells on active EAE

	Incidence	Mortality	Day of onset	Mean maximum clinical score
PBS	95% (19/20)	10% (2/20)	12.2 ± 2.0	3.2 ± 0.8
Rat IgG	87.5% (14/16)	18.8% (3/16)	12.8 ± 1.4	3.1 ± 1.2
Anti-CD25	100% (20/20)	85% (17/20)[a]	9.2 ± 1.2[a]	4.8 ± 0.5[a]

EAE in SJL mice was induced with 60 μg $PLP_{139–151}$ in CFA plus two injections of 150 ng pertussis toxin on day 0 and 2. These mice were injected with 200 μl PBS, 0.4 mg normal rat IgG or 0.4 mg anti-CD25 mAb (PC61) i.p. on day 1, 4 and 7 post-immunization, and scored daily for signs of EAE for 30 days.
[a]$P < 0.001$ versus PBS or rat IgG.

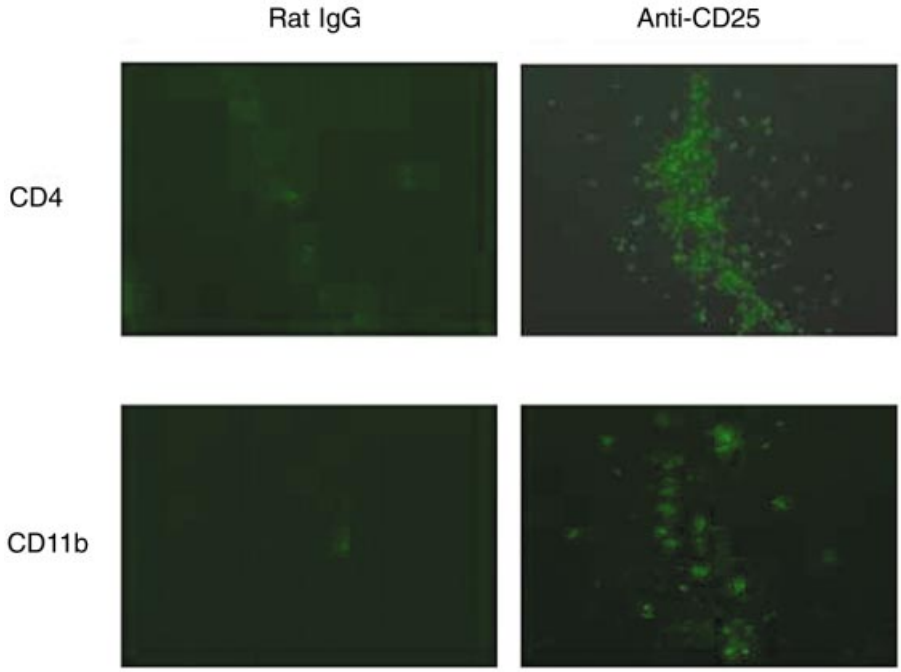

Fig. 2. Infiltration of CD4⁺ cells and CD11b⁺ cells in cerebral cortex. SJL mice were injected with 0.4 mg rat IgG or 0.4 mg anti-CD25 mAb on day 1, 4, and 7 after immunization with 60 μg $PLP_{139–151}$ in CFA and two injections of pertussis toxin. The mice were sacrificed on day10 post-immunization. Cryosections of brains of mice were prepared as described in Methods and stained with anti-CD4 (1:20) or anti-CD11b (1:50) for 1 h at room temperature, followed by FITC-conjugated goat anti-rat IgG (1:500) for 1 h at room temperature.

there were few inflammatory foci in the spinal cord of anti-CD25 antibody-treated mice (data not shown).

In vivo reduction of CD25⁺CD4⁺ regulatory T cells increases IFN-γ secretion and decreases IL-10 production by LN cells

To investigate the effect of CD25⁺CD4⁺ regulatory T cells on immune responses of LN cells, pLN cells from anti-CD25 antibody-treated, rat IgG-treated or PBS-treated mice were stimulated with $PLP_{139–151}$. As shown in the Fig. 3, upon *in vitro* stimulation with PLP 139–151, IFN-γ production by LN cells from anti-CD25 antibody-treated mice was significantly increased as compared to rat IgG-treated or PBS-treated mice (3468 ± 412 versus 614 ± 124 or 869 ± 95 pg/ml in a concentration of $PLP_{139–151}$ of 100 μg/ml, $P < 0.001$ respectively). However, IL-10 production was significantly decreased compared with control groups ($P < 0.001$), suggesting that CD25⁺CD4⁺ T cells may represent one of the sources of IL-10 *in vivo* or facilitate IL-10 production by other cell subsets. In addition, the proliferative response of LN cells of anti-CD25 antibody-treated mice to $PLP_{139–151}$ was significantly higher than that of PBS or rat IgG-treated mice in the presence of stimulation with 100 μg/ml of PLP 139–151 ($P < 0.01$). There was no significant difference in the production of IL-2 and TGF-β by LN cells of anti-CD25 antibody-treated mice (data not shown).

Transfer of CD25⁺CD4⁺ regulatory T cells from naive SJL mice suppresses EAE

To address whether CD25⁺CD4⁺ T cells from naive SJL mice have the suppressive activity *in vivo*, purified CD25⁺CD4⁺ or CD25⁻CD4⁺ T cells from naive SJL mice were injected i.v. into mice 2 days before induction of EAE. As shown in Fig. 4, the mice receiving CD25⁺CD4⁺ T cells developed mild EAE with a decreased severity of disease compared to those receiving CD25⁻CD4⁺ T cells or HBSS. The mean maximum clinical score in CD25⁺CD4⁺ T cell-transferred mice was significantly

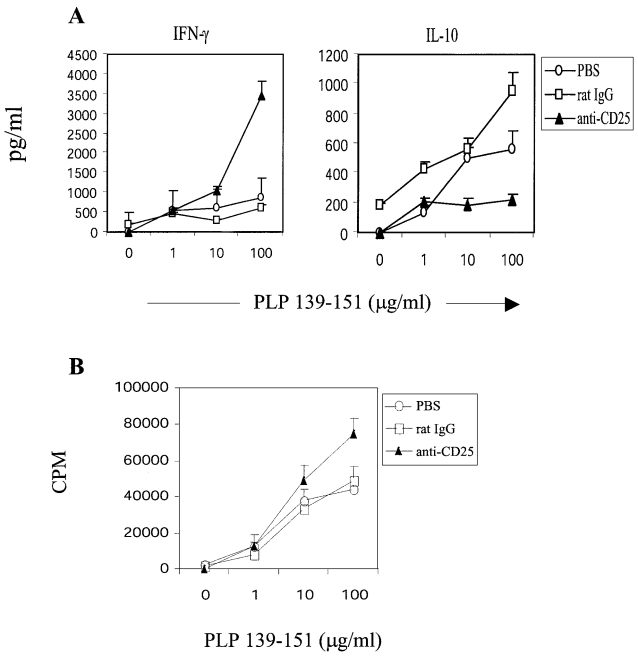

Fig. 3. Immune responses of LN cells in anti-CD25 antibody-treated mice. SJL mice were injected i.p. with PBS (open circles), 0.4 mg normal rat IgG (open squares) or 0.4 mg anti-CD25 antibodies (black triangles) on day 1, 4 and 7 post-immunization and immunized with 60 μg of PLP 139–151 in CFA. pLN of the mice were removed on day10 post-immunization. (A) pLN cells (1.0×10^6/well) were stimulated with the indicated concentrations of $PLP_{139–151}$ *in vitro*. ELISA for IL-2, IFN-γ and IL-10 was performed. (B) For proliferation of pLN cells, 5.0×10^5 cells/well were cultured with the indicated concentrations of $PLP_{139–151}$ and pulsed with 1 μCi [^{3}H]thymidine/well at 72 h and harvested 12 h later. Results are the mean values of three independent experiments.

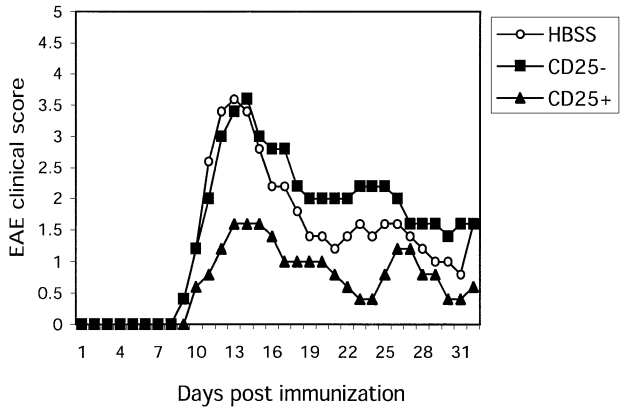

Fig. 4. Suppression of EAE by transfer of CD25⁺CD4⁺ regulatory T cells from naive SJL mice. HBSS (open circles), CD25⁻CD4⁺ T cells (black squares) or CD25⁺CD4⁺ T cells (black triangles) freshly isolated from naive SJL mice were injected i.v. into SJL mice (2.5×10^6 cells/mouse) 2 days before immunization with 60 μg $PLP_{139–151}$ in CFA in the flank. Each mouse received 150 ng of pertussis toxin on day 0 and 2 post-immunization, and scored daily for 32 days. Data are shown as the mean clinical scores of disease of five or six mice per group and are representative of three independent experiments.

lower than CD25⁻CD4⁺ T cell- or HBSS-transferred mice (2.3 ± 0.7 versus 3.9 ± 0.9 or 3.5 ± 0.5, $P < 0.01$). However, transfer of CD25⁺CD4⁺ T cells did affect the incidence and onset of the disease (data not shown).

IL-10 is involved in the suppressive activity of CD25⁺CD4⁺ regulatory T cells in vitro and in vivo

As described above, CD25⁺CD4⁺ regulatory T cells suppress EAE. To investigate the role of immunoregulatory cytokines in the action of CD25⁺CD4⁺ T cells *in vitro*, the cytokine profile of naive CD25⁺CD4⁺ or CD25⁻CD4⁺ T cells T cells was measured. CD25⁺CD4⁺ and CD25⁻CD4⁺ T cells were first purified from naive SJL mice and stimulated with plate-bound anti-CD3 *in vitro*. As shown in Fig. 5(A), CD25⁻CD4⁺ T cells from naive SJL mice secreted IFN-γ and IL-2, but not IL-10, whereas CD25⁺CD4⁺ T cells secreted IL-10 and no detectable levels of IFN-γ or IL-2. To test the role of IL-10 *in vitro*, we added IL-10 sR to the co-culture of CD25⁺CD4⁺ and CD25⁻CD4⁺ populations, and found that IL-10 sR reversed by 70% the suppressive activity of CD25⁺CD4⁺ T cells stimulated with anti-CD3 *in vitro* in the presence of APC (Fig. 5B).

In order to assess the role of IL-10 *in vivo*, we adoptively transferred CD25⁺CD4⁺ regulatory T cells from wild-type or IL-10-deficient mice into mice that were actively induced for EAE. As shown in Fig. 5(C), transfer of CD25⁺CD4⁺ T cells from wild-type mice inhibited active EAE induced by immunization of MOG 35–55 in CFA, whereas transfer of CD25⁺CD4⁺ T cells from IL-10-deficient mice was unable to suppress EAE. The mean maximum EAE score of mice receiving CD25⁺CD4⁺ T cells from wild-type mice was significantly decreased compared to mice receiving either CD25⁻CD4⁺ T cells from wild-type mice or CD25⁺CD4⁺ T cells from IL-10-deficient mice (2.38 ± 0.5 versus 3.25 ± 0.8 or 3.1 ± 0.4, $P < 0.05$).

Discussion

In the present study, we found that CD25⁺CD4⁺ T cells in EAE-susceptible strains of mice had a regulatory role in that they down-regulated antigen-induced EAE through a mechanism that involves IL-10. First, mice with reduced numbers of CD25⁺CD4⁺ regulatory T cells following treatment with anti-CD25 antibody developed hyperacute and atypical EAE with high mortality. Second, this reduction of CD25⁺CD4⁺ regulatory T cells *in vivo* was associated with increased antigen-stimulated production of IFN-γ and decreased secretion of IL-10 by lymph node cells when stimulated *in vitro*. Third, IL-10 is involved in the *in vitro* and *in vivo* suppressive activity of CD25⁺CD4⁺ regulatory T cells in EAE as we can partially reverse *in vitro* suppression by IL-10 sR and CD25⁺CD4⁺ T cells from IL-10-deficient mice do not suppress EAE.

It is becoming clear that CD25⁺CD4⁺ regulatory T cells may use multiple mechanisms to suppress immune responses *in vivo* and that the relative importance of these mechanisms depends on the experimental models (16). There is evidence suggesting regulatory cytokines (IL-10 and TGF-β) play a role in mediating the suppressive activity of CD25⁺CD4⁺ T cells in some systems, although other mechanisms are involved as well (19,24–30). It has been shown that inhibition of immune pathology by CD25⁺CD4⁺ regulatory T cells may result from controlling the expansion of CD25⁻CD4⁺ T cells via a process that requires IL-10, because CD25⁺CD45RB^{low}CD4⁺ T cells from IL-10-deficient mice are not able to protect from colitis

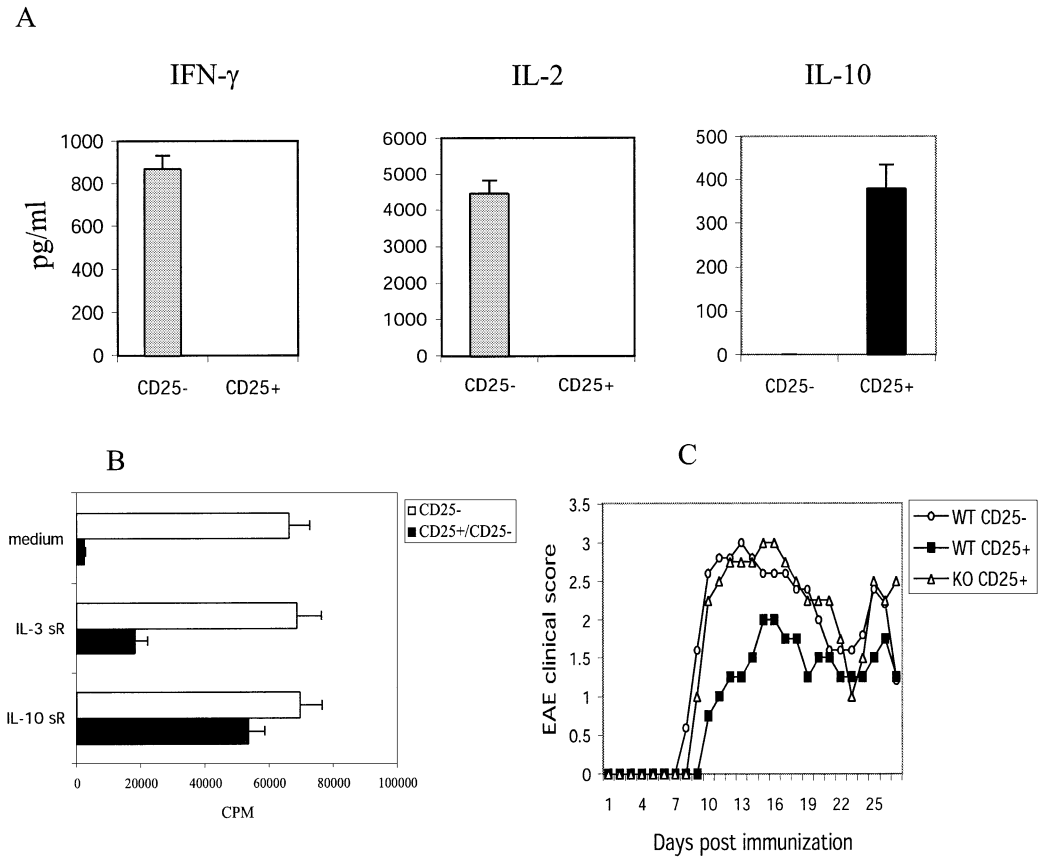

Fig. 5. Involvement of IL-10 in the *in vitro* and *in vivo* suppression by CD25⁺CD4⁺ regulatory T cells. (A) Cytokine profile of purified CD25⁻CD4⁺ T cells (shaded columns) or CD25⁺CD4⁺ T cells (black columns). CD25⁺CD4⁺ or CD25⁻CD4⁺ T cells from naive SJL mice were purified as described in Methods and cells (1.0×10^6/well) were cultured in 48-well plates with plate-bounded anti-CD3 (10 μg/ml) for 48 h. IFN-γ, IL-2 and IL-10 were measured by ELISA. Results are the mean values ± SD of three independent experiments. (B) Purified CD25⁻CD4⁺ T cells (5.0×10^4/well) alone (white bars) or with equal numbers of CD25⁺CD4⁺ T cells (black bars) were cultured with APC (5.0×10^4/well) in the presence of 0.5 μg/ml of soluble anti-CD3 and 10 μg/ml of human IL-3 sR or mouse IL-10 sR for 48 h. Results are the mean values ± SD of three independent experiments. (C) Purified CD25⁻CD4⁺ T cells and CD25⁺CD4⁺ T cells from wild-type (WT) mice or CD25⁺CD4⁺ T cells from IL-10-deficient (KO) mice were injected i.v. into C57BL/6J mice (3.0×10^6 cells/mouse) 2 days before immunization with 200 μg $MOG_{35–55}$ in CFA in the flank. Each mouse received 150 ng of pertussis toxin on day 0 and 2 post-immunization, and was scored daily for 28 days. Data are shown as the mean clinical scores of disease of four or five mice per group and are representative of three independent experiments.

(26). In addition, IL-10 has been reported to be important in prevention of graft rejection by CD25⁺CD4⁺ regulatory T cells (27).

IL-10 is a crucial cytokine in down-regulation of autopathogenic T_h1 responses in EAE. IL-10-deficient mice develop a severe EAE and are unable to recover from the disease (31). T cells from IL-10-deficient mice exhibit a strong antigen-specific proliferation, produce high levels of IFN-γ and tumor necrosis factor-α when stimulated with an encephalitogenic peptide, and induce very severe active EAE upon transfer into wild-type mice. In addition, IL-10 transgenic mice are resistant to the induction of EAE (31) and IL-10 mRNA expression is increased in the CNS during the recovery phase of EAE (32). IL-10 produced by PLP-specific T memory cells suppressed EAE when adoptively transferred to PLP peptide-immunized mice (33).

Studies by Belkaid *et al.* demonstrate that CD25⁺CD4⁺ T cells from naive mice express elevated levels of IL-10 mRNA and can secrete IL-10 when stimulated *in vitro*, and IL-10 produced by regulatory T cells contributes directly to parasite persistence (34). Consistent with these findings, we found that CD25⁺CD4⁺ T cells from naive SJL mice secreted high levels of IL-10 and inhibited proliferation by CD25⁻CD4⁺ T cells in the presence of stimulation with anti-CD3. LN cells from animals treated with anti-CD25 antibody in which CD25⁺CD4⁺ regulatory T cells were reduced produced less IL-10 and more IFN-γ compared to rat IgG-treated mice when stimulated with antigen *in vitro*. Our histologic data showed that on day 10 post-induction there was an infiltration of CD11b⁺ and CD4⁺ cells in brains of anti-CD25 antibody-treated mice compared to control animals. Therefore, *in vivo* elimination of CD25⁺CD4⁺ regulatory T cells may result in increased migration of pathogenic T cells and activated macrophages into the CNS, which exacerbates EAE. Kohm *et al.* (23) have recently reported that CD25⁺CD4⁺ regulatory T cells from conventional C57BL/6J mice suppress active and passive EAE which is associated with a decrease of autoreactive T_h1 cells, but an increased frequency of $MOG_{35–55}$-specific T_h2 cells, and that

$CD25^+CD4^+$ regulatory T cells inhibit both the proliferation and IFN-γ production by a $MOG_{35–55}$-specific T_h1 cell line *in vitro*. Our results are consistent with Kohm *et al.*'s findings and begin to define mechanisms by which $CD25^+CD4^+$ regulatory T cells regulate EAE. In addition to protection by adoptive transfer of $CD25^+CD4^+$ regulatory T cells, we have shown that depletion of these cells in SJL mice *in vivo* exacerbated EAE. Furthermore, animals depleted of $CD25^+CD4^+$ regulatory T cells produced high amounts of IFN-γ and decreased amounts of IL-10 *in vitro* compared to control animals. $CD25^+CD4^+$ T cells from IL-10-deficient mice were unable to suppress active EAE.

TGF-β has also been shown to play a role in suppression of $CD25^+CD4^+$ regulatory T cells in some systems. Powrie *et al.* have shown that protection from colitis by $CD25^+CD45RB^{low}CD4^+$ T cells is dependent on CTLA-4 and TGF-β (19). The action of TGF-β on T cells is revealed in mice expressing a T cell-specific dominant-negative form of the TGF-β receptor II. These mice develop inflammatory infiltrates in both colon and lung (35). We previously reported that $CD25^+CD4^+$ T cells from orally tolerized animals produced more TGF-β than those from control animals, and that adding TGF-β sR into co-culture of $CD25^+CD4^+$and $CD25^-CD4^+$ T cells partially abrogated the function of regulatory T cells (24). In this study, TGF-β1 did not appear to play a role in suppression of EAE by $CD25^+CD4^+$ regulatory T cells. Of note, $CD25^+CD4^+$ T cells are not a unique population of regulatory T cells *in vivo*. Lafaille *et al.* showed that $CD25^-CD4^+$ T cells from naive wild-type mice suppressed spontaneous EAE (36). It is possible that $CD25^-CD4^+$ T cells may also have regulatory properties. We have found that $LAP^+CD25^-CD4^+$ T cells suppress colitis induced by transfer of $CD45RB^{high}$ $CD4^+$ T cells in SCID mice (37). $DX5^+$ T cells, but not $CD25^+CD4^+$ T cells, protected animals from autoimmune diabetes (38) and $CD25^-CD4^+$ T cells also mediated dominant transplantation tolerance (39).

In summary, our data demonstrate that $CD25^+CD4^+$ regulatory T cells suppress the immune responses of pathogenic T cells *in vivo* in an animal model of autoimmune demyelinating disease and that the mechanism of this suppression involves IL-10.

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Abbreviations

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
LN	lymph node
MOG	myelin oligodendrocyte glycoprotein
PE	phycoerythrin
pLN	popliteal lymph node
PLP	proteolipid protein
sR	soluble receptor
TGF	transforming growth factor

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