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Suppressor role of Rat CD8⁺CD45RC^{low} T cells in Experimental Autoimmune Uveitis (EAU)

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Abstract

To determine whether decreased regulatory T cell activity contributes to the pathogenesis of recurrent experimental autoimmune uveitis (EAU), we compared the immunoregulatory activity of CD8⁺CD45RC^{low} T cells isolated from rats that had recovered from acute EAU with those from rats with the progressive, recurrent disease. Our results showed that CD8⁺CD45RC^{low} T cells isolated from the recovered rats showed suppressive activity in vitro, whereas those from rats with progressive, recurrent EAU do not. Depletion of CD8⁺CD45RC^{low} T cells from T cells used for adoptive transfer of EAU increased the pathogenic activity of the T cells. Co-transfer of CD8⁺CD45RC^{low} T cells with uveitogenic T cells prevented the relapse of disease in the recipient rats. The suppressive CD8⁺CD45RC^{low} T cells expressed increased levels of Foxp3 after stimulation in vitro with the autoantigen, and inhibited the production of IFN- γ by autoreactive T cells. Our data indicate that the decreased suppressive activity of CD8⁺CD45RC^{low} T cells is correlated with disease development in this autoimmune disease. Further studies on the biology of this T cell population should provide much needed insights into disease pathogenesis.

Keywords

autoimmunity; CD8 autoreactive T cell; EAU; uveitis

Introduction

Uveitis is a common cause of human visual disability and blindness. The animal model, experimental autoimmune uveitis (EAU), provides an essential tool for studying the pathogenesis of the human disease (Chan et al., 1987, Donoso et al., 1989). Previous studies in our laboratory have established two prototypic rat EAU models. In one, the disease is monophasic and the animal recovers completely after an acute episode lasting for 5–7 days (designated as recovered rats), while, in the other, the disease is progressive and recurrent and the animals have repeated exacerbations of disease for at least two-three months (Shao et al., 2003b). The availability of these two disease models provides us with a good opportunity to

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determine whether disease progression is caused by increased activity of pathogenic T cells or decreased activity of suppressor T cells.

The immune system has evolved complex mechanisms to avoid an autoimmune response while providing protective immunity and transgenic animals studies have shown that animals can keep auto-aggression in check despite the presence of huge numbers of self-reactive cells (Katz et al., 1993, Goverman et al., 1993). Further studies are required to determine whether the effects of T cells on the autoimmune response and protective immunity are controlled by distinct regulatory T cell subsets or by shared Treg subsets activated to different levels. Recent studies have demonstrated that, among the CD4 T cells, a subset expressing CD25 has strong suppressor activity (Shevach, 2000, Suripayer et al., 1998). There is also evidence that some subsets of CD8 T cells are functionally suppressive (Sun et al., 1988b, Sun et al., 1999b, Jiang et al., 1992, Endharti et al., 2005, Najafian et al., 2003)(Bloom et al., 1992, Jiang et al., 1992, Rifa'i et al., 2004, Sun et al., 1999a). It is important to note that, during the late phases of chronic autoimmune diseases, CD8 autoreactive T cells accumulate in the damaged organ and in the periphery (Friese and Fugger, 2005, Bradl et al., 2005, Sun et al., 2001). Given that recurrent uveitis causes major clinical problems, including vision loss, in humans, the emphasis of the present study was to determine the role of enhanced pathogenic activity or decreased suppressor activity of T cells in the mechanisms that lead to either self-limited, monophasic disease or progressive, recurrent disease.

CD8⁺ T cells can be divided into two subsets on the basis of the level of the surface molecule CD45RC expressed (Endharti et al., 2005, Rifa'i et al., 2004). Approximately 80% of CD8 T cells are CD45RC^{high} and the rest (< 20%) CD45RC^{low}. The CD8⁺CD45RC^{low} T subset possesses suppressive activity for effector T cells rejecting transplanted organs (Endharti et al., 2005, Rifa'i et al., 2004). In the present study, we investigated whether this T cell population also has suppressive activity in autoimmune diseases, such as EAE and EAU. Our results showed that, while, in the rat, pathogenic T cells in EAE and EAU expressed CD4⁺CD45RC^{high}, a regulatory T cell line derived from a rat with EAE expressed CD8 and CD45RC^{low}. Moreover, CD8⁺CD45RC^{low} T cells isolated from recovered rats, but not from rats with progressive recurrent disease, had strong suppressive activity. These suppressor T cells expressed increased levels of Foxp3 and inhibited the production of IFN- γ by autoreactive T cells. Furthermore, the suppressor activity of CD8⁺CD45RC^{low} T cells correlated directly with EAU disease status. Our data indicate that manipulation of regulatory T cell activity may prevent progression or relapses in this autoimmune disease.

Materials and Methods

Animals and reagents

Pathogen-free female Lewis rats (5- to 6-week-old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were housed and maintained at the animal facilities of the University of Louisville (Louisville, KY).

All animal studies conformed to the Association for Research in Vision and Ophthalmology statement on the use of animals in ophthalmic and vision research. Institutional approval was obtained and institutional guidelines regarding animal experimentation followed. The IRBP peptide, R16 (residues 1177–1191 of bovine IRBP; ADGSSWEGVGVVPDV), was synthesized by Sigma-Aldrich (St. Louis, MO).

Animal Model of EAU—The induction of uveitis in Lewis rats by immunization with peptide R16 has been described previously (Shao et al., 2004, Shao et al., 2003a, Shao et al., 2003b). Briefly, the rats were immunized subcutaneously with 200 μ l of an emulsion containing 50

μg of R16 and 500 μg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) in incomplete Freund's adjuvant (Sigma, St Louis), distributed over six spots on the tail base and flank.

For induction of uveitis by adoptive transfer of R16-specific T cells, naïve Lewis rats were injected intravenously either in vitro re-stimulated R16-specific T cells prepared from rats with uveitis (induced by either antigen immunization or T cell transfer) or R16-specific T cell lines in 0.5 ml of PBS, and examined daily for clinical signs of uveitis by slit-lamp biomicroscopy. Intensity of uveitis was scored blind on an arbitrary scale of 0 to 4 (Shao et al., 2003a) with 0 as no disease, 1 as engorged blood vessels in the iris and an abnormal pupil configuration, 2 as a hazy anterior chamber, 3 as a moderately opaque anterior chamber, with the pupil still visible, and 4 as an opaque anterior chamber, obscured pupil, and frequently, proptosis. Inflammation in the eye was confirmed by histopathology.

Preparation of IRBP-specific T cells

Briefly, T cells from R16-immunized rats were isolated at 9 days post immunization (p.i.) from pooled lymph nodes and spleens by passage single cell preparation through a nylon wool column, then 1×10^7 cells in 2 ml of RPMI medium in a 6-well plate (Costar) were stimulated with 10 $\mu\text{g}/\text{ml}$ of IRBP1-20 in the presence of 1×10^7 irradiated syngeneic spleen cells as antigen presenting cells (APCs). After 2 days, the activated T cell blasts were isolated by gradient centrifugation on Lymphoprep (Robbins Scientific, Mountain View, CA) and cultured in RPMI 1640 medium supplemented with IL-2-containing medium (10 ng/ml).

Isolation of the CD8⁺CD45RC^{low} T-cell subset

The monoclonal antibodies (mAbs) used for the purification of T cell subpopulations and for flow cytometry were OX35 (mouse anti-rat CD4), OX6 (mouse anti-rat major histocompatibility complex [MHC] class II), OX8 (mouse anti-rat CD8), OX12 (mouse anti-rat kappa light chain), and OX22 (mouse anti-rat CD45RC) [BD Biosciences, CA]. Rat CD8⁺ T cells were purified from pooled draining lymph nodes and spleen cells by removing B cells, CD4 T cells, class II-positive cells, and NK cells using a cocktail of mAbs containing OX-12, OX35, OX6, and NKR-P1a (CD161a) [BD Biosciences, CA] using MACS magnetic column. CD8⁺CD45RC^{high} T cells were enriched by magnetic beads after labeling with the mAb OX-22, whereas CD8⁺CD45RC^{high}-depleted cells were designated as CD8⁺CD45RC^{low} T cells. The purity of the sorted populations was confirmed by flow cytometry analysis and was always greater than 95%.

Proliferation assay

R16-specific T cells (3×10^5 cells/well) were seeded in 96-well microtiter plates with or without R16 in the presence of APCs (1×10^5) in a total volume of 200 μl for 48 h. [³H]thymidine was added to each culture for the last 8 h and incorporation was assessed using a microplate scintillation counter (Packard Instruments, Meriden, CT). For the in vitro suppression assay, unfractionated T cells depleted of CD8⁺CD45RC^{low} T cells were used as responder cells because they gave a stronger response than total unfractionated T cells. Such responder T (3×10^5 cells/well) were prepared from recovered rats and stimulated with R16 antigen (0.1 $\mu\text{g}/\text{ml}$) and APC in the presence or absence of varying numbers of CD8⁺CD45RC^{low} T cells, then processed as above. The proliferative response was expressed as mean cpm $\pm$ SD.

Flow cytometry analysis

Aliquots of 2×10^5 cells were double-stained with combinations of FITC- or PE-conjugated monoclonal antibodies against rat $\alpha\beta$ TCR (R73), CD45RC, CD8, Foxp3, or CTLA-4 (all from BD Biosciences, La Jolla, CA). For intracellular staining, cells were resuspended with anti-CD8 or isotype control antibodies, then fixed overnight with 1 ml of fixation buffer (Fix &

Perm cell permeabilization kit; eBioscience). After washing, the fixed cells were incubated for 30 mins with anti-rat-Foxp3 or anti-CTLA-4 antibodies. Data collection and analysis were performed on a FACS_{Calibur} flow cytometer using CellQuest software (San Jose, CA).

ELISA

IFN- γ was measured using a commercially available ELISA system (R&D Systems).

Statistics

The data are expressed as the mean $\pm$ SD. Statistical analyses were performed using Student's *t* test. A *p* value of 0.05 or less was considered to be statistically significant.

Results

A rat regulatory T cell line (anti-S1) with a suppressive effect on encephalitogenic T cells in EAE expresses very low levels of CD45RC

We previously reported the isolation of a rat regulatory T cell line (anti-S1) from rats that had recovered from induced EAE and shown that it neutralized the pathogenic activity of encephalitogenic T cells *in vivo* and prevents encephalitogenic T cell activation *in vitro*. (Sun et al., 1988b, Sun et al., 1988a). To determine whether regulatory T cell lines express different levels of CD45RC, we compared the CD45RC expression of an encephalitogenic T cell line, S1 (Sun et al., 1988b, Sun et al., 1988a), a uveitogenic T cell line, S22 (Shao et al., 2003b), and the anti-S1 line (Sun et al., 1988b, Sun et al., 1988a). As shown in Fig. 1, both the encephalitogenic and uveitogenic rat T cell lines expressed CD4 and CD45RC^{high}, whereas the regulatory anti-S1 line expressed CD8 and CD45^{low}.

CD8⁺CD45RC^{low} T cells from rats that had recovered from EAU are suppressive *in vitro*

To determine whether the CD8⁺CD45RC^{low} T cells have a suppressive effect on autoreactive T cells in EAU, we have determined the abundance of this T subset in monophasic and recurrent disease phases of EAU. As we previously reported, Lewis rats immunized with the uveitogenic peptide R16 develop an acute and monophasic uveitis, showing overt clinical symptoms at 8–9 days post-immunization (p.i.) which persist for 4–5 days and subside subsequently, whereas the disease induced by adoptive transfer of *in vitro* activated IRBP-specific T cells develop an acute uveitis with several recurrences (Shao et al., 2003b). We found that in monophasic EAU rats, the numbers of CD8⁺CD45RC^{low} T cells were significantly increased in recovered phase of disease (Fig. 2A), whereas in recurrent rats, the number of CD8⁺CD45RC^{low} T cells remained largely unchanged (Fig. 2B). Functionally tests showed, removal of the CD8⁺CD45RC^{low} T cells greatly enhanced the proliferative response of the total T cells of monophasic EAU rats (2C), but not those of recurrent EAU rats (2D), indicating that CD8⁺CD45RC^{low} T cells from monophasic EAE rats are functionally suppressive. In addition, while co-culture of R16-specific T responder cells with the CD8⁺CD45RC^{low} T cells derived from monophasic EAU rats completely abolished the proliferation of the R16-specific responder T cells *in vitro* (Fig. 3A). CD8⁺CD45RC^{low} T cells from rats suffering from recurrent EAU did not show suppressor activity (Fig. 3B). In addition, the IFN- γ producing ability of the responder R16-reactive T cell correlated with the disease status of uveitis, namely, responder T cells derived from rats with progressive, recurrent EAU produced much higher levels of IFN- γ after *in vitro* stimulation by autoantigen than the same T cell population isolated from monophasic rats (Fig. 3C). We then tested the inhibitory effects of CD8⁺CD45RC^{low} T cells on the production of IFN- γ by R16-specific responder T cells. When these responder cells were co-cultured with CD8⁺CD45RC^{low} T cells at a ratio of 2:1, IFN- γ production by the responder T cells was significantly decreased by the CD8⁺CD45RC^{low} T cells derived from monophasic EAU, but not those isolated from progressive, recurrent disease (Fig. 3D). In all

above suppression assay, unfractionated T cells depleted of CD8⁺CD45RC^{low} from recovered EAU rats were used as responder cells as described in material and methods. Similar results were obtained when purified CD4 T cells were used as responder cells (not shown).

We also tested whether a same regulatory T cell population was able to suppress responder T cells of the monophasic and recurrent EAU. Our results showed that responder T cells from monophasic and recurrent EAU rats were similarly inhibited (not shown). Kinetic studies have shown that the suppressive CD8⁺CD45RC^{low} T cells persisted for at least several weeks after the recovery of monophasic EAU but remained undetectable in recurrent EAU rats (Fig. 3E).

CD8⁺CD45RC^{low} T cells from rats recovered from monophasic EAU suppressed adoptively transferred EAU

To further assess the suppressor activity of CD8⁺CD45RC^{low} T cells in vivo, we performed adoptive transfer. R16-specific T cells were isolated from rats recovered from monophasic EAU (15–20 days p.i.). Recipient rats received either unfractionated T cells or the CD8⁺CD45RC^{low}-depleted T cells after an in vitro stimulation with immunizing antigen. As shown in Fig. 4A, depletion of the CD8⁺CD45RC^{low} T cell subset significantly enhanced the disease-inducing effect of the transferred T cell population. To further prove the suppressive activity of CD8⁺CD45RC^{low} cells, we also performed co-transfer assay. As demonstrated in Fig. 4B, co-transfer of CD8⁺CD45RC^{low} cells significantly inhibited the pathogenic activity of R16-specific T cells (Fig. 4B).

Suppressor CD8⁺CD45RC^{low} T cells express increased levels of Foxp3, but not CTLA4, after in vitro stimulation,

We then examined whether the suppressor activity of CD8⁺CD45RC^{low} T cells was associated with increased intracellular expression of Foxp3. Our results showed that Foxp3 levels expressed by freshly isolated CD8⁺CD45RC^{low} T cells from naïve, recovered, or recurrent rats did not differ significantly (Fig. 5A). However, after stimulation with R16 in vitro, only the CD8⁺CD45RC^{low} T cells from recovered rats expressed upregulated levels of Foxp3 (Fig. 5B&C).

Since a previous study suggested that the suppressor activity of CD8 T regulatory cells is related to the level of CTLA-4 expression (Takahashi et al., 2000), we examined whether suppressor CD8⁺CD45RC^{low} T cells expressed increased levels of CTLA-4. Our results showed that CD8⁺CD45RC^{low} T cells from recurrent or recovered rats expressed comparable levels of CTLA-4, regardless of whether they were freshly isolated (Fig. 5D) or after stimulation with autoantigen in vitro (Fig. 5E).

To determine whether direct cell-cell contact is required for the suppression of CD8⁺CD45RC^{low} T cells, we have tested the suppressive activity in the culture supernatants of the CD8⁺CD45RC^{low} T cells. Culture supernatants were collected 24h after the co-culture of CD8⁺CD45RC^{low} and the responder T cells. Our results showed that culture supernatants did not contain suppressive activity (data not shown).

Discussion

Although many studies have shown that CD8⁺ T cells play important roles in the pathogenesis of autoimmune diseases (Bloom et al., 1992, Jiang et al., 1992, Rifa'i et al., 2004, Sun et al., 1988b, Sun et al., 1999a, Endharti et al., 2005), the cellular and molecular mechanisms involved remain largely unclear. Moreover, CD8 autoreactive T cells may act either as pathogenic T cells (Bloom et al., 1992, Jiang et al., 1992, Rifa'i et al., 2004, Sun et al., 1988b, Sun et al., 1999a) or suppressive T cells, which inhibit disease development (Endharti et al., 2005, Rifa'i

et al., 2004). In the present study, we studied the role of the CD8⁺CD45RC^{low} T cell subset in the pathogenesis of the autoimmune diseases, EAE and EAU. Our previous results and the present ones show that such T cells are functionally suppressive in both EAE (Sun et al., 1988b) and EAU.

The overall goal of this study was to determine whether disease progression was due to enhanced pathogenic T cell activity or decreased regulatory T cell activity. Comparison of the CD8⁺CD45RC^{low} T cells isolated from recovered rats and from rats with progressive, recurrent disease showed that the former possessed strong suppressor activity (Fig. 3A), while the latter did not (Fig. 3B), suggesting that the loss of suppressor activity of the regulatory T cells may contribute to disease progression. However, the mechanisms responsible for hindering the development of suppressor activity of these T cells during progressive disease needs further study.

CD8⁺CD45RC^{low} T cells have previously been reported to possess suppressor activity for T cells rejecting transplanted organs (Rifa'i et al., 2004). Our study supports this observation by showing that these T cells also have suppressor activity in the autoimmune diseases EAE and EAU. An in vitro proliferation assay showed that CD8⁺CD45RC^{low} T cells isolated from recovered rats failed to proliferate in response to immunizing autoantigen (data not shown), whereas the unfractionated T cells showed an increased antigen-specific response when CD8⁺CD45RC^{low} cells were depleted, compared to unfractionated T cells (Fig. 2C). When CD8⁺CD45RC^{low} T cells were co-cultured with R16-specific responder T cells, a significant inhibitory effect on the proliferation of, and IFN- γ production by, the R16-T cells was seen (Fig. 3A & D). Moreover, the suppressor activity could also be demonstrated in in vivo studies, as adoptive transfer of IRBP-specific T cells induced more severe uveitis in naïve recipients if the CD8⁺CD45RC^{low} T cells were removed from the transferred T cells, (Fig. 4A). As someone else may argue that the group injected with CD8 depleted cells might have received more CD4⁺ cells per rat than the group injected with unfractionated cells, we believe that the numbers of adoptively transferred T cells are important for the induced disease. However, if the injected cell numbers vary within the limit (> or < 20%), the severity of induced disease will not be significantly affected (data not shown). Further, co-transfer of CD8⁺CD45RC^{low} cells and R16-specific T cells neutralized the pathogenic activity of the latter cells (Fig. 4B).

To determine the mechanism by which suppressor T act, we assessed the expression of Foxp3 and CTLA-4. Our results showed that cells from recovered rats with strong suppressor activity or rats with recurrent EAU with no suppressor activity (Fig 3) did not express high levels of Foxp3 (Fig. 5A and B). However, after an in vitro stimulation with immunizing autoantigen, increased Foxp3 levels were seen with the cells from recovered rats, but not the rats with recurrent disease (Fig. 5C).

We have also performed suppression test using CD8⁺CD45RC^{low} T cells derived from naïve donors. Our results showed that CD8⁺CD45RC^{low} cells from naïve rats also possess suppressive activity in vitro (not shown). At this point, we believe CD8⁺CD45RC^{low}. Unfortunately, we are not able to identify the specificity of the cell.

Finally, we compared CTLA-4 expression by CD8⁺CD45RC^{low} T cells showing varying degrees of suppressor activity. Our results showed no correlation between suppressor activity and CTLA-4 levels, with or without in vitro antigen stimulation (Fig. 5D and E), suggesting that the loss of suppressor activity of CD8⁺CD45RC^{low} T cells in recurrent EAU was not due to decreased CTLA-4 expression. The mechanisms by which CD8⁺CD45RC^{low} T cells lose their suppressor capability in recurrent EAU rats remain to be determined.

In summary, our data showed that CD8⁺CD45RC^{low} T cells have suppressor activity and are involved in the pathogenesis of autoimmune uveitis. The suppressor activity of

CD8⁺CD45RC^{low} T cells in inhibiting is correlated to the proliferation of, and pro-inflammatory production by, autoreactive T cells correlates with their Foxp3 expression. Further studies on the biology of this T cell population should provide much needed insights into disease pathogenesis.

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Abbreviations

EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveitis
IRBP	interphotoreceptor retinoid-binding protein
R16	residues 1177–1191 of bovine IRBP
recovered rats	rats that have recovered from acute monophasic EAU
T_{reg}	regulatory T cell

Fig. 1. EAE Suppressor, but not EAE pathogenic, and EAU pathogenic T cells express low levels of surface CD45RC

A rat encephalitogenic T cell line (S1), uveitogenic T cell line (IRBP22), and EAE suppressor T cell line (anti-S1) were double-stained with antibodies against CD4 or CD8 and CD45RC, then analyzed by FACS. The values represent the purity of each cell population. Our results showed that both pathogenic T cell lines expressed CD4 and high levels of CD45RC and the suppressor anti-S1 cell line expressed CD8 and low level of CD45RC.

Fig. 2. Rat R16-specific T cells show an enhanced proliferative response after CD8⁺CD45RC^{low} T cells were removed

(A&B) T cells were separated from pooled spleens and draining lymph nodes of disease-inducing rats. The abundance of CD8⁺CD45RC^{low} T cells was tested by flow cytometry analysis. Remitting rats were chosen after the acute episode and before the first relapse of the disease. (C) Proliferative response of unfractionated T cells and T cells depleted of CD8⁺CD45RC^{low} T cells from recovered EAU rats. (D) Proliferative response of unfractionated T cells and T cells depleted of CD8⁺CD45RC^{low} T cells from recurrent EAU rats, harvested at the peak of the first relapse. T cells (4×10^5 , from pooled spleens and lymph nodes of rats) were stimulated for 48 h in the presence of irradiated APCs. [³H]thymidine being added to each cultured well for 8 h before assessing incorporation. The data shown are representative of those obtained in 3 independent experiments.

Fig. 3. CD8⁺CD45RC^{low} T cells isolated from recovered rats suppress the proliferation of, and IFN-γ production by, R16-specific responder T cells

(A) Unfractionated T cells depleted of CD8⁺CD45RC^{low} were used as responder cells.

Responder T cells were prepared from pooled spleens and draining lymph nodes of recovered rats (18 days p.i.) as described in the *Materials and Methods*, then 3×10^5 cells were stimulated with a suboptimal dose of immunizing antigen (R16, 0.1 μg/ml) in the presence of irradiated syngeneic spleen (APCs) and graded numbers of CD8⁺CD45RC^{low} T cells from recovered rats and proliferation were assessed. (B) As in (A), but using CD8⁺CD45RC^{low} T cells from rats with progressive, recurrent disease, harvested at the peak of the first relapse. The results shown are representative of those for 3 independent experiments. (C) R16-specific T responder cells from recovered or progressive, recurrent EAU rats were stimulated with R16 and APCs, then the supernatants were collected 48h later for IFN-γ measurement. (D) R16-specific responder cells from recovered rats were co-cultured with CD8⁺CD45RC^{low} T cells from recovered rats or recurrent rats, harvested at the peak of the first relapse at a ratio of 2:1 and the supernatants collected after 48h culture and analyzed for IFN-γ production by ELISA. The data shown are representative of those obtained in 3 independent experiments (**, $p < 0.01$). (E) As in A, CD8⁺CD45RC^{low} T cells were isolated from monophasic or recurrent EAU rats at different time point after the initiation of disease. The results shown are representative of those for 3 independent experiments.

Fig. 4. CD8⁺CD45RC^{low} T cells from rats that had recovered from EAU are suppressive in vivo (A) Adoptive transfer of IRBP-specific T cells depleted of CD8⁺CD45RC^{low} T cells induces more severe uveitis in recipient rats.

R16-specific T cells were isolated from rats recovered from EAU (18 days p.i.), then the unfractionated T cells or T cells depleted of CD8⁺CD45RC^{low} T cells were stimulated with R16 and APCs for 48 h before the activated T cell blasts were separated with Ficoll gradient centrifugation and injected i.v. into naive rats. Clinical signs of uveitis were scored as reported previously. This study was repeated twice, with three rats in each group.

(B) Co-transfer CD8⁺CD45RC^{low} cells with R16-specific T cells prevented disease recurrent. R16-specific T cells were stimulated with R16 and APCs for 48 h before the activated T cell

blasts were separated with Ficoll gradient centrifugation. Then blast T cells alone (2.5×10^6), or blast T cells (2.5×10^6) plus $CD8^+CD45RC^{low}$ (2.5×10^6) were injected i.v. into naive rats ($n=3$, in each group). Clinical signs of uveitis were scored as reported previously. The data are one of two independent assays, in which mean clinical score + SD of three rats in each group were shown.

Fig. 5. CD8⁺CD45RC^{low} T cells isolated from recovered rats express increased levels of Foxp3 after in vitro stimulation

(A) CD8⁺CD45RC^{low} T cells were isolated from naive, recovered, and recurrent rats. After surface staining of FITC-labeled CD8, Foxp3 was detected as described in the *Materials and Methods*.

(B and C) Freshly purified unfractionated CD8 T cells (>95%, data not shown) were stained directly with antibodies against CD45RC and FoxP3 (B) or were first stimulated with R16 antigen and APCs for 48 h in a 12-well plate, then the activated T cells were separated by Ficoll centrifugation and assessed for intracellular Foxp3 expression (C). (D and E) As in B and C

using anti-CTLA4 antibodies instead of anti-Foxp3 antibodies. The results shown are representative of those for 3 independent experiments.