

# Retina-Specific T Regulatory Cells Bring About Resolution and Maintain Remission of Autoimmune Uveitis

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**Experimental autoimmune uveitis (EAU) induced in mice by immunization with the retinal Ag interphotoreceptor retinoid-binding protein (IRBP) is a model of human autoimmune uveitis. We examined whether T regulatory cells (Tregs) found in uveitic eyes are IRBP specific, functionally suppressive, and play a role in natural resolution of disease and in maintenance of remission. Progressive increase of Foxp3<sup>+</sup> Treg to T effector cell (Teff) ratio in uveitic eyes correlated with resolution of disease. At peak disease, up to 20% of Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>) and up to 60% of Teffs (CD4<sup>+</sup>Foxp3<sup>-</sup>) were IRBP specific, whereas in lymphoid organs retina-specific T cells were undetectable. Tregs isolated from eyes of mice with EAU efficiently suppressed IRBP-specific responses of Teffs from the same eyes. Importantly, systemic depletion of Tregs at peak disease delayed resolution of EAU, and their depletion after resolution triggered a relapse. This could be partially duplicated by depletion of Tregs locally within the eye. Thus, the T cell infiltrate in uveitic eyes of normal mice with a polyclonal T cell repertoire is highly enriched in IRBP-specific Tregs and Teffs. Unlike what has been reported for Tregs in other inflammatory sites, Tregs from uveitic eyes appear unimpaired functionally. Finally, Foxp3<sup>+</sup> Tregs play a role in the natural resolution of uveitis and in the maintenance of remission, which occurs at least in part through an effect that is local to the eye. *The Journal of Immunology*, 2015, 194: 3011–3019.**

**N**atural T regulatory cells (Tregs) that are released from the thymus of normal animals soon after birth are essential for maintenance of tissue homeostasis (1, 2). Treg depletion in adulthood precipitates multiorgan autoimmune disease and death (3). Induced Tregs arise as a result of contact with Ag during adult life, but their ability to control active autoimmunity has been debated. Although Tregs were shown to be present in the target organ affected by inflammation, isolated Tregs from inflammatory sites may be deficient functionally. Inflammatory cytokines have been implicated in this impairment of Treg function, possibly permitting ongoing and chronic inflammation in the target organ (4, 5).

In the case of the eye, the situation is colored by the unique needs and properties of the ocular tissues. Due to the necessity to preserve vision that might sustain collateral damage as a result of inflammation, the healthy eye is an immunoregulatory environment that resists inflammatory processes (6). By using T cells expressing a transgenic retina-specific TCR, we demonstrated directly that the living eye converts naive retina-specific T cells into functionally competent Tregs (7). This process, which is part of the

phenomenon known as ocular immune privilege, is promoted by high concentrations of TGF- $\beta$  and retinoic acid that are constitutively present in the eye. Other studies indicated that a tiny population of resident Tregs is present in the healthy eye and may constitute part of ocular immune privilege (8). Nevertheless, the eye is subject to destructive inflammation precipitated by uveitogenic T cells activated outside the eye, which have acquired the ability to actively cross the blood-retinal barrier. Our previous data demonstrated that such committed T effector cells (Teffs) are impervious to the inhibitory ocular microenvironment, explaining why uveitis can be induced despite ocular immune privilege. However, even though the ocular microenvironment cannot prevent uveitis, the acute phase of experimental autoimmune uveitis (EAU) disease is typically of short duration and starts resolving spontaneously after ~1 wk to 10 d. This self-limiting nature of EAU is in apparent contradiction with the demonstrated inability of the ocular microenvironment to directly control activated uveitogenic T cells (7).

Although the small population of Tregs within the healthy eye raises the threshold of development of retinal autoimmunity (8), its presence does not clarify the role of Tregs after this threshold has already been broken and autoimmunity has begun. The present study deals with this question. We examine the hypothesis that the spontaneous resolution of EAU and maintenance of remission involve the activity of functionally competent Tregs. Using gene-manipulated C57BL/6 mice in which Tregs can be identified by Foxp3-driven GFP expression and can also be deleted by Foxp3-driven diphtheria toxin (DT) receptor (DTR) expression, we examine the behavior of Tregs in the uveitic eye and the consequences of their depletion peripherally as well as locally. In complementary experiments using B10.RIII mice, in which availability of MHC–Ag multimers and retina-specific TCR transgenic mice permit the identification and analysis of retina-specific T cells and their responses, we study the specificity of eye-infiltrating Tregs and their functional competence in an Ag-driven setting. Our data indicate that the T cell infiltrate in uveitic

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Abbreviations used in this article: AC, anterior chamber; DT, diphtheria toxin; DTR, DT receptor; EAU, experimental autoimmune uveitis; IRBP, interphotoreceptor retinoid-binding protein; LN, lymph node; Tconv, conventional T cell; Teff, T effector cell; Treg, T regulatory cell; TSDR, Treg-specific demethylation region; WT, wild-type.

eyes of normal mice with a polyclonal T cell repertoire is highly enriched in interphotoreceptor retinoid-binding protein (IRBP)-specific Tregs and Teffs. Interestingly, unlike what has been reported for Tregs in other inflammatory sites, Tregs from uveitic eyes are able to inhibit not only naive, but also bona fide uveitogenic Teffs that are present in eyes during uveitis (9–12). Systemic and local Treg depletion studies support the notion that Foxp3<sup>+</sup> Tregs play a role in the natural resolution of uveitis as well as in the maintenance of remission, and that this is achieved, at least in part, through local mechanisms.

## Materials and Methods

### Mice

C57BL/6 and B10.RIII were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 DEREg mice, which express a GFP-DTR fusion protein under control of the Foxp3 promoter, were a gift of T. Sparwasser (Hanover, Germany) (13, 14). GFP-Foxp3 reporter mice obtained from A. Y. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) (15) were crossed onto the B10.RIII background for 10 generations to produce Foxp3-GFP B10.RIII reporter mice. Some investigators reported an altered *in vivo* function of Tregs expressing this construct, in which Foxp3-GFP is present as a fusion protein (16, 17). However, *in vivo* studies in the EAU model did not reveal any evidence for changes in Treg function in the Rudensky mice crossed onto the B10.RIII background, as judged by EAU scores and frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in their uveitic eye (Supplemental Fig. 1), so that this effect might be particular to the strain and disease model under study. IRBP TCR transgenic mice (R161H) on the Rag2<sup>-/-</sup> B10.RIII background (18) were bred in-house. All strains of mice were tested and found to be free of the rd8 mutation of the *crbl* gene, except for the DEREg mice on C57BL/6 background. The rd8 mutation manifests as focal areas of disorganization within the retina, without infiltration of inflammatory cells. Because the DEREg mice were compared with their own littermates as controls, and because the rd8 lesions cannot be confused with the lesions and inflammation typical of uveitis, presence of the rd8 gene does not affect the interpretation of our experiments. Mice were housed under pathogen-free conditions, fed standard laboratory chow ad libitum, and used at 6–12 wk of age. Treatment of animals was in compliance with institutional guidelines. The National Eye Institute Animal Care and Use Committee approved all animal study protocols.

### Reagents

Human IRBP peptide residues 1–20 (H-2<sup>b</sup> restricted) and 161–180 (H-2<sup>f</sup> restricted) were synthesized by Anaspec (Fremont, CA). Whole IRBP was purified from bovine retinas by Con A-Sepharose affinity chromatography and HPLC (19). IRBP-specific T cells were identified with the fluorescently labeled IRBP161–180/MHC class II dimer reagent (20). Tregs were identified by flow cytometry either by detection of GFP or by Foxp3 staining (eBioscience, San Diego, CA). All additional Abs for flow cytometry and cell sorting were purchased from eBioscience or BioLegend (San Diego, CA).

### EAU induction and scoring

EAU in Foxp3-GFP B10.RIII reporter mice was induced with 10–12 µg human IRBP161–180 (21). Mice on the C57BL/6 background were immunized with a mixture of 150 µg IRBP plus 300 µg IRBP1–20. As additional adjuvant, 0.2 µg *Bordetella pertussis* toxin (List Biologicals, Campbell, CA) was given *i.p.* on day 0 and again on day 2. All IRBP Ags were emulsified 1:1 v/v in CFA that had been supplemented with *Mycobacterium tuberculosis* strain H37RA (Sigma-Aldrich, St. Louis, MO) to 2.5 mg/ml. A total of 200 µl emulsion was injected *s.c.*, divided among three sites: base of tail and both thighs. Inflammatory activity was evaluated in a masked fashion in anesthetized mice by fundus examination under a binocular microscope after dilation of the pupil. Clinical inflammation scores ranged from 0 to 4 according to the following criteria: 0 = no inflammation; 0.5 = trace disease; 1 = minimally active, localized; 2 = moderately active, multiple lesions; 3 = active, multiple diffuse lesions; and 4 = very active, often with retinal detachment or hemorrhage. Histology scoring followed previously published criteria (22).

### Isolation of T cells for sorting and analysis

To isolate T cells from eyes of uveitic mice, enucleated eyes were first trimmed of external tissue and then the globe was opened along the limbus

to remove the lens. The remaining tissue was minced in HL-1 media (Lonza, Walkersville, MD). Cell/Tissue separation was accomplished with 1.0 mg/ml collagenase D treatment (Roche, Indianapolis, IN) for 40 min at 37°C. After washing, tissue was dispersed through a 40-µm strainer, resuspended in PBS plus 2% FBS plus 2 mM EDTA sorting buffer, and then stained. For lymph nodes (LN), tissue was dispersed through a 40-µm strainer and then resuspended in sorting buffer. Cell populations were analyzed using a BD FACSCalibur (BD Biosciences, San Jose, CA) and MACSQuant Analyzer (Miltenyi Biotec, Auburn, CA) or were sorted on a FACS Aria II (BD Biosciences) to 99% purity. To determine the number of Tregs present in eyes of mice months after EAU challenge, eye cells were incubated with CD4<sup>+</sup> isolation beads (Miltenyi Biotec) prior to staining and then passed through a magnetic column that was integrated into a MACS-Quant Analyzer. Because the frequency of Tregs at these late time points was expected to be low, utilization of the column permitted the enrichment of the cells before analysis. All data were analyzed using FlowJo (Tree Star, Ashland, OR).

### DNA methylation analysis of the Treg-specific demethylation region in Tregs

Eyes from (male) Foxp3-GFP B10.RIII reporter mice were harvested 17 d after EAU immunization and were prepared for sorting, as described above. The following populations were collected: IRBP Dimer<sup>+</sup> CD4<sup>+</sup>GFP<sup>+</sup>, IRBP Dimer<sup>+</sup> CD4<sup>+</sup>GFP<sup>-</sup>, IRBP Dimer<sup>-</sup> CD4<sup>+</sup>GFP<sup>+</sup>, and IRBP Dimer<sup>-</sup> CD4<sup>+</sup>GFP<sup>-</sup>. Methylation analysis followed the protocol of Kim et al. with modifications (23). Briefly, the cells were processed through the following steps: genomic DNA extraction (QIAmp DNA Micro Kit), bisulfate conversion (EpiTect Bisulfite kit), preparation of PCR (PyroMark PCR Kit), and then pyrosequencing (PyroMark Gold Q96) on a PyroMark ID 1.0 instrument (Biotage, Charlotte, NC). Analysis of 11 CpGs of the mouse Foxp3 promoter Treg-specific demethylation region (TSDR) was performed. The mean of all 11 CpGs of each sample constituted the percent methylation of each sample. All kits were from Qiagen (Germantown, MD).

### IRBP-specific Treg suppression assays

The suppressive ability of Tregs (CD4<sup>+</sup>GFP<sup>+</sup>) sorted from uveitic eyes on proliferation and cytokine production of target cells was tested in coculture experiments using two types of responder cells: 1) naive (CD62L<sup>high</sup> CD44<sup>low</sup>) sorted LN cells from R161H Rag2<sup>-/-</sup> mice, and 2) Teffs (CD4<sup>+</sup>GFP<sup>-</sup>) sorted from uveitic eyes of Foxp3-GFP B10.RIII reporter mice 17 d postimmunization. A total of 5 × 10<sup>4</sup> responder cells was cultured in HL-1 media supplemented with 2% normal mouse serum plus 1 × 10<sup>5</sup> T cell-depleted syngeneic (irradiated) APCs ± 1.0 µg/ml IRBP161–180 as stimulant. Various numbers of Tregs sorted from the same uveitic eyes were added to the cultures. Cultures were incubated in round-bottom plates for 60 h, and proliferation was evaluated by [<sup>3</sup>H]thymidine uptake in the last 18 h. IFN-γ, IL-17, and GM-CSF levels were measured from 48-h coculture supernatants by ELISA (R&D Systems, Minneapolis, MN). Each suppression experiment used 30–40 EAU-challenged mice. Nevertheless, only small numbers of cells could be sorted from the uveitic eyes so that only one replicate per dilution could be performed. Therefore, reproducibility of the experiments was demonstrated in repeat experiments rather than intraexperiment replicates.

### Systemic and local Treg depletion

Foxp3-expressing Tregs in DEREg mice were depleted systemically in mice during the peak of EAU (day 21) or, after disease resolution (day 64 or 88), by the administration of 0.5 µg DT (EMD Millipore, Billerica, MA) *i.p.* on successive 2 d. Experiments established that this depletion regimen eliminated systemic as well as eye-infiltrating Tregs for about a period of 1 wk (14) (Supplemental Fig. 2 and data not shown). Treg depletion in DEREg mice was verified in all experiments by assessment of GFP-expressing Tregs in the blood (tail bleeds) after administration of DT. Local Treg depletion in the eye was performed following the protocol of Lehmann et al. (24) with modifications. A total of 25 ng DT was injected in a volume of 1.5 µl into the anterior chamber (AC) of one eye, and an equal volume of PBS was injected into the contralateral eye three times during 1 wk, either starting during the peak (day 19) or after the resolution of disease (day 69 or 77). To evaluate inflammation, eyes were harvested 36 h after the last AC treatment, prefixed in 4% phosphate-buffered glutaraldehyde for 1 h, and then transferred to 10% phosphate-buffered formaldehyde. Fixed and dehydrated tissue was embedded in methacrylate, and 4- to 6-µm sections were stained with standard H&E. Cellular infiltrates within the entire vitreous of the DT- and PBS-injected eyes were enumerated from the histology sections in a masked fashion. In all systemic

and local depletion experiments, both DEREK and wild-type (WT) littermates received DT treatment.

**Statistics**

Statistics were performed using the unpaired or paired *t* test, as indicated. A two-tailed *p* value <0.05 was considered significant.

**Results**

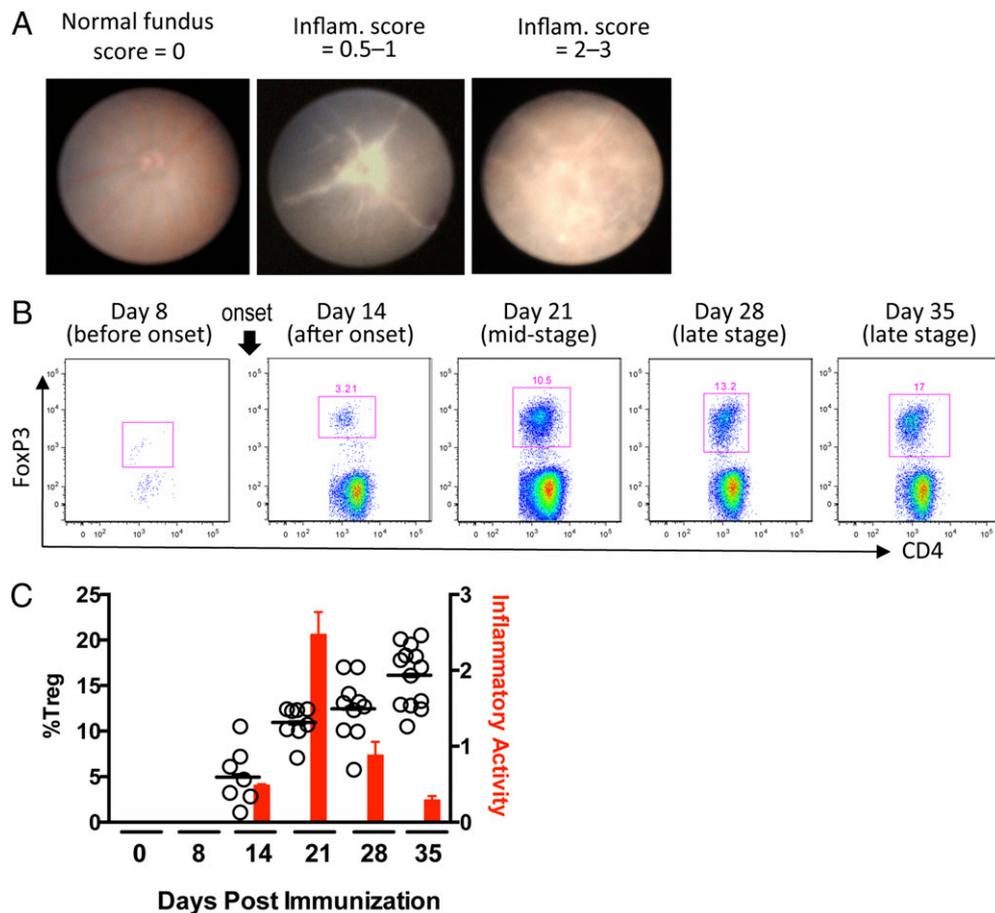
*Foxp3<sup>+</sup> Tregs accumulate in the eye during the course of EAU*

Immunization with retinal Ag gives rise to uveitogenic CD4<sup>+</sup> Teffs in the periphery that are capable of penetrating the blood retinal barrier, migrating into the eye, and inducing uveitis with the aid of other recruited leukocytes. Inflammatory activity in the eye is defined by the degree of cellular infiltrate. To determine whether Tregs are a component of the infiltrating population of cells, C57BL/6 mice were challenged for EAU, and the eye infiltrate was examined for viable CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs at weekly intervals. The inflammatory activity was monitored by fundus exams at the same time points. Fig. 1A shows examples of inflammation visible in the fundus. C57BL/6 mice were chosen for this experiment because their disease development progresses to peak activity in an incremental fashion in the first 3 wk after EAU challenge, in contrast to B10.RIII mice whose disease advances explosively, peaks early, and is accompanied by severe anterior inflammation that precludes fundus exams for 7–10 d after onset. Disease onset occurred about day 14 with inflammation peaking from days 19 to 21 and then rapidly resolving. The proportion of CD4<sup>+</sup>Foxp3<sup>+</sup>

Tregs increased progressively throughout the mid and late stages of disease (Fig. 1B) and correlated with resolution of inflammation (Fig. 1C). In addition, the transcription factor Helios, which has been associated with T cell activation (25, 26), was expressed in ≥80% of Foxp3<sup>+</sup> Tregs at all time points by intracellular staining (data not shown).

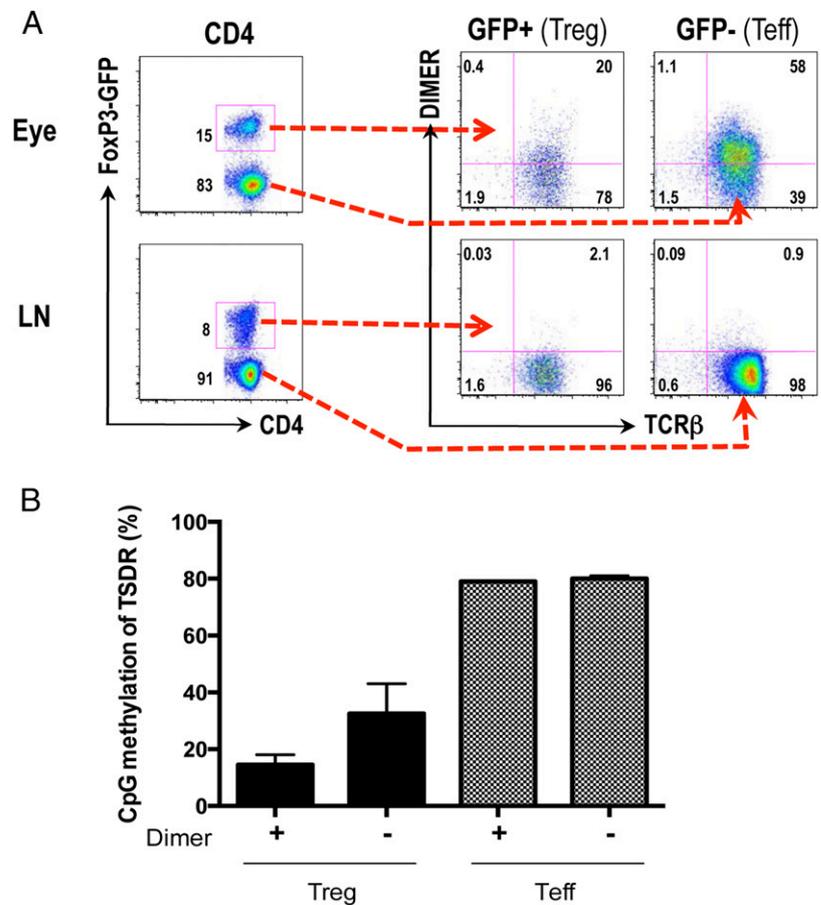
*IRBP-specific Tregs and Teffs are highly enriched in the inflamed eyes compared with LNs*

Because the expected frequency of IRBP-specific T cells in WT mice is initially very low, experiments were conducted to determine whether the T lymphocytes that accumulated in the uveitic eye and draining LNs were specific for IRBP. IRBP-specific cells were detected by flow cytometry after staining with labeled IRBP161–180 MHC class II dimer, which binds to cells bearing TCRs specific to IRBP (20). Ocular cells were harvested from Foxp3-GFP B10.RIII reporter mice 17 d postimmunization. IRBP-specific Tregs (GFP<sup>+</sup>) and Teffs (GFP<sup>-</sup>) were highly enriched in the inflamed eyes compared with the LNs that drain the immunization site (Fig. 2A). Notably, 20% of the total CD4<sup>+</sup>GFP<sup>+</sup> Treg population was specific for IRBP. Of the infiltrating GFP<sup>-</sup> population, 60% were IRBP specific. Furthermore, these numbers are likely to represent an underestimation, as T cells exposed to their cognate Ag (present in the eye) downregulate TCR expression (27, 28). In contrast, only low numbers of IRBP-specific Tregs were found in the peripheral LNs that drain the site of immunization,



**FIGURE 1.** Accumulation of Tregs in the eye during the course of EAU is associated with resolution of disease. C57BL/6 mice were challenged for EAU. At each time point, inflammatory activity was evaluated by fundus examination, and eye-infiltrating cells were collected after perfusion for flow cytometry. **(A)** Shown are examples of normal, mild, and severe inflammation in the fundus of EAU-challenged mice. **(B)** Percentage of viable CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in the pooled eyes of one representative mouse at each time point. **(C)** Bars: average inflammatory activity of mice at each time point. Circles: percentage of viable CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs. Each circle represents one mouse (both eyes averaged). Shown are results from two repeat experiments combined.

**FIGURE 2.** IRBP-specific Tregs are highly enriched in inflamed eyes and have a demethylated TSDR. B10.RIII Foxp3-GFP reporter mice were challenged for EAU. **(A)** Seventeen days postimmunization, cells from the eyes and draining peripheral LNs (draining the immunization site) were harvested and stained with CD4 and the IRBP161–180 MHC class II dimer and analyzed by flow cytometry. Shown is one representative experiment of five. **(B)** Seventeen days post-immunization, eye cells were stained and sorted for the designated populations. Methylation status of Foxp3 TSDR was assessed per *Materials and Methods*. Shown is a combination of two experiments.



and IRBP-specific effector cells in the periphery were below detection.

#### *The TSDR of IRBP-specific Tregs in eyes with uveitis is demethylated*

It has been reported that epigenetics plays a role in Foxp3 gene expression. The TSDR is an evolutionary conserved CpG-rich element in the Foxp3 locus. Previous studies reported that demethylation of this region is associated with stability of the Foxp3 phenotype. In contrast, this region in conventional T cells (Tconv) cells remains heavily methylated (29). We wanted to assess the phenotypic stability of the Tregs found in the uveitic eyes, in particular those that are IRBP specific. We therefore examined the methylation status of the TSDR region in sorted Foxp3<sup>+</sup> versus Foxp3<sup>-</sup> T cells contained within the IRBP-specific versus nonspecific CD4<sup>+</sup> T cell populations from the ocular inflammatory infiltrate of Foxp3-GFP B10.RIII reporter mice with EAU on day 17 after immunization.

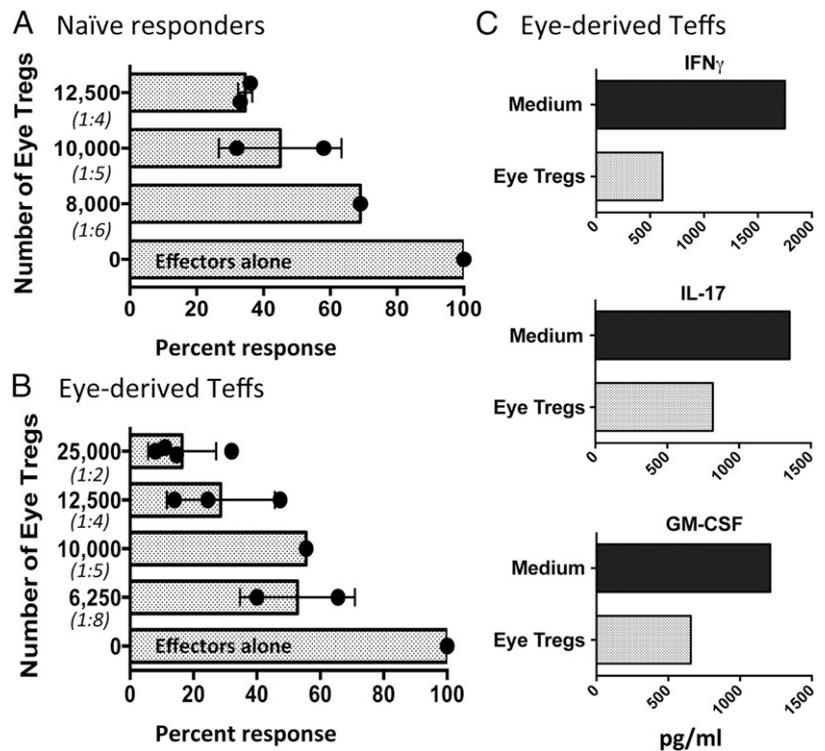
IRBP-specific Tregs (GFP<sup>+</sup>Dimer<sup>+</sup>) were demethylated (14.5%) indicative of a stable Treg phenotype (Fig. 2B). Tregs that were not IRBP specific (GFP<sup>+</sup>Dimer<sup>-</sup>) exhibited an intermediate methylation pattern (32.5%), suggesting that this population is a mixture of stable and unstable Tregs that may express Foxp3 transiently. Both IRBP-specific and nonspecific Teffs (GFP<sup>-</sup>Dimer<sup>+</sup> or GFP<sup>-</sup>Dimer<sup>-</sup>) were highly methylated (80%), a status characteristic of Tconv cells (Fig. 2B). These data suggest that, at peak disease, the polyclonal T cell infiltrate of the eye does contain an IRBP-specific population of Tregs with stable Foxp3 expression, indicative of a fully differentiated and stable regulatory phenotype.

#### *Tregs from inflamed eyes suppress IRBP-specific Teffs*

Considering that the methylation status of the IRBP-specific uveitic Tregs that are found in uveitic eyes implied a stable phenotype, and by inference, suppressive function, we next asked whether suppression by the eye-infiltrating Tregs could be demonstrated functionally. To maintain relevance to disease, which targets IRBP, we used an IRBP-specific suppression assay to examine the ability of eye-derived Tregs to inhibit naive cells and Teffs. Toward that end, Tregs sorted from eyes of uveitic Foxp3-GFP B10.RIII reporter mice were cocultured with naive IRBP-specific R161H T cells from Rag2<sup>-/-</sup> donors (18), or with CD4<sup>+</sup>Foxp3<sup>-</sup> T cells (Teffs) sorted from the same eyes, in the presence of APCs (irradiated splenocytes) and IRBP161–180 Ag. The eye-derived Tregs were able to inhibit proliferation of 50,000 responder R161H T cells (Fig. 3A) dose dependently, with 65% inhibition achieved at 1:4 Treg/responder ratio and progressively less inhibition at lower ratios. More importantly, Tregs from inflamed eyes of the Foxp3-GFP B10.RIII reporter mice suppressed the IRBP161–180-specific proliferation of 50,000 Teffs sorted from the same eyes (Fig. 3B). Again, a dose response was apparent in which better than 80 and 70% suppression was achieved at 1:2 and 1:4 Treg/Teff ratio, respectively. Lower numbers of eye Tregs were also inhibitory.

The proinflammatory cytokines IFN- $\gamma$  and IL-17 are produced in the eye by Th1 and Th17 effector cells, both of which infiltrate uveitic eyes and each of which constitutes a pathogenic effector cell type (30). Both of these cell types produce GM-CSF, which also has been shown to be a pathogenic cytokine in autoimmune disease (31, 32). We therefore measured the effect of eye-derived Tregs on IRBP-driven production of these three cytokines on

**FIGURE 3.** Tregs from inflamed eyes are functionally competent to inhibit activation and effector function of IRBP-specific T cells. Graded numbers of sorted CD4<sup>+</sup>GFP<sup>+</sup> Tregs from pooled eyes of EAU-challenged B10.RIII Foxp3-GFP reporter mice, harvested 17 d after immunization, were cultured with  $1 \times 10^5$  T-depleted APCs + 1.0  $\mu$ g/ml IRBP161–180 +  $5 \times 10^4$  sorted naive (CD62L<sup>high</sup>CD44<sup>low</sup>) R161H Rag2<sup>-/-</sup> responder cells (A), or CD4<sup>+</sup>GFP<sup>-</sup> Teffs sorted from eyes of the same EAU-challenged mice (B). Treg to T responder (Treg/Tresp) ratio is shown in parentheses. Proliferation was measured by [<sup>3</sup>H]thymidine uptake. Each circle is the result of one replicate at that cell dilution, and each bar represents the average  $\pm$  SEM for all the experiments. [Responder cell cpm range for (A): 12,495–25,492. Responder cell cpm range for (B): 10,505–20,230.] (C) Cytokine content in supernatants from cocultures described in (B) was measured by ELISA (Treg/Teff ratio = 1:5). Shown is one representative experiment of two.

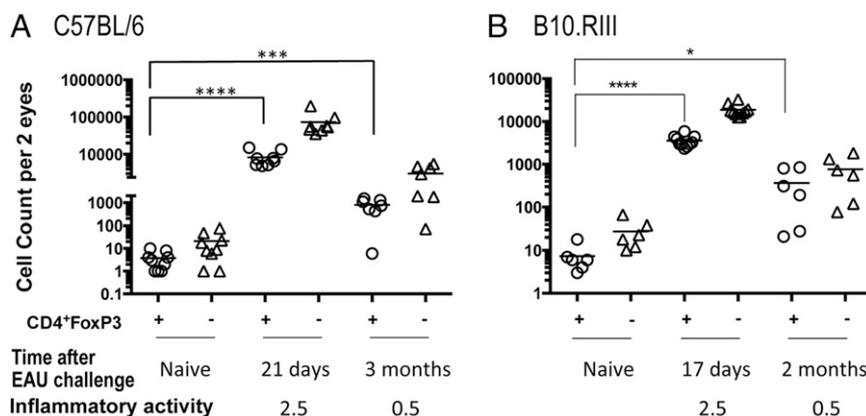


supernatants from eye-derived Teffs responding to Ag. Ten thousand eye-derived Tregs suppressed Ag-driven production of IFN- $\gamma$ , IL-17, and GM-CSF by 50,000 eye-derived Teffs (Fig. 3C). Taken together, these data demonstrate that the Tregs from uveitic eyes have suppressive ability and can inhibit both activation and effector function of IRBP-specific T cells.

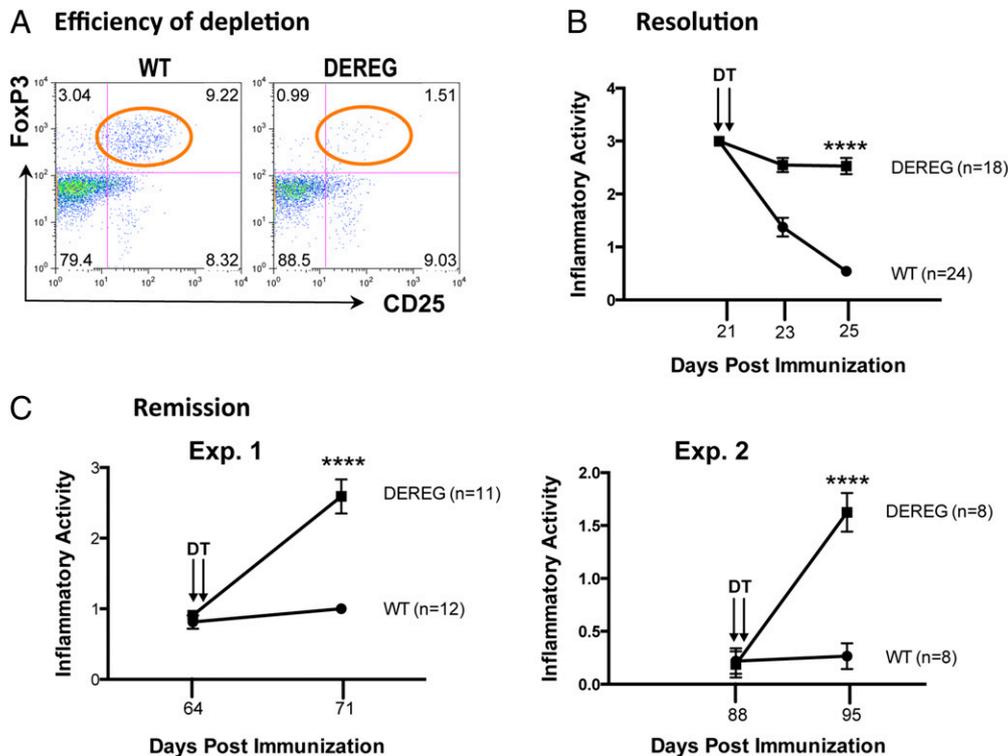
*Tregs continue to persist in postuveitic eyes after disease resolution*

We next asked whether Tregs continued to persist in eyes of C57BL/6 and B10.RIII mice after resolution has occurred. Fundus exams showed that the high inflammatory activity that was present in both strains at peak disease had subsided to trace activity in the early months after disease induction (Fig. 4). At each time point, both eyes of each mouse strain were pooled and the CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs and CD4<sup>+</sup>FoxP3<sup>-</sup> Teffs were enumerated by flow cytometry.

Counts were compared with those of age-matched naive controls. Although eyes were thoroughly perfused before harvesting, some cells may have adhered to the vasculature and may not have been flushed out. Therefore, the naive cell counts were considered as background and averaged fewer than 80 cells for each T cell population. Cell numbers in individual animals were highly variable. Nevertheless, whereas at the peak of disease the average of total CD4<sup>+</sup> infiltrating cells was in the tens of thousands per eye, with Tregs composing anywhere between 10 and 20% of the infiltrate, at 3 mo the average of total infiltrating cells had diminished by an order of magnitude and Treg frequency nearly doubled on average (Fig. 4, Supplemental Table I). These data demonstrate that Tregs as well as Tconv cells continue to be present locally for several months after the acute inflammation has resolved, with the progressive increase of Treg/Teff ratio observed during the earlier stages of disease (Fig. 1) continuing to show the same trend.



**FIGURE 4.** Foxp3<sup>+</sup> Tregs persist in eyes of post-EAU mice long after resolution. C57BL/6 and B10.RIII mice were challenged for EAU. At each time point, the fundus was examined for inflammatory activity, and then both eyes of each mouse were harvested after perfusion and pooled for cellular analysis. CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs (circle) and the CD4<sup>+</sup>FoxP3<sup>-</sup> Teffs (triangle) were enumerated by flow cytometry. Age-matched naive mice served to determine background counts. Number of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in challenged mice compared with background counts: (A) C57BL/6: 21 d postchallenge, \*\*\*\**p* < 0.0001; 3 mo postchallenge, \*\*\**p* < 0.0005. (B) B10.RIII: 17 d postchallenge, \*\*\*\**p* < 0.0001; 2 mo postchallenge, \**p* < 0.04. Statistical significance determined by unpaired *t* test. Cell counts for all mice are shown in Supplemental Table I.



**FIGURE 5.** Systemic depletion of Tregs at the peak of EAU, or after resolution, has functional consequences on disease. DERE mice and their WT littermates were challenged for EAU. The average inflammatory activity of all mice  $\pm$  SEM is graphed at each time point. **(A)** Systemic depletion efficiently depleted Tregs from the blood of DERE mice 48 h after DT administration. **(B)** Systemic depletion of Tregs at the peak of EAU delayed resolution. DT was administered i.p. on days 21 and 22 (unpaired *t* test: \*\*\*\**p* < 0.0001 for day 25). Shown is a combination of three experiments. **(C)** Systemic depletion of Tregs after EAU resolution reversed remission. DT was administered i.p. on days 64 and 65 (Exp. 1) or days 88 and 89 (Exp. 2) (unpaired *t* test: \*\*\*\**p* < 0.0001 for day 71 (Exp. 1) and day 95 (Exp. 2)).

#### *Depletion of Tregs at the peak of uveitis delays resolution, and depletion after resolution precipitates relapse of disease*

From the data described above, it was logical to hypothesize that Tregs play a role in remission of the disease. To examine this directly, we used mice expressing a DTR under the Foxp3 promoter (DEREG mice) (13, 14). Preliminary experiments established that systemic injection of DT depletes Tregs peripherally as well as in the eye (Fig. 5A, Supplemental Fig. 2). DERE mice and their WT littermates were challenged for EAU. An initial fundus examination to determine inflammatory activity was performed at peak disease, which in the C57BL/6 strain is between days 19 and 21. Afterward, the Tregs were depleted by i.p. administration of DT. Subsequent fundus exams showed that, whereas the eye inflammation in the WT mice resolved rapidly, as expected, the inflammation continued to remain active in the DERE mice whose Tregs had been depleted (Fig. 5B).

We next investigated whether Treg depletion after resolution of disease would precipitate a relapse. Both DERE and WT mice were immunized for EAU. Presence of full-blown uveitis was confirmed by fundus examination 3 wk later. The mice were then allowed to recover from disease until day 64 in one experiment, and until day 88 in the second experiment. The mice continued to maintain a very low-grade chronic inflammation throughout this time period. After DT was administered, an immediate relapse of inflammation was observed in the eyes of mice whose Tregs were depleted (Fig. 5C). These data suggest that Tregs play a role not only in the resolution of disease but also in the maintenance of remission.

#### *Treg activity to bring about and maintain resolution is in part due to local mechanisms*

Because systemic injection of DT depletes Tregs peripherally as well as in the eyes (Fig. 5A, Supplemental Fig. 2), it was therefore

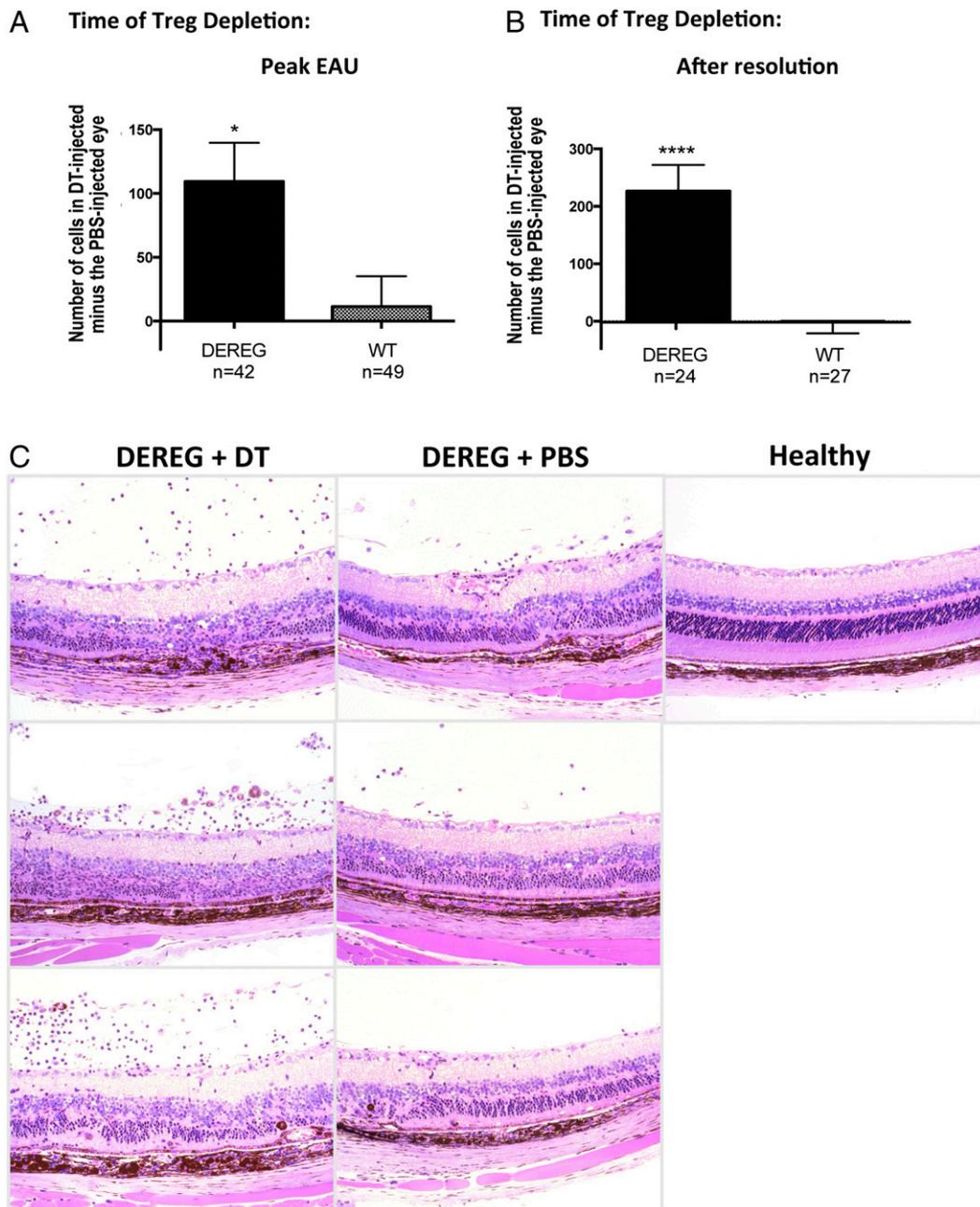
relevant to examine whether Tregs controlled disease activity at least in part by acting locally. We used the same experimental paradigm as in the systemic depletion experiments described above, except that DT was injected into the AC. This route was preferred to injecting into the vitreous to avoid the confounding effects of the microtrauma of injection at the site of inflammation. Small molecules can percolate from the AC into the back of the eye, although the efficiency of this process is difficult to estimate. DT was injected into one eye, and PBS was injected into the contralateral eye to control for the nonspecific proinflammatory effects of the AC injection. The functional efficacy of such AC injection has been reported (8). Each mouse received three DT injections during a 1-wk period. Eyes were collected for analysis 1 d after the third injection.

We first confirmed that this AC regimen of DT administration reduced the Treg population only in the eye, but had no effect on peripheral Treg numbers (Supplemental Fig. 2). By flow cytometry, the AC depletion protocol depleted  $\sim$ 50% of Tregs in inflamed eyes of DERE mice (Supplemental Fig. 2C). In WT mice with EAU, AC DT treatment had no effect (data not shown). Importantly, although Tregs were reduced by half in the DT-injected eye of DERE mice, no difference in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs was observed in the (eye-draining) submandibular lymph nodes of the same mice (Supplemental Fig. 2D). As well, there was no difference in percentage of Foxp3-GFP<sup>+</sup> Tregs in the blood of individual mice before and 36 h after DT treatment (Supplemental Fig. 2E).

Because AC injections interfered with the ability to perform optimal fundus examination, inflammatory activity in the back of the eye was evaluated by counting the number of infiltrating cells in histology sections in multiple fields in a blind fashion. For each

mouse, the total number of cells counted in the PBS-injected eye was subtracted from the total number of cells in the contralateral DT-injected eye. The differences for each mouse were averaged. It should be noted that the architectural damage is present in both the DT- and the PBS-injected eyes, as they have already undergone disease, and that even after resolution there is a residual infiltration in PBS-treated eyes, which does not completely disappear for many months. The intensity of inflammation can, however, be quantitated by counting the number of inflammatory infiltrating cells. There was a significantly higher retention of infiltrating cells in eyes in

which Tregs were depleted at the peak of EAU (Fig. 6A). When local Tregs were depleted from the eye after resolution, a significant increase in the number of infiltrating cells occurred (Fig. 6B, 6C). Supplemental Table II displays individual cell counts for all mice, emphasizing that, whereas in DERE mice there was a substantial increase in cells in the eye that received the DT injection, in eyes of WT mice the total number of cells in the two eyes was quite close. These data suggest that the effect of Tregs in bringing about and maintaining remission can at least in part be attributed to Tregs acting locally within the eye.



**FIGURE 6.** Local depletion of Tregs within the eye has functional consequences on disease. DERE mice and WT littermates were challenged for EAU. DT was injected into the AC of one eye, and PBS was injected into the contralateral eye, as described in *Materials and Methods*. Cellular infiltrates within the entire vitreous were counted from histology sections. The number of cells in the PBS-injected eye was subtracted from the number of cells in the DT-injected eye for the same mouse. Shown is the mean of all cell count differences for DERE and WT mice  $\pm$  SEM. **(A)** Local depletion of Tregs at the peak of EAU delayed resolution of inflammation. Eyes were treated with DT on days 19, 21, and 23 and harvested for histology on day 25. Shown is a combination of four experiments (unpaired *t* test: \**p* < 0.01). **(B)** Local depletion of Tregs after resolution induced a relapse of inflammation. DT treatment was started 10 wk after EAU challenge, and eyes were harvested for histology 1 wk later. Shown is a combination of two experiments (unpaired *t* test: \*\*\*\**p* < 0.0001). **(C)** Eye histology of DERE mouse. AC of one eye was injected with DT, and AC of the contralateral eye was injected with PBS. The pictures represent **(B)**.

## Discussion

The healthy eye has the ability to induce Tregs. This property has been postulated for a long time based on the ability of ocular fluids to induce Treg function in conventional T cells (6), and has recently been demonstrated directly to be an inherent property of the living eye by using mice that express a TCR recognizing an Ag present within the retina (7, 8). The question then arises: how can uveitis occur if the eye can convert conventional T cells to Tregs? Our previous data indicated that the ability of the eye to control already primed Teffs, such as are involved in inducing uveitis, is quite limited compared with its ability to control naive retina-specific T cells. Such T cells, which have been primed in the periphery and have acquired the ability to actively break down the blood-retinal barrier, cannot be converted to Tregs within the eye. Although recently evidence has been presented in favor of a tiny eye-resident population of Tregs that raise the threshold of susceptibility to uveitis (8), the activated Teffs can clearly overcome local inhibitory mechanisms and induce disease. Furthermore, the inflamed eye is less efficient in converting even naive T cells to Tregs (7). Therefore, we hypothesized that successful resolution of disease requires additional mechanisms, such as influx of Tregs from the periphery.

In keeping with the scenario that, for EAU to develop, the Teffs must be the first cells on the scene, the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> to CD4<sup>+</sup>Foxp3<sup>-</sup> cells among CD4<sup>+</sup> T cells in eyes with developing uveitis starts out low. However, that proportion kept rising throughout the disease process, and when Foxp3<sup>+</sup> T cells reached 10% of the total CD4<sup>+</sup> cells in the eye on day 21, acute inflammation rapidly resolved. The mechanisms that act to progressively change the Teff/Treg ratio in the uveitic eye are unclear and may involve additional cell types, such as myeloid-derived regulatory cells and regulatory B cells (33, 34). Nevertheless, the functional role of Tregs in the resolution process is upheld by failure of the disease to resolve during the period of observation if Tregs were depleted at peak disease by DT treatment. Conversely, depletion of Tregs after resolution precipitated a recurrence of inflammation. This led us to the conclusion that Foxp3<sup>+</sup> Tregs are instrumental in bringing about spontaneous resolution and in maintaining remission.

In contrast to findings in some other experimental and clinical autoimmune diseases, in which Tregs showed reduced ability to suppress Teffs from the site of inflammation, Tregs isolated from uveitic eyes were competent in suppressing Teffs from the same location in an Ag-specific suppression assay. The reason for this apparent discrepancy is not clear, but it has been reported that high levels of inflammatory cytokines, particularly TNF- $\alpha$  and possibly IL-6, can compromise Treg function and reduce their ability to suppress (9, 11, 12). In this context, it might be of note that uveitogenic Teffs isolated from the eye produced relatively little IL-6 and TNF- $\alpha$  (data not shown).

In interpreting these experiments, it has to be taken into account that the expression of the DTR on Foxp3<sup>+</sup> T cells in DEREG mice is "leaky" and there is a small population of Foxp3<sup>+</sup> cells that do not express DTR. These cells are not depleted. They undergo homeostatic expansion and repopulate the mouse, leaving ~1-wk window when functional consequences of Treg depletion can be reliably measured (14) (our unpublished data). This property of DEREG mice is both a limitation and an advantage. On the one hand, the "window of opportunity" in which biological effects can be observed is fairly short (but in our case, sufficient). In contrast, the mice remain healthy and do not develop an autoimmune disorder that can confound the situation, as does another strain of Foxp3-DTR mice, in which depletion of Foxp3<sup>+</sup> Tregs is permanent and the mice progress to spontaneous systemic autoimmunity (3).

Our data demonstrate a novel finding that a very high proportion of Foxp3<sup>+</sup> and an even higher proportion of Foxp3<sup>-</sup> CD4<sup>+</sup> T cells in uveitic eyes of WT mice with a normal polyclonal repertoire are IRBP specific. In contrast, outside the eye, including the eye-draining submandibular lymph nodes, the frequency of IRBP-specific dimer-binding cells was negligible. This could imply that IRBP-specific T cells are either retained in the eye, or that they actively proliferate there. We and others have previously presented data indicating that retinal Ag recognition by uveitogenic T cells occurs *in situ* (and in fact is a prerequisite for elicitation of EAU) (35, 36). Furthermore, naive IRBP-specific R161H T cells (but not polyclonal T cells) injected into the eye proliferate and dilute a T cell proliferation dye as they acquire Foxp3 expression and acquire an Ag-experienced phenotype (7). This supports the notion that Ag recognition in the eye is accompanied by specific functional consequences, both for effector and for regulatory cells. The finding that local depletion of Tregs within the eye promptly reignites EAU suggests that at least some of the postrecovery homeostasis occurs within the eye and involves an active control of Teffs by Tregs that may be present there.

To examine this hypothesis directly, we attempted to deplete Tregs locally within the eye, without affecting the peripheral Tregs, by injecting DT into the AC using a method described by Lehmann et al. (24). Although AC administration of DT indeed had no demonstrable effects outside the eye, it did not deplete Tregs within the eye as effectively as did systemic DT administration. After three consecutive DT injections over the course of 1 wk, only 50% of Tregs within the eye appeared to have been depleted. It is unknown whether the amount of DT reaching the vitreous from the AC was insufficient to deplete all the Tregs, or whether they were rapidly replaced in the eye from the periphery, as has been proposed by a recent study (8). Nevertheless, the changes in inflammatory activity within the eye after local Treg depletion paralleled those seen after systemic administration. This supports the notion that the ameliorating effect of Tregs on uveitis was, at least in part, being exerted locally within the eye. Extrapolating these findings to human uveitis, which may relapse due to mechanisms that remain obscure, it is conceivable that one such mechanism could be the systemic or local failure of Tregs.

In summary, the present data identify Foxp3<sup>+</sup> Tregs as an essential regulatory cell type in ocular autoimmunity. Although presence of a resident Foxp3<sup>+</sup> Treg population in the healthy eye has been suggested, any protective effects of such Tregs are clearly overcome by retina-specific Teffs that had been activated in the periphery and can enter the eye actively through the blood-retinal barrier. Thus, although Tregs are unable to prevent uveitis, they are nevertheless instrumental in bringing about resolution of the disease and in maintaining a state of remission. This appears to be achieved, at least in part, by Tregs acting locally within the eye to dampen acute inflammation and prevent its recurrence.

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## Disclosures

The authors have no financial conflicts of interest.

## References

- Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
- Piccirillo, C. A., and E. M. Shevach. 2004. Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin. Immunol.* 16: 81–88.
- Kim, J. M., J. P. Rasmussen, and A. Y. Rudensky. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8: 191–197.
- Buckner, J. H. 2010. Mechanisms of impaired regulation by CD4(+)/CD25(+) FOXP3(+) regulatory T cells in human autoimmune diseases. *Nat. Rev. Immunol.* 10: 849–859.
- Sawant, D. V., and D. A. Vignali. 2014. Once a Treg, always a Treg? *Immunol. Rev.* 259: 173–191.
- Streilein, J. W. 2003. Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation. *J. Leukoc. Biol.* 74: 179–185.
- Zhou, R., R. Horai, P. B. Silver, M. J. Mattapallil, C. R. Zárate-Bladés, W. P. Chong, J. Chen, R. C. Rigden, R. Villasmil, and R. R. Caspi. 2012. The living eye “disarms” uncommitted autoreactive T cells by converting them to Foxp3(+) regulatory cells following local antigen recognition. *J. Immunol.* 188: 1742–1750.
- McPherson, S. W., N. D. Heuss, and D. S. Gregerson. 2013. Local “on-demand” generation and function of antigen-specific Foxp3+ regulatory T cells. *J. Immunol.* 190: 4971–4981.
- Ehrenstein, M. R., J. G. Evans, A. Singh, S. Moore, G. Warnes, D. A. Isenberg, and C. Mauri. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF $\alpha$  therapy. *J. Exp. Med.* 200: 277–285.
- You, S., M. Belghith, S. Cobbold, M. A. Alyanakian, C. Gouarin, S. Barriot, C. Garcia, H. Waldmann, J. F. Bach, and L. Chatenoud. 2005. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes* 54: 1415–1422.
- Korn, T., J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T. R. Petersen, B. T. Bäckström, R. A. Sobel, K. W. Wucherpfennig, T. B. Strom, et al. 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat. Med.* 13: 423–431.
- Valencia, X., G. Stephens, R. Goldbach-Mansky, M. Wilson, E. M. Shevach, and P. E. Lipsky. 2006. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood* 108: 253–261.
- Lahl, K., C. Lodenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, and T. Sparwasser. 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 204: 57–63.
- Lahl, K., and T. Sparwasser. 2011. In vivo depletion of FoxP3+ Tregs using the DEREK mouse model. *Methods Mol. Biol.* 707: 157–172.
- Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.
- Darce, J., D. Rudra, L. Li, J. Nishio, D. Cipolletta, A. Y. Rudensky, D. Mathis, and C. Benoist. 2012. An N-terminal mutation of the Foxp3 transcription factor alleviates arthritis but exacerbates diabetes. *Immunity* 36: 731–741.
- Bettini, M. L., F. Pan, M. Bettini, D. Finkelstein, J. E. Rehg, S. Floess, B. D. Bell, S. F. Ziegler, J. Huehn, D. M. Pardoll, and D. A. Vignali. 2012. Loss of epigenetic modification driven by the Foxp3 transcription factor leads to regulatory T cell insufficiency. *Immunity* 36: 717–730.
- Horai, R., P. B. Silver, J. Chen, R. K. Agarwal, W. P. Chong, Y. Jittayasothorn, M. J. Mattapallil, S. Nguyen, K. Natarajan, R. Villasmil, et al. 2013. Breakdown of immune privilege and spontaneous autoimmunity in mice expressing a transgenic T cell receptor specific for a retinal autoantigen. *J. Autoimmun.* 44: 21–33.
- Pepperberg, D. R., T. L. Okajima, H. Ripps, G. J. Chader, and B. Wiggert. 1991. Functional properties of interphotoreceptor retinoid-binding protein. *Photochem. Photobiol.* 54: 1057–1060.
- Karabekian, Z., S. D. Lytton, P. B. Silver, Y. V. Sergeev, J. P. Schneck, and R. R. Caspi. 2005. Antigen/MHC class II/Ig dimers for study of uveitogenic T cells: IRBP p161-180 presented by both IA and IE molecules. *Invest. Ophthalmol. Vis. Sci.* 46: 3769–3776.
- Agarwal, R. K., P. B. Silver, and R. R. Caspi. 2012. Rodent models of experimental autoimmune uveitis. *Methods Mol. Biol.* 900: 443–469.
- Caspi, R. R. 2003. Experimental autoimmune uveoretinitis in the rat and mouse. *Curr. Protoc. Immunol.* Chapter 15: Unit 15.6.
- Kim, Y. C., R. Bhairavabhotla, J. Yoon, A. Golding, A. M. Thornton, D. Q. Tran, and E. M. Shevach. 2012. Oligodeoxynucleotides stabilize Helios-expressing Foxp3+ human T regulatory cells during in vitro expansion. *Blood* 119: 2810–2818.
- Lehmann, U., N. D. Heuss, S. W. McPherson, H. Roehrich, and D. S. Gregerson. 2010. Dendritic cells are early responders to retinal injury. *Neurobiol. Dis.* 40: 177–184.
- Gottschalk, R. A., E. Corse, and J. P. Allison. 2012. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J. Immunol.* 188: 976–980.
- Akimova, T., U. H. Beier, L. Wang, M. H. Levine, and W. W. Hancock. 2011. Helios expression is a marker of T cell activation and proliferation. *PLoS One* 6: e24226.
- Cai, Z., H. Kishimoto, A. Brunmark, M. R. Jackson, P. A. Peterson, and J. Sprent. 1997. Requirements for peptide-induced T cell receptor downregulation on naive CD8+ T cells. *J. Exp. Med.* 185: 641–651.
- Niedergang, F., A. Dautry-Varsat, and A. Alcover. 1997. Peptide antigen or superantigen-induced down-regulation of TCRs involves both stimulated and unstimulated receptors. *J. Immunol.* 159: 1703–1710.
- Polansky, J. K., K. Kretschmer, J. Freyer, S. Floess, A. Garbe, U. Baron, S. Olek, A. Hamann, H. von Boehmer, and J. Huehn. 2008. DNA methylation controls Foxp3 gene expression. *Eur. J. Immunol.* 38: 1654–1663.
- Luger, D., P. B. Silver, J. Tang, D. Cua, Z. Chen, Y. Iwakura, E. P. Bowman, N. M. Sgambellone, C. C. Chan, and R. R. Caspi. 2008. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J. Exp. Med.* 205: 799–810.
- El-Behi, M., B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G. X. Zhang, B. N. Dittel, and A. Rostami. 2011. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat. Immunol.* 12: 568–575.
- Codarri, L., G. Gyölvézi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, and B. Becher. 2011. ROR $\gamma$ t drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat. Immunol.* 12: 560–567.
- London, A., I. Benhar, M. J. Mattapallil, M. Mack, R. R. Caspi, and M. Schwartz. 2013. Functional macrophage heterogeneity in a mouse model of autoimmune central nervous system pathology. *J. Immunol.* 190: 3570–3578.
- Wang, R. X., C. R. Yu, I. M. Dambuzza, R. M. Mahdi, M. B. Dolinska, Y. V. Sergeev, P. T. Wingfield, S. H. Kim, and C. E. Egwuagu. 2014. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat. Med.* 20: 633–641.
- Prendergast, R. A., C. E. Iliff, N. M. Coskuncan, R. R. Caspi, G. Sartani, T. K. Tarrant, G. A. Luty, and D. S. McLeod. 1998. T cell traffic and the inflammatory response in experimental autoimmune uveoretinitis. *Invest. Ophthalmol. Vis. Sci.* 39: 754–762.
- Thurau, S. R., T. R. Mempel, A. Flügel, M. Diedrichs-Möhrling, F. Krombach, N. Kawakami, and G. Wildner. 2004. The fate of autoreactive, GFP+ T cells in rat models of uveitis analyzed by intravital fluorescence microscopy and FACS. *Int. Immunol.* 16: 1573–1582.

## Corrections

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