The Living Eye "Disarms" Uncommitted Autoreactive T Cells by Converting Them to Foxp3+ Regulatory Cells following Local Antigen Recognition


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Immune privilege was once thought to be the property of a few select sites that include the eye, testis, the pregnant uterus, and (of all things) the hamster cheek pouch, and it was believed to be based mainly on sequestration behind blood–tissue barriers. This view has changed over the years. Immune privilege is now considered a general phenomenon through which many tissues are able to actively direct and control immune responses to preserve their physical and functional integrity in the face of inflammatory processes (1, 2). Not only the eye and testis, but also the brain, the liver, and mucosal sites, including the gut, lung, and female reproductive tract, are examples of organs that have recently been intensely studied in this regard (3–8).

The eye, perhaps more than any other tissue, must control local expression of immunity. Vision is a very strong evolutionary selective pressure, and to sustain it, multiple mechanisms have evolved to regulate immune responses affecting the eye. The healthy eye is sequestered behind an efficient blood–retina barrier (BRB) and has a virtual absence of lymphatic drainage and a profoundly immunosuppressive ocular microenvironment (3, 9).

Under some circumstances, the eye can also elicit systemic regulatory circuits known as anterior chamber-associated immune deviation (ACAI) (10). These safeguards are necessary because the very sequestration of the eye from the immune system impedes peripheral tolerance to retinal Ags (11), allowing persistence in the circulation of nontolerant retina-specific T cells, which can gain entry into the eye passively (as a result of trauma and bleeding into the eye) or actively (following a priming event in the periphery).

Experimental autoimmune uveitis (EAU), elicited in mice by retina-specific T cells, is a model for human autoimmune uveitis, which is often accompanied by responses of patient lymphocytes to retinal Ags. Uveitis has an incidence and prevalence similar to multiple sclerosis and is thought to be responsible for 10–15% of blindness in the United States (12). Adoptive transfer experiments in laboratory rodents revealed that infiltration of as few as 10 activated retina-specific uveitogenic T cells into a healthy eye is sufficient to start the inflammatory process leading to EAU (11). It has been an open question why immune privilege, which protects the eye efficiently from day-to-day minor inflammatory insults and traumas and is thought to underlie the extraordinary success of retinal (allo) grafts, which enjoy close to 90% acceptance at the 1-year mark without any tissue matching (13), is unable to prevent onset of uveitis.

Local induction of regulatory T cells (Tregs) by the eye as a manifestation of immune privilege has been a topic of much interest and even more debate. There is a considerable body of data showing that ocular fluids and ocular resident cells can inhibit activation of T cells in culture and can even induce them to become Tregs (14–19). However, although systemic induction in the spleen of Tregs as part of the eye-driven ACAID phenomenon is based on in vivo findings (10), the notion that Tregs can be induced locally within the eye has been based entirely on in vitro
data that were never critically examined in vivo, because the tools for this have simply not been available.

In the current study, we used newly developed retina-specific TCR transgenic (Tg) mice and stringent experimental paradigms to demonstrate for the first time, to our knowledge, that the living eye efficiently converts naive retina-specific T cells to Foxp3+ Tregs. Recognition of retinal Ag is required for this process. This indicates that T cell priming can occur locally within the tissue and can effectively “disarm” uncommitted T cells with the potential to cause pathology. Despite the presence of TGF-β in ocular fluids, conversion of T cells to Tregs in vivo requires retinoic acid (RA), which is normally present in the eye due to its function in the visual cycle. Thus, RA in the living eye plays a dual role: in vision and in immune privilege. Notably, Ag-experienced T cells appear to be resistant to the immunoregulatory effects of the ocular microenvironment; additionally, Treg conversion of uncommitted cells is hindered in inflamed eyes. These findings may explain why uveitis can be induced by retina-specific T cells activated in the periphery, in the face of ocular immune privilege.

**Materials and Methods**

**Mice**

The following five mouse strains on B10.RIII background were used in this study: Foxp3-GFP reporter mice on an otherwise conventional background, used as donors of polygonal T cells; interphotoreceptor retinoid-binding protein (IRBP) TCR Tg mice on a conventional background, used as cell donors; IRBP TCR Tg mice on a Rag2–/– background, used as cell donors; and CD90.1 congenics, otherwise on a conventional background, used as recipients of cells from the first three strains; and IRBP+/- mice, used as cell recipients in Fig. 2B. C57BL/6 wild-type (WT) mice were used only to analyze tissue expression of retinaldehyde dehydrogenase (RALDH) enzymes (Fig. 4A, 4B).

B10.RIII Foxp3-GFP reporter mice were produced by backcrossing Foxp3-GFP reporter mice on 129×C57BL/6 mixed background (Dr. A. Rudensky, Sloan-Kettering Cancer Center, New York, NY) (20) onto the B10.RIII background. IRBP TCR Tg Foxp3-GFP reporter mice on conventional B10.RIII or Rag2–/– B10.RIII background were bred in-house, as were IRBP+/- B10.RIII (21) and CD90.1-congenic B10.RIII mice. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions, fed standard laboratory chow ad libitum, and used at 6–10 wk of age. Treatment of animals was in compliance with Institutional Guidelines, and all animal study protocols were approved by the National Eye Institute Institutional Animal Care and Use Committee, Animal Study Protocol No. NEI-581.

**CD4+ T cell isolation and analysis**

T cells were enriched from pooled splenocytes and lymph nodes by using T cell columns (R&D Systems). From these, CD4+GFP T cells were sorted on FACS Aria (Becton Dickinson, San Jose, CA) (99% purity). Abs used for cell sorting and flow cytometry analysis were all from eBioscience (San Diego, CA).

**Immunofluorescent staining and confocal microscopy**

Eye sections (10 μm) were fixed in 4% paraformaldehyde, and blocked with normal goat serum. The sections were stained with a polyclonal Ab against RALDHs (detecting all isoforms ALDH1a1, ALDH1a2, ALDH1a3; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, followed by incubation for 30 min in Alexa Fluor 568-conjugated secondary Ab (Invitrogen-Molecular Probes) and DAPI (1 μg/ml; Invitrogen-Molecular Probes).

**In vivo Treg conversion**

CD4+GFP T cells were purified from lymph nodes and spleens of IRBP TCR Tg mice expressing a Foxp3-GFP reporter gene. Donor mice were on a Rag2–/– background where specified; otherwise, mice on a conventional background were used when not required by the experiment for reasons of availability (~30% TCR Tg T cells) (R. Horai, unpublished observations) (Figs. 1D, 2B, 4C, 5). About 0.5 million cells were injected through the pars plana into the posterior chamber of each eye of CD90.1 congenic recipients. Eyes that sustained unintentional damage to the lens or other structures as a result of the injection were excluded from analysis. To block RA signaling, CD4+GFP T cells were pretreated with LE540 (50 μM or 100 nM) for 1 h before injection. On the indicated days after cell injection, recipient eyes were harvested and dispersed into single-cell suspension, as described (22).

**Immunization of mice for uveitis**

To prepare WT recipients with uveitis or to prepare primed cells from IRBP TCR Tg (Rag2–/–) donors, mice were immunized with 10 μg IRBP161–180 peptide emulsified in CFA (Difco, Detroit, MI) that had been supplemented with Mycobacterium tuberculosis strain H37RA to 2.5 mg/ml, as described (23). The WT mice received an intraocular cell transfer 2 wk after immunization, after confirmation of disease by fundus examination (23), or their eyes were collected on day 8 for isolation and analysis of infiltrating T cells.

**Treg-suppression assay**

CD4+Foxp3+ T cells isolated from IRBP TCR Tg Foxp3-GFP reporter mice were injected into eyes of CD90.1 congenic recipients. After 8 d, Foxp3+ or Foxp3+ CD4+CD90.2+ donor T cells were sorted out (~2,000–4,000 cells/well) and cocultured with sorted naive CD4+CD44hi CD62L+hi responder cells from IRBP TCR Tg mice (Rag2–/–; 50,000/well). Cells were stimulated with 1 μg/ml IRBP161–180 peptide in the presence of irradiated T cell-depleted spleen cells (100,000/well) for 72 h. Proliferation was determined by [3H]thymidine incorporation and scintillation counting.

**Donor cell proliferation within the eye**

CD4+Foxp3+ T cells isolated from IRBP TCR Tg Foxp3-GFP reporter mice were labeled with eFluor 670 (5 μM; eBioscience) and injected into the eyes of naive congenic recipients or naive recipients fed a vitamin A (VitA)-deficient diet or VitA control diet. On day 4, recipient eyes were harvested, dispersed into single-cell suspension, and stained for CD4, CD90.2. Propidium iodide-excluding (live) cells were analyzed for Foxp3 expression and eFluor 670 dilution in CD4+CD90.2+ cells by flow cytometry.

**Cytokine production by donor cells**

CD4+Foxp3+ T cells from IRBP TCR Tg Foxp3-GFP reporter mice (Rag2–/–) background were injected into eyes of CD90.1 congenic recipients. Six days after injection, cells from recipient eyes were isolated and stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (GolgiPlug; BD Pharmingen, San Diego, CA). After 4 h, cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% BSA and 0.05% Triton X-100, and stained with PerCP-Cy5.5-conjugated anti-CD4, PE-conjugated CD90.2, allophycocyanin-conjugated anti-IFN-γ, or allophycocyanin-conjugated anti–IL-17. Up to 500,000 events were acquired using BD CellQuest software and analyzed using FlowJo software (TreeStar, Ashland, OR). As a positive control, Foxp3-GFP reporter mice were actively immunized for EAU, as described above. On day 16 after immunization (7 d after onset), inflamed eyes were prepared for single cells, stained with PMA and ionomycin, and analyzed for cytokine production.

**Real-time PCR for cytokine mRNA expression by nonconverted cells**

CD4+GFP+ T cells from IRBP TCR Tg Foxp3-GFP reporter mice were injected into eyes of CD90.1 congenic recipients. Eight days after injection, cells from recipient eyes were isolated, and donor CD4+CD90.2+ GFP+ cells were purified by sorting and subjected to real-time PCR. As positive control, Foxp3-GFP reporter mice were actively immunized for EAU, as described above. On day 17 after immunization (8 d after onset), inflamed eyes were prepared for single cells, stained with PMA and ionomycin, and analyzed for cytokine production.

**Induction of VitA deficiency**

VitA-deficient mice were produced, as described (24). Briefly, pregnant dams were placed on a diet lacking VitA (cat. #TD.09838; Harlan, Madison, WI) as soon as pregnancy could be detected (~14 day of gestation).
Control mice were fed the same diet supplemented with 20,000 IU VitA/kg (cat. #TD.09839; Harlan). Pups were maintained on the same diet after weaning. Mice were used after 9 wk of age.

Statistics and experimental reproducibility

Values are presented as mean ± SD, where indicated. Statistical differences were calculated with an unpaired Student t test, two-tailed (GraphPad Prism version 5.0b). Statistical significance was set at \( p \leq 0.05 \). Experiments were repeated at least two, and usually more, times. Groups contained three to seven mice, depending on the experiment (usually four to five). Results were highly reproducible.

Results

Retina-specific conventional T cells convert to Foxp3+ Tregs in the living eye

Naive, as well as Ag-experienced, T cells can gain entry into the eye as a result of trauma and bleeding into the eye or during inflammation after the BRB is disrupted. Thus, their ability to convert to Tregs in vivo is highly relevant to maintenance of ocular homeostasis. To examine this issue, we used T cells from Foxp3-GFP reporter mice, which also express a Tg TCR specific for a retinal protein (IRBP) (R. Horai, unpublished observations). IRBP is an abundant protein in the retina whose role is to bind and transport retinoids within the interphotoreceptor space, and it serves as an autoimmune target in uveitis (23).

The experimental approach is depicted in Fig. 1A. CD4+GFP− cells (non-Tregs) were purified from IRBP TCR Tg Foxp3-GFP RAG2−/− CD90.2+ donors or from control Foxp3-GFP non-Tg donors, and ~0.5 million cells were injected into each eye of CD90.1 congenic recipients. GFP-Foxp3 expression in donor-derived (CD90.2+) cells became detectable on day 4 after injection; by day 8, up to one third of donor cells expressed Foxp3 (Fig. 1B). Very few donor T cells could be detected during this period.
of time in the eye-draining submandibular lymph node, spleen, or pooled peripheral lymph nodes distant from the eye (Fig. 1C), supporting the notion that conversion to Foxp3 positivity occurred locally within the eye, without involving recirculation of the cells through the periphery.

Although T cell expression of Foxp3 is practically synonymous with regulatory function in mice, we wished to confirm that the converted T cells are indeed suppressive. To examine this, we injected IRBP TCR Tg Foxp3-GFP$^+$ T cells into eyes of CD90.1 congenic recipient mice, as depicted in Fig. 1A. After 8 d, the GFP$^+$ and the GFP$^-$ donor-derived cells were sorted from these eyes and used in an Ag-specific suppression assay with fresh IRBP TCR Tg target T cells. In two repeat experiments, 2,000 and 4,000 sorted GFP$^+$ T cells (all that could be obtained from 18 and 29 eyes, respectively) suppressed 50,000 responder T cells in an Ag-specific assay, whereas the GFP$^-$ donor cells that had not been converted did not suppress (Fig. 1D). Notably, 50% and 57% suppression was achieved at 1:25 and 1:12.5 Treg/responder

**FIGURE 2.** Conversion requires local Ag recognition and is accompanied by proliferation. A. Eyes of CD90.1 congenic recipients were injected with CD4$^+$GFP$^+$ T cells from IRBP TCR Tg (Rag2$^{-/-}$) or non-TCR Tg Foxp3-GFP reporter donors and were analyzed on day 8 after injection. Representative experiment of three is shown. B. CD4$^+$GFP$^+$ T cells sorted from IRBP TCR Tg Foxp3-GFP reporter mice were injected into eyes of naive CD90.1 congenic recipients who were either WT ($n = 4$ eyes) or IRBP$^{-/-}$ ($n = 8$ eyes). On day 7 after cell injection, eyes were analyzed for Foxp3 expression on donor cells. Representative experiment of three is shown. C. Conversion in vivo involves proliferation. Donor cells were labeled with eFluor 670 and were injected into the eyes of naive recipients. On day 4, Foxp3 expression and eFluor 670 dilution in CD4$^+$CD90.2$^+$ cells were analyzed by flow cytometry. Data are representative of at least three independent experiments.

**FIGURE 3.** Nonconverted cells are primed but are restricted from expressing effector function in the eye. CD4$^+$GFP$^+$ T cells from naive IRBP TCR Tg Foxp3-GFP reporter mice (Rag2$^{-/-}$) were injected into eyes of CD90.1 congenic recipients. A. Donor cells injected into the eye express activation markers. Seven days after injection, cells from the recipient eyes were analyzed for CD44 versus CD62L expression on donor cells by flow cytometry. Starting population contained $<1\%$ CD44$^{hi}$CD62L$^{lo}$ cells. Shown is one representative of three independent experiments. B. Eye-injected donor T cells express low effector function compared with T cells from eyes with actively induced EAU. Seven days after injection, cells isolated from the recipient eyes were pulsed ex vivo with PMA/ionomycin for 4 h in the presence of brefeldin A. Cells stained intracellularly were analyzed for IFN-$\gamma$ and IL-17 expression in Foxp3$^-$ and Foxp3$^+$ populations. Cells from eyes of GFP-Foxp3 reporter mice (non-TCR Tg), in which EAU was actively induced by IRBP161–180 immunization, were similarly analyzed 7 d after disease onset. Shown are representative plots of four (eye injected) and three (EAU) repetitions. C. Nonconverted GFP$^+$ IRBP TCR Tg donor T cells were purified by sorting 8 d after injection into eyes and were analyzed by real-time PCR for expression of pro- and anti-inflammatory cytokines (white columns) compared with CD4$^+$ GFP$^-$ cells sorted from eyes of WT mice 8 d after onset of EAU induced by active immunization (black columns). Cytokine mRNA expression is normalized to freshly isolated WT naive CD4$^+$Foxp3$^-$ cells (gray columns).
ratios, respectively, indicating that the in vivo-converted cells were highly functional suppressors.

Conversion requires local Ag recognition and is accompanied by proliferation

We wished to examine whether in situ Ag recognition was required for conversion. CD4+GFP− cells from IRBP TCR Tg Foxp3-GFP or WT Foxp3-GFP reporter mice were injected into eyes of CD90.1 congenic recipients, and conversion was examined 7–8 d later. As before, IRBP TCR Tg T cells efficiently converted to Tregs in the eye, whereas polyclonal CD4+ T cells completely failed to convert (Fig. 2A). Polyclonal cells did not convert even if they were incubated with anti-CD3/CD28 before injection into the eye (data not shown), indicating that a sustained Ag stimulation is required. Similarly, there was no conversion in eyes of IRBP−/− recipient mice, which lack the target Ag (21, 25) (Fig. 2B).

We next asked whether the Ag-dependent Foxp3 conversion within the eye involved proliferation. Our recent study in which we examined conversion of naive T cells to Tregs in vitro by aqueous humor (AH) demonstrated that although proliferation took place, it was not necessary for conversion (19). To address this question in vivo, GFP− donor cells labeled with the proliferation dye eFluor 670 were injected into eyes of healthy recipients. GFP expression versus proliferation dye dilution was examined on day 4 after injection. In IRBP TCR Tg cells, Foxp3-GFP expression was detected only in cells that had undergone several rounds of division (Fig. 2C). Polyclonal cells again did not convert and did not proliferate.

T cells that had not been converted are primed but do not express effector function in the eye

In all of the experiments described thus far, it stands out that only a part of the donor cells became converted to Tregs. Therefore, we were curious about the functional status of the nonconverted cells. To address this, converted and nonconverted CD90.2+ IRBP TCR Tg donor cells from eyes of CD90.1 recipients were examined a week after injection for the expression of activation markers and for the ability to produce uveitis-relevant cytokines IFN-γ and IL-17 ex vivo (Fig. 3). Most nonconverted (as well as converted) cells expressed CD44 and were CD62L−, both parameters indicating that they had been primed (Fig. 3A). Nevertheless, expression of IFN-γ and IL-17 by these cells was consistently lower than in Foxp3+CD4+ T cells isolated from eyes of mice with actively induced EAU approximately a week after onset (Fig. 3B). At the

FIGURE 4. RA is required for optimal Treg conversion in vivo. A, Immunofluorescence staining for RALDHs in the healthy eye. Eyes from C57BL/6 mice were stained with a polyclonal Ab against all RALDH isoforms (red color, arrows) or with DAPI nuclear stain (blue) (original magnification ×25). B, Expression of RALDHs (Aldh1a1, Aldh1a2, and Aldh1a3) in the eye compared with spleen and liver of C57BL/6 mice by quantitative real-time PCR. Target gene expression was normalized to its own GAPDH expression and then standardized to expression in a spleen (set as 1). Each symbol represents an individual mouse. A representative experiment of three is shown. C, Donor CD4+GFP− cells from IRBP TCR Tg Foxp3-GFP reporter mice were pretreated with LE540 (50 μM or 100 nM) for 1 h before injection. Foxp3 expression in CD4+CD90.2+ cells was examined 7 d later. Shown is a representative experiment of five. D, Reduced Treg conversion in mice fed a VitA-deficient diet. CD4+GFP− T cells from IRBP TCR Tg mice were injected into the eyes of naive congenic recipients fed a regular diet, VitA control diet, or VitA-deficient diet. On day 7 after cell injection, eyes were analyzed for Foxp3 expression on donor cells. IRBP TCR Tg donors were on a conventional background. Shown is one representative experiment of three with three to seven mice per group. **p ≤ 0.01. E, Donor cell proliferation in eyes of VitA-deficient versus control mice. Donor CD4+GFP− cells from IRBP TCR Tg Foxp3-GFP reporter mice were labeled with eFluor 670 and injected into the eyes of naive recipients fed a VitA control diet or a VitA-deficient diet. On day 4, Foxp3 expression and eFluor 670 dilution in CD4+CD90.2+ cells were analyzed by flow cytometry. Shown is a representative experiment of two.
transcriptional level, nonconverted donor cells expressed, across
the board, less message for proinflammatory cytokines (IFN-γ,
IL-17, GM-CSF, and TNF-α) and more message for anti-inflam-
matory cytokines (IL-10 and TGF-β1) (Fig. 3C). These results
suggest that IRBP-specific cells that had not been converted to
Foxp3 positivity are restricted from expressing effector function in
the eye. This conclusion is borne out by fundoscopic examination
of the injected eyes, which showed retinal architecture with only
minor changes even 3 wk after IRBP TCR Tg cell injection, a time
when mice actively immunized for uveitis are already past the
peak of their disease.

RA is required for conversion of T cells to Foxp3+ Tregs in vivo
Numerous studies in vitro and in vivo have substantiated the role of
TGF-β in conversion of T cells to Tregs (26). The eye contains
high levels of TGF-β, mostly as TGF-β2 and mostly in latent form
(9, 27). The eye also contains high levels of RA, because RA and
its derivatives function in the visual cycle (28). Conversion of
the VitA metabolite, retinal, into RA is accomplished by the en-
zyme RALDH, which is present in three isoforms: ALDH1a1, ALDH1a2, ALDH1a3 (29). By immunohistochemical staining,
RALDH activity was present in the retina and cornea, and all three
isoforms were detected in ocular tissues by PCR (Fig. 4
b,4)

In a recent study, we showed that both TGF-β and RA in AH
contribute to Treg conversion by ocular fluids in vitro (19). To
explore whether RA was needed for the Ag-specific Foxp3 con-
version in the more complex ocular environment, in vivo (28). Conversion of
The data described above are compatible with the interpretation
that a retina-specific T cell that enters the eye and is exposed to its
cognate Ag there may be induced to adopt a Treg fate and/or is
inhibited from expressing effector function. If so, the question
immediately arises: How can uveitis develop? Because uveo-
egenic T cells are primed in the periphery before they encounter the
ocular microenvironment, we decided to examine the effect of the
ocular microenvironment on Ag-experienced, as opposed to Ag-
naive, T cells. Naive (CD44lowCD62Lhigh) cells were sorted
from lymph nodes and spleens of unmanipulated IRBP TCR Tg
RAG2−/− Foxp3-GFP reporter mice. Primed (CD44highCD62Llow)
T cells were similarly sorted from spleens of donors that had been
immunized with IRBP161–180 10–14 d earlier. Both populations
of cells were then injected into eyes of CD90.1 congenic recipi-
ents; 6 d later, eyes were examined by fundoscopic examination
and collected for cell analysis. Although the naive population
efficiently converted to Foxp3 positivity, as expected, the in vivo-
primed IRBP-specific T cells failed to convert to Foxp3 positivity
(Fig. 6A, 6B) and, instead, induced severe inflammation in the
recipients’ eyes. In contrast, eyes injected with naive cells retained
a largely normal appearance of the retina, even 3 wk after cell
injection. Fig. 6C shows illustrative examples of the clinical and
histopathological appearance of eyes at the observed scores of
inflammation. These results provide an in vivo correlate to our
recent finding that effector/memory T cells resist conversion into
Tregs by ocular fluids (19) and demonstrate that this has func-
tional consequences that are directly relevant to disease.

Taken together, the data suggest that T cells specific to retinal
Ag, with the potential to cause uveitis, can be “disarmed” upon
entry into the ocular microenvironment by conversion to a Foxp3+
Treg phenotype and/or inhibition of their function. However, in
contrast to uncommitted cells, previously primed cells are resis-
tant to the immunosuppressive and Treg-inducing effects of the
ocular microenvironment. They retain their effector function and
can participate actively in the induction of uveitis.

Discussion
To our knowledge, the present study provides the first in vivo
evidence that the local ocular microenvironment of the eye can
“disarm” uncommitted T cells with the potential to cause pa-
thology by converting them to Foxp3+ Tregs and/or by dampening
their expression of effector function. Importantly, because conver-
sion of naive T cells to Foxp3+ Treg phenotype requires Ag
recognition and priming (32, 33), our data support the interpre-
tation that naive T cells that gain access to the tissue can be
primed in situ. This finding contrasts with the prevailing notion
that naive T cells are preferentially primed in lymph nodes. Al-
though some would argue that naive T cells are normally excluded from healthy tissues, minor or even major internal bleeding, as a consequence of a fall or contusion, can and does occur. However, in most tissues, in situ priming cannot be easily studied, because cell migration cannot be excluded as a factor. As a relatively closed compartment of small dimensions, the eye is uniquely suited to addressing this question. Our data suggest that if and when naive cells do gain access to the tissue, under some conditions they may indeed be primed there.

We considered the possibility that the converted T cells were, in fact, primed in the eye-draining lymph node. Although we cannot completely exclude that a few injected T cells may leave the eye, become converted in the lymph nodes, and come back, exceedingly few donor-derived Foxp3+ cells are detectable in the eye-draining submandibular lymph node or other peripheral lymphoid organs (Fig. 1C), and they do not express Foxp3. Furthermore, T cells that leave the eye would have to recirculate through the body to return to the eye. Experimental data indicate that an activated retina-specific T cell in the peripheral circulation has a 0.000015 chance of finding its way to the uninflamed eye (11, 34). Finally, our recent in vitro data show that Tregs converted by AH of the eye are initially unstable and lose Foxp3 expression if removed from the AH-containing environment (19). Thus, for all of these reasons, exit from the eye and re-entry as a prerequisite for conversion seem very unlikely. It would be of considerable interest to identify the APCs in the eye that participate in the priming and conversion process, but this is a massive undertaking in itself, which is beyond the scope of the current study. That said, a study by Heuss et al., performed in parallel with the present work and reported in the preceding issue of The Journal of Immunology, identified a population of CD11c+ dendritic cells in the retina that are likely to be involved in this process (35).

The biological relevance of Treg conversion within the eye hinges on the Ag-exposure history of the infiltrating T cell. It is accepted that naive T cells do not cross blood vessels to enter into tissues. However, naive T cells can, and do, gain entry into the eye passively. As stated above, bleeding into the eye can be induced by trauma or by vascular abnormalities accompanying conditions such as macular degeneration, diabetic retinopathy, and retinopathy of prematurity (36). In addition, naive T cells also gain entry into the eye under conditions.
of inflammation. During spontaneous EAU in IRBP TCR Tg mice, up to 10% of IRBP161–180-specific T cells that infiltrate inflamed eyes are naive by CD44 and CD62L expression (R. Horai, unpublished). This is in line with reports of other investigators (37), who showed that naive T cells are actively recruited into various chronically inflamed tissues through CCL21 expressed by endothelial cells. Our data show that conversion of T cells to Tregs is severely impaired in the inflammatory environment; therefore, it is possible that rather than being “disarmed,” naive T cells entering the eye during inflammation may become effector cells and, at least initially, actively contribute to disease. If that is the case, Tregs recruited into the eye from the periphery may be critical to tip the balance and (together with local conversion, however limited) help bring about resolution of the disease.

It is notable that, despite evidence of having been primed, the nonconverted donor T cells within the eye appear to be restricted from expressing effector function and are not precipitating acute uveitis. Nonconverted donor T cells might in part be kept in check by the converted Foxp3+ Tregs, which appear to be extremely potent suppressors. Furthermore, increased TGF-β1 and IL-10 message in the nonconverted population after 8 d in the eye implies that they themselves could contain some Tregs that do not express Foxp3. However, because both time and proliferation are required for Tregs to be primed and differentiated, it is likely that additional inhibitory influences in the eye might contribute directly to dampening their effector potential. Ocular fluids contain TGF-β as well as neuropeptides, such as α-melanocyte-stimulating hormone, vasoactive intestinal peptide, calcitonin gene-related peptide, and somatostatin (38). The ability of ocular resident cells, including retinal glial Müller cells, choroidal cells, and various pigment epithelial cells, to inhibit T cell activation and function in culture has been known for some time (14, 17, 39). Both Müller cells and retinal pigment epithelial (RPE) cells in the back of the eye are reported to suppress effector function of primed T cells in culture (14, 40). However, only coculture with RPE cells was reported to convert T cells to Tregs, partly through contact and culture (14, 40). Nevertheless, it was not a given that RA would be needed in the eye in vivo, for at least two reasons. First, in the in vitro study, the AH had been acid treated by necessity, in latent form, because as a result of its profibrotic effects, TGF-β activation must be tightly controlled (44). Second, it is conceivable that the presence of other potential Treg-inducing factors in the eye (i.e., inhibitory neuropeptides and ocular resident cells) (14–19) might replace the need for RA. Although we were not able to tease out the relative contribution of ocular resident cells versus ocular fluids and their complex components to the conversion process in vivo, our data demonstrate that RA is a required component for optimal Treg conversion in the living eye. We hypothesize that, because the amount of bioactive TGF-β in the eye is limiting, the role of RA takes on particular importance. Thus, RA functions in visual signal transduction, as well as in maintaining immune homeostasis within the eye.

An issue that has perplexed the field for many years is why does immune privilege, which efficiently protects the eye from consequences of day-to-day minor insults and traumas and is believed to underlie the extraordinary success of corneal (allo)grafts (3, 13), apparently fall short of preventing an autoimmune attack on the eye. Our current data may help to explain this conundrum. First, we demonstrate that, in contrast to uncommitted cells, Ag-experienced T cells are resistant to conversion in the ocular microenvironment and instead induce uveitis. Although accidental or surgical disruption of a blood vessel within the eye would be expected to mostly bring naive (easily converted) T cells into the eye, uveitis is elicited by effector T cells that had been activated outside of the eye, which then acquire the ability to penetrate the BRB and start the inflammatory process (34). Second, de novo conversion of uncommitted T cells to Tregs is hindered in eyes that are already inflamed, further compounding the situation. Impaired conversion to Tregs may be attributed, at least in part, to profound changes in the composition of ocular fluids during uveitis. During uveitis there is a reduction in inhibitory mediators, such as TGF-β, and likely other small-molecule components, including RA, as a result of loss of the BRB (45). At the same time, the presence of inflammatory mediators, including IFN-γ, IL-1, and IL-6, would tend to tip the balance to Th1 and Th17, rather than to Treg differentiation (45, 46).

Our findings shed new light on the phenomenon of immune privilege and on its role, as well as its limitations, in actively controlling inflammatory responses within the tissue and maintaining immune homeostasis.

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Disclosures
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References


