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Peripheral Induction of Tolerance by Retinal Antigen Expression

Dale S. Gregerson, Neal D. Heuss, Ute Lehmann, and Scott W. McPherson

The contribution of peripheral expression of tissue-specific CNS Ags to the generation of tolerance is uncertain. To study this question, we examined mice transgenic (Tg) for expression of β-galactosidase (βgal) on the retinal photoreceptor cell arrestin promoter, in conjunction with TCR Tg mice producing CD4+ T cells specific for βgal (βgalTCR). Several strategies were used to test the hypothesis that βgal expressed in the retina supported thymus-independent tolerance and regulatory T cell development. Retinal expression generated an immunoregulatory response that depressed development of immune responses to βgal following systemic immunization with βgal. This regulation was transferable to naive mice by CD3+ 450 T cells from naive retinal βgal+ donors. Experiments that removed the βgal+ retina by enucleation showed that subsequent development of a regulatory response was lost. Adoptive transfer of CD25+ βgalTCR T cells into retinal βgal Tg mice on the Rag2−/− background led to regulatory activity that limited lymphopenia-induced proliferation of βgalTCR T cells in mice with retinal expression of βgal and inhibited the ear-swelling assay for delayed type hypersensitivity. These results show that retinal expression of very small amounts of a tissue-specific Ag can generate tolerance that includes regulatory T cells. The Journal of Immunology, 2009, 183: 0000–0000.

Immunologic self-tolerance is required for an effective immune system and is provided by the concerted activities of central and peripheral tolerance. A growing body of evidence describes mechanisms underlying the generation and activity of regulatory T cells (Tregs) that bear the CD4+25+Foxp3+ phenotype (1, 2). These Ag-specific Tregs play an important role in tolerance to tissue-specific self-Ags (TSA) (3). Expression of TSA in thymus, including aire gene-directed expression of TSA in medullary thymic epithelial cells, leads to negative selection of T cells specific for TSA (4, 5) and to positive selection of Tregs specific for TSA (6), providing protection from autoimmune disease. Although the development of Tregs in young mice is largely thymus dependent (7), CD4+25+ Tregs redevelop spontaneously several months following thymectomy (8, 9). It has also been demonstrated that CD4+25+ Tregs can develop from mature, peripheral CD4+ T cells in vivo in response to exogenous Ag administered by i.v. or oral routes (10, 11).

Experimental autoimmune uveoretinitis (EAU) is a retinal autoimmune disease mediated by CD4 (12) or CD8 (13) T cells directed to retinal Ags, including interphotoreceptor retinoid binding protein (IRBP). Through use of IRBP-deficient and wild-type (wt) mice, thymic expression of IRBP was shown to provide central tolerance to IRBP, through negative selection (14), and generation of CD25+ Tregs (15). Aire-deficient mice developed autoimmune retinitis that was dependent on retinal IRBP expression; mice deficient in aire and IRBP did not develop retinal inflammation, but other organs maintained targets of autoimmune disease (16). Because thymic expression of IRBP was not required to generate Tregs that protected from retinal inflammation (15), it is possible that Tregs with specificity for other retinal TSA could contribute to protection from retinal autoimmunity.

We propose that Tregs result from contact with retinal Ags in the periphery, contributing to the generation of tolerance, separate from the contribution of thymic expression. Using Escherichia coli β-galactosidase (βgal) transgenic (Tg) mice to achieve Ag expression from the arrestin promoter in retinal photoreceptor cells, we found spontaneous immunoregulation that altered the immune response to βgal (17). Although analysis of retinal βgal Tg mice has not revealed detectable levels of βgal in thymus, whether by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining, RT-PCR, or evidence of thymic selection, very low levels could contribute to thymic generation of Tregs. The present results show that intracellular expression of Ag in neurons (photoreceptor cells) in normal, quasi-senescence retina led to peripheral generation of Tregs that could be attributed to retinal-derived Ag.

Materials and Methods

Mice

βgal-expressing Tg mice have been described elsewhere (17). βgal expression in rod photoreceptor cells of arrβgal mice produces 150 ng of βgal retina and <0.5 ng/pineal gland. GFAPβgal mice express βgal in CNS astrocytes (175 ng/brain). βgal expression in adult ROSA26 mice was low but widespread. TCR Tg mice carrying an αβ-TCR conferring specificity for a class II MHC-restricted response to a βgal peptide were described previously (βgalTCR mice (18)). All βgal Tg mice and Rag2−/− mice were backcrossed onto the B10.A background (Charles River Laboratories). Mice were housed under specific pathogen-free conditions on lactose-free chow and handled in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research, and University of Minnesota animal use and care guidelines.
Ags and immunization

βgal was purchased from Prozyme. A class II-restricted immunodominant epitope of βgal (YVVDEANIETHGMV) was prepared by the Microchemical Facility at the University of Minnesota. Some mice received a s.c. injection on a hind thigh with 50–200 μg of βgal in CFA containing 1 mg of killed Mycobacterium tuberculosis H37Ra (Sigma-Aldrich). Others were inoculated i.p. with 1.25–5 × 10^6 PFU/mouse of live, recombinant vaccinia virus (VSC 56) expressing βgal under control of the synthetic early–late promoter (19). VSC 56 was obtained from Dr. J. Yewdell (National Institute of Allergy and Infectious Diseases/National Institutes of Health, Bethesda, MD).

T cells and T cell culture

Naive CD4 T cells specific for βgal were isolated from βgal TCR Tg mice. Cultures were done with or without βgal peptide (1–10 μM), APC, and βgal TCR T cells as indicated, in RPMI 1640 with 10% FCS. Cytokines in culture supernatants were determined by cytometric bead array. Abs and cytokine standards were purchased from BD Biosciences and eBioscience, R&D Systems, and Millipore.

Positive and negative selection of cells

Isolation of CD25+ (clone 7D4), CD4+, CD8+, or Thyl.2+ cells or depletion of these populations was done using the MACS system (Miltenyi Biotec). Cell suspensions were incubated with Abs conjugated with paramagnetic beads and applied to MS+, LS+, or LD columns according to the manufacturer’s protocols. For transfer, cells were washed and resuspended to 5 × 10^6 cells/ml in saline. Recipient mice received up to 5 × 10^6 cells in 0.1 ml by i.v. injection. Larger numbers or volumes of cells were given by i.p. injection as indicated.

Ear test measurement of delayed-type hypersensitivity (DTH)

The difference in ear thickness due to a DTH response to Ag was measured with a micrometer (Mitutoyo), before and after Ag inoculation. A 30-gauge needle was used to give an intrapinna injection of 50–100 μg of βgal in 10 μl of saline.

Enucleations

To perform bilateral enucleations to remove all retina-associated Ag, mice were anesthetized with xylazine/ketamine HCl. The lids were retracted to proptose the eye. The globes were grasped with curved forceps, and scissors were used to do a blunt dissection, followed by cutting the optic nerve to free the eye. Pressure was applied with a cotton swab to control bleeding. The lids were closed with a single gut suture, and antibiotic ointment was applied. Buprenorphine was used postoperatively.

RT-PCR method

mRNA was prepared using μMACS mRNA isolation kit (Miltenyi Biotec) and DNase I (Ambion) per manufacturer’s protocol. One to 5 μl of mRNA was reverse transcribed in a 20-μl reaction containing 100 ng of reverse primer for the indicated gene (see below) and 10 U of RNase inhibitor (Ambion) with dNTPs (0.5 mM final), reaction buffer (1× final), and reverse transcriptase (4 μl of final) from Omniscript RT kit (Qiagen). The reverse transcription reaction was incubated at 37°C for 60 min and terminated at 93°C for 7 min. One microliter of the reverse transcription reaction was then amplified by PCR using 100 ng of the forward and reverse primers, 1 mM MgCl2, 0.2 mM dNTPs, and 1× reaction buffer plus 1.25 U of HotStarTaq DNA polymerase (Qiagen) in a final volume of 50 μl. The PCR were subjected to an initial denaturation at 95°C for 15 min followed by 40 cycles of 92°C for 30 s, 60°C for 45 s, 72°C for 60 s, and then a final extension at 72°C for 7 min. The RT-PCR products were analyzed by Gel Star (Lonza)-stained agarose gels. The forward and reverse primers were as follows: rod photoreceptor cell arrestin, 5′-CTCTCAGTCTCTCCCTCCAGCAAAGAC-3′ and 5′-CTCACCTGGGTATTACGAC-3′; acidic ribosomal binding protein, 5′-AACACGATGTAGCAACCAGTTTGG-3′ and 5′-CTGCAACTGCTCATCACC-3′; and β-actin, 5′-GGTGGGCCCCGGCTTAGC-3′ and 5′-CTTCTTTGATGTACCCAGCAGTATTTC-3′.

Results

Evidence for tolerance in retinal Ag Tg mice

We previously reported that expression of βgal in retinal rod photoreceptor cells led to spontaneous tolerance to βgal, but the tolerance was overwhelmed by immunization with βgal in CFA containing a high dose of M. tuberculosis. H37Ra and concurrent i.v. pertussis toxin, as required to induce EAU (17). Given this sensitivity to a highly aggressive immunization protocol, we asked if the regulatory activity was relevant to physiological challenges. Mice vaccinated with live VSC 56 virus showed that DTH to βgal in convalescent arrβgal mice was inhibited (Fig. 1A). The GFAPβgal and βgal+ROSA26 control mice also demonstrated reduced DTH following viral challenge. Control virus lacking βgal expression induced no evidence of immunity or tolerance to βgal (data not shown). No autoimmune disease was seen in βgal+ tissues of βgal Tg mice vaccinated with virus. Ag-specific inhibition in arrβgal mice was shown by use of BSA in CFA immunization and ear-swelling assays to BSA in control and arrβgal mice (Fig. 1B).

Tolerance was transferred by splenocytes (SPL) and acted on the afferent response to Ag

Adaptive transfer of immunoregulation induced by peripheral, intracocular inoculation of exogenous Ag has been reported to inhibit

FIGURE 1. Transgenic expression of βgal inhibited the immune response to βgal elicited by infection with VSC 56 (A) or by immunization with Ag in CFA (B and C). A, Analysis of the DTH response to βgal. Mice were ear tested with purified βgal 21 days postinfection. B, Analysis of the specificity of tolerance in arrβgal mice. Mice were immunized with BSA/CFA, and the DTH response to BSA was assayed 21 days later. C, The regulatory activity of arrβgal mice acted on the afferent response in vivo. Recipient mice were immunized with βgal, and 30 × 10^6 spleen cells from control B10.A or arrβgal donor mice were transferred i.p. as indicated. DTH was analyzed by ear test 7 days postimmunization. All results are given as the mean ± SD. n = 5–7 mice/group. *p < 0.01 (t test) for arrβgal vs B10.A mice.
established immune responses to retinal Ags in immunized recipients (20). To test naïve arrβgal mice for transferable activity, SPL from naïve, B10.A, and arrβgal mice were transferred into βgal-negative recipients. After 1 day, the recipients were immunized with βgal in CFA and ear tested 7 days later. SPL from arrβgal donors, but not wt donors, depressed the DTH response (Fig. 1C). However, the regulatory activity was only effective if transferred before Ag priming.

The tolerogenic activity of arrβgal mice was transferred by CD3^+4^+25^- T cells

If inhibition of DTH in arrβgal mice was mediated by Tregs, depletion of CD3^+^ cells should abrogate the transfer of inhibition of βgal DTH. CD3-depleted or unseparated SPL (Fig. 2A) were transferred, followed by infection with VSC 56, and ear testing (Fig. 2B). B10.A mice that received CD3-depleted arrβgal SPL had full-scale ear swelling, relative to controls, showing that splenic CD3 cells from arrβgal mice transferred inhibition of DTH. To determine whether the inhibitory activity was carried by CD4^+^ or CD8^+^ T cells, these T cell populations from arrβgal mice were prepared as described in Materials and Methods (Fig. 2C). The CD4^+^ population from arrβgal mice clearly inhibited the expression of DTH to βgal after VSC56 vaccination, but the CD8^+^ population did not (Fig. 2D). The association of inhibitory activity with CD3^+^4^+^ cells suggested the activity of CD4^+^25^-^ Tregs. SPL and lymph node (LN) cells from arrβgal mice were enriched for CD3^+^4^+^ cells as above and separated into CD25^-^ and CD25^+^ fractions (Fig. 2E). Recipient B10.A mice were vaccinated and ear tested after 26 days. Whereas unseparated cells transferred inhibition of ear-swelling, CD25-depleted cells did not (Fig. 2F).

Removal of the retina alters tolerance in arrβgal mice

The thymus produces CD4^+^25^-^Foxp3^+^ Tregs to many self-Ags, as shown by day 3 thymectomy (8), Foxp3 deficiency (21), and aire deficiency (5, 6). Thymic expression of βgal was not detected in arrβgal mice by RT-PCR (Fig. 3A). Conversely, arrestin mRNA was detected in retina and thymus (Fig. 3B). The control ROSA26 mice expressed readily detectable βgal mRNA in thymus and retina. The results suggested that the recombinant arrestin promoter construct in the arrβgal Tg mice had reduced activity in the thymus, raising the possibility that retinal βgal expression could contribute to the observed tolerance.

Approximately 99.9% of βgal in arrβgal mice is expressed in the retina; the remainder is expressed in the pineal gland. Therefore, enucleation may test the role of retinal Ag expression in generation of tolerance. B10.A and arrβgal mice were enucleated and irradiated with 800 Gy 2 days later to ablate existing T cells. Control mice were either irradiated only or unmanipulated. Five months later, the mice were immunized with βgal in CFA and ear tested. Enucleated arrβgal mice had reduced ability to inhibit DTH elicited by ear-testing βgal-immunized mice (Fig. 4A). Since the B10.A mice. A total of 7 × 10^6^ CD4^+^ or CD8^+^ T cells or 30 × 10^6^ unseparated cells were transferred to normal B10.A mice. E and F, Transfers of CD25-depleted cells failed to inhibit the βgal ear-swelling response. E, FACS analysis of spleen/LN cells depleted of CD25^-^ Different clones of anti-CD25 were used for depletion (PC-61) or detection (7D4). CD25^-^ cells (50 × 10^6^) or unseparated cells (30 × 10^6^) were transferred. F, CD25^-^ cells did not inhibit ear swelling. For all assays, the cells were transferred to B10.A mice on day 0, and mice were immunized on day 1 by vaccination with VSC 56 and ear tested on day 26–28 with βgal. Values of p (t test) for comparisons with vaccinated B10.A control groups are given. Error bars indicate SD.
Functional Tregs can be peripherally induced in βgal and arrestin mRNA in the normal background, carry the arrestin and extraocular expression of βgal, was inhibited after recovery from enucleation and irradiation (Fig. 4).

The tolerogenic activity of arrβgal SPL acts on naive βgalTCR T cells in vitro

Since the regulatory activity acted on afferent responses in vivo, it was tested on naive βgalTCR T cells in vitro. Approximately 60% of mature T cells, and 80–90% of CD4+ T cells in these mice on the normal background, carry the αβ-TCR conferring specificity for a class II MHC-restricted epitope of βgal (18). The effect of arrβgal SPL on the T cell response to Ag was assessed by secretion of IFN-γ in vitro. Enriched, naive βgalTCR T cells were obtained by positive selection for CD4+ SPL and LN cells from βgalTCR mice and cultured with and without Ag in the presence of nonirradiated arrβgal or normal B10.A SPL. Nonirradiated arrβgal SPL inhibited Ag-induced IFN-γ production by naive βgalTCR T cells (Fig. 5) but had less effect on production of IL-2, IL-4, IL-10, or TNF-α (data not shown).

Functional Tregs can be peripherally induced in βgalTCR T cells

Oral, i.v., and i.p. administration of Ags has been reported to induce CD4+25+ Tregs in the periphery (10, 22, 23). As proof of principle that peripheral βgal can generate Tregs, the i.v. route to generation of βgal-specific CD4+25+ Tregs in normal B10.A mice was tested (Fig. 6). Ten days after i.v. inoculation of 200 μg of soluble βgal, SPL and LN cells were pooled, and CD4+25+ T cells were isolated. Regulatory activity was assessed by testing cocultures for effects on Ag-dependent cytokine production by purified CD4+25+ naive βgalTCR T cells. IL-2 and IFN-γ production by βgalTCR T cells was inhibited by addition of CD4+25+ cells from mice pretreated with βgal, but there was little effect of adding CD4+25+ cells from untreated B10.A mice (Fig. 6D). Although elevated levels of TGF-β1 were found in cocultures of βgal-induced Tregs and βgalTCR T cells, Tregs from either donor did not make significant amounts when cultured separately and made no detectable IL-2, IL-10, or IFN-γ.

Tolerance is found in arrβgal × βgalTCR double Tg mice on the Rag−/− background

We previously showed that arrβgal × βgalTCR double Tg mice spontaneously developed CD25+ Tregs that inhibited DTH to βgal

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Examination of thymus and retina for expression of βgal and arrestin mRNA in βgal Tg and control B10.A mice. A. RT-PCR for βgal mRNA in samples from thymus and retina. B. Detection of endogenous arrestin mRNA in thymus and retina from B10.A and arrβgal mice. ARBP, acidic ribosomal binding protein.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Retinal βgal expression contributed to the production of tolerance. A. Arrβgal and control mice were enucleated and/or irradiated to eliminate the retina as a source of Ag and to deplete existing Tregs. Five months later, all mice were immunized with βgal, and the DTH response was analyzed by ear testing with βgal 7 days postimmunization. B. ROSA26 and control mice were similarly treated and then ear tested. Results are given as the mean ± SD, and p values (t test) are indicated.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Spleen cells from arrβgal mice inhibited the in vitro Ag response of naive βgalTCR T cells. A total of 5 × 10^6 nonirradiated SPL from arrβgal mice or control B10.A mice were cultured with/without 1 × 10^5 βgalTCR CD4+ T cells and 50 μg/ml βgal as indicated. Supernatants were collected at 72 h after Ag stimulation and assayed by ELISA. The results are given as the mean ± SD, and p values (t test) are indicated.
A reduced frequency of T cells with a regulatory phenotype developed in unstimulated cultures was at back-
ground levels and is not shown.

Results are given as the mean ± SD of duplicate assays (three wells per sample in each assay) of 72-h supernatants. BD = below detection. Cytokine produc-
tion in unstimulated cultures was at back-
ground levels and is not shown.

FIGURE 6. Induction and analysis of CD4⁺ Tregs induced by periph-
eral administration of βgal in B10.A mice. A–C, FACS analysis of CD4⁺ (A), CD4⁺ (B), and Thy-1-depleted cells (C) for use in the functional assays. The cells in A and B were depleted with anti-CD19, -CD11b, -CD11c, and -CD8 followed for selection for CD4⁺ T cells. D, Ag-stimulated cyto-
kine production by naïve CD4⁺ βgalTCR T cells in coculture with popu-
lations of CD4⁺ T cells from normal B10.A mice or βgal-inoculated B10.A mice. Irradiated, Thy1-depleted C57BL/6 mice were depleted with T cell-depleted T cells as a

factor and regulatory T cells depending on whether they were gen-
erated in wt or arrβgal mice, respectively.

CD25⁺Foxp3⁺ T cells are found in wt and Tg mice

It has been reported that generation of CD4⁺ Tregs in TCR Tg mice on the Rag⁻/⁻ background requires positive thy-
mic selection by cognate Ag (25). In the case of TSA, this is
thought to be dependent on the aire gene (26, 27). CD4⁺ T cells were poorly selected, at least numerically, in arrβgal × βgalTCR double Tg mice on the Rag⁻/⁻ background (Fig. 7B). The results suggest an Ag source other than thymus,
because there was no evidence of enhanced positive selection of CD25⁺ Foxp3⁺ cells in the arrβgal mice, unlike the βgalTCR × ROSA26 double Tg mice, which exhibit thymic expression and produced an elevated number of Tregs. No discrete population of CD25⁺ Foxp3⁺ cells was found in thymus from either the βgalTCR × Rag⁻/⁻ mice or the arrβgal × βgalTCR × Rag⁻/⁻ mice (data not shown).

Rag⁻/⁻ mice reveal a peripheral contribution of retinal Ag to tolerance in mature T cells

The peripheral induction of a regulatory response was further tested by adoptive transfer of purified, mature, peripheral CD25⁺ βgalTCR T cells into Rag⁻/⁻ recipients that cannot thymically generate mature CD4⁺ Tregs (28, 29). Backcrossing the arrβgal mice onto the Rag⁻/⁻ background provided βgal⁻ retina as the peripheral source of specific Ag for peripheral generation

and/or maintenance of Tregs from the transferred T cell popu-
lations. Analysis of CD25⁺ βgalTCR donor cells before inoculation showed that contamination by Foxp3⁺ CD25⁺ double-positive cells was reduced to 0.5% from a starting point of 10⁻¹⁵% (Fig. 8A). Several populations of CD25⁺ T cells were also prepared for transfer and contained ~75% Foxp3⁺ CD25⁺ double-positive cells (Fig. 8A).

FIGURE 7. CD25⁺ Tregs were generated endogenously in arrβgal × βgalTCR double Tg mice. A, Tolerance developed spontaneously in the arrβgal × βgalTCR double Tg mouse on the Rag⁻/⁻ background. Ear testing was done with βgal without prior immunization. Number of mice (N) as indicated. Results are given as the mean ± SD, and p values (t test) are indicated. B, A reduced frequency of T cells with a regulatory phenotype developed in arrβgal × βgalTCR mice. FACS analysis for CD25⁺ Foxp3⁺ T cells as a percentage of the total CD3⁺ splenic T cells, N ≥ 3 for all groups. The results are given as the mean ± SD.

(24). These mice were backcrossed onto the Rag⁻/⁻ background, so that both βgal-specific effector T cells capable of mediating DTH, and Tregs specific for βgal, would be generated from the same precursors. Inhibition of DTH was found in these mice with retinal βgal expression on the Rag⁻/⁻ background (Fig. 7A), consistent with peripheral generation of tolerance due to retinal Ag expression. The βgalTCR T cells gave rise to populations of ef-

fector and regulatory T cells depending on whether they were gen-
erated in wt or arrβgal mice, respectively.

CD25⁺Foxp3⁺ T cells are found in wt and Tg mice

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Rag⁻/⁻ mice reveal a peripheral contribution of retinal Ag to tolerance in mature T cells

The peripheral induction of a regulatory response was further tested by adoptive transfer of purified, mature, peripheral CD25⁺ βgalTCR T cells into Rag⁻/⁻ recipients that cannot thymically generate mature CD4⁺ Tregs (28, 29). Backcrossing the arrβgal mice onto the Rag⁻/⁻ background provided βgal⁻ retina as the peripheral source of specific Ag for peripheral generation

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CD25− cells inhibited EAU in arrβgal × Rag−/− mice

Although inclusion of CD25+ cells from wt mice in transfers to arrβgal recipients did not inhibit DTH, they were not without effect. We recently found that adoptive transfer of naive, CD25-depleted βgalTCR T cells to arrβgal × Rag−/− mice led to a high incidence of severe EAU (18). If 5 × 10^6 CD25− cells from B10.A mice were added to 2.5 × 10^5 naive, CD25-depleted βgalTCR T cells in the inoculum, pathogenesis of EAU was substantially inhibited in arrβgal × Rag−/− recipients (Table I). Similarly, isolation and transfer of the CD4+ population from βgalTCR mice, which also contains ~6% CD25+ T cells, led to a substantial reduction in incidence and severity of EAU relative to transfer of the CD25− population only.

Manipulation of the retina alters tolerance in arrβgal mice

If development of Tregs from circulating, mature CD4 T cells required recognition of retinal Ag, preventing this process should reduce tolerance. The enucleation strategy shown in Fig. 4 supported the hypothesis that retinal Ag contributed to the regulatory activity, but the irradiation used to lymphoablate the mice could damage the retina of control mice and promote Ag trafficking. To avoid radiation damage, the Tg T cells and arrβgal mice on the

Table I. Adoptive transfer of CD25+ T cells inhibits induction of EAU by LIP-activated βgalTCR T cells

<table>
<thead>
<tr>
<th>Cells Transferred</th>
<th>Recipient Mice</th>
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<td></td>
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<td>&lt;0.01</td>
<td>&lt;0.001</td>
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a CD25− cells were 6% of the total.

b Purified CD25− cells were added to CD25− T cells to comprise 17% of the total T cells.

*Values of p for the comparison of EAU incidence and severity relative to CD25− T cells alone. Eyes were collected 66–77 days postransfer.

FIGURE 9. Enucleation prevents induction of tolerance in CD25− βgalTCR T cells transferred into arrβgal × Rag−/− mice. A, Resting CD25− T cells (2.5 × 10^5/mouse) from βgalTCR × Rag−/− mice were inoculated into the indicated recipients. Mice were ear tested after 56 days. B, After ear testing, the mice were rested for 9 days and treated with anti-CD25. Three days later, the mice were again ear tested with βgal; measurements at 48 h are shown. Results are given as the mean ± SD, and p values (t test) are indicated. enucl’d = enucleated.
Rag−/− background were used. The mice were enucleated and given naive CD25+ T cells from βgalTCR mice on the Rag−/− background. After 8 wk, the mice were tested for evidence of Treg development from the donor cells (Fig. 9A). The recipients with intact, βgal+ retinas gave inhibited DTH responses, whereas the enucleated recipients developed full-scale responses similar to Rag−/− controls, showing that Ag in the retina elicited the regulatory response. Because there was no irradiation of the mice or manipulation of the thymus, the outcome was imposed on mature peripheral T cells by retinal Ag. This result was followed by the demonstration that the cells responsible for the inhibited DTH in the control recipients that were not enucleated were sensitive to anti-CD25 depletion, which restored their DTH to full-scale levels (Fig. 9B).

Discussion

Our goal was to determine whether retinal expression of Ag in arrβgal mice was a factor in generating tolerance. Attempts to detect thymic expression of βgal by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining for enzymatic activity and RT-PCR yielded no evidence for expression. Functional assays for thymic selection have also failed to implicate thymic βgal. Grafting bone marrow from the βgalTCR mice into irradiated arrβgal vs B10.A recipients gave no evidence of activation in peripheral populations from LN, spleen, or blood, nor differences in maturation of cells from thymus (18). Conversely, βgalTCR T cells were substantially affected when βgalTCR bone marrow was grafted into ROSA26 mice, consistent with their thymic and systemic βgal expression.

We show here the presence of an Ag-specific activity from arrβgal mice that transfers with CD3+ CD4+ CD25+ T cells and inhibits the afferent response to βgal immunization. The generation of the tolerance was significantly dependent on the presence of a βgal− retina. Although the Ag is localized to the retina, its presence in normal and injured retina altered the systemic DTH response in naive, arrβgal × βgalTCR double Tg mice, showing that the effect was not limited to local (retinal) mediators or mechanisms. Elsewhere, we showed that systemic tolerance exhibited by the double Tg mice was reversed by treatment with anti-CD25 in vivo (24). Double Tg mice on the Rag−/− background also developed tolerance. Foxp3+ T cells were generated in vivo from βgalTCR T cells during lymphopenia-induced proliferation (LIP), and βgal-specific Tregs could be generated in adult βgalTCR mice by i.v. inoculation of βgal, confirming the ability to induce regulatory activity in mature βgalTCR T cells. In addition to inhibiting CD4 T cell-mediated DTH, as described here, the regulatory activity generated in arrβgal mice was also able to inhibit the ear swelling mediated by βgal-specific CD8 T cells raised by the VSC 56 vaccination (30).

Ear-swelling assays for DTH in lymphopenic recipients of CD25− βgalTCR T cells were inhibited if βgal was present in the retina, but βgal− retina was also susceptible to autoimmune destruction, showing a dissociation between the mechanisms of DTH and the pathogenesis of retinal autoimmune disease. Inclusion of exogenous CD25− T cells from βgal− donors in the lymphopenia experiments showed no effect on βgal induction of ear swelling, but they inhibited the EAU, again indicating the dissociation between EAU susceptibility, and the systemic readout of tolerance. Exogenous Tregs limited the LIP expansion of the βgalTCR T cells, similar to findings in other models (31), but no more so than having βgal in the retina.

A similar fraction of CD25− Foxp3+ T cells was recovered from βgalTCR × Rag−/− mice, with or without retinal βgal expression, suggesting that positive selection did not contribute significantly to the size of the Treg population. Our results are consistent with peripheral tolerance and induction of Tregs to βgal in naive arrβgal mice, supporting our previous observation that elicitation of DTH is inhibited in βgal-immunized arrβgal mice (17). The findings provide evidence that extends the mechanisms of retinal immune privilege to include the extrathymic development of CD4+ CD25+ Tregs that are dependent on local production of an endogenous retinal Ag. We do not disregard the possibility that thymic expression of βgal may occur at some point in development and contribute to the overall production of Tregs. But, we found that in adult mice, significant generation of the regulatory response to βgal could be accomplished by Ag from the retina.

There is substantial strain and species heterogeneity in the repertoire of TSA expressed in thymus, including retinal TSA (32). Curiously, thymic expression of a single retinal protein, IRBP, not only negatively selects the repertoire for IRBP reactivity (33) but also serves to protect the retina from spontaneous autoimmune attack against a variety of retina-specific self-Ags (16). It was also shown that thymic expression of IRBP may not be required to generate Tregs that protect retina (15). One interpretation is that tolerance to other retinal Ags is not required. Alternatively, it raises the possibility that other retinal Ags contribute to induction of peripheral tolerance. We have not found evidence for arrestin-promoted βgal expression in thymus from arrβgal mice, even though there is evidence for arrestin expression in thymus (32). We can only speculate about βgal expression, because of uncertainty in the mechanism by which aire gives expression of TSA. The arrestin promoter:βgal construct used to make the arrβgal mice uses only a portion of the relatively uncharacterized promoter region of arrestin, and it serves to give a low level of expression of βgal, even in retinal photoreceptor cells. Furthermore, the location effects on the activity of the transgenic arrestin promoter are unknown and may strongly affect its activity. In any case, the possible role of thymus is not being disputed; we have asked if Ag of retinal origin can generate regulatory T cells and found evidence for it. The mechanisms that maintain tolerance in the periphery, including the extrathymic expression of aire (34) and the extrathymic generation of new regulatory cells specific for peripheral self-Ags, are increasingly important topics.

The literature on the generation of Foxp3+ Tregs in Rag−/− mice is unclear, with evidence both for (35) and against (36) their production. The role of cognate Ag is an obvious variable. Our results suggest that very little Ag in a privileged site altered the response of Ag-specific donor CD25+ T cells in lymphopenic mice. Clearly, oral or i.v. administration of large doses of Ags can give rise to Tregs (37), but these conditions are not physiologically relevant to the arrβgal Tg mice used in this study where nanograms of intracellular Ag are expressed in neurons behind the blood-retina barrier. Models for peripheral induction of Ag-specific Tregs that are potentially relevant to our observations are based on the presentation of very low doses of Ag by dendritic cells (DC) under subimmunogenic conditions (29). Using Ab to target delivery of subnanogram doses of Ag to DC (28) or release of submicromolar doses of Ag via an osmotic pump (38), induced Tregs indistinguishable from Tregs developed in thymus.

How retinal Ag is gathered by APC in normal retina and where it is delivered so that it can induce tolerance are fundamental questions. There has been no demonstration that naive T cells enter retina and encounter retinal Ag on APC that could lead to development of a regulatory response. Analysis using labeled T cells has found them undetectable except in inflamed retina or if the T cells were activated (39). Regardless of the mechanism, the retinal origin of the Ag, rather than thymic, was the issue examined by our experiments. Although there is no direct evidence that Ag-laden APC leave the retina, we propose that trace amounts of retinal Ag
are gathered by cells with APC potential. Parenchymal microglia have a very low rate of turnover (40) and may not perform this function. Other candidates include retinal perivascular cells, which may turn over with kinetics similar to that in brain (~50% in 2–3 mo (41, 42)), possibly providing slow, ongoing delivery of retinal Ag to peripheral lymphoid tissues. Previously, we used the optic nerve crush (ONC) to induce apoptosis in retinal ganglion cells (24). ONC is a well-known model for glaucoma that leads to retinal microglial migration and activation in the retina (43). Although the βgal+ photoreceptor cells are not directly affected by the ONC, we found that 2 wk after an ONC, the inhibition of DTH was lost in arrβgal × βgalTg or Tg mice. The ONC experiment suggests that changing the interaction of innate immune cells with the neural retina can alter the balance of effector/regulatory activity to a local Ag.

Another candidate population for delivery of retinal Ag to lymphoid tissue is circulating monocytes that have transiently exited the circulation. The possibility for monocyte “surveillance” is based on observations that activated T cells, without specificity for Ag in the tissue, migrated into retina (44). Their entry into retina was accompanied by ED1+ MHC class II+ monocytes. Local, minor breakdown of the blood/retinal barrier was found, together with local activation of retinal microglia. The migration and reverse transmigration of human monocyte-derived DC has been described previously (45). Randolph et al. (45) found that CD16+ monocytes underwent abluminal migration. If the cells encountered activation factors, CD80/86 and class II were up-regulated. In the presence of TGF-β, their differentiation into DC was completed, and reverse migration back into the circulation was found. DC are now well known to possess the ability to promote differentiation of Tregs (46, 47). The differentiation resulting from DC are now well-known to possess the ability to promote differentiation of Tregs (46, 47). The differentiation resulting from TGF-β exposure has been shown to induce some DC to be regulatory or tolerizing (48, 49), and TGF-β is known to promote the generation of CD4+Foxp3+ Tregs (50). On the basis of these observations, it is possible that generation of activated T cells to ongoing environmental challenges provides opportunities for small numbers of activated T cells to enter retina, bringing monocytes with them. These cells have the opportunity to gather Ag and, in the TGF-β-rich retinal environment, may develop the regulatory phenotype, exit in the circulation, and travel to spleen. Over time, even a low level of this migration may produce a regulatory response to Ags of retinal origin. Treatment of immature bone marrow DC with TGF-β and IRBP, and transfer to naive mice, made them resistant to EAU induction by IRBP (51). Previous studies from our lab suggest that the APC that support pathogenesis of retinal autoimmunity can be recruited (52). Where the retinal Ag-bearing cells interact with T cells to produce the regulatory response is unknown at this time. Recent results show that very low, systemic cell-associated Ag levels can produce functional changes in the response to an Ag, even when the Ag is already present in the eye (53–55).

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References